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# Role of Free Radicals in Mutation, Cancer, Aging, and the Maintenance of Life

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Mutations—and hence evolution—cancer and aging may be in part caused by side effects of some of the free radicals produced in the course of normal metabolism (*1, 2*). This possibility, suggestive of chemical means of modifying these phenomena in a beneficial manner, arose from a consideration of these spontaneous processes in the light of present-day free radical chemistry and radiobiology. The purpose of this paper is to detail as much as is conveniently possible the data currently available supporting this hypothesis, to indicate how free radicals may produce these effects, and, lastly, to suggest how free radicals may be involved in the maintenance of life.

Irradiation of living things by ionizing radiation can result in mutation, cancer, and aging (*3, 4*). Inasmuch as these effects also arise spontaneously in nature, it is natural to inquire if the processes might not be similar. One means by which radiation is believed to produce its biological effects, and probably the major one, is through induced dissociation of water with the resultant formation of the chemically active free radicals, $HO\cdot$ and $HO_2\cdot$ (*5*). These same free radicals might be expected to be produced during the course of the major energy-gaining reactions employed by living things—the reduction of molecular oxygen to water, and the conversion of light energy to chemical energy. Also, since reactions involving free radicals are in general not "clean-cut" (i.e., not specific), it would be anticipated that some of these expected free radicals would produce side effects through more or less random reaction with cellular components. That free radicals are actually present in living systems has been demonstrated by electron spin resonance (ESR) absorption studies (*6–8*). Further, it has been shown that the concentration of free radicals increases with increasing metabolic activity in conformity with the postulation set forth earlier that free radicals are involved in biologic oxidation-reduction reactions (*9–10*). Are some of these free radicals the expected $HO\cdot$ and $HO_2\cdot$ radicals and/or radicals of a similar high order of chemical reactivity, and where might they arise in the cell? A number of lines of evidence taken together

leave little, if any, doubt that free radicals of the nature of HO· are actually present in living things and in amounts sufficient to simulate the effects of radiation.

In the first place, the reaction of molecular oxygen with organic compounds in the presence of oxidation catalysts such as iron salts has been known for many years (*11–13*); these reactions are believed to occur by a free radical path involving the HO· radical (*10*). Hence, by analogy, the reduction of oxygen in an animal cell catalyzed by respiratory enzymes, particularly those containing iron, might also proceed with the intermediate formation of HO·. By analogy also, the conversion of hydrogen peroxide to $O_2$ and $H_2O$ by catalase might be expected to parallel to some extent the same over-all decomposition catalyzed by iron salts in which HO· and $HO_2$· are involved (*14*).

In the case of plants, studies of chlorophyll-sensitized photoöxidation and of the Hill reaction are highly suggestive that free radicals such as HO· can arise in them in the course of the conversion of light to chemical energy (*15*).

Further evidence that HO· and $HO_2$· are produced in the animal cell comes from the study of vitamin E-deficient animals. The mitochondria and microsomes of such animals contain lipid peroxidation products (*16, 17*). Formation of these compounds would be expected to proceed by a free radical path involving HO·, and in addition their subsequent breakdown also should produce active free radicals including −HO·, RO·, and $RO_2$·, where R is an organic group. These peroxidation products are formed in potentially biologically significant amounts, for calculation shows that decomposition of the lipid peroxide in the liver of rabbits, formed during a 4- to 5-week period on a vitamin E-deficient diet, would yield 0.26 $\mu$M of free radicals per gram—about fifty times the amount produced by 800 rads (*16*). Cells with a normal amount of the natural fat antioxidant, vitamin E, would also be expected to produce lipid peroxidation products but at a slower rate owing to a shorter chain length of the free radical oxidation reaction.

Oxygen toxicity studies are likewise consistent with the hypothesis that free radical reactions involving HO· and $HO_2$· are present in the normal cell (*18*); the toxic effects appear to arise when the oxygen concentration is sufficient to overcome the natural "antioxidant defenses" of the cell.

Studies of simpler systems also suggest that the radicals HO· and $HO_2$·, and RO· and $RO_2$·, are produced. For example, investigation of the action of xanthine oxidase, with either sulfite oxidation (*19*) or luminal (*20*) used to detect free radicals, indicate that HO· and/or $HO_2$· are formed. The organic free radicals, detected by ESR arising in several dehydrogenase systems (*7*), in the action of peroxidase and $H_2O_2$ on a number of substrates (*21*), and in illuminated chloroplast preparations (*22*), would be expected to react, to a greater or lesser degree, depending on the availability of oxygen and the resonance stability of the free radical, with oxygen with the formation of radicals such as $RO_2$· and $HO_2$·. In the case of the interaction of succinate with aerated particles derived from the sarcosomes of pig heart

muscle, the concentration of unpaired electrons was $10^{-8}$ mole per gram wet weight —presumably due to the presence of organic free radicals (*23*).

The foregoing, taken as a whole, strongly indicates that chemically active free radicals of the nature of $HO\cdot$ and $HO_2\cdot$ are produced in living things in the course of normal metabolism. Hence it is possible that a part, or all, of the processes leading to the production of spontaneous mutation, cancer, and aging are essentially the same as those induced by radiation, the initiating $HO\cdot$ and $HO_2\cdot$ radicals arising on the one hand in the metabolic processes, and on the other by the dissociation of water. From this point of view spontaneous mutations, cancer, and aging can be looked on as a result of continuous "internal radiation," whereas these same processes produced by external radiation are largely the result of an increment in the amount of total "radiation" to which the organism is exposed.

The hypothesis that endogenously produced free radicals are involved in mutation, cancer, and aging leads to the following suggestion concerning the maintenance of life in general: The continued survival of living things in a changing environment is largely a result of the selection by them of reactions that not only serve as major sources of energy but also, through the side effects of some of the free radicals produced in the course of these reactions, provide on the one hand for mutation—and hence of evolution and survival—and on the other, and by essentially the same mechanism, for cancer and aging. The latter two phenomena can be considered to play a useful role, and possibly a necessary one, in the evolutionary process by aiding in the disposal of old organisms after they have provided new "experiments" for evaluation against the environment.

The effects produced by endogenously formed free radicals would not be expected to be identical in all respects to those resulting from similar radicals arising by irradiation because of differences in concentration and distribution of radicals, and of local availability and concentration of substances capable of inhibiting their effects. Thus, radiation-induced free radicals are concentrated along paths randomly distributed throughout the entire cell, whereas those of endogenous origin would be expected for the most part to arise in and be concentrated around relatively localized areas such as the mitochondria. Hence, because the mean free paths of radicals of the nature of $HO\cdot$ are short, endogenous radicals might be anticipated to have a lesser chance to reach and to react with material in some areas of the cell, for example the DNA of the nucleus, than the same number of radicals of radiation origin; some of the latter might arise in the immediate vicinity of the DNA and be able to react with it even in the presence of high concentrations of antioxidants. If we consider DNA further, it is likely that the pattern of chemical attack would be different in the two instances; a radiation track near a DNA molecule could result in a number of radicals reacting with a localized segment, whereas endogenous radicals would be expected to react with DNA in a more diffuse manner. These considerations would also be expected to account for the longer period of time generally required to produce a given effect by radicals of metabolic origin

than by those formed by irradiation even though the total number of the former—as suggested by studies of vitamin E-deficient animals (*16*) and ESR investigations (*7, 23*)—might be considerably greater. Further, these considerations should also be involved in the explanation of the poor correlation between radiosensitivity and life span of living things; it is also necessary to take into account additional variables including differences in temperature, in ploidy, in oxygen concentration, and in the amount and kind of antioxidants present (for example, the intracellular concentration of amino acids, *24,* in insects is high relative to that of mammals) and of substances capable of catalyzing the homolytic scission of peroxides into free radicals.

The manner in which free radicals such as $HO\cdot$ and $HO_2\cdot$ produce their effects on cells is obscure. In general, however, they would be expected to react for the most part near where they are formed—particularly in the case of $HO\cdot$ because of its high reactivity. Since most oxygen utilization occurs in the mitochondria, it would be anticipated that these radicals could initiate oxidation-polymerization reactions involving the unsaturated mitochondrial lipids—it seems likely that this may be the origin of the pigments formed in vitamin E deficiency (*16*) and of the so-called "age pigments" (*25*); possibly the mitochondrial changes observed with increasing age, both structurally (*26*) and biochemically (*27, 28*), are caused by this. Further, they would be expected to react most readily with the more easily oxidized compounds such as DPNH and those containing the mercaptan group (SH). They would also be expected to react to a certain extent with other cellular constituents including the nucleic acids; owing to the long biological half-life of deoxyribonucleic acid (DNA) in the intermitotic phase, plus the fact that changes may be transmitted, attacks on DNA would be expected to be particularly important in aging. The organic radicals formed in this manner (by removal of a hydrogen atom) could then undergo further reactions—e.g., addition of oxygen leading to the formation of peroxides and other oxygenated compounds, degradation into smaller units, dimerization—much as has been observed in simpler free radical and polymer systems (*29, 30*).

Although active free radicals should react for the most part near where they are formed, their effects at a distance could be enhanced by diffusion of less-reactive hydroperoxides (ROOH) and peroxides (ROOR′) whose formation they may initiate—for example, hydroperoxides of amino acids and peptides (*31*). Subsequent decomposition of these compounds would result in liberation of $RO\cdot$ and $HO\cdot$ radicals at some distance (for example, in the nucleus) from the site of formation of the initially formed free radical.

If we apply the foregoing general concepts of the mode of action of active free radicals to mutation, cancer, and aging, it would seem that mutation, since it is due to DNA changes, could be initiated by free radicals through attack on the enzymes or precursors involved in the synthesis of DNA and/or chemical alteration of

existing DNA. An example of the latter might be the formation of hydroperoxides (and of their degradation products) of DNA as has been observed in simpler systems (*32*). Similarly, it is likely that cancer, at least in some cases, may arise through alteration of DNA as a result of the action of endogenous free radicals.

DNA changes (some, or all, of which may be initiated by free radicals) may also be involved in the aging process. This is suggested by a number of observations, including the following: (1) The life span of the offspring of irradiated male mice may be shortened almost as much as that of their fathers (*33*). (2) The effect of 120 r of whole-body irradiation on life span shortening in rats is the same whether the radiation is given all at once or in divided doses of 20 r (*34*). (3) Immature developing animals are more sensitive to radiation damage than are adult animals (*34*). (4) The life span of various species is roughly inversely proportional to the germ line mutation rate of the species (*35*). And (5) a genetically determined relationship between parental and filial life expectancy has been demonstrated from family data as well as from twin investigations (*36*). In keeping with the foregoing observations which suggest that DNA changes are involved in aging are two recent successful mathematical formations of the aging process based on the premise that DNA changes accumulate with time (*37, 38*).

Alterations occurring in the DNA of cells of a multicellular organism would be expected to impair eventually their functional ability and, collectively, the harmonious interrelationships necessary for the life of the cells as a whole. Thus, changes in the properties of newly formed connective tissue with the age of an organism (*39*) may reflect mutations accumulating with time in the stem cells of the fibroblasts. Since all the cells of the body are dependent on the connective tissue, changes in it with time, due to mutations in cells primarily responsible for its maintenance, may have a deleterious effect on the organism. Likewise, in the case of long-lived cells such as those of the skeletal muscle or of the nervous system of man, accumulated changes in the DNA would be expected to gradually decrease their functional ability—is this the major reason for the senile heart in the first case, and of senility in the latter?

In addition to the postulated changes in the DNA occurring with time, due to action of free radicals, other free radical-induced changes in the cell may also contribute to the decreased functional ability of the cell (*40–42*); the decreased oxidative phosphorylation observed in the liver mitochondria of vitamin E-deficient animals (*16*) suggests that the similar effects noted in aged animals (*27*) may also be due to mitochondrial oxidative changes.

In a multicellular organism the process of aging could be going on at different rates in different tissues and organs so that death of the organism could occur because of impaired function of one part—for example, the heart—even though the other cells of the organism were capable of functioning satisfactorily.

In addition to changes in the cells themselves, it would be expected that the

connective tissues of the organism would also slowly undergo deleterious changes with time, in part secondary to oxidation reactions involving molecular oxygen sensitized by metals, such as copper, iron, or cobalt salts, or metalloörganic enzymes containing such elements. These effects would be expected to be most pronounced in those components having a long biological half-life, such as collagen and elastin; recent studies on elastin are in keeping with this expectation (*43*).

Non-free radical changes can be postulated to account for the aging process (*44, 45*) but the effects produced by radiation, coupled with the foregoing discussion, indicate that it is more likely that the major processes producing aging are initiated by free radicals.

The foregoing concept of the free radical origin (at least in part) of mutation, cancer, and aging arose from a study of the aging process. This process has been the source of considerable speculation (*44–49*), prompted in part by man's desire to prolong his effective life span.

If aging does involve attack on the organism by free radicals as suggested above, it would be anticipated that an increase in the concentration in the organism of compounds, such as mercaptans, capable of reacting rapidly with free radicals might decrease the rate of attack on constituents such as DNA and thus lead to a decrease in the rate of aging and therefore a prolongation of effective life. Thus far, two experiments based on this possibility have been carried out. The first experiment (*50*) employed AKR (male) and C3H (female) mice. These mice were used because their life span is short. Although for the most part they die of lymphatic leukemia (AKR) or mammary carcinoma (C3H), it was believed that they should be suitable for testing the postulated influence of free radicals on aging, since, if the theory was correct, the average age at which the neoplastic process appeared might be increased. Mice were obtained shortly after weaning, divided into groups of about 30 (10 per cage), and then fed daily (ad libitum) a powdered diet to which was added nothing, or the hydrochloride of an antioxidant. The mice were weighed and counted every month.

AKR mice on cysteine hydrochloride (1.0% w) (w = by weight), 2-mercaptoethylamine hydrochloride (1.0% w), or 2,2′-diaminodiethyl disulfide dihydrochloride (0.5% w) had a half-survival time (age at which 50% of the animals were dead) of 10.5, 10.5, and 10.6 months, respectively, whereas that of the controls was 7.6 months—a prolongation of about 35% ($p < 0.01$). The influence of these compounds is not through an effect on the leukemia which the AKR's develop, as shown by the fact that blood smears taken at 12 and 13 months were all normal except one, and in this case the mouse was moribund. Further, the weight gains of the control and treated mice were essentially the same. Ascorbic acid (2% w) and 2-mercaptoethanol (0.5% w) were also studied; neither was effective in prolonging life. None of the five compounds evaluated had any certain effect in the C3H mice.

The second experiment (*51*) utilized Swiss male mice in addition to the AKR (males) and C3H (female) strains. Pelleted food—to which either nothing or the hydrochloride of one of four antioxidants was added before pelleting—was used instead of powdered in hope of achieving longer elevated tissue levels of the additives. Fresh batches of food were made up at intervals of one to three months.

Cysteine hydrochloride (1% w) and hydroxylamine hydrochloride (2% w) increased the half-survival time of the AKR mice from the control figure of 9.6 months to 11.0 and 11.2 months, respectively—a prolongation of about 15%. 2-Mercaptoethylamine hydrochloride (1% w) did not increase the life span—for some reason the members of this group of AKR mice had a relatively high mortality in the early months of the experiment. 2,2′-Diaminodiethyl disulfide was apparently toxic at the level employed, 1% w, for this as well as for the other two strains of mice.

2-Mercaptoethylamine hydrochloride (1% w) increased the half-survival time of C3H mice from a control value of 14.5 months to 18.3 months—an increase of 26% ($p < 0.01$). There was some indication (decreased mortality rate) during the early months of the experiment in the earlier work that 2-mercaptoethylamine might have a beneficial effect on the life span; the marked effect in the second experiment may be in part due to the difference in the mode of feeding. Hydroxylamine hydrochloride (1% w) produced a slight prolongation of the half-survival time; from 14.5 months to 15.5 months—a prolongation of about 7%. In this, as in the first experiment, cysteine hydrochloride (1% w) did not prolong life.

None of the antioxidants studied had a beneficial effect on the life span of the Swiss mice; there is no apparent explanation for the difference between the Swiss mice and the other two strains.

These two experiments are quite encouraging and justify further exploratory studies of antioxidants as antiaging agents; several additional antioxidants, shown to protect mice against multiple sublethal doses of X-irradiation (*52*), are now under study in the AKR, C3H, and Swiss mice. In addition, the magnitude of the life-span prolonging effect, particularly that produced by 2-mercaptoethylamine hydrochloride, suggests that endogenously produced free radicals do play a major role in the aging process.

The foregoing results are in agreement with a human study (*53*) indicating that older persons with low blood levels of vitamin A and ascorbic acid (both easily oxidized compounds) have a higher mortality rate than do persons with greater amounts of these substances in the blood.

The concept that free radicals are involved in the aging process was applied to the fact that the rate of aging, as measured by the log of the mortality rate, increases with advancing age. Part of this effect could be due to a decrease in the level of antioxidants, for example, mercaptans, in the body with increasing age. A study of the serum mercaptan concentration as a function of age was in agree-

ment with this possibility (*54*). The serum concentration of mercaptan groups of normal males decreased with age from a level about 55 $\mu$M per 100 ml of serum at age 20 to 40 to about 40 $\mu$M per 100 ml at age 80. The data for women are of the same magnitude and show the same trend. The serum concentration of another antioxidant, ascorbic acid, likewise has been reported to decrease with age in males (*55*).

Thus, on both theoretical and experimental grounds there is firm support for the hypothesis that endogenously produced free radicals play a prominent role in mutation, cancer, and aging.

## SUMMARY

Mutation, cancer, and aging are attributed basically to the side effects of endogenously formed free radicals. Evidence is presented for the formation of these radicals along with considerations of how they might produce their effects as well as possible differences in the mode of action of endogenous and radiation-induced radicals.

Experimental work on the aging process based on the above hypothesis has been encouraging: Several antioxidants were found to increase the half-survival time of AKR and C3H mice; the prolongation with 2-mercaptoethylamine was around 20%. Serum mercaptan levels, as predicted by the free radical hypothesis, were found to decrease with age.

This free radical hypothesis raises the possibility that the continued survival of living things in a changing environment is fundamentally a result of the selection by them of reactions that not only serve as major sources of energy but also, through the intermediate formation of active free radicals, provide, on the one hand, for mutation—and hence of evolution and survival—and, on the other, and by essentially the same mechanism, for cancer and aging. The latter two phenomena can be considered to play a useful role, and possibly a necessary one, in the evolutionary process by aiding in the disposal of old organisms after they have provided new "experiments" for evaluation against the environment.

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