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BASE MODIFICATION IN ADULT ANIMAL LIVER DNA AND SIMILARITY TO RADIATION-INDUCED BASE MODIFICATION

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SUMMARY

Analysis of the hydrolysis products of DNA from young and adult animals showed an age-dependent increase of a modified base peak. The modified material was similar to that produced in calf thymus and normal bases irradiated with ^{60}Co gamma rays in the presence of oxygen. However, the estimation of the amount of modified product by ultraviolet absorption and radioactive measurements showed that the modified component has an unusally high ultraviolet absorption. Ultraviolet absorption spectra of the modified component from DNA and its bases are quite similar. These results suggest that oxidative modification of DNA bases in animal liver has occured, and support the proposed DNA oxidation hypothesis for aging and carcinogenesis.

Oxidative modification of nucleic acid bases by ionizing radiation (1-5), and by oxidizing agents such as nitrous acid, peracids and hydrogen peroxide (6-10) has been widely studied. The radicals produced by ionizing radiation can act as oxidizing agents that may modify biomolecules, including DNA, in vivo. The intermediates formed during progressive reduction of a molecule of oxygen are the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) (11). Living systems have defensive mechanisms to overcome the deleterious effects of these intermediates, such as superoxide dismutase for O_2^- (12), and catalase and peroxidase for H_2O_2 (13,14). According to Fridovich (15), oxygen toxicity is normally held check by a balance among the rates of formation and destruction of reactive forms of oxygen, which means that oxygen-dependent modification are improbable, but not impossible, in aerobic mammalian systems. The physiological oxygen tension has been shown to be mutagenic towards Salmonella typhimurium (16). Hyperbaric oxygen is also known to be mutagenic for E. coli (17,18). Karlson (19) has suggested that oxygen acts not only as an acceptor of electrons or of hydro-

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gen, but can also enter organic molecules directly by the catalytic action of oxygen-activating enzymes. Recently, Totter (20) has shown a possible relationship between spontaneous cancer and oxygen metabolism. This suggests that the reduction of oxygen in normal metabolism to O_2^-, and subsequent production of more harmful radicals (21), may also be a source of DNA modification. On the other hand, cells have various DNA repair systems (22-24). A decline in repair capability has been correlated with the life-span of mammals and the process of aging (25,26).

The present study was done to learn whether DNA bases modified by radicals are completely repaired or remain partially unrepaired and accumulate with age. Calf thymus DNA and normal bases were irradiated with ^{60}Co gamma rays and the results compared with those obtained for DNA from young and adult animals.

MATERIALS AND METHODS

The animals used were 3 and 28 week old mice (JCL-ICR, female) and 3 and 31 week old rats (JCL-ICR, female). Number of livers per treatment were: 40 and 20 for 3 and 28 week old mouse, and 20 and 5 for 3 and 31 week old rat, respectively. DNA was extracted from liver of five groups of mice and rats.

Isolation of nuclei from liver cells. Fresh wet liver was cut into small pieces and suspended in 2.3 M sucrose containing 3.3 mM $CaCl_2$ (1:1, w/v). The suspension was homogenized in a tissue grinder (K-885303, Kontes Glass CO., New Jersey) and filtered through five layers of gauze. The filtrate was then centrifuged at 23 000 rpm for 40 min at 4° in a Beckman L5-50 ultracentrifuge. The supernant was discarded and the clear jelly-like pellets of nuclei were washed with 2 ml of 0.34 M sucrose and recentrifuged at 23 000 rpm for 15 min at 4°. DNA was extracted from these cleaned pellets. The yield of nuclei was about 6% of fresh wet liver tissue.

Extraction and purification of DNA from nuclei. Marmur's method (27), up to the step of deproteinization with chloroform-isoamylalcohol, was used for the extraction of DNA. The following enzymatic treatements were done to remove RNA, glycogen and protein: 1) RNA was digested by incubating the DNA preparation with bovine pancreatic ribonuclease (20 μg/ml) and TI ribonuclease (10 units/ml) for 1 h at 37°, 2) glycogen was removed with α- and β- amylases (each 0.4 mg/ml) for 1 h at 37°, and 3) protein was removed by adding pronase (0.5 mg/ml) and incubating for 2 h at 37°. After the enzymatic treatments, sodium dodecyl sulphate (0.5%, in final) was added and the mixture stirred gently for 20 min at room temperature with equal volume of phenol-m-cresol-water (7:1:2, v/v/v) containing 0.1% 8-hydroxyquinoline. The mixture was centrifuged at 5000 rpm for 10 min and the upper aqueous layer containing DNA was transferred to a beaker. This phenol extraction step was repeated three times and the aqueous phase containing DNA was subjected to dialysis with 100 volumes of standard saline-citrate solution (SSC, 0.15 M NaCl-0.15 M Na citrate) at 4° for 3 days, renewing the SSC every day.

DNA was precipitated from the dialysate by layering 1 volume of isopropanol with gentle shaking. The thread-like white precipitate was dissolved in cold SSC, and DNA was reprecipitated by isopropanol. This purified DNA was washed with the

small volumes of 95% ethanol, 99.5% ethanol and ether, respectively, and finally dried in a desiccator.

Contaminations of RNA, protein and polysacchride in the purified DNA were measured by Orcinol (28), Biuret (28) and Anthrone reactions (29), respectively, and found to be less than 1% for RNA and protein. However, the DNA from adult animals contained a few per cent of glycogen compared to young (< 1%)

Gamma ray irradiation of DNA and its bases. Calf thymus DNA (Sigma Chemical CO.) dissolved in SSC was irradiated with ^{60}Co gamma rays (Shimadzu, 10000 S) at a dose rate of 10 000 rad/min at 0^{o}. It was then hydrolysed. Solutions were bubbled with either O_2 or N_2 for 15 min prior to irradiation. The SSC solutions of [^{14}C]bases (1 mM each) were also irradiated under similar conditions.

Hydrolysis and gel filtration. The glass tubes containing equal volumes of DNA solution and 12 N HCl was sealed in the presence of N_2 and hydrolysed by heating at 100^{o} for 3 h. The solution was dried by evaporation in vacuo, absorbing the HCl gas into KOH. The sample, after dissolving in 2 ml water, was applied onto a Sephadex G-10 column (1.2 x 80 cm) and the A_{260} of each fraction (2 ml) was measured. Gamma-irradiated base solutions were separated on a starch column (30,31).

RESULTS

Base modification in adult animal liver DNA. Sephadex gel-filtration patterns of mouse (3- and 28-week-old) and rat (3- and 31-week-old) liver DNA after acid hydrolysis are shown in Figs. 1 and 2. A very small fraction of modified bases was observed in the young mouse and rat liver DNA, which increased clearly in adult animal liver DNA. This modified peak accounted for about 10% and 20% of the total DNA from the liver of old mice (28 week) and rats (31week), respectively. This surprisingly high yield of modification led us to question whether it reflects true modification or is due to some unique properies of the modified component. Further experiments reported here suggest a high ultraviolet absorption by this component.

Base modification in gamma-irradiated calf thymus DNA. In order to see whether the modified material could be produced by irradiation, calf thymus DNA was irradiated in the presence of oxygen with ^{60}Co gamma rays. A peak, similar to the modified base peak observed in adult animal liver DNA, also appeared and increased with dose (Fig. 3). Formation of the modified material by gamma rays was much less in the presence of N_2 (data not given), suggesting a role of oxygen in production of the modified material. However, one could question the existence of

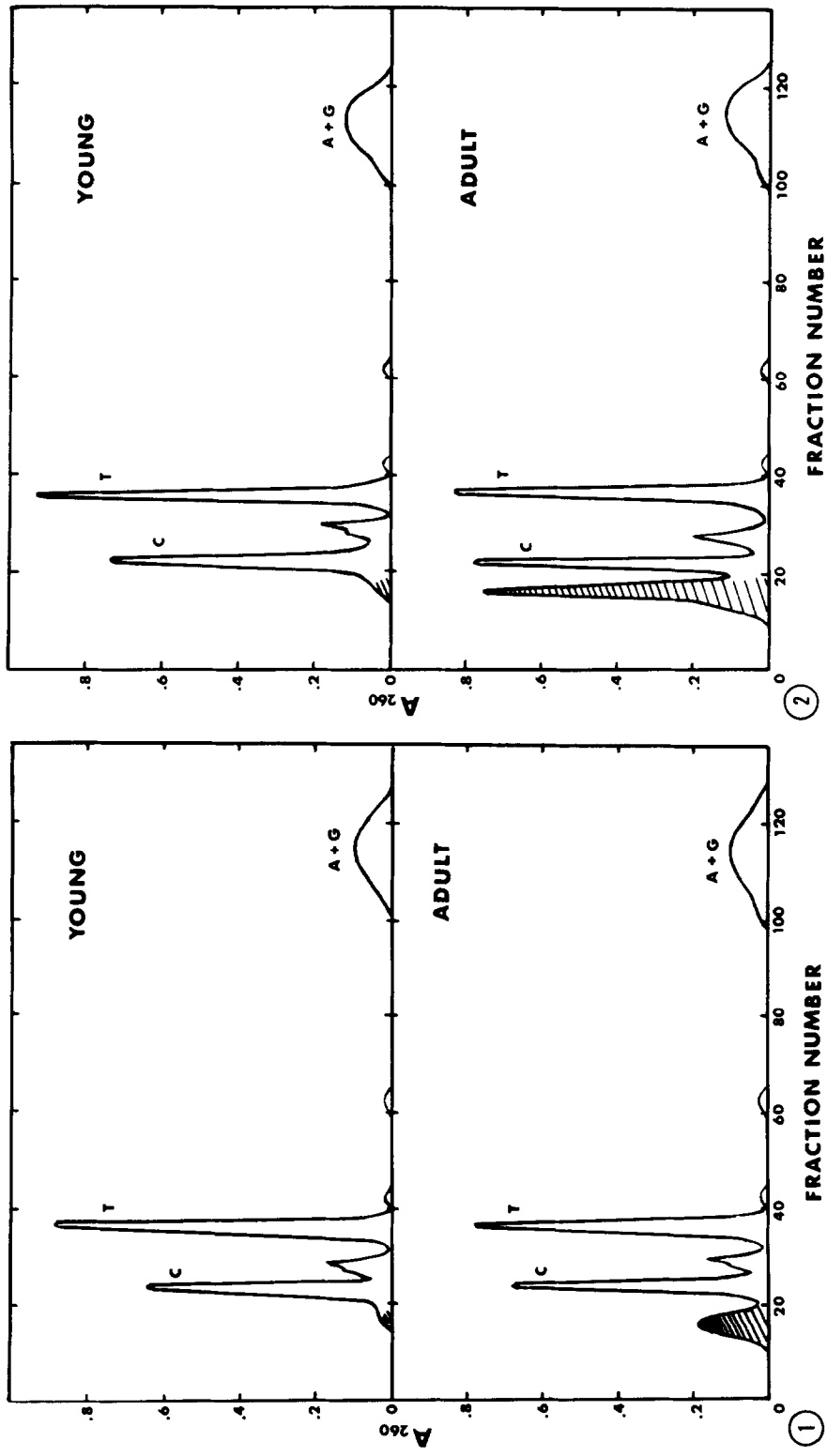

FIG. 1. Elution pattern of the bases obtained after acid hydrolysis of mouse liver DNA of 3 week and 28 week old on a Sephadex G-10 column (1.2 X 80 cm). The column was eluted with water and 2 ml fractions were collected. The shaded peak indicates modified bases.

FIG. 2. Elution pattern of the bases obtained after acid hydrolysis of rat liver DNA of 3 week and 31 week old on a Sephadex G-10 column. Otherwise as in Fig. 1.

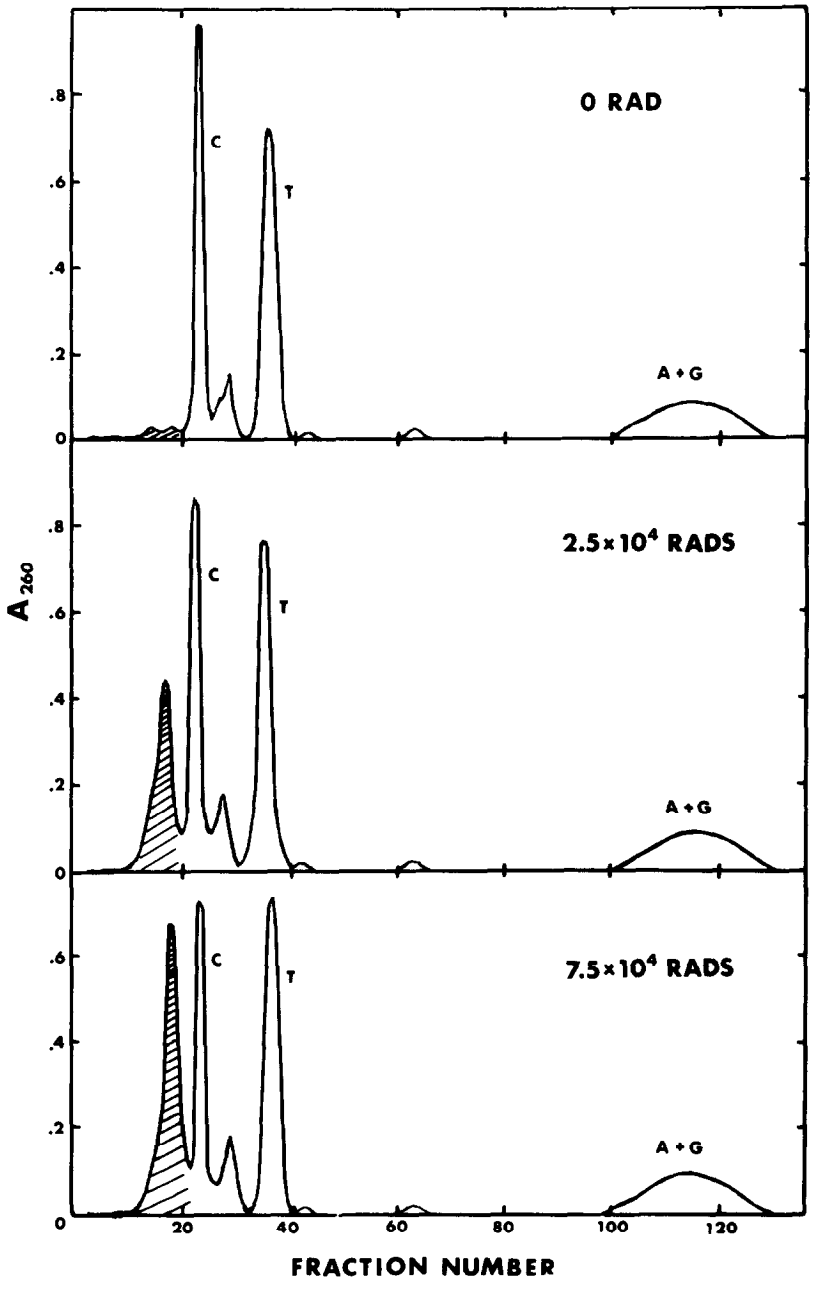

FIG. 3. Elution pattern of the bases obtained after acid hydrolysis of calf thymus DNA irradiated with gamma rays in O_2. Otherwise as in Fig. 1.

a single modified peak and could assume that there was more than one modified product, but we did not observe this under our experimental conditions. This might also be due to small amounts of other modified products and/or to their

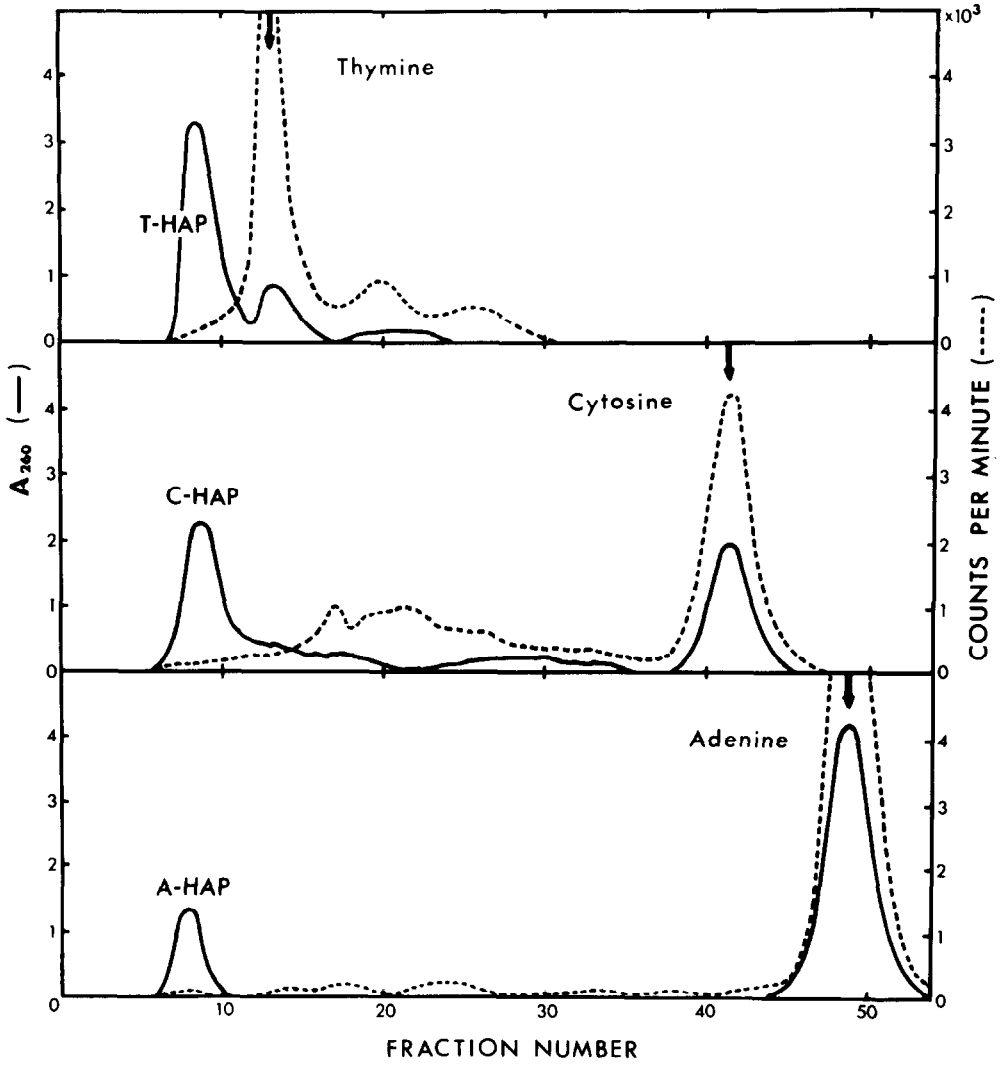

FIG. 4. Elution pattern of [^{14}C] thymine, [^{14}C] cytosine, and [^{14}C] adenine (10^{-3} M, 0.5 µCi/ml of SSC) irradiated with gamma rays in O_2 on a starch gel column. The column was eluted with n-propanol-0.5 N HCl (2 : 1, v/v) and 2 ml fractions were collected. The arrow mark indicates the original base position. Radiation dose was 2.5×10^5 rads.

normal UV absorbancy. This single modified peak is not an experimental artifact, as we observed it repeatedly and at the same position on different columns. It seems to be a polymerized product with unique properties, described in the following experiments.

Oxygen-dependent modification of bases. The column chromatograms of [^{14}C]thymine, [^{14}C]cytosine and [^{14}C]adenine irradiated with gamma rays in the presence

of oxygen, are shown in Fig. 4. It was interesting to find a modified peak very similar to the one observed in adult animal liver DNA (Figs. 1 and 2). and gamma-irradiated calf thymus DNA (Fig. 3). They all are formed only in the presence of oxygen. When monitored at 260 nm, their yield seems to be very high. However, the actual yield, monitored by radioactivity, was found to be quite low (Fig. 4, dotted line). This finding suggests a high UV absorbancy of this material. Their UV spectra (Fig. 5) are quite similar to each other and to the modified base peak from adult animal and gamma-irradiated calf thymus DNA (data not given). These results suggest the formation of oxygen-dependent modified product(s) having high UV absorbancy. The high-UV-absorbing product (HAP) did not move from the origin on thin-layer-chromatography plates with solvent systems of 1) chloroform-methanol water (4:2:1, v/v/v) (32), 2) ethylacetate-isopropanol-water (75:16:;9, v/v/v) (32) and 3) isopropanol-28% ammonium hydroxide (9:1, v/v), or on chromatographic paper with n-butanol-water (86:14, v/v) (33) as a solvent system.

DISCUSSION

The findings reported here suggest the accumulation of oxidative modifications of bases in adult animal liver DNA which were present in very low amounts in the

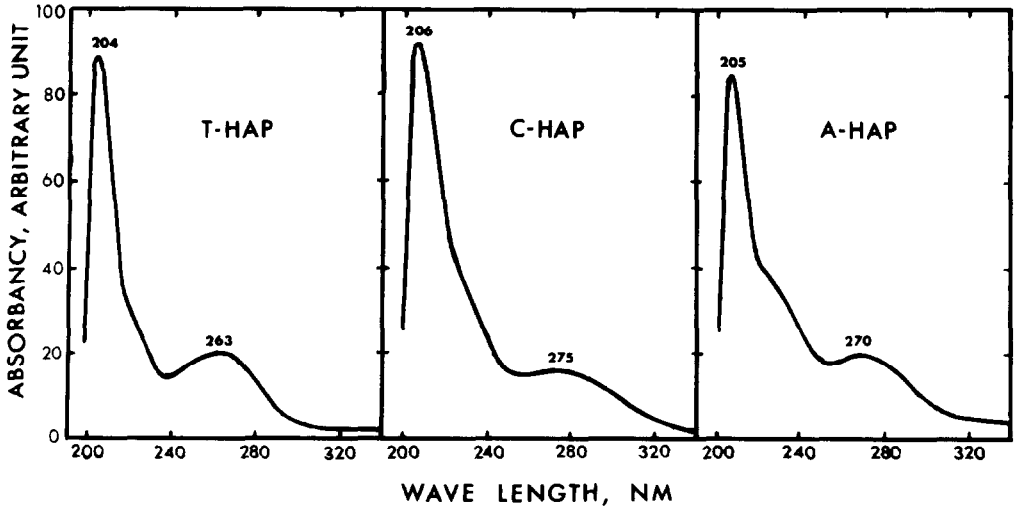

FIG. 5. UV spectra of T-HAP, C-HAP, and A-HAP in n-propanol-0.5N Hcl (2 : 1, v/v).

livers of young animals. The modified bases were readily detected because of their high UV absorption. The modified material was also formed in calf thymus DNA (or in the solution of free bases) irradiated with gamma rays in the presence of oxygen, suggesting a role of oxygen in modification. All samples of modified material whether from adult animal liver DNA or from irradiated DNA have similar UV absorption spectra and do not move from the origin on thin-layer-chromatography plates or on chromatographic paper suggesting that this high absorbancy product may be polymerized forms of oxidized bases. The age dependent accumulation of modified bases in DNA may be the result of metabolic activities.

Several chemical carcinogens are activated by metabolic oxidations (34-39). The autooxidation in general has been suggested to be capable of producing all the reactive species derived from the reduction of molecular oxygen (40). Such oxygen-reactive species could oxidize, not only carcinogen, but also other biomolecules including DNA. However, it is not clear to what extent such oxidative modifications in DNA are repairable.

Previous findings and the data reported here support the DNA oxidation hypothesis for aging, mutagenesis and carcinogenesis. Normal DNA when modified (e.g., by base oxidation) may result in replication errors which can lead to mutagenesis or carcinogenesis. The modified DNA may also produce abnormal RNA transcripts resulting in the formation of abnormal proteins which in turn can lead to abnormal metabolism and aging. The rate of accumulation of modifications might affect the life span and oncogenic age which in turn may depend on the DNA repair ability and oxidation suppression ability. These capabilities differ in different animal species and even in different organs of the same animal. Recently, Tolmasoff et al. (41) have suggested that longer-lived species have a higher degree of protection against by-products of oxygen metabolism. Orgel (42) proposed that translation errors explain how abnormal protein synthesis is related to aging. Other workers (43,44) suggest that modified DNA may induce mutagenesis or carcinogenesis, and abnormal protein molecules may cause metabolic disorders that impair cellular functions, resulting in aging. The slowing of aging process by antioxidants (38,

45,46) and the effect of hyperbaric oxygen on aging (47) further support the DNA base-oxidation theory. Several theories of aging and carcinogenesis have been proposed, however, we conclude that DNA base modification would be essential. Further studies aimed at elucidating the chemical structure of the modified material and its rate of accumulation are in progress.

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