MITOCHONDRIA, OXIDATIVE DNA DAMAGE, AND AGING

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ABSTRACT

Protection from reactive oxygen species (ROS) and from mitochondrial oxidative damage is well known to be necessary to longevity. The relevance of mitochondrial DNA (mtDNA) to aging is suggested by the fact that the two most commonly measured forms of mtDNA damage, deletions and the oxidatively induced lesion 8-oxo-dG, increase with age. The rate of increase is species-specific and correlates with maximum lifespan.

It is less clear that failure or inadequacies in the protection from reactive oxygen species (ROS) and from mitochondrial oxidative damage are sufficient to explain senescence. DNA containing 8-oxo-dG is repaired by mitochondria, and the high ratio of mitochondrial to nuclear levels of 8-oxo-dG previously reported are now suspected to be due to methodological difficulties. Furthermore, MnSOD +/- mice incur higher than wild type levels of oxidative damage, but do not display an aging phenotype. Together, these findings suggest that oxidative damage to mitochondria is lower than previously thought, and that higher levels can be tolerated without physiological consequence.

A great deal of work remains before it will be known whether mitochondrial oxidative damage is a "clock" which controls the rate of aging. The increased level of 8-oxo-dG seen with age in isolated mitochondria needs explanation. It could be that a subset of cells lose the ability to protect or repair mitochondria, resulting in their incurring disproportionate levels of damage. Such an uneven distribution could exceed the reserve capacity of these cells and have serious physiological consequences. Measurements of damage need to focus more on distribution, both within tissues and within cells. In addition, study must be given to the incidence and repair of other DNA lesions, and to the possibility that repair varies from species to species, tissue to tissue, and young to old.

INTRODUCTION

It has been more than 40 years since the Free Radical Theory of Aging was first put forward by Denham Harman (1). In a seminal paper, he proposed that free radicals would be produced in the utilization of molecular oxygen by animal cells, and that as a consequence of free

radical reactions with nucleic acids and other cellular components, the animal would develop mutations and cancer. He also suggested that damage by endogenous free radicals was the fundamental cause of aging. A second theory, proposed three years afterward by Leo Szilard, postulated specifically that time-dependent changes in somatic DNA, rather than other cellular constituents, were the primary cause of senescence (2). Both authors based their theories in large part on the belief, common at the time, that radiation accelerated aging independently of its effects on carcinogenesis. In addition, the discoveries that ionizing radiation produces the hydroxyl radical (3) and that radicals are produced in biological systems (4) had both recently been made.

Unless they are removed by cellular defense systems, reactive oxygen species (ROS) and free radicals may react with cellular components including proteins, lipids, and DNA. While all such damage is likely to have harmful effects, modifications to DNA are particularly threatening to the organism because they may lead to permanent and heritable changes via the formation of mutations and other types of genomic instability. Reaction of DNA with ROS causes many potentially mutagenic or lethal lesions including strand breaks, abasic sites, and oxidized bases. At least two of the most common DNA lesions, 8-oxo-7-hydro-2'-deoxyguanosine (8-oxo-dG) and 5'-hydroxy-2'-deoxycytidine (5-OH-dC), are mutagenic (5, 6). There are many defense systems to prevent damage from leading to cancer, however, and whether endogenous ROS are actually of biological consequence in cancer formation is still being questioned (7, 8).

A brief discussion is perhaps warranted to clarify the commonly used terms "ROS" (reactive oxygen species) and "free radical." Chemically, a free radical is an atom or molecule that exists independently (is free) and which has an unpaired electron. Many, but not all, ROS are also free radicals. The converse is also true: not all radicals are "ROS" since many do not contain an oxygen atom. Although this review will focus on the harmful effects of ROS and other radicals, it is worth mentioning that not all ROS and radicals that are produced in biological systems are accidents of nature: many, in fact, are necessary for life. Superoxide is released by phagocytes as part of the immune response (9), for example, and nitric oxide is of major importance in many signal transduction pathways (10). (The Nobel Prize was recently awarded to Robert F. Furchgott, Louis J. Ignarro

and Ferid Murad for their discovery that nitric oxide acts as a messenger to control blood vessel dilation.) Another free radical, ubisemiquinone, plays an integral role in mitochondrial electron transport. Even hydrogen peroxide has been reported to play a biological role, serving as a second messenger in adipocytes (11-14). Thus, while protection from unwanted ROS and radicals is desirable, if a "magic pill" were developed that would eliminate them completely from the body, it would be a deadly poison.

For simplicity the term "ROS" will be used in place of "ROS and free radicals" throughout the remainder of this review, since based on what is currently known concerning mitochondrial DNA damage, they are the most relevant players.

It now seems likely that if ROS lead to aging, it is by a more complex mechanism than the simple induction of somatic mutations. For example, survivors of radiation exposure from the World War II atomic blasts in Japan developed high levels of somatic mutations and cancer, but mortality due to causes other than cancer and phenotypic aging were not accelerated (15). Nevertheless, modified versions of the theory have gathered a great deal of support. These predict specific targets or chronic exposure as the keys to the relevance of endogenous ROS, as opposed to the "shotgun blast" induced by radiation. It remains an open question as to whether resistance to damage by endogenous ROS is sufficient to explain why one species lives longer than another. However, it has been conclusively demonstrated that protection from such damage is at least necessary to a long lifespan. For an excellent review, the reader is referred to (16).

The Free Radical Theory of Aging was modified in 1972 by its creator to suggest that mitochondria might be the "clock" that determines lifespan (17). The early evidence for the role of mitochondria in aging was exhaustively reviewed in 1980 (18). It was noted that cells are resistant to large amounts of ROS generated by radiation induced radiolysis of water, and suggested that only specific targets within the cell would be critical to damage by ROS. Based on discoveries showing that mtDNA is replicated at the mitochondrial membrane (19), in close proximity to the electron transport chain, they concluded that this DNA was likely to be the most critical target. It was postulated that the resulting loss of mitochondrial function would be an early event in a cascade that would lead, ultimately, to aging.

MtDNA has several unique properties that make this hypothesis attractive (20). First, it is sequestered in organelles which, due to their nature, also tend to sequester any positively charged, membrane permeant species. If a drug or chemical having these properties is introduced into the cell, it will tend to accumulate in the mitochondria. If the drug is reactive with DNA, the mtDNA is then a convenient target. Second, as noted above, mtDNA is closely associated with the mitochondrial electron transport chain. This is one of the most significant sites of oxygen radical production in the cell.

In addition, the activities of enzymes in the electron transport chain may also lead to the activation of reactive mutagens while they are in close proximity to the mtDNA. Third, mtDNA is not protected by the nucleosomes found in nuclear DNA. Fourth, replication of mtDNA occurs at the inner mitochondrial membrane (19) and only in the perinuclear mitochondria (21). Approximately one half of the H strand is completed prior to initiation of L strand replication. As a result, mtDNA exists partly in a single stranded conformation, suggesting that it might be more vulnerable than nuclear DNA to attack. Fifth, the negative supercoiling of the genome also makes it sensitive to damage. Finally, the presence of damage or mutations in mtDNA could lead to an altered function in the electron transport chain. In a vicious circle, decreased function in the electron transport chain may lead to an increase in oxygen radical production (18, 22).

As mentioned earlier, one of the most well studied lesions to result from interactions between DNA and oxygen radicals is 8-oxo-dG. The addition of an OH group at the eighth position in guanine by reducing agents in the presence of oxygen was first described by Kasai and Nishimura in 1983 (23). These workers investigated the induction of this lesion by various agents, including X-irradiation (24-27). At that time, they noted that X-irradiation could not induce the lesion in solutions containing ethanol, and concluded that the reaction involved the hydroxyl radical.

The use of Electrochemical Detection (ECD) in conjunction with high performance liquid chromatography (HPLC) to detect the oxidatively induced DNA lesion 8-oxo-dG was pioneered by Floyd in 1986 (28). ECD of 8-oxo-dG is approximately one thousand fold more sensitive than optical methods of detection, and it was the first technique to allow detection and quantification of an oxidatively modified DNA base at physiologically relevant levels. Since that time, hundreds of studies have been dedicated to this lesion, and more has been learned about it than about any of the other oxidative DNA lesions.

8-oxo-dG is a mutagenic lesion, not a blocking lesion: that is, it does not stop polymerases during transcription and replication. 8-oxo-dG will often adopt the syn conformation (29, 30) and mispair with adenine during DNA replication and transcription, as shown in Figure 1. Most DNA polymerases fail to recognize this mismatch, and so 8-oxo-dG in the template strand results in a G:A mismatch and eventually, if the mismatch is not correctly repaired, in G→T transversions (31). The mitochondrial polymerase gamma is no exception (32). 8-oxo-dG is not the most mutagenic lesion, based on the mutation spectrum resulting from oxidative stress, which favors G→A transitions (33, 34). However, its biological relevance is demonstrated by the fact that enzymes for its repair and to prevent its incorporation into DNA are found in organisms ranging from *E. coli* (35) to human (36).

It is worth noting that although mutations are frequently the result of damage, the degree to which they

Figure 1: Base Pairing of 8-oxo-dG

This cartoon shows a cross-section of the DNA strand, with the sugar backbone represented by dark circles. While the DNA lesion 8-oxo-dG is capable of forming a normal base pair with cytosine (left panel), it also is prone to “flip” into the syn conformation, which allows it to form an incorrect basepair with adenine, leading ultimately to a mutation if left unrepaired.

occur and the biological consequences which they might have will for most part not be discussed in this review. Furthermore, DNA damage can have direct consequences (for example, nuclear DNA damage influences cell cycle and apoptosis). This is also a topic that is beyond the scope of this review. Instead, the focus of the current work will be primarily on the types and levels of DNA alterations that are present in mitochondria and on the means by which the cells remove them.

Methods Used for the Measurement of Oxidative DNA Damage

The methods commonly used for the measurement of oxidative damage to DNA can be divided into two groups: those that measure damage, and those that measure undamaged DNA. If damage is distributed randomly, the distinction is not important. Since the *in vivo* distribution has lately been shown *not* to be random (37), however, it is necessary to understand the assumptions that are made when each type of measurement is used. The consequences of each assumption are illustrated graphically in Figure 2.

The three most frequently used methods for the direct measurement of damage (as depicted on the left-hand side of Figure 2) are high performance liquid chromatography with electrochemical detection (HPLC/ECD 38), micro-HPLC coupled with mass spectrometry (micro-HPLC/MS 39), and gas chromatography with mass spectrometry (GC/MS 40). As shown in Figure 3, each of these assays begins with hydrolysis of the DNA, followed by chromatographic separation of the resulting nucleosides (or bases, depending on the method of hydrolysis).

Most methods that measure undamaged DNA (as shown on the right hand side of Figure 2) rely on the removal of damage by repair enzymes, followed by any of several methods for quantitation. Two of these methods are alkaline elution, which is based on the fact that shorter DNA fragments elute more rapidly from a filter than long ones (the alkalinity both denatures the DNA and breaks alkaline sensitive sites), and measurement of MW shifts in agarose gels (41). Damage to circular DNA such as the mitochondrial genome can be detected

using repair enzymes followed by observation of changes in form (supercoiled to circular, etc.) (42, 43). A fourth method, used since 1985 to measure damage for the purposes of repair but only recently to measure endogenous damage, is the enzymatic/Southern blot method, more commonly referred to as the gene specific repair assay (44, 45). As illustrated in Figure 4, a repair enzyme is used as a tool to generate single strand nicks at sites of damage in restricted DNA. The DNA is then run on a denaturing gel, where the cleaved strands move forward, away from strands that contained no damage. The DNA is transferred to a nylon membrane and probed for genes or sequences of interest. The loss of damaged DNA from the main band allows quantitation of the undamaged DNA. Finally, PCR based methods amplify a sequence that does not contain lesions or strand breaks (46, 47). These methods are often combined with the use of repair enzymes to create strand breaks at the site of damage, which removes the damaged fragments from the amplified pool (48-50). In addition, primer extension and Ligation-Mediated PCR (LMPCR) can both be used to map the site of damage and (to some extent) to quantitate the relative abundance of damage at several sites (51, 52).

There are other methods for measuring damage which have potential applications to studies of mtDNA, including ^{32}P postlabeling, Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS), and immunoassays. Each has advocates and critics, but a full description and analysis of each of these methods would be beyond the scope of this review. Worthy of special mention, however, is the development of a recombinant Fab which recognizes 8-oxo-dG. It was shown that DNA damaged by H_2O_2 and ionizing radiation contained antigenic sites, and such sites co-localized with nuclear and mitochondrial DNA. In addition, the antigenic sites were removed when the cells were treated with the bacterial repair enzyme Fpg, which strongly indicates that the antigen is, indeed, 8-oxo-dG (53).

No discussion of methods for the measurement of oxidative DNA damage would be complete without consideration of the tremendous controversy which surrounds the true level of oxidative damage in DNA. This

Figure 2: The Effect Of A Non-Random Distribution Of Damage On The Measured Levels Of A Lesion

A cartoon to illustrate the effect of a non-random distribution of damage on quantitation methods such as HPLC/ECD or GC/MS that depend on detection of damage (left) or on methods such as the enzymatic/Southern blot assay which depend on detection of undamaged DNA (right).

Note that neither method alone describes the true situation very well, but that together (8 damaged bases, 3/4 undamaged fragments), the two methods reveal the non-random distribution.

is due primarily to the observation that the levels measured by various methods do not agree with one another (reviewed in 54, 55). This problem is so severe that a consortium known as the European Standards Committee for Oxidative DNA Damage (ESCODD) was established in 1997 to try to resolve the methodological difficulties. Most recently, they have published a comparison of the ability of 21 different laboratories to determine the level of damage in a series of standards. (Many failed.) Based on this, they have listed minimal criteria that must be met before accurate results can be obtained. These include having a coefficient of variation of less than 10% for identical samples, and less than 5% for duplicate samples prepared following a single protocol and measured multiple times to obtain a "best value." In addition, differences between a series of standards containing increasing amounts of damage of physiologically relevant levels should be recognized (56). The fact that the amount of damage induced by photoactive dyes such as methylene blue and light is linearly dependent on the light exposure makes such standards simple to prepare (57, 58).

Measurements of Oxidative DNA Damage in MtDNA

The measurement of damage in mtDNA faces an additional challenge, above those faced in the measurement of nuclear DNA damage. In most methods, the mitochondria must first be isolated from the cells, and DNA

then isolated from this purified and concentrated suspension. Clearly, this procedure itself had the potential for inducing oxidative damage, but no alternative was available. In 1988, HPLC/ECD was applied for the first time to mtDNA. Using mitochondria isolated from rat liver, it was found that the DNA contained 117, 8-oxo-dG per 10^6 dN, in comparison to only 7.2, 8-oxo-dG per 10^6 dN in nuclear DNA from the same animals (59). This finding added a tremendous amount of support to the mitochondrial theory of aging, and has at the time of this writing been cited nearly 500 times in various studies and reviews.

There is a tremendous variability in the values reported for 8-oxo-dG in mtDNA, as might be expected given the difficulties in measuring oxidative DNA damage that have already been discussed. They range from a low of 0.08, 8-oxo-dG per 10^6 dN in HeLa cells (60) to an astonishing high of 4840 per 10^6 dN in heart tissue taken from a 100 week old rat (61). If that amount of damage were randomly distributed, each mitochondrial genome would carry an average of 160 lesions. If not randomly distributed, then a subset of the genomes would be even more oxidized. At the (perhaps unlikely) extreme, this same number would be obtained if one out of every 46 genomes was so damaged that each and every dG had been converted to 8-oxo-dG. The two distributions would be expected to have very different physiological consequences.

Figure 3: HPLC/ECD, micro-HPLC/MS, And GC/MS: Methods Flowcharts

The major steps involved for the three most frequently used methods in the direct analysis of oxidatively induced DNA damage.

Even within a single system, reported values cover a wide range. Levels reported for mtDNA isolated from rat liver range from 4 per 10⁶ bases (43) to 110 per 10⁶ bases (61) (reviewed in 62, 63).

It is noteworthy that the largest change with age is found within a single method, and indeed, by a single laboratory: using HPLC/MS, values for mtDNA are reported to increase 250 fold with age, from 5.7, 8-oxo-dG per 10⁶ dN for human cardiac tissue from 30 year olds to 1430, 8-oxo-dG per 10⁶ dN for cardiac tissue from 90 year olds (64). One problem in interpreting such results is that very few tissues have been examined by more than one laboratory. Thus, it is difficult to say with certainty whether the observed 250 fold increase seen with age in human heart is due to the fact that it is an unusual tissue, or to some interaction with the HPLC/MS methodology. Although the former possibility is made more likely by a report from the same laboratory that age related increases are not seen in rat liver (61), a 250 fold increase is truly remarkable. As already noted, discrepancies due to methodology are of major concern in the measurement of 8-oxo-dG. For those tissues which

have been examined by more than one laboratory, there is disagreement over both absolute levels and changes with age. MtDNA from porcine liver has been variously reported to contain 2, 8-oxo-dG per 10⁶ dN (43) or 682, 8-oxo-dG per 10⁶ dN (65). In our own laboratory, using mtDNA from rat liver mitochondria, values from young rats were found to be as low as 5, 8-oxo-dG per 10⁶ dN using HPLC/ECD or as high as 135, 8-oxo-dG per 10⁶ dN using GC/MS (57, 66, 58). There are clearly methodological problems remaining to be solved, and the clearest demonstration that solutions have been found will be direct methods comparisons.

Our laboratory attempted to circumvent some of the difficulties in measuring 8-oxo-dG by using the HPLC-Coularray system, which allows identification of the measured species not only by retention time but also by ionization potential, and also by creating standards by damaging DNA with photoactivated methylene blue to ascertain the sensitivity of the system. The levels of damage detected in the standards were linear over a wide range, including the lower levels which were comparable to those observed in liver DNA. There was no

Figure 4: The Fpg/Southern Blot Assay

Top panel: the principle behind the Fpg/Southern Blot Assay. Fpg is used to generate a single strand nick at the site of damage in a restricted DNA fragment. In a denaturing gel, the cleaved strand moves forward, away from the main band. After blotting and probing, this loss of damaged DNA from the main band allows quantitation of the undamaged DNA. The number of incisions is calculated using the Poisson distribution: Incisions = $-\ln(\text{Fpg treated/untreated})$. Bottom Panel: A sample blot. From left: Control -Fpg, Control + Fpg, Damaged -Fpg, Damaged +Fpg.

change in the levels of 8-oxo-dG levels in the nuclear DNA from 6-month (young) and 23-month-old (senescent) rat liver DNA. However, at 6 months, the level of 8-oxo-dG in mtDNA was 5-fold higher than nuclear and increased to approximately 12-fold higher by 23 months of age (57). This is in agreement with most other studies which have been published. In almost every case in which both nuclear and mitochondrial values were presented, mtDNA was more damaged than nuclear DNA and the ratio increased with age. That the increase with age is real is also suggested by two studies which also found that age-related increases in the level of mitochondrial 8-oxo-dG were lower in calorically restricted animals (67, 68). Other markers of oxidative damage were also reduced (69). The effect of caloric restriction is important since caloric intake is the only known factor to directly alter the rate of aging (70).

To be applied, most assays of 8-oxo-dG levels require prior isolation of the mitochondria. The Fpg/Southern blot method does not, however. This fact was exploited to compare damage in isolated vs. not-isolated mitochondria (58). It was found that nuclear and mitochondrial levels of oxidative damage differed in young animals only after the mitochondria have been separately isolated from the tissue in question. This has implications for studies using isolated mitochondria to compare young with old animals: is damage in vivo higher in older animals, or is susceptibility to damage during mitochondrial isolation higher in older animals? The same ques-

tion arises for any studies which have compared one group with another using isolated mitochondria.

The question of oxidative damage in mtDNA, or in nuclear DNA, has not been conclusively answered and cannot be until the methodological problems have been solved. Once methods are developed which allow different laboratories to independently obtain the same number of lesions when given the same sample, questions regarding the endogenous levels will need to be revisited. The reader with serious interest in the measurement of oxidative damage to DNA is once again urged to follow the work of the European Standards Committee on Oxidative DNA Damage (ESCODD) (56).

Relationship Between Deletions and Oxidative Damage

Perhaps the most well known mutation to occur in mtDNA is the "common" deletion, occurring in humans between nucleotide positions 8470 and 13459 of the mitochondrial sequence. First discovered in patients suffering from mitochondrial myopathies, this deletion was later found to occur in all adult humans and to increase exponentially with age (71, 72). The tissues that exhibit the highest levels of the mtDNA deletions are usually post-replicative and tend to have the highest metabolic rate (73-77). For example, increases in the level of the common deletion with age have been reported in most brain regions and the regions showing the greatest increases are those involved in dopamine metabolism: the caudate (78), putamen (79, 78), striata (80) and substantia nigra (78). Dopamine is a neurotransmitter that in some circumstances reacts to produce ROS (81). The association of high levels of the common deletion and a potentially high concentration of ROS suggests that ROS may be involved in the genesis of the deletion.

It has been proposed that poorly respiring mitochondria containing damaged DNA will be inefficiently degraded, an effect termed the "Survival of the Slowest" (82). This hypothesis was built around the suggestion that normal mitochondria incur more oxidative damage than defective mitochondria (82), based on the notion that without electron flow through the electron transport chain there will be no leakage of electrons from the chain. An objection has been raised, however, to the idea that oxidative damage is the signal for mitochondrial degradation (83). It seems likely that mitochondrial defenses are ordinarily sufficient to prevent oxidative damage. However, the essence of the model – that slower, poorly functioning mitochondria are subject to less turnover than active, normally functioning ones – is not affected by this criticism. In fact, the model would easily accommodate the reverse causality. It could be that defective mitochondria have higher levels of oxidative damage, and that it is excessive levels of oxidative damage that prevent recognition by the normal "turn-over" machinery.

The complementary suggestion has also been made that in addition to being inefficiently degraded, damaged mitochondria would also be poorly replicated due to an

energy shortage and diminished proton gradient. As a result, damaged mitochondria in a population of dividing cells would be rapidly diluted away (84). Together, these two attractive hypotheses would explain the finding that non-dividing cells such as neurons are more likely than rapidly dividing cells to contain damaged mitochondria.

The deletion accumulates exponentially with age even in species with drastically different lifespans, e.g., mice and men: thus, in absolute terms, it occurs much faster in mice, and presumably with fewer mitochondrial divisions for amplification of the event (85). Almost all studies quantifying the number of mitochondrial genomes within a tissue which contain the common deletion have found that the incidence remains well under 1%. (It has been reported to reach 12% in the putamen (79).) Before the remaining genomes are assumed to be full length and normal, however, it should be considered that the common deletion is merely one of many that occur in the mtDNA (86-94), and tandem duplications in the noncoding region have also been detected in some human tissues including muscle (95, 96) testis and skin (96). It is possible that the impact of any single change is low, but cumulatively, rearrangements and deletions may alter mitochondrial function.

When only a few cells are used for the PCR reaction, the amount of the deletion detected is a function of the number of cells used, showing that the deletions are not evenly distributed among the cells (97). In situ hybridization is also useful to address this question. In extraocular muscles from elderly humans, the distribution of fibers with mitochondrial dysfunction (as determined by cytochrome c oxidase activity) was compared with the distribution of the common deletion. While fibers bearing the deletion did show dysfunction, many dysfunctional fibers lacked the deletion (98), and the authors concluded that mitochondrial dysfunction was poorly explained by mtDNA deletions. Whether this conclusion is correct is arguable, since as is discussed above, other deletions and rearrangements are possible. Noteworthy, however, was the demonstration that the common deletion was distributed in a mosaic pattern at the cellular level, so that cells either carried deleted DNA, or did not. A similar effect is seen in progressive external ophthalmoplegia, a disease which is characterized by the formation of a variety of mtDNA deletions throughout the patient's lifetime. These deletions were found to vary from cell to cell, showing clonal expansion (99). Yet another disease state with possible implications for the interpretation of data concerning the common deletion in aging is Hashimoto's thyroiditis. In this disease, the activity of cytochrome-c oxidase is lost in a scattered subset of cells. The common deletion was detected only in the affected areas (100). In mitochondrial myopathies, there is a mosaic distribution to the deletion even within a single muscle fiber, with clusters of defective mitochondria present along the length of the fiber (101). Similar distributions have been seen in muscle from aging rhesus monkeys (102). Thus, several lines of

evidence suggest that the deletion occurs in a mosaic pattern, which could influence its physiological relevance.

Preliminary data in our laboratory suggest that oxidative damage to mtDNA in older animals may, like deletions, be distributed non-randomly. Age-related increases are seen in isolated mtDNA using HPLC/ECD, but are not observed when the Fpg/Southern blot method is used (Anson, unpublished observation). Since the two methods show remarkable agreement when DNA that has been randomly damaged in vitro or when mtDNA from the isolated mitochondria of young animals, is used as a substrate (58), the most likely explanation for the discrepancy in measuring damage in samples from older animals is that the damage is not randomly distributed. (The explanation for this reasoning is shown in Figure 2.) A more recent study from the Richter laboratory has demonstrated that even in mtDNA preparations from young animals, the lesion is not randomly distributed. It is confined for the most part to fragmented mtDNA (37).

Several studies have looked at both oxidative damage and mtDNA deletions, based on the hypothesis that if the two increase as a function of age at the same rate, they are causally connected. 8-oxo-dG and deletion levels both increased as a function of age in human diaphragm (103), and human heart (104, 64). Accumulation of mtDNA deletions and of malondialdehyde (a marker of oxidative damage to lipids), and increases in MnSOD in human liver mitochondria were found to occur together as a function of age (105). Lipid peroxidation correlates with deletion formation and level in various human tissues (106). In cortex, age related increases in 8-oxo-dG correlate with the rise in the common deletion (107) but only the deletion increases in Alzheimer's Disease, suggesting that if the effect is causal, it is not a direct relationship. This is also suggested by findings in mouse heart, where mtDNA deletions were found to accumulate with age, while 8-oxo-dG in mtDNA peaked in middle aged animals (108). This does not rule out a role for oxidative damage *per se*, since there is no reason to believe that oxidized bases such as 8-oxo-dG would lead *directly* to a deletion: the connection would probably be through errors occurring during repair, or through other, concurrent forms of oxidative damage.

A more direct connection between oxidative damage and mitochondrial deletions has been examined in other studies. Copper is well known to induce oxidative damage to DNA (109, 110, 49), and accumulation of copper in the mitochondria, a condition known as Wilson's Disease, leads to a high level of mtDNA deletions (111). Perhaps the strongest evidence comes from a study which used x-rays to induce the deletion in cultured cells. That the level of induction varied with cell type, and that the deletion was induced at all, are both of great interest (112).

A prediction of one version of the mitochondrial theory of aging is that mitochondrial dysfunction will lead to increased radical formation and, thus, to still more

dysfunction (18, 22). It has also been independently suggested that the deletions are an effect, rather than a cause, of mitochondrial dysfunction (113), due to their apparent low abundance. However, neither prediction was supported by the finding that the presence of pre-existing mitochondrial defects (genetic disorders) did not predict the formation the common deletion (114). This is not a trivial question, and the issue may not be closed. However, the finding that levels of 8-oxo-dG are low in young animals does support the suggestion that the lesions themselves are not the initiating event in a damage cascade (58). It seems more likely that any age-related increase that occurs is due to a failure elsewhere in the system, such as in the antioxidant defense system, the repair system, or perhaps in the inherent peroxidizability of the membranes due to alterations in lipid composition.

Mechanisms For Mitochondrial DNA Maintenance

The steady state level of 8-oxo-dG, or of any lesion within a cell, is controlled by the rate of formation and by the rate of removal. The cell protects itself in two ways: protective enzymes and chemicals are used to decrease the rate of damage formation, and repair and replacement pathways exist to remove any damage that occurs (115).

It has been known since the discovery in 1969 of superoxide dismutase (SOD) (116) that mechanisms have evolved to minimize the amount of damage incurred due to ROS. SOD is the enzyme responsible for dismutating superoxide to hydrogen peroxide, the first step in its detoxification. Recently, new evidence for the critical importance of these mechanisms has begun to emerge. Mice lacking the cytosolic form of the enzyme, CuZnSOD, show a phenotype only when stressed (117, 118). However, two different strains of mice lacking the mitochondrial enzyme, manganese SOD, fail to survive more than a few weeks (119). The mitochondrial form thus seems more important. The more viable of the two strains develops neuropathology before dying. The less viable dies of cardiomyopathy before neuropathology can develop. Intriguingly, if these mice are given MnTBAP, an SOD mimetic, they are protected from the cardiomyopathy and hepatic degeneration they would normally experience, but since MnTBAP does not cross the blood brain barrier, it does not protect the brain. As a result, the pups develop severe neuropathologies (120).

Recently, yet another level of protection from damage was discovered: an alteration in succinate dehydrogenase cytochrome b which results in decreased electron transport through complex II leads to extreme hypersensitivity to oxygen in *C. elegans* (121). This supports an earlier finding by Guidot et al (122) showing that mitochondria play a role not only in the production of ROS, but in their ultimate disposal.

Removal of Damage from MtDNA (and Removal of Damaged MtDNA)

The original finding that pyrimidine dimers are not repaired in mtDNA (123) is often cited without a discussion of the other results presented in the same study. Not only were the lesions not excised, they were not repaired by photoreactivation (which differs from *X. laevis*, in which UV damage to mitochondrial DNA is repaired by a photolyase (ref. 19 in 123, 124)), and there was good evidence that the genomes containing the lesions were neither replicated nor degraded. This implies that in mammalian mitochondria, genomes containing pyrimidine dimers are simply diluted away as the mitochondria themselves are replicated and replaced (123). A later study using cell lines which selectively incorporate bromodeoxyuridine (BrdU) into mtDNA when the BrdU is included in the culture medium (125), revealed a second pathway available to cells in dealing with mtDNA damage. Exposure of BrdU-containing DNA to near-UV light causes debromination of the BrdU and subsequent formation of strand breaks and protein:DNA crosslinks. This damage was not repaired in mammalian mtDNA, but also was not diluted away. Instead, the amount of damaged mtDNA that could be recovered from the cells declined sharply, so that within 8 hours of damage, the majority of the mtDNA is missing. This is not accompanied by a decreased yield of mitochondria themselves, suggesting that the DNA itself was degraded (125). Thus, depending on the type of damage, damaged mtDNA may be removed by dilution or degradation. Which factors determine the pathway that will be used in response to mtDNA damage, however, have not been established. A very recent study examined mutations induced by UV light in mtDNA. Cells that lack nuclear repair of UV induced lesions show characteristic GC→AT mutations. Interestingly, this study confirmed the lack of repair or degradation for mitochondrial genomes containing UV induced damage, but found that roughly 13 fold more lesions were required per mutation induced than in the nuclear DNA of repair-defective cells (126). The simplest explanation would be if the mitochondrial polymerase gamma is less able to bypass lesions than nuclear polymerases.

In view of the high copy number of mtDNA, the early findings showing that damage could lead to dilution or degradation led many to regard the mitochondrial genome as disposable. It was assumed that mitochondria completely lacked the ability to repair DNA. Although it has been confirmed that mitochondria cannot repair pyrimidine dimers (127, 126), it is now known that DNA repair is indeed present in mitochondria, and that many other types of damage are repaired quite efficiently.

During the last decade there have been tremendous advances in the area of DNA repair. DNA repair measurements can now be done at the level of individual genes (128), individual DNA strands (129) and in individual nucleotides (130). The widely used terminology for these approaches is an analysis of the fine structure

of DNA repair. The developments in our understanding of DNA repair in nuclear DNA and the role of DNA repair in aging have been reviewed elsewhere (131-133). Several of these techniques have been applied to mitochondria, however, and have proved extremely informative.

Two of the earliest reports of DNA repair in mitochondria dealt with alkylation. Both O6-methyl-2'-deoxyguanosine (134) induced by N-nitrosodimethylamine and O6-ethyl-2'-deoxyguanosine induced by N-ethyl-N-nitrosourea (135) are repaired in hepatic mtDNA, and the kinetics of lesion removal from mtDNA were very similar to the kinetics of removal from nuclear DNA. However, the ability of mitochondria to repair O6-ethyl-2'-deoxyguanosine was tissue specific: it was removed from rat liver or kidney mtDNA, but little removal was observed from the DNA of brain mitochondria.

One of the most valuable tools in the study of DNA repair in mitochondria is the enzymatic/Southern blot assay, more commonly referred to as the gene specific repair assay (44, 45). (This assay was described in a previous section of this review: it is summarized in Figure 4.) This assay and its variations have been used to measure DNA repair of mtDNA in human and hamster cells without the need for mitochondrial isolation, and a variety of mtDNA repair activities have been observed. Alkali-labile sites induced by nitrosourea streptozotocin (136), N-methylpurines following exposure to methyl-nitrosourea (127), and N7 methylguanine adducts induced by treatment with methyl methanesulfonate (MMS) (137) are all lesions for which removal from the mitochondrial genome has been observed.

In contrast to the above lesions, mitochondria cannot repair complex alkylation damage generated by N-nitroso-N-butylurea (134), or by bis(2-chloroethylmethyl)amine (127), nor can they repair 6-4 photoproducts (126) or psoralen induced interstrand crosslinks (138), and there is only minimal repair of cisplatin intrastrand crosslinks (127). The latter finding is consistent with their inability to repair pyrimidine dimers. However, they can repair cisplatin interstrand crosslinks (127) and also repair adducts caused by treatment with 4-nitroquinoline-1-oxide (4NQO) (139), perhaps through a recently described recombinational pathway (140). Thus, while mitochondria seem to be proficient at base excision repair, with the exception of adducts caused by 4NQO, they seem unable to repair the type of bulky lesions which are repaired in the nucleus by nucleotide excision repair.

Despite intensive study of cellular responses to oxidative damage in general, very little was known until recently about the cellular response to oxidative damage in mtDNA (141). To study the removal of damage from DNA, it is necessary to induce high levels of, and then to monitor the subsequent removal of, the lesion of interest. Ideally, the levels should at least be ten-fold above background. As counter-intuitive as it seems at first, this is a difficult condition. It is not, of course, because the damage itself is difficult to induce. Rather,

it is due to complications that result from treatments which induce the desired level of damage. Often, the cells are either killed in great numbers, or other forms of damage prevent precise quantification of the lesion of interest.

The first successful study of the repair of 8-oxo-dG in mtDNA was published in 1993 (142). They used alloxan to generate between 96 and 106 lesions per 10^6 dN, of which 45 to 50 were likely to have been 8-oxo-dG based on resistance to short term exposure to mild alkali and sensitivity to the *E. coli* repair enzyme Fpg. They also demonstrated the repair of 31 pyrimidine lesions per 10^6 dN, based on sensitivity to the *E. coli* repair enzyme Endonuclease III.

Unfortunately, in later studies the same laboratory failed to induce base damage using the same agent. It seems possible that the spectrum of damage induced by alloxan is dependent on other variables. (The variability in damage caused by a single agent is a problem we have encountered in our own laboratory, with photosensitizers: this will be discussed in more detail below.) A slightly higher concentration of alloxan than the one used in the original study was used to test the response of a normal fetal lung fibroblast line (WI38) and of XP-A(20S), an SV-40 transformed cell line from a patient having xeroderma pigmentosum group A (XPA). 8-oxo-dG is resistant to mild alkali (143) but readily cleaved by Fpg, and alloxan induced only 6 Fpg sensitive sites per 10^6 dN in the WI38 fibroblasts, after subtraction of alkali sensitive sites (144). Worth noting, however, is that a dramatically effective mitochondrial repair of strand breaks or alkaline sensitive sites was observed in the normal fetal lung fibroblasts, but not in the transformed skin fibroblasts. (Whether the difference was due to the transformation, the tissue source, the XPA related defect, or merely to the differences between two individuals was not demonstrated.) Mitochondrial repair of strand breaks was also shown in a study using bleomycin as the damaging agent (145).

While removal of damage from a single-copy nuclear sequence can only be due to repair, there are a thousand copies of the 16 kb, circular mitochondrial genome per cell, and damage to any particular copy could be expected to be irrelevant. In fact, although the kinetics suggested that a component of the damage removal observed in these initial studies was due to repair, it remained an open question as to whether at least a portion of it might be due to selective degradation of damaged genomes. Two studies have addressed this issue. In one, WI38 cultured fetal lung fibroblasts were treated with photoactivated methylene blue (146). While photoactivated methylene blue does not induce high levels of damage in nuclear DNA, presumably due to the cell's ability to reduce it to its photo-insensitive, colorless form, it was found to be highly effective when used to induce oxidative damage in mtDNA. Following damage, the amount of full length mtDNA as a fraction of nuclear DNA was monitored for several hours thereafter. If in fact the removal were due to degradation of damaged mtDNA,

the mtDNA:nuclear DNA ratio would decrease. In fact, no decrease was seen in the ratio although damage decreased from an initial level of approximately 74, 8-oxo-dG per 10^6 dN, which is on average over 2 lesions per genome, to less than half of that value during the time period monitored. Thus, selective degradation of mtDNA did not occur. Replication of mtDNA was also ruled out as a variable in that study. The second study used alloxan to generate approximately 70 alkaline sensitive sites per 10^6 dN as well as approximately 16 enzyme sensitive sites per 10^6 dN. The mtDNA to nuclear DNA ratio remained constant throughout the repair period (50).

Even after the removal of 8-oxo-dG from mtDNA was demonstrated, several basic questions remained. The first was whether repair in the mitochondrial genome and in a nuclear sequence proceeded at the same rate. This was addressed using transformed Chinese hamster ovary fibroblasts, with photoactivated acridine orange as the damaging agent (147). 34 - 47 lesions per 10^6 dN were induced. Repair was essentially complete within 5 hours in both nuclear and mitochondrial sequences. The specificity of photoactivated acridine orange for the induction of 8-oxo-dG is quite good (148, 149), but the toxicity was rather high. This limited the number of lesions that could be induced and the cell types that could be used.

In our laboratory, attempts to use methylene blue to investigate changes in repair capacity with age have been confounded by the variability in the response of cells to methylene blue: treatment with methylene blue led to a subsequent loss of mtDNA even in cells that were not exposed to light in at least some skin fibroblasts and proved fatal to hepatocytes (unpublished results). This may have been due to the effect of methylene blue on cellular energetics.

The notions that steady-state levels of 8-oxo-dG are high in mtDNA, that mtDNA does not show the mutation pattern expected of the lesion (150), and that mitochondria can repair the lesion are difficult to reconcile.

One possibility would be that damage is induced at such a high rate that repair simply is not sufficient to maintain it at low levels. If that were true, however, then mutations and functional consequences would be expected, since 8-oxo-dG is a mutagenic, non-blocking lesion. Another possibility was that repair was in some way dependent on transcription or replication. Transcription-coupled repair is a well-known phenomenon in the nucleus. If a similar mechanism existed in the mitochondria and the lesion were only removed from genomes during transcription or replication, then functional consequences could be avoided even though steady-state levels could reach high levels. To address this possibility, the rate of repair was measured in both DNA strands of the frequently transcribed ribosomal region of the mitochondrial genome and in both strands of the non-ribosomal region. No difference in the rate of repair between strands or between two different regions of the genome that differ substantially with regard to

transcriptional activity was observed. Mitochondrial replication was prevented during the repair period, and so repair was able to proceed in the absence of replication and without any correlation with transcription (146). An independent and concurrent study also examined the strand specificity of repair for alkaline sensitive sites, and found no difference between the two strands (50).

The studies discussed so far in this section all used variations of the gene specific repair assay. However, two PCR based assays have also recently been used to examine mitochondrial repair of oxidative damage. A quantitative PCR (QPCR) assay, which depends on the ability of a lesion to prevent amplification of a fragment during PCR, was applied to the repair of mitochondrial damage in SV40 transformed fibroblasts following treatment with hydrogen peroxide (47). They found that treatment with 200 micromolar hydrogen peroxide for 15 min induced damage in mtDNA that was more extensive than damage in a nuclear sequence and was repaired rapidly, but that treatment for 1 hour, while not causing more damage, resulted in the selective loss of repair capability in the mitochondria. They found, furthermore, that the persistence of mtDNA damage correlated with WAF1/CIP1 induction, growth arrest, and apoptosis. Other studies also suggest that mitochondria may serve as a damage sensor. In HA-1 cells exposed to oxidative stress, mitochondrial RNA is selectively degraded - other cytoplasmic RNAs are not damaged (151). This suggests that the degradation may be due to a cellular response, rather than to direct RNA damage. A later study reported that mtDNA is also selectively degraded in response to hydrogen peroxide in that cell type (HA-1 cells) (152). This degradation coincided with elevation of growth arrest mRNAs and with growth arrest. Higher doses and longer exposures lead to apoptosis. The authors speculate that the degradation products act as intracellular signals. An independent study found that rho(0) cells (which lack mtDNA) are resistant to radiation, suggesting that mtDNA plays a role in radiation sensitivity and the induction of apoptosis (153).

Ligation Mediated PCR is yet another tool that is now available to examine damage and repair at the single nucleotide level (130). One study has examined the damage and repair of lesions at the nucleotide level in mitochondria using LMPCR. After treatment with alloxan, damage was highest at guanine, followed by thymine, cytosine, and with the least damage, adenine. (Damage frequencies were calculated on a per site basis: that is, damaged guanine was expressed per normal guanine, and so on.) Repair rates were consistent with those seen using the gene specific assay (50).

One major feature that has been reported for the repair of mitochondrial oxidative damage is inducibility. Several studies found that there was a small amount of endogenous damage present in cultured cells at the onset of the study. Cells that were treated with a damaging agent and allowed to repair the induced damage, also repaired the endogenous damage (142, 144). Studies in our own laboratory failed to confirm this, due to the

absence of endogenous damage observed in the mtDNA of control cells (147, 146). The reason for the discrepancy is not known. However, different methods for calculating lesion frequency were used in the two studies. In one, lesion frequency was calculated based on the ratio of damaged to control samples, whereas in the other, one of two identical aliquots was treated with a repair enzyme, and lesion frequency calculated based on the ratio between the two aliquots. The latter method factors out alkaline sensitive sites, leaving only enzyme sensitive sites in the calculation. Thus it may be that there are alkaline-sensitive sites, but not Fpg-sensitive-alkaline-insensitive sites, present in mtDNA of cultured cells, and that the repair of these sites is inducible. In line with this possibility, in response to asbestos at nontoxic concentrations, the induction of AP-endonuclease mRNA and protein as well as enzyme activity was shown in rat pleural mesothelial cells. (AP-endonuclease is involved in the repair of abasic sites, which are alkali-labile.) Confocal scanning laser microscopy showed that AP-endonuclease was primarily localized in the nucleus, but was also present in mitochondria (154).

There is a specific increase in 8-oxo-dG endonuclease activity with age in rat liver and heart. Age-related changes in mtDNA repair capacity were measured by assessing the cleavage activity of mitochondrial extracts towards an 8-oxo-dG-containing substrate. Enzymatic activity is higher in 12 and 23 month-old rats than in 6 month-old rats in both tissues, while other activities showed little change with age, or even a decrease in activity. This suggests an induction of the 8-oxo-dG-specific repair pathway with age (155). The possibility that repair in mitochondria is inducible is exciting, and deserves further attention in the future.

Mitochondrial Repair Proteins

Many of the proteins involved in mtDNA repair remain to be identified, but some have already been discovered. Uracil DNA glycosylase was one of the first repair activities detected in mitochondrial extracts (156). This activity, which excises uracil from DNA, was later named UDG1 when it was purified from human cells by affinity chromatography. It is a 30kd protein, encoded by the same gene which encodes the 36kd nuclear form of the protein (157, 158).

Several other repair enzymes have been detected in mammalian mitochondria, including a methyltransferase (134), an AP endonuclease (159), and an 8-oxo-dG glycosylase termed mtODE, for mitochondrial oxidative damage endonuclease (160). The latter enzyme excises 8-oxo-dG when it is paired with dC, but not when it is paired with dA. Unlike the bacterial repair enzyme Fpg, mtODE does not recognize 4,6-diamino-4-hydroxy-5-formamidopyrimidine. In addition to these enzymes, the mouse and human homologs of the yeast OGG1, which excises 8-oxo-dG from DNA, have been reported to contain mitochondrial localization sequences (161): whether or not OGG1 and mtODE are the same protein has not yet been determined. Three of the 4 isoforms of

hOGG1, the human homolog of the yeast OGG1, have been shown in immunohistochemical studies to be present in the mitochondria. It was shown in the same study that the human homolog of the *E. coli* nth gene product, hNTH1, which excises oxidized pyrimidines such as thymine glycol from DNA, is also present in mitochondria. So too is hMYH, which excises adenine from DNA when it occurs opposite 8-oxo-dG (162).

The mitochondrial DNA polymerase itself plays at least two roles in repair. First, its exonuclease activity is critical to its fidelity. A mutation generated in the 3'-5' exonuclease domain of the yeast mitochondrial DNA polymerase caused a 104 fold decrease in the 3'-5' exonuclease activity of the enzyme, and led to mitochondrial genomic instability. The frequency of mtDNA point mutations also rose, to a level 1500 times higher than in the wild-type strain (163). Second, once damaged bases have been excised, mitochondrial DNA polymerase is necessary for their replacement. An *in vitro* repair system for abasic sites using *X. laevis* mitochondrial extracts has been established. It consists of a mitochondrial apurinic/apyrimidinic (AP) endonuclease, a deoxyribosephosphodiesterase, and mitochondrial DNA polymerase gamma. It also contains a newly isolated mtDNA ligase, a 100-kDa enzyme which is suspected to be related to nuclear DNA ligase III (164).

At least in yeast, there appears to be active mismatch repair as well. Disruption of the *Saccharomyces cerevisiae* MSH1 caused mutagenesis and large-scale rearrangement of mtDNA (165). MSH1 is a yeast homolog of the *E. coli* Mut S mismatch repair protein.

Yet another repair activity may be present in mitochondria. Although the existence of homologous recombination in vertebrate mitochondria remains controversial (reviewed in 166), evidence continues to mount for its existence (167, 168). Mitochondrial protein extracts from mammalian cells have been reported to catalyze homologous recombination of plasmid DNA substrates. This activity required ATP, although non-hydrolyzable analogs could be used instead without complete loss of activity. Affinity-purified anti-recA antibodies inhibited the reaction, suggesting that activity is dependent on a mammalian mitochondrial homolog of the bacterial strand-transferase protein. It seems likely that this process is involved in mtDNA repair (140).

Finally, there are some lesions that are not repaired in mtDNA. One possibility that has recently come to light is that the degradation of damaged genomes may be mediated by the mitochondrial endonuclease G (endo G). Strand breaks induced by oxygen radicals greatly enhanced the susceptibility of the damaged mtDNA to nucleolytic attacks from endo G. The enzyme cleaved at or near sites where single-strand breaks were present in the opposite strand. Cisplatin-mediated intrastrand crosslinks also facilitated Endo G digestion (169).

There are several unanswered questions in terms of the mitochondrial response to DNA damage. One issue that hasn't been fully explored is the relationship between repair and turnover (degradation and replace-

ment) in the maintenance of mtDNA. In fetal lung fibroblasts, no degradation of mtDNA was observed following mtDNA damage with photoactivated methylene blue, and maintenance depended on repair (146). To what extent this is true in general is not known.

If damage varies from tissue to tissue and cell to cell, the question arises: does repair, also? At least for O6-ethyl-2'-deoxyguanosine, the answer is yes. This lesion was repaired efficiently in rat liver mitochondria, less so in rat kidney mitochondria, and not at all in brain mitochondria (135). If this phenomenon were found to be general, it would provide a possible explanation for the heterogeneity of mtDNA damage, and more importantly, suggest that it might be possible to discover ways to up-regulate repair in tissues or cells in which it is inefficient. Along the same line of thought, repair of thymine glycol in nuclear DNA was inducible by low dose radiation prior to a damaging dose (170). Is this true for repair in mtDNA?

Aging, Oxidative Damage, And MtDNA

The evidence discussed above makes it clear that mtDNA damage does, in fact, increase with age. The amount of damage that occurs is a function of species and tissue, and at least in some species is also affected by environmental variables such as caloric intake and activity levels. An age-related increase has been most clearly established for mitochondrial deletions, but there is some evidence that these may, in turn, be linked to oxidative stress. Although there is disagreement about absolute levels, most direct measurements of oxidative damage to the mtDNA do suggest that it, too, increases with age. However, much remains to be learned concerning distribution and physiological relevance, particularly at the cellular level. In addition, a great deal of study has been dedicated to a single oxidative DNA lesion: 8-oxo-dG. There are other lesions, any of which may have physiological consequences. And what of secondary reactions - protein/DNA and lipid/DNA crosslinks? Is mtDNA isolated from old animals more damaged, or more susceptible to damage because of changes in mitochondrial membrane lipids? If more damaged, why?

The discoveries concerning the repair of 8-oxo-dG in mtDNA make an accumulation of such damage rather mysterious. It's likely that there are a small number of genomes, contributing most of the 8-oxo-dG seen when average levels are measured. Even if so, the question remains: why? Is the rate of damage induction so severe that repair doesn't suffice? Or has repair been compromised in some way? Furthermore, if 8-oxo-dG is extremely high, how are G → T transversions avoided? Is replication selective for undamaged genomes? If so, does this occur at the level of the genome, at the level of the organelle, or at the level of the cell? Learning more about the distribution of damage will provide insight into this issue, also.

Even if there is an increase in oxidative damage with age, the question concerning its biological consequences

remains unanswered. Does such damage actually cause aging? Evidence that mitochondrial radical formation may be involved in aging comes from comparative studies between species of different maximum lifespan. It has for some time been a puzzle that species of approximately the same size would differ drastically in lifespan. For example, a pigeon lives approximately ten times as long as a rat, a hummingbird at least three times as long as a mouse. Recently, two groups have shown that mitochondrial ROS production is lower in the longer lived avian species (171, 172), and in addition, avian renal epithelial cells are extremely resistant to growth under 95% oxygen, and to treatment with hydrogen peroxide, paraquat, or gamma irradiation (173). Herrero and Barja (174, 175) reported recently that mtDNA oxidative damage is lower in several long lived species than in short lived species. The problems inherent in this measurement have been discussed above: nevertheless, the differences were found, and while the DNA oxidation reported may have reflected events that occurred during the isolation procedure, the fact remains that long lived species seemed to be more resistant to the effect.

While most of the studies described so far have been correlational, the actual functional relevance of oxidative damage at the mitochondrial level was evaluated in a recent study. It was found that oxidative damage accumulates in the mtDNA (and lipids and proteins) of mice heterozygous for the mitochondrial form of superoxide dismutase (MnSOD-/+), and that mitochondrial function was decreased by this damage (176). On the other hand, the mice themselves fail to show a visible phenotype even in old age and live as long as, or slightly longer than, wild type (T.T. Huang, personal communication). Taken together, these facts argue that oxidative damage in excess of that seen in wild type animals is not sufficient to increase the rate of aging, although they do not rule out the effect that greatly increased levels, unevenly distributed, might have.

SUMMARY AND CONCLUSIONS

A great deal of time has elapsed since the "Free Radical Theory of Aging" and the "Mitochondrial Theory of Aging" were proposed, and a great deal of information has accumulated which supports the contention that protection from ROS and mitochondrial damage are necessary to longevity. The two most commonly measured forms of damage to mtDNA, deletions and the oxidatively induced lesion 8-oxo-dG, have been found by many groups to increase with age. The rate of increase has been found to be species-specific and to correlate with maximum lifespan. However, recent discoveries have shown that DNA containing 8-oxo-dG is repaired by mitochondria. Repair occurs independently of replication and transcription, and so the high levels of 8-oxo-dG that have been frequently reported remain unexplained. To some extent, the high levels can be attributed to methodological difficulties, and it was re-

cently shown that in the livers of young rats, oxidative damage to full length mtDNA is no higher than in nuclear DNA. However, the increased levels seen with age in isolated mitochondria still need explanation. While a great deal has been learned, there are many challenges ahead. Measurements of damage need to focus more on distribution, both within tissues and within cells. In addition, study must be given to the incidence and repair of other DNA lesions, and to the possibility that repair varies from species to species, tissue to tissue, and young to old.

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