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Relationship Between Oxygen Metabolism, Aging and Development

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OUTLINE

ABSTRACT

Evidence concerning the involvement of metabolic rate, prooxidants and antioxidants in processes of aging and development of animals is examined. Life span of poikilotherms and homeotherms is apparently dependent on a genetically-determined metabolic potential (i.e., total amount of energy expended during life per unit weight) and the rate of metabolic expenditure. Metabolic potential may vary in different species and under different environmental conditions. The relationship between metabolic potential, metabolic rate and duration of life is most demonstrable in organisms with a variable basal metabolic rate, such as poikilotherms and mammalian hibernators. Experimental regimes which reduce metabolic rate prolong life span and tend to retard the rate of age-related physiological and biochemical changes and vice versa. Effects of metabolic rate on aging may be mediated by oxygen free radicals. Antioxidant defenses tend to decline

during aging, whereas, free radical induced damage seems to increase with age. Intracellular environment becomes progressively less reducing during the course of development and aging. We have postulated that such a shift in redox potential may play a role in the modulation of gene activity during development and aging.

KEY WORDS

Aging, metabolic rate, free radicals, differentiation, gene regulation.

INTRODUCTION

The life cycle of all multicellular organisms can be divided into two rather incongruous phases. The initial phase, starting with the union of gametes, involves processes of cellular differentiation and growth, leading to the achievement of sexual maturity and reproductive activity. In the next phase, organisms undergo a progressive and irreversible decline in physiological efficiency, whereby, their vulnerability to death increases logarithmically with the passage of time. Death occurs when the ability of the organism to maintain homeostasis is exceeded by the severity of the destabilizing challenges of the environment.

Cellular differentiation involves a sequential repression and derepression of specific genes, which leads to a phenotypic transition of the cell. Aging has been viewed as either a continuation or a deterioration of the differentiated state. In the former view, aging results from a genetically programmed repression of specific genes or a derepression of "geronto" genes, whose products induce cellular deterioration.[1] Although this is an appealing concept, to date, no specific product of such aging genes has been detected. Alternatively, epigenetic theories view aging to result from the inadequacy of protective and reparative mechanisms. In the latter view, the functional decline of differentiated cells in the postreproductive phase of life is due to the accumulation of unrepaired damage, which leads to a gradual loss of genic control.[2] Cutler [3,4] has postulated that aging is due to a generalized deterioration of gene regulatory functions - a phenomenon he termed "dysdifferentiation".

The nature of the factors that induce cellular differentiation and that are responsible for the functional decline of differentiated cells during the aging process is presently not well understood. Nevertheless, there is considerable evidence to suggest that oxidative metabolism plays an important role in processes of development and aging. Whereas, the influence of metabolic rate (rate of oxygen utilization) on the aging process has been recognized since the beginning of this century, the

possible involvement of oxygen metabolites in developmental processes has only recently been suspected. A well coordinated series of changes occur during cellular differentiation and aging that seem to involve oxygen free radicals and cellular antioxidant defenses. In this review, we examine the evidence implicating the role of oxidative metabolism in processes of aging and development. A unified hypothesis which proposes that oxygen free radical mediated events are involved in aging and differentiation is presented.

Although aging occurs after the completion of the process of development, the relationship between oxidative metabolism and aging shall be examined first because the bulk of the available information deals with this phenomenon. The choice of this sequence may indeed be helpful in providing a rationale for the critical evaluation of the studies implicating oxidative processes in development.

I. THE RATE OF LIVING THEORY

The concept that basal metabolic rate of organisms is a determinant of longevity was first introduced by Rubner in 1908.[5] He noted that the total amount of energy metabolised per gram body weight, from maturity to death, in five different mammalian species (horse, cow, dog, cat and guinea pig) was relatively similar, ranging from 170-226 kcal, whereas, the life spans of these animals exhibited up to five-fold differences. Rubner postulated that living matter expends a discrete amount of biological energy during life and the duration of life was determined by the time spent to transform this energy.

The first experimental evidence supporting Rubner's postulate was provided by Loeb and Northrop,[6] who studied the effects of ambient temperature on development and life span of *Drosophila melanogaster*. Durations of larval, pupal and adult stages were found to be inversely proportional to ambient temperature. For example, total duration of life from egg to death was 177 days at 10^{o} and only 21 days at 30^{o}C. Life span of the adult fly was 120 days at 10^{0} and 14 days at 30^{o}C. Between 15^{o} to 25^{o}C, where development was normal, the temperature coefficient for the duration of developmental stages (i.e. larval and pupal) was identical to that for the life span of the adult stage. This led the authors to define aging in chemical terms. They postulated that duration of life is determined by the production of an unknown substance leading to the aging effect or by the destruction of substances which prevent aging.

On the basis of further studies on the effects of ambient temperature on development and life span of D. melanogaster and cantaloupe seedlings as well as survivorship curves of starved populations of wild and mutant D. melanogaster, Pearl and coworkers[7-9] introduced the expression "rate of living" and proposed the theory named after it. As originally stated, this theory postulated that duration of life is a function of two variables:[7,8]

1. The inherent vitality of the individual, which is genetically determined, as was proposed by Rubner.[5]

2. The average rate of metabolism or rate of energy expenditure during life, or as stated by Pearl,[8] "in general the duration of life varies inversely as the rate of energy expenditure during life."

The main implication of this theory was that if metabolic potentials of a group of organisms belonging to the same species were identical, life spans would depend on the rate of metabolism. Studies on poikilotherms (cold-blooded animals) and hibernating mammals have in general supported the concept that metabolic rate and longevity of organisms, belonging to the same species, are inversely correlated.[10,11] More specifically, a decrease in metabolic rate of poikilotherms has a life-lengthening effect. This relationship is strikingly evident in the differential expression of the trait "longevity" in poikilotherms and homeotherms. The characteristic species-specific life span (e.g., 2 years for mouse and 100 years for man) is a feature of homeotherms (warm-blooded animals) only, which have a stable metabolic rate. In contrast, life spans of poikilothermic species are highly variable under different environmental conditions, which influence metabolic rate. For example, under wild conditions houseflies live about 3 weeks in the summer, but in the winter they retreat to dark areas, reduce their muscular activity and remain alive for 6 months or longer.[12] Similarly, summer worker honey bees have a life span of about 35 days while winter bees live up to 8 months.[13] The effect of metabolic rate on life span of social insects, where queens live for 10-15 years, was described by Wheeler[14] as follows:

> "All the subsocial and social insects live in small cavities of the soil or wood, in hives or, in the more exceptional cases of social wasps and certain tropical ants, in the cavities of carton nests. The environment is, therefore, one which restricts or inhibits muscular movement and is dark, poor in oxygen, and of rather low and uniform temperature. All of these conditions would necessarily favor a lowered rate of metabolism and activity and an accumulation of fat in the insect body. The queens, or mothers of insect societies certainly impress one as having acquired their physiological and some of their morphological peculiarities as responses to just such an environment,

for they are very sluggish and tend to lose the powers of flight (Meliponinae) or even the wings (ants and termites) and to acquire an accentuated anabolism as shown in the accumulation of fat and of yolk-laden eggs. Certainly the life-span of the three castes of ants and social bees would seem to be roughly proportional to their respective expenditures of energy."

A. Testing the Rate of Living Theory

Studies testing the validity of the rate of living theory have attempted to verify: 1) the existence of a fixed metabolic potential or Rubner's constant, and 2) if life spans are inversely correlated with metabolic rate.

Most of the experimental studies dealing with the relationship between metabolic rate and life span have been performed in poikilotherms, especially insects, using ambient temperature as a means to vary metabolic rate.

(i) Ambient temperature and life span. In poikilotherms, ambient temperature not only affects the basal metabolic rate, but, more significantly, has a profound effect on the level of physical activity of organisms. In general, within the viable range, poikilotherms are more active physically at warmer temperatures. For example, using a radar-Doppler device, to measure physical movement of houseflies at different ambient temperatures, it was found that elevation in the ambient temperature from 17° to 26°C induced a 15-fold increase in walking activity and a 10-fold increase in flying activity of the flies.[15] In insects, flying exerts extremely high metabolic demands, e.g., rate of oxygen consumption in houseflies and blowflies increases 60 to 100-fold during flying as compared to resting or walking state.[16,17]

A striking confirmation of both postulates of the rate of living theory was provided by MacArthur and Baillie[18] in the crustacean Daphnia magna. They compared the life span and heart rate of male and female Daphnia at different environmental temperatures, ranging from 8° to 28°C. The heart rate of males was about 20% faster than that of females. Elevation of temperature from 8° to 28°C increased heart rate of males 412% and shortened life span by 77%. Length of life multiplied by heart rate was a constant (around 15,400,000 heart beats per life span), regardless of temperature or gender. The authors inferred that organisms possess a fixed sum of genetically-determined vitality and the length of life is condensed or lengthened inversely with metabolic rate. They concluded that "it is not time but tempo of life that best measures the rate of aging of an organism."

Similarly, Smith-Sonneborn and Reed[19] found that life span of paramecium cultures grown at 24° or 27°C were significantly different; however, the number of divisions observed in the two groups was identical.

Several investigators, employing temperature to vary metabolic rate, have confirmed the general validity of the rate of living theory. For example, Miquel et al.[20] found that life spans of D. melanogaster were inversely related to ambient temperature and the average amount of oxygen consumed by flies during life was relatively constant. Byzova[21] reported that total life-time oxygen consumed by the beetle Tenebrio molitor (mealworm) at 20°, 25°, and 30°C was constant, whereas, life span decreased 2.5 times between 20° and 30°C. Effects of different ambient temperatures on metabolic rate and life span of adult milkweed bugs and houseflies were examined in this laboratory. In milkweed bugs, average longevity was 70% and 200% longer at 18° than at 25° and 30°C, respectively; whereas, metabolic potential was statistically similar at all 3 temperatures.[22] A similar relationship between ambient temperature, metabolic rate and life span was observed in the houseflies. At 20°C, the average life span of flies was 44% and 190% longer than at 25 and 30°C, respectively. However, unlike milkweed bugs, metabolic potential tended to be higher at lower temperatures (Fig. 1). Analyses of mortality of milkweed bugs and houseflies, using Gompertz plots, indicated that the slopes of Gompertz plots were steeper and the intercepts were higher at warmer temperatures.[11,22] Sacher[23] has inferred that slopes of Gompertz plots, obtained by plotting the logarithms of age-specific death rates (calculated as the ratio of the number of organisms dying during a given interval to the number alive at the beginning of that interval) versus age, depict aging rates, and the intercepts represent vulnerability to death from age-independent causes. Thus, in milkweed bugs and houseflies, elevation in ambient temperature increases both the aging rate and the vulnerability to age-independent mortality. Increase in longevity at low temperatures has also been noted in a variety of diverse organisms including annual fish,[24] butterflies[25] and nematodes,[26] lending credence to the rate of living theory.

In an effort to provide a precise, mathematical relationship between temperature and chemical aging, Shaw and Bercaw[27] reformulated the rate of living theory, based on the assumption that longevity depended on the exhaustion of a hypothetical longevity substance, as originally hypothesized by Loeb and Northrop.[6] Shaw and Bercaw postulated that if Drosophila are kept at a low temperature for a certain length of time and then transferred

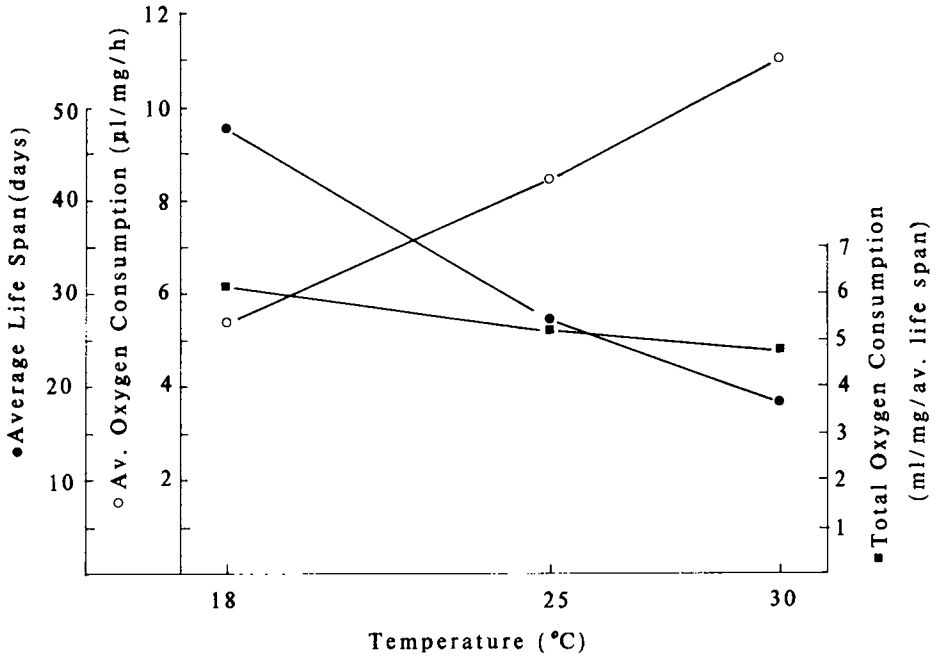

Fig 1. Effect of ambient temperature on average life span, metabolic rate (O_2 consumption/mg/ml/hr) and metabolic potential (total O_2 consumed during average life span) of male houseflies. Metabolic potential was measured on the basis of measurements of O_2 consumption at several different ages. Metabolic rate was measured in flies kept in groups of 100 in one cubic foot (0.027 cubic meter) cages.[91]

to a higher temperature, their expectation of life (L_3) is given by:

$$L_3 = L_2 + x(1 - \frac{L_2}{L_1})$$

where L_1 and L_2 are life spans at lower and higher temperatures, respectively. Using this equation, Clarke and Maynard Smith[28] and Maynard Smith [29] analysed the mortality of adult Drosophila, transferred from a lower to a higher temperature, and vice versa, at different ages. They interpreted their results to suggest that only the latter portion or about 1/3 of life span, the "dying phase," was actually inversely related to temperature, whereas, the earlier 2/3 of the life span, the "aging phase," was independent of ambient temperature and also, implicitly, of metabolic rate. This interpretation was called the "threshold theory".

Subsequent studies by Lamb[30] in D. subobscura and Hollingsworth[31] in D. melanogaster did not confirm the predictions of the threshold theory. However, based on the equation derived by Shaw and Bercaw,[27] some of their results were not in complete agreement with the predictions of the rate of living theory. The sources of the conflict between the threshold and rate of living theories have been discussed in detail previously[10,11,32-34] and will thus be mentioned here only briefly. Apparently, in studies employing ambient temperature to modify metabolic rates, two questionable assumptions have been made:

1. It was widely believed that ambient temperature is strictly proportional to metabolic rate of insects at various temperatures and ages, and after transfer from one temperature to another. There is a large body of literature indicating the existence of temperature-compensative abilities in insects and other poikilotherms.[35,36] For example, at 20°C, oxygen consumption of cockroaches was higher in those previously kept at 10°C as compared to those previously maintained at 26°C,[37] indicating that previous thermal history of insects influences their subsequent metabolic rate. Similarly, in the last trimester of life, oxygen consumption by milkweed bugs kept at 25°C was higher than those kept at 30°C.[22] Hence, the assumption that metabolic rate of poikilotherms can be invariably equated with ambient temperature is erroneous.

2. The assumption, made by Shaw and Bercaw[27] as well as others, that constancy of metabolic potential of organisms at different temperatures is a valid test for the veracity of the rate of living theory is untenable for the following reasons. Many important in vivo biological functions such as membrane permeability, rates of enzyme synthesis and degradation, proportions of isozymes, and balance between metabolic pathways are temperature-dependent.[35] Furthermore, depending on species-specific preference, overall physiological efficiency of organisms varies at different temperatures. It is therefore unreasonable to expect that metabolic potential of organisms will remain unchanged at different ambient temperatures. Obviously, metabolic potential would be lowered by suboptimal conditions. For example, the total amount of oxygen consumption, until average life span, was found to be 12-15% greater in houseflies maintained at 18°C than at 25°C.[38] Since it cannot be reasonably established that any two experimentally varied conditions are equally optimal for the expression of the metabolic potential, it would seem that metabolic potential of an organism remains a hypothetical amount of biological energy

expended under a specific experimental condition. This amount would differ under various environmental conditions.

To summarize, studies dealing with the effects of temperature on life span of poikilotherms have invariably confirmed the inverse relationship between life span and ambient temperature. However, metabolic potential at different temperatures may or may not be quantitatively similar. This fact, however, should not detract from the main implication of the rate of living theory, namely that metabolic rate and life span are inversely correlated.

(ii) Physical activity and life span. To further explore the relationship between metabolic rate and life span, and to avoid complications due to secondary effects of varied ambient temperatures, we altered the metabolic rate of houseflies by manipulations of flying activity. Since flying increases the metabolic rate of houseflies 60 to 100-fold,[17] variations in flying activity provide a highly effective means to alter the rate of oxygen consumption. Levels of physical activity of houseflies were altered by a variety of methods, including variations in the size of housing containers, population density and sex ratios, as well as surgical removal of wings.[32,39,40] In general, it was found that experimental regimes which decreased the level of physical activity tended to increase the life span of flies, and vice versa. Average and maximum life spans of male flies kept under conditions of low physical activity in bottles (250 ml), where flying is not permitted, were about 2.5 times longer than those kept under conditions of high physical activity, in one cubic foot cages, where flying is possible[39] (Fig. 2). Life spans of flies were also prolonged by surgical removal of wings.[40] Similarly, an increase in the proportion of females in the population increased male life span due to a reduction in their physical activity, in pursuit of sexually receptive females .[39,40]

To investigate whether individual differences in life spans of cohorts are related to differences in the levels of spontaneous physical activity, walking and flying activity of flies was monitored by radar-Doppler. Flies which were more active in walking and flying tended to die earlier.[40] Recently, Lints et al.[41] reported the results of a study which they interpreted to indicate that spontaneous physical activity in D. melanogaster is not correlated with life span. However, these authors measured only the walking tendency of flies confined in a petri dish for about 6 minutes on a single day in a fly's life. This and other flaws in

their experimental design, pointed out elsewhere,[42] do not permit validation of their claim.

To study the relationship between metabolic rate and life span, Trout and Kaplan[43] employed "shaker" mutants of D. melanogaster. These mutants suffer from a neurological impairment. They are highly active physically and have shorter life spans than the wild controls. Metabolic rate and life spans of "shakers" were found to be inversely correlated, but the metabolic potential of all groups was similar, around 6 ml oxygen/mg wet weight. The authors inferred that metabolic rate, i.e., basal plus induced, is the major variable determining longevity in Drosophila.

Life spans of worker honey bees are reportedly modulated by physical activity related to foraging activity. Life span of bees is prolonged in proportion to the period spent in relative inactivity in the hive.[44] Even in the non-flying insects, physical activity has been shown to affect life span. For example, Kern[45] reported that adult male silkmoths, which do not fly or eat as adults, become very active physically, by frequent shaking of the body, in response to stimulation by the female pheromones. Removal of the antennae, which act as chemoreceptors, reduces the level of physical activity and causes a significant prolongation in their life span.

(iii) Metabolic rate and life span in mammal. Because the basal metabolic rate of mammals cannot be varied experimentally for prolonged periods, mammalian studies have mainly dealt with the effects of exercise on longevity, and with correlations between species life spans and basal metabolic rates.

Contrary to Rubner's assumption, Cutler[3] has reported three separate categories of metabolic potential in mammals. Non-primate mammals expend about 200-, non-human primates about 400-, and humans about 800-kcal/g body weight/life span. Within each of these categories, the basal metabolic rate is apparently inversely related to species-specific life span, which supports the rate of living theory.

Results of studies on the effects of physical activity on the aging process of mammals are rather ambiguous. Several studies have shown that voluntary exercise prolongs the life span of laboratory rodents;[46] however there is no report, to our knowledge, documenting the effects of various levels of mild to strenuous chronic exercise on longevity. The age of the organisms appears to modulate the effects of physical activity. Forced exercise is beneficial to young organisms, but deleterious to older ones.[47] A serious limitation in the experimental design of existing mammalian studies, dealing with physical activity and life span, is that comparisons

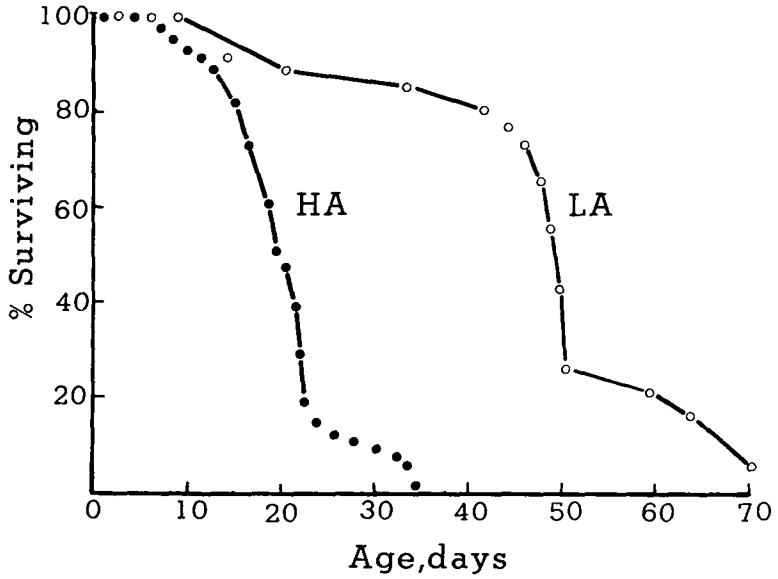

Fig 2. Survivorship curves of houseflies kept under conditions of high activity (HA ;100 flies/1 cubic-foot cage; 4 males: 1 female) and low physical activity (LA ;one fly/250 ml glass bottle). (Adapted from 39).

of experimental animals are made with sedentary controls. Such studies only demonstrate that lack of muscular activity under the highly confined laboratory conditions is detrimental to animals. It should be borne in mind that under natural conditions rodent species are highly active physically. It is also well known that certain minimal levels of physical exercise are essential for the prevention of tissue atrophy in mammals. It is entirely possible that physical activity beyond this critical level is deleterious.

Decrease in the ambient temperature of mammals results in an increase in the rate of oxygen consumption. Animals maintained at low temperatures have been found to have significantly shorter life spans than controls maintained at room temperature.[48-50]

Mammalian hibernators can be considered to constitute a physiological link between poikilotherms and homeotherms in their ability to maintain a stable basal metabolic rate. Lyman et al.[51] examined the relationship between hibernation and longevity in Turkish hamsters that hibernated for 0 to 33% of their lives. Metabolic rate was lower in hibernators kept at 5°C than in controls maintained at 22°C. In general, life spans of hibernators were longer than non-hibernators. Furthermore, animals that hibernated

longer also lived longer. Maximum life span was greater in hibernating than in non-hibernating animals.

In summary, there appears to be strong evidence that in simpler model systems, such as poikilotherms, longevity is inversely related to the level of physical activity. Temperature effects on life span in poikilotherms are largely mediated by changes in physical activity. The relationship between temperature, metabolic rate and longevity is more complex in mammals due to homeothermy and the physiological necessity for physical activity to prevent atrophy. Nevertheless, basal metabolic rate of mammals is inversely correlated with species-specific life span, within phylogenetic groups having similar metabolic potentials.[3]

II. EFFECTS OF METABOLIC RATE ON AGE-RELATED CHANGES

It is reasonable to expect that factors purporting to affect the rate of aging would retard or accelerate the biochemical and physiological changes accompanying the aging process. Some of the most ubiquitous age-related changes, in widely divergent species, are the accumulations of lipofuscin and thiobarituric acid (TBA)-reactants, and increased exhalation of alkanes.[52] As described below, the rate of these age-related changes is influenced by metabolic rate of the organisms.

A. Lipofuscin

Most cell types exhibit an age-related increase in the amount of characteristic cytoplasmic structures, often referred to as "lipofuscin". Lipofuscin granules are membrane-bound lysosomal structures which contain lipoidal moieties, exhibit yellow to brown coloration, emit yellow to greenish autofluorescence under UV and accumulate with age.[53] A closely related structure, termed "ceroid", has similar characteristics but is formed under pathological conditions traceable to a specific biochemical impairment.[54] Lipofuscin has been the subject of several recent reviews[53-57] as well as a recent compendium.[58] In the current thinking, lipofuscin is believed to be formed by the involvement of two distinct processes, which are: 1) autophagocytosis, and 2) peroxidation of lipids, followed by copolymerization of lipids and proteins.[57] Some of the fluorescent material in lipofuscin granules is extractable in organic solvents[59] and exhibits blue emittance.[60] Although there is some disagreement concerning the chemical nature of fluorophores, it is generally believed that oxygen-derived free radicals play a major role in the formation of fluorescent material.[60,61] According to a widely accepted scheme developed by Tappel

and coworkers (for references, see 60,61), the blue-emitting fluorescent material in lipofuscin granules arises by the peroxidation of polyunsaturated fatty acids. Lipid peroxides break down into a variety of products including malondialdehyde and alkanes, e.g., ethane and pentane. Malondialdehyde reacts with amine-containing molecules, such as proteins, nucleic acids and certain phospholipids, to form Schiff-base compounds with the structure: RN=CH-CH=CH=NHR. Tappel's inference that blue-emitting, lipofuscin fluorophores are formed as an end-product of free radical-induced lipid peroxidation, has provided a conceptual link between oxygen consumption, free radicals, lipofuscin and aging. A highly attractive feature of Tappel's hypothesis is that the chloroform-soluble fluorescent material (SFM) provides a marker for studying the involvement of free radicals and oxidative damage in the aging process. However, blue-emitting fluorophores also exist in the extra-lipofuscin compartments within the cell; therefore, the concentration of soluble fluorescent material and volume of lipofuscin granules may not be proportionately related.[62] For the sake of clarity, the term "lipofuscin" will be applied here to the *in situ*, morphologically-detectable, autofluorescent granules, and the term "soluble fluorescent material "(SFM) will refer to the substances present in tissue extracts.

The relationship between oxygen free radicals and formation of lipofuscin was convincingly demonstrated by Thaw et al.[63] in cultured glial cells. The amount of lipofuscin was shown to increase in the presence of $FeCl_3$/ascorbate in the medium as well as elevated ambient oxygen concentration, and to decrease in the presence of antioxidants in the medium. A variety of other studies (for references, see 56,64) have also indicated a relationship between ceroid accumulation and antioxidant deficiency.

There is considerable evidence indicating that the rate of lipofuscin accumulation is dependent on metabolic rate and the rate of aging. For example, rate of lipofuscin accumulation in the hearts of dogs is approximately 5.5 times faster than in humans, which roughly corresponds to the difference in their life spans.[65] Friede[66] compared the distribution of oxidative enzymes, such as succinate dehydrogenase and DPN-diaphorase, with the relative amount of lipofuscin in 66 different loci in the aged human brain. Nerve cells exhibiting relatively high oxidative enzyme activity contained more lipofuscin than nerve cells characterized by relatively low activity of oxidative enzymes. A fortuitous insight into the relationship between functional activity, oxidative enzyme activity, and the amount of

lipofuscin was provided by studies on two persons who had lost an eye. Neurons of the lateral geniculate body, receiving terminals from the blind eye, showed a marked decrease in DPN-diaphorase activity and in the amount of lipofuscin as compared to the neurons connected to the seeing eye. According to the author, the presence of "wear and tear" pigment appeared to be related to the functional "wear and tear" of a given region as reflected by the intensity of oxidative enzymes. Dolman and Macleod[54] have cited several other examples of a relationship between functional activity of cells and their lipofuscin levels. Postural muscles of humans have lesser amounts of lipofuscin than muscles involved in movement.[67] Paralyzed muscles of stroke victims have relatively little lipofuscin.[67]

The relationship between metabolic rate and lipofuscin accumulation was experimentally demonstrated in this laboratory. Average as well as maximum life spans of adult houseflies were prolonged approximately 2.5 times by elimination of flying activity. The rate of lipofuscin deposition, measured in three different tissues by quantitative electron microscopy, was faster in the short-lived, high activity flies as compared to the long-lived, low activity flies.[68,69] However, the maximum level of lipofuscin reached in the two groups was nearly equal. Increase in ambient temperature and in oxygen tension also increases the rate of lipofuscin formation in various tissues of D. melanogaster.[70]

Further experimental evidence indicating the relationship between lipofuscin deposition and metabolic rate was provided by Papafrangos and Lyman[71] in Turkish hamsters. As also mentioned above, Lyman et al.[51] had reported earlier that Turkish hamsters that spent part of their lives in the depressed metabolic state of hibernation had 23% longer average life spans than non-hibernators. A comparison of lipofuscin content in the brain and the heart of hamsters indicated that animals which hibernated 11-23% of their lives had a slower rate of lipofuscin accumulation than those which hibernated only 0-7% of their lives. It was also found that the differences between the hibernators and the non-hibernators became more marked with age, especially in the heart. Although the rate of lipofuscin deposition was not found to be directly proportional to alterations in life spans, the total volume of lipofuscin reached at the end of life was similar in the two groups. Thus, studies in both poikilotherms and hibernating mammals show that lipofuscin deposition corresponds to alterations in metabolic rate and life span. However, this relationship should not be interpreted to imply that lipofuscin is causally related to aging. Rather, lipofuscin should be considered a manifestation of cellular senescence.

B. Soluble Fluorescent Material

The concentration of fluorescent material (SFM) in chloroform-methanol extracts of tissues exhibiting Schiff base-like fluorescent characteristics has been shown to increase with age in a variety of organisms.[57] Environmental conditions such as ambient temperature and physical activity, which enhance metabolic rate, tend to increase the rate of SFM accumulation. In milkweed bugs[2] and fruitflies,[55,72] the rates of SFM accumulation have been found to be faster at higher than at lower ambient temperatures. The maximum levels were reached earlier in insects kept at higher temperatures. A comparison of houseflies, kept under conditions of high and low levels of physical activity, indicated that SFM accumulation was faster in the former than in the latter group, but the maximal level reached was similar in the two groups.[73] Individual flies which exhibited a greater tendency for spontaneous flight activity (measured by radar-Doppler) tended to have a shorter life span and contained more SFM than the relatively inactive, lazy flies.[74] Basson et al.[75] have also reported that the rate of SFM accumulation is faster in rats undergoing treadmill physical training than in sedentary controls.

C. Thiobarbituric Acid-Reactants

One of the consequences of free radical interactions with cellular structures can be the peroxidation of polyunsaturated lipids, which is detectable by the evolution of alkanes such as ethane and n-pentane, from the animal and by the production of TBA-reactive material.[61,75] However, Gutteridge[77] has reported that in addition to lipid peroxidation, TBA-reactants or malondialdehyde-like substances can arise from free radical damage to other organic molecules, such as amino acids, DNA and carbohydrates. In milkweed bugs[22] and houseflies,[78] the concentration of TBA-reactants increased with age at significantly faster rates in organisms kept at relatively higher ambient temperatures. These results can be interpreted to suggest that increased metabolic rate heightens the *in vitro* susceptability of tissues to peroxidative changes, and may reflect *in vivo* damage.

D. Alkane Production

Alkane exhalation has been proposed as a sensitive indicator of *in vivo* lipid peroxidation.[79,80] Ethane and n-pentane, which are scission products of .-3 and -6 polyunsaturated fatty acids, respectively, have been the most commonly used indicators. An increase in alkane production has

been reported in rats with age[81] and in response to vitamin E-deficiency.[61] Dillard et al.[82] have reported an increase in the level of pentane exhalation in humans during physical exertion. Studies in the housefly have indicated that in vivo n-pentane production increases 1.7-fold during the average life span of the fly.[83] The amount of n-pentane, generated by the flies in vivo, was 2.7 times greater at 28°C than at 20°C, which clearly demonstrated that an increase in metabolic rate causes an increase in the in vivo rate of lipid peroxidation. Furthermore, homogenates of houseflies, aged at a higher temperature, exhibited a greater susceptibility to undergo lipid peroxidation, as indicated by n-pentane production, in response to tert-butyl hydroperoxide-induced oxidative stress than those aged at a lower ambient temperature. Age-associated increases in the in vivo evolution of n-pentane and in response to tert-butyl hydroperoxide in vitro are indicative of the increased vulnerability of flies to free radical-induced damage as a function of age.

III. METABOLIC RATE AND FREE RADICAL GENERATION

Although the existence of a relationship between metabolic rate and life span has been known for a long time, and, as also pointed out above, originally formed the basis of the "rate of living" theory, the possible mechanism underlying this relationship remained obscure until recently. A link between oxygen utilization and generation of oxygen-centered free radicals was first proposed by Gerschman et al.[84] Later, Harman[85] suggested that free radical-induced damage may be the cause of gradual physiological attrition underlying the aging process.

There is some evidence to indicate that enhanced metabolic rate increases the intracellular concentration of free radicals, which in turn increases the magnitude of lipid peroxidative and other damage to cellular organelles. Davies et al.[86] have reported a 2- to 3-fold increase in free radical ($R^{\bullet}$) concentration in homogenates of muscle and liver of rats following submaximal exercise until exhaustion. A similar $R^{\bullet}$ signal ($g = 2.004$) was detected in homogenates from vitamin E-deficient animals. Mitochondrial respiratory control values were lower in exercise-exhausted and vitamin-E deficient rats than in controls. State 4 (idling) respiration was increased in exercised and vitamin E-deficient rats, while state 3 (ADP-stimulated) respiration appeared to be unaffected, suggesting leakage of protons from mitochondria. Concentrations of conjugated dienes and TBA-reactants were greatly increased in both vitamin E-deficient and exercised animals, indicating enhanced lipid peroxidation. Similarly, the exhalation

of n-pentane is significantly higher at 28°C than at 20°C suggesting that free radical production is greater under conditions of higher metabolic rate.[83]

IV. TESTING THE FREE RADICAL THEORY

Although the hypothesis, that free radicals generated during cellular metabolism are the main cause of cellular damage occurring during aging, was advanced about three decades ago, and considerable knowledge about free radical reactions has since accumulated, experimental studies testing this hypothesis have been rather desultory. The main approach for the investigation of free radical involvement in the aging process has been to study the effects of exogenous antioxidants on life span of organisms. Harman[87,88] has cited the life-lengthening effects of antioxidant intake to constitute experimental support for his hypothesis; however, results of a variety of studies have not clearly supported this claim. For example, 2-mercaptoethylamine hydrochloride and butylated hydroxytoluene were reported by Harman[87] to increase the average life span of mice; however, in a reinvestigation Kohn[89] reported that when survival of control mice was optimal, the same antioxidants had no life-lengthening effect. Antioxidants were found to lengthen the average life span of mice only when the life spans of controls were below the optimal level. Furthermore, antioxidant administration does not prolong the maximum life span, which is widely believed to be the main indicator of the rate of aging of organisms. Parenthetically, it may be added that the failure of antioxidants to extend life span is not surprising even if free radicals were indeed the causal agents in aging. It is not feasible to achieve sufficient intracellular concentrations of antioxidants to counteract a significant proportion of hydroxyl radicals generated in cells. However, more importantly, cells seem to exert a homeostatic control over their antioxidant levels and, as discussed below, administration of exogenous antioxidants causes a compensatory depression of endogenous antioxidant defenses.[3,90]

The free radical theory of aging has been frequently criticized, justifiably, for lack of direct supportive evidence. Nevertheless, one can also pose the question: Is it possible to provide unambiguous direct evidence linking free radicals with the aging process, even if they were actually involved with the aging process? In our opinion, it may be practically impossible to do so because of the requirements of direct proof in science. It is presently unrealistic to establish a cause and effect relationship between oxygen free radicals, present in extremely low

concentrations in cells, and the aging process, which occurs very gradually and has poorly defined markers, while excluding all other changes occurring during aging.

A rational and productive investigative approach may be to test the predictions of the free radical theory of aging. Within this scope, three lines of inquiry have been followed: 1. Age-related changes occurring in organisms suggesting free radical involvement. 2. Relationship between life expectancy and antioxidant defenses or degree of free radical-induced damage. 3. Experimental effects of prooxidants and antioxidants on aging. Evidence concerning these aspects is discussed below.

A. Age-related Changes and Antioxidant Defenses

As described above, there is much evidence to suggest that free radical reactions play a causal role in the formation of lipofuscin, TBA-reactive substances and alkanes. In mammals[81] as well as insects,[83] alkane production increases with age. In addition, in the housefly, *in vivo* concentrations of inorganic peroxides and GSSG increase with age,[11] which suggests that tissues of older organisms are relatively more vulnerable to free radical-induced damage. This may be the result of a decline in antioxidant defenses and/or an increase in the rate of free radical generation. It should be noted here that various components of the antioxidant defense system often possess overlapping functions and may undergo compensatory changes to maintain a stable balance.[90]

A comprehensive analysis of age-related changes in antioxidant defenses of the housefly indicated that SOD activity decreased during the last one third of life; catalase activity steadily declined with age and was approximately half the level in the old flies as compared to the young.[91] Glutathione (GSH) level sharply declined in older flies, whereas, the concentration of chloroform-soluble antioxidants (vitamin E) greatly decreased during the first part of life and remained relatively constant thereafter. *In toto*, results of these studies indicated that enzymatic and non-enzymatic defenses against free radicals and hydroperoxides in the adult housefly tend to deteriorate with age (Fig. 3), whereas, levels of the products of free radical reactions such as H_2O_2, GSSG, TBA-reactants, *n*-pentane production and lipofuscin increase with age (Fig. 4).

Attempts to determine if age-related decline in antioxidant defenses is a widespread phenomenon have produced varied results. Kellogg and Fridovich[92] measured total SOD activity in Sprague-Dawley rats at various ages. A slight age-dependent decrease was detected in the liver, but not in

the brain. Conversely, Massie et al.[93] reported a 36% decline in total SOD activity in the brain of C57BL/6J mice between 50 and 900 days of age. In the brain of male albino Wistar rats, Vanella et al.[94] found that cytosolic SOD activity declined during the first 30 months of age, however, mitochondrial SOD activity increased at a proportional rate so that the total SOD activity remained relatively stable. Catalase activity has been reported to decrease in aging Drosophila[95,96] and the housefly.[91] GSH content of various tissues of the mouse undergoes significant decrease in the latter half of life.[97,98] Activities of glutathione peroxidase, glutathione reductase and glutathione S-transferase also decline in old mice.[98,99]

To summarize, results of the studies cited above suggest that overall antioxidant defenses tend to decline with age. However, with few exceptions,[91] no attempts have been made to obtain a comprehensive profile of antioxidant protection of cells with age. It is imperative to obtain such comprehensive information because of the compensatory interdependence among various components of the antioxidant system. It is possible that some antioxidant defenses may remain at fairly high levels throughout life to compensate for the age-related loss of other defenses.

The question whether or not the rate of free radical generation increases with age in the rat was investigated by the group of Nohl and Hegner.[100-102] A comparison of 3- and 23-month old rats indicated that generation of the superoxide radical and H_2O_2, in both intact mitochondria and mitochondrial fragments from the heart, was higher in older rats. The concentration of dienes, aldehydes and ketones was also higher in old rats. The ratio of unsaturated to saturated fatty acids in the inner mitochondrial membrane decreased with age. Using glutamate, malate, 3-hydroxybutyrate and succinate as substrates, it was found that respiratory activity, respiratory control values and P:O ratios of mitochondria were lower in old rats. The authors inferred that free radical generation in mitochondria increases with age and can contribute, via lipid peroxidation, to changes in lipid-dependent enzyme systems.

B. Antioxidants and Life Expectancy

Cutler[3] has made comparative studies of life span potential (LSP; age of oldest survivor), life span energy potential (LEP; metabolic potential) and antioxidant capacity in various mammalian species. While these species exhibited up to 30-fold differences in the LSP, only 3 distinct classes of

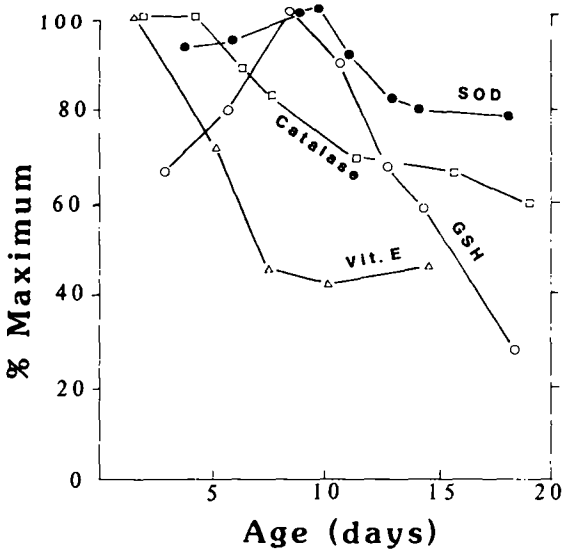

Fig 3. Age-related changes in antioxidant defenses in the male housefly.[91] SOD, superoxide dismutase; GSH (reduced glutathione). (Reproduced from 11).

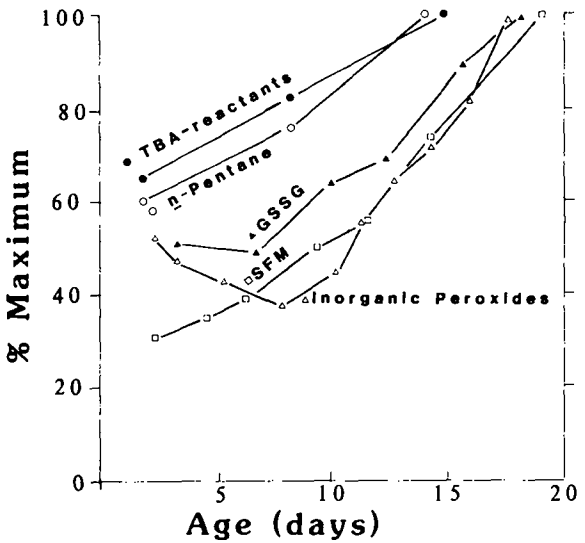

Fig 4. Age-associated changes in the concentration of various products of free radical reactions in male housefly. TBA-reactants, GSSG (oxidized glutathione), SFM (chloroform-soluble fluorescent material presumably derived from lipofuscin), and inorganic peroxides (primarily H_2O_2) were measured in whole body homogenates of flies.[91] n-Pentane production was measured in vivo.[83] (Reproduced from 11).

LEP were evident. It was hypothesized that rate of oxygen utilization was related to aging, and animals with higher LEP values were more resistant to the deleterious effects of oxygen utilization due to the presence of higher concentrations of antioxidants in relation to their metabolic rate. Antioxidants such as SOD,[103] uric acid,[104] carotenoids and tocopherol were found to be positively correlated with LEP while ascorbate, glutathione, glutathione peroxidase and S-transferases were negatively correlated with LEP.[3] Brain homogenates of organisms with a high LEP were found to be more resistant to autoxidation (determined with TBA) than homogenates of organisms with a low LEP.[3] Serum levels of TBA-reactants were inversely correlated with LEP.[3] Results of these studies suggested that certain antioxidant defenses are correlated with LSP and LEP values in mammals.

Recently, we examined the relationship between antioxidant defenses and life expectancy in the housefly (unpublished). All flies lose flight ability prior to death, hence, on the basis of presence or absence of flight activity, flies destined to die earlier can be separated from their longer lived cohorts of the same age. Flies with a shorter life expectancy contained significantly lower levels of SOD activity, catalase activity and glutathione, and higher concentrations of inorganic peroxides and TBA-reactants as compared to flies with longer life expectancy.

Studies by Munkres[105] on conidial longevity of Neurospora crassa indicate that longevity of various mutants is positively correlated with antioxidant enzymes SOD, catalase, and peroxidases.

C. Effects of Oxidative Stress and Antioxidants on Aging

Most of the evidence concerning the cellular effects of oxidative stress has been derived from in vitro studies. Such studies have yielded a wealth of theoretical information; however, the effects of chronic exposure to oxidative stress, which would be relevant to aging studies, are difficult to study due to: 1) narrow limits of tolerance by living organisms, and 2) homeostatic compensatory controls.

A comprehensive examination of the effects of experimentally-induced oxidative stress, employing a variety of approaches, was conducted in the housefly in this laboratory. Putative changes in the level of free radicals were induced by the administration of free radical generators or by the inhibition of endogenous free radical defenses. Diamide[106] (an -SH oxidant) and paraquat[107] (a herbicide believed to generate O_2^-) were used to enhance the production of free radicals. Diethyldithiocarbamate (DDC)[108] (an

inhibitor of SOD), 3-amino-1,2,4-triazole (3-AT)[109] (a specific inhibitor of catalase), and L-buthionine-SR-sulfoximine[110] (an inhibitor of glutathione synthesis) were employed to depress endogenous cellular antioxidant defenses against free radicals. Iron was administered to catalyze the interaction between O_2^- and H_2O_2 and the decomposition of lipid peroxide.[111] In addition, antioxidants such as ascorbate, β-carotene and α-tocopherol were administered to the flies in order to reduce the putative levels of free radical.[90] Endogenous levels of glutathione were increased by the administration of L-2-oxothiazolidine-4-carboxylate.[110]

Results of these studies are summarized in Table 1. In general, these studies indicated that oxidative stress induces a compensatory decrease in metabolic rate and an increase in GSH concentration, while exogenous antioxidants depress one or more components of the endogenous antioxidant defense system. Although diamide and 3-AT did not alter average life span, the metabolic potential of the flies, which is an indicator of total vitality, was decreased. DDC slightly increased life span but did not affect metabolic potential. However, DDC is also an effective antioxidant due to its metal-binding properties. Other treatments decreased life span and metabolic potential

Administration of exogenous antioxidants did not increase the life span of flies.[91] Results of numerous other studies, which have employed antioxidant administration as a means to test the free radical theory of aging (reviewed by Balin,[112] and Cutler[113]), have also indicated that exogenous antioxidants are ineffective in prolongation of the maximum life span of organisms. Relatively high intake of ascorbate and α-tocopherol was in fact toxic to the flies. Exogenous antioxidants had a compensatory effect on endogenous antioxidants. For example, administration of ascorbate, which has an overlapping function with glutathione and SOD was found to depress cellular levels of both. α-Tocopherol and β-carotene tended to depress SOD activity.[91]

Administration of a relatively low concentration of the above prooxidants (except iron) or antioxidants to the housefly did not affect the rates of age-associated changes such as accumulation of SFM and TBA-reactive material.[114] Iron administration increased the level of SFM and lipofuscin. Exposure of flies to relatively high concentrations of prooxidants caused rapid mortality, which prevented the measurement of age-related parameters. Overall, results of these studies indicated that a complex balance exists between prooxidants and antioxidants within cells. This is consistent with the hypothesis that augmentation or depression of one antioxidant defense

Table 1. Effects of Prooxidant and Antioxidant Regimes on Life Span, Metabolic Rate, Metabolic Potential, H_2O_2, and Endogenous Antioxidants in the Adult Male Housefly.

Treatment	Action	Parameter*						
Prooxidants:		Life span	Metabolic Rate	Metabolic Potential[1]	H_2O_2	SOD[2]	Catalase	GSH[3]
Diamide	GSH-oxidant	0	-	-	+	-	+	+
Paraquat	O_2^--generator	-	-	-	+	0	0	+
DDC[4]	SOD-inhibitor	+	-	0	-	-	-	+
3 AT[5]	Catalase-inhibitor	0	-	-	+	+	-	+
BUS[6]	Inhibitor of GSH synthesis	-	0	-	+	0	-	-
$FeCl_2$	OH· formation	-	0	-	+	0	+	0
High Activity	Increased metabolic rate	-	+	0	+	0	0	+
Antioxidants:								
LOC[7]	Stimulant of GSH synthesis	0	0	0	+	0	+	+
Ascorbate	Antioxidant	-	-	-	-	-	0	-
β-carotene	Antioxidant	0	0	0	+	-	0	-
α-Tocopherol	Antioxidant	-	0	-	+	-	-	-

* + increase; 0 no change; - decrease

[1]Metabolic potential is the total, average ml of O_2 consumed/mg wet wt/life span.

[2]SOD, superoxide dismutase; [3]GSH, reduced glutathione; [4]DDC, Diethyldithiocarbamate;

[5]3AT, 3 Amino-1,2,4-triazole; [6]BUS, L-Buthionine-SR-sulfoximine; [7]LOC, L-2-Oxothiazolidine-4-carboxylate

causes a compensatory change in another related or overlapping antioxidant mechanism.

V. FREE RADICALS, DYSDIFFERENTIATION AND AGING

Although free radicals are present in cells under steady state conditions and can cause molecular damage, it is unlikely that aging is solely due to the accumulation of physical damage. A survey of age-associated changes clearly indicates that aging is not accompanied by a ubiquitous attrition of structural components of cells. Cutler[3,4] has suggested that aging may at least be partly due to free radical-induced changes in the differentiated state of cells, whereby, normally repressed genes become derepressed during the senescent phase. In his view, the optimal state of differentiation gradually degenerates into a state of "dysdifferentiation" as a result of genomic damage by long-term exposure to free radicals. According to this line of reasoning, the relationship between differentiation and aging would be governed by factors which control repression and derepression of genes during developmental and post-developmental stages.

Cellular differentiation ultimately results from the differential expression of genes; however, this process is modulated by cytosolic factors. Transplantation of cultured somatic cell nuclei into oocytes has been found to derepress embryonic genes and repress genes normally expressed in differentiated somatic cells.[115] In at least one case, transplantation of nuclei from dedifferentiated cells, i.e., nuclei from cancer cells, to oocytes results in the formation of normal tissues.[116] Such studies indicate the existence of cytosolic factors which reversibly influence gene expression.

The hypothesis that aging is due to changes in gene expression does not necessarily mean that such changes are due to genomic damage; however, diminished control of genomic expression would result in cellular inefficiency and may ultimately lead to death.[3,4,117] Several lines of evidence tend to support Cutler's dysdifferentiation hypothesis. Non-histone proteins are believed to play an important role in the regulation of gene expression.[118,119] It is well documented that non-histone proteins are extremely sensitive to surrounding charges and ion balance, and that they undergo age-related alterations in overall charge.[120,122] The murine leukemia virus is normally not expressed in brain or liver tissue; however, a greater fraction of the viral genome is expressed in the brain and liver of older animals than in young animals.[123,124] Furthermore, the number of

globin messenger RNA molecules has been found to increase in the brain and liver with age. Since globin is not normally synthesized by the brain or liver, the observed increase may indicate that mechanisms controlling gene expression become less effective in older organisms.[123] It would also seem significant that many carcinogens are more effective in altering gene expression than in causing mutations.[125,126] Cross-linking of chromatin proteins may be one of the causes of the age-related decline in protein synthesis observed in a variety of organisms.[127,128]

On the basis of existing evidence, it is reasonable to assume that gene expression changes with age. Such changes may result from alterations in controlling mechanisms rather than damage to the genome. If dysdifferentiation plays a causal role in aging, a higher rate of metabolism should accelerate this process. The mechanism by which metabolic rate may bring about dysdifferentiation is presently obscure. In the context of existing knowledge, two different mechanisms can be suggested: 1. Free radicals generated by metabolic processes may damage gene-regulatory sites as suggested by Cutler[3,4] 2. In our view, it is also possible that shifts in the balance between cellular oxidants and reductants may represent the cytosolic factors which affect the patterns of gene expression associated with processes of development and aging. Relative concentrations of oxidized forms of glutathione, NAD, and NADP in the rat skeletal muscle are higher, at the expense of reduced forms, in old rats than in young rats.[129] We have observed a similar pattern in the whole body homogenates of the houseflies (unpublished). Such findings suggest that the intracellular environment of old cells is less reducing than in the young cells.

VI. INVOLVEMENT OF OXYGEN RADICALS IN DEVELOPMENT

A variety of metabolic fields and gradients are known to affect developmental processes in multicellular organisms. Many of the changes during embryonic development appear to correspond, either directly or indirectly, to alterations in oxygen metabolism.[130] Child[131] postulated that in regenerating organisms the regions of higher metabolic activity influenced the development of regions with lower metabolic activity. Differential vascularization, which would presumably lead to unequal oxygenation of tissues, is believed to influence developmental patterns in higher organisms.[132-134] Furthermore, phenotypic expression in cultured embryonic chick cells can be experimentally controlled by variations in oxygen tensions.[135]

Several lines of evidence suggest that oxygen-derived free radicals may be involved in the process of cellular differentiation. Polytene chromosomes of salivary glands in insects exhibit a characteristic puffing pattern during development.[118] Uncouplers of mitochondrial respiration, e.g., dinitrophenol, menadione, oligomycin and antimycin A have been observed to induce chromosomal puffing.[136,137]

An important clue to the involvement of oxygen radicals in the process of differentiation is provided by the fact that alterations in the differentiated state are invariably accompanied by changes in the level of cellular free radical defenses. Notably, cancer cells appear to exhibit a reduction in the activity of mitochondrial SOD (mangano-isozyme).[138-140] In many cases, the activity of cytosolic SOD (Cu/Zn isozyme) is also greatly reduced.[141] The rate of cell division, which is indicative of the extent of dedifferentiation, has been found to vary indirectly with SOD activity, i.e., the highest rates of cell division occur in cells with the lowest SOD activity.[139,142] Other antioxidant enzymes in cancer cells also exhibit decreased activity.[143] Conversely, SOD activity has been observed to increase during metamorphosis in insects[144] and during differentiation of the cellular slime mold, Didymium iridius.[145]

We have observed increases in Mn-SOD activity of up to 46-fold during the differentiation of various strains of the slime mold, Physarum polycephalum.[146] Increased SOD activity was accompanied by an elevation in cyanide-resistant respiration (Fig. 5). The rate at which Mn-SOD activity increases roughly corresponds to the rate of differentiation. Under identical culture conditions, strains of Physarum, which did not differentiate, failed to exhibit increased SOD activity. The only other enzyme in Physarum previously reported to exhibit a large increase during spherulation is glutamate dehydrogenase (9-fold). Other enzymes examined in Physarum exhibit approximately constant or decreased activity during differentiation.[147] Of the enzymes examined, only superoxide dismutase activity increases so strikingly, and only the changes in superoxide dismutase activity parallel the rate of differentiation (Fig. 5).

Inorganic peroxide concentration was greatly elevated in differentiating strains of Physarum, but not in a non-differentiating strain.[146] It is noteworthy that rates of H_2O_2 generation and lipid peroxidation are lower in cancer cells than in normal cells. The decrease in the rate of H_2O_2 generation is believed to be due to low SOD activity,[138] and the decrease in lipid peroxidation has been postulated to result from alterations in membrane composition of tumor cells.[142,148] The rate of

tumor growth has also been reported to be inversely related to lipid peroxidation,[148] which is consistent with the observation that lipid peroxides inhibit mitosis.[149] In regenerating rat liver, the level of lipid peroxides decreases during the mitotic phase of regeneration and increases during redifferentiation.[150]

Glutathione has been implicated in a number of developmental processes. Cell state transitions are frequently accompanied by alterations in GSH concentration. Dedifferentiated cells, such as cancer cells, contain high levels of GSH.[151] For example, the growth rate of human skin tumors is reported to be proportional to GSH concentration.[152] In vertebrates, GSH increases during the mitotic phase of regeneration and subsequently declines as the cells redifferentiate.[153] γ-Glutamyl transpeptidase (GGT), an enzyme which can catalyze GSH oxidation, appears to decrease in developing systems and to be lowest in differentiated cells.[154] Elevation of GGT activity has been observed in dedifferentiated and premalignant cells.[155,156] Once differentiated, cells contain a constant low level of GGT activity.

The antioxidant function of GSH as well as its role in the maintenance of cellular ion balance could potentiate many of the effects observed at different times during development. Antioxidants such as dihydrobenzoic acid and brief periods of anoxia retard or completely inhibit the development of Drosophila.[157] High concentrations of antioxidants[158] and changes in cellular ion balance[159] have also been implicated as factors leading to dedifferentiation and cancer in mammals. Free radicals, particularly O_2 have been reported to greatly affect membrane permeability to ions by increasing the level of lipid peroxidation and the oxidation of -SH groups in membrane ATPases.[160]

Changes in nuclear concentration of ions, such as K^+, Na^+ and Mg^{++}, have been found to cause chromosomal puffing in insects.[161,162] Variations in GSH concentration can greatly affect the ion distribution in cells.[163] GSH is also a modulator of cell redox state.[164] A large change in intracellular GSH concentration may thus affect the distribution of charges in the cell and will markedly affect the ratio of reducing to oxidizing equivalents.

GSH concentrations decrease about 80% during differentiation in Physarum (Fig. 5).[146,165-167] Furthermore, the rate at which GSH concentration decreases appears to inversely correspond to the rate of differentiation.[165] Increased free radical production during differentiation may result in GSH oxidation and extrusion from cells,

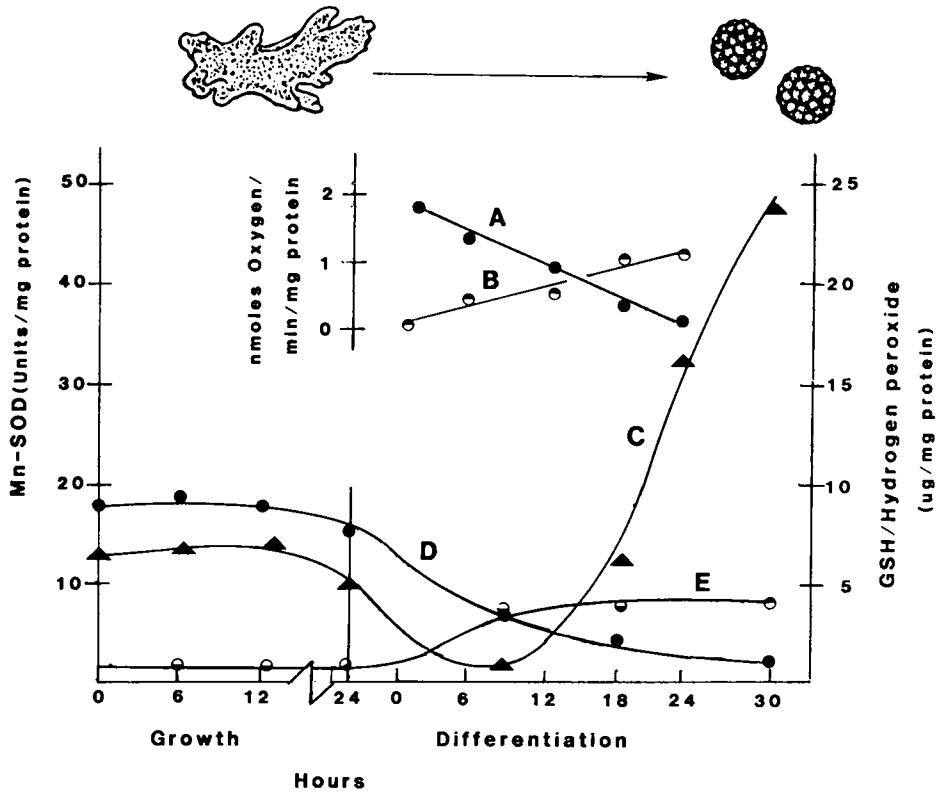

Fig 5. Parameters of oxygen metabolism in Physarum polycephalum during the cell cycle (growth) and during differentiation into spherules (induced by starvation). Oxygen consumption was measured in plasmodial homogenates in the presence of 1.3mM KCN in pH 7.1 buffered glucose (B), and in salts medium without KCN at pH 3.8 (A). Superoxide dismutase activity (C), and concentrations of hydrogen peroxide (E) and glutathione (D) were measured during the growth of plasmodia from the second (MII) through the third (MIII) post-fusion mitosis and during starvation-induced differentiation of microplasmodia grown in shake flasks. (Adapted from 146, 167).

albeit, GSH is also known to bind with proteins. Binding of GSH to proteins, which can affect enzyme activities, may also account for part of the decrease in GSH concentration observed during differentiation.[168] Treatment of Physarum with chemical agents that increase or decrease GSH concentration results in a corresponding decrease or increase in the rate of differentiation.[165] Interestingly, the free radical-generating herbicide, paraquat was found to accelerate differentiation in Physarum.[166] Treatment of a non-differentiating strain of Physarum with paraquat and buthionine sulfoximine (to decrease GSH level) induced the formation of immature differentiated structures.[166]

In housefly larvae, GSH concentration is high during development but decreases dramatically during metamorphosis.[169] GSH concentration is relatively high immediately following metamorphosis. It decreases during the next week and then sharply increaes around the ninth day of adult life. Thereafter, it declines steadily until death.[91,169] The cause of GSH decline observed in adult insects appears to be due to the loss of γ-glutamylcysteine synthetase activity, and thus results from decreased synthesis rather than increased oxidation.[170] The age-related decline in GSH may also result from a decrease in glutathione reductase activity since the level of GSSG has been observed to increase in aging houseflies.[91]

Cyanide-resistant respiration in the housefly remains relatively constant during larval stages (approximately 11% of total respiration). The cyanide-resistant respiration is twice as great in pupae as in larvae and five times greater in pupae than in adults.[167] Superoxide dismutase (SOD) activity is low in larvae and increases very markedly during metamorphosis.[144] Increased SOD activity and cyanide-resistant respiration would seem to indicate that changes in the rate of free radical generation occur during metamorphosis. A large increase in free radical generation in pupae would account for increased SOD activity and decreased GSH concentration during this period.

We have also observed a 2-fold increase in Mn-SOD activity and a one-third decrease in GSH concentration in differentiating mammalian myoblasts.[171] Interestingly, free radical-generators such as X-radiation and benzo(a) pyrene induce cytodifferentiation, i.e. adipogenesis, in embryonic mouse cells.[172]

On the basis of the above-cited evidence, we hypothesize that differentiation is associated with high SOD activity and low GSH concentration.

The influence of metabolic rate on life span, and the changes which occur in the free radical defenses during differentiation and dedifferentiation, raise the possibility that a dynamic equilibrium exists between prooxidants, antioxidants and cellular charge distribution, which acts as a set point for the regulation of gene expression. We hypothesize that differentiation, in part, results from the establishment of this equilibrium and senescence is due to a shift in the equilibrium in favor of prooxidants. An important function of the antioxidants is to maintain cellular redox state and ion balance, both of which affect chromatin configuration and gene expression.[11,163]

Non-histone proteins are believed to play an important role in the regulation of gene expression[118] and must migrate from the cytosol into the nucleus before chromosomal puffing can occur. It is well documented that nonhistone proteins are extremely sensitive to surrounding charges and ion balance, and that they undergo age-related alterations in overall charge.[120-122] It would seem possible that changes in cellular redox state and ion balance, which appear to occur during differentiation, may affect the migration of non-histone proteins into the nucleus as well as the binding properties of these proteins to chromatin. In aging individuals, alterations in the cellular redox state and ion balance may initiate events which ultimately lead to decreased regulatory control and dysdifferentiation. According to our model, antioxidant defenses modulate nuclear-cytoplasmic interaction. Aging is due to attrition of this type of regulation and cancer is due to the loss of the optimal equilibrium between antioxidants and prooxidants. High concentrations of prooxidants would be associated with differentiation, whereas, low concentrations of prooxidants or high levels of nonenzymic antioxidants are related to mitotic activity. Only with further study can the validity of these hypotheses be assessed.

CONCLUSIONS AND SUMMARY

We have reviewed the evidence suggesting the involvement of metabolic rate and oxygen metabolites in processes of aging and development. There is now little doubt that the rate of aging and metabolic rate of organisms are inversely correlated in poikilotherms as well as homeotherms. In the former, a variety of experimental studies have demonstrated that regimes which lower metabolic rate extend life span and retard the rate of age-related physiological and biochemical changes. In homeotherms, apparently, there are three categories of metabolic potential (i.e. total energy

consumed during life). Within each category, basal metabolic rate is inversely correlated with species-specific longevity.

In the current belief, the biochemical effects of metabolic rate are mediated by the active oxygen species, generated as a result of univalent reduction of oxygen. Experimental increase in physical activity has been shown to stimulate the production of free radicals and their subsequent reaction products.

Efforts to demonstrate, unambiguously, a direct causal relationship between oxygen radicals and the aging process have so far been unsuccessful because steady state concentrations of free radicals are very low and the aging process is extremely slow. Furthermore, it is also difficult to rule out the causal involvement of other biological factors in aging especially when virtually every physiological function exhibits an age-related alteration. It is our suggestion that a more fruitful experimental approach would be to focus on the predictions of the free radical theory of aging.

Antioxidant administration does not seem to prolong longevity in many cases probably because organisms exert a homeostatic control over their endogenous antioxidant levels. Administration of exogenous antioxidants tends to depress levels of endogenous antioxidants.

Age-related increase in exhalation of alkanes *in vivo*, which are products of free radical induced lipid peroxidation, suggests that free radical-induced damage tends to increase with age. There is some evidence that this enhanced vulnerability is due to both an age-dependent decline in antioxidant defenses as well as increased production of oxygen free radicals. The metabolic potential (which is a measure of aging rate) of mammals appears to be directly correlated with the efficiency of antioxidant defenses in relation to per unit metabolic rate and inversely related to *in vitro* auto-oxidizability of tissues.

We postulate that aging is due to the loss of a balance between prooxidants and antioxidants in cells, which is necessary for the maintenance of differentiated state of cells.

We have discussed some intriguing experimental evidence that oxygen free radicals may also play an inductive role in developmental events. Intracellular environment becomes less reducing during differentiation. High levels of SOD activity and low levels of GSH are in general associated with differentiation while the reverse is true during dedifferentiation. It is postulated that changes in cellular redox state may be responsible for altered gene expression during differentiation and aging.

The main implication of the arguments presented in this review is that oxygen metabolites play a causal role in the induction of cellular diferentiation and senescence.

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ABBREVIATIONS

DDC, diethyldithiocarbamate; DPN, diphosphopyridine nucleotide; LEP, life span energy potential; LSP, life span potential; SFM, soluble fluorescent material; SOD, superoxide dismutase; TBA, thiobarituric acid