136

July 31, 1972

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I am submitting herewith a dissertation written by Salma Fidaali Halai entitled "The Effects of Dietary Free Radical Inhibitors and Feed Restriction on Age Associated Biochemical Parameters and Lifespan of AKR Male Mice." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

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THE EFFECTS OF DIETARY FREE RADICAL INHIBITORS AND FEED RESTRICTION ON AGE ASSOCIATED BIOCHEMICAL PARAMETERS AND LIFESPAN OF AKR MALE MICE

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Salma Fidaali Halai

August 1972

ACKNOWLEDGEMENT

The author wishes to express her appreciation and thanks to the following people:

Dr. Roy E. Beauchene for his guidance during the course of this study, his help in the preparation of this thesis and for his paternal interest in her welfare;

Dr. Mary Rose Gram for her counselling in both academic and personal matters and her continued interest in the author's work;

Dr. Jane R. Savage for her friendship and encouragement throughout the author's stay at the University of Tennessee;

Dr. John T. Smith for his unfailing help in solving problems in the lab;

Dr. Mary Jo Hitchcock for her cooperation in serving on the thesis committee;

Sarah Tucker, her roommate, for her willing assistance in all phases of the research and her ever present shoulder to cry on;

Mrs. Rossie L. Mason for her uncomplaining assistance in doing the lab work;

Jay Beauchene and Debbie Hemphill for their willing and cheerful help in the lab and with the care of the animals;

Mrs. Barbara Boston and Mrs. Betty Debord, the secretaries in the Nutrition Department, for their willingness to assist in any way possible;

Dr. William L. Sanders for his kind help in doing the statistical analyses;

Mrs. Wiley Thomas for her cooperation and personal interest in the preparation of the manuscript for printing;

Dr. Elizabeth C. Hillier and the staff of the Oklahoma-Pakistan Home Economics Program for their concern for her progress in her graduate work; and

Her mother and family for their faith and moral support and the Hunts for being her family away from home.

The author was granted study leave by the Governing Council of the College of Home Economics, Karachi, to accept a Ford Foundation Fellowship for three years. She was also a recipient of a University of Tennessee Non-Service Fellowship and a P.E.O. International Peace Scholarship for one year, as well as a research grant from the Ford Foundation and one from the Altrusa Foundation. She is grateful to each of these organizations for making possible the furtherance of her graduate education.

ABSTRACT

The effects of dietary free radical inhibitors and of feed restriction on selected age associated biochemical parameters and on life span of AKR male mice were investigated. The control animals (n = 58) were fed a nutritionally adequate basal diet ad libitum; the restricted mice (n = 40) were allowed the basal diet 15 hours out of every 48 hours; the BHT (n = 40) and the vitamin E mice (n = 40) were fed the basal diet containing either 3 meq of butylated hydroxytoluene (0.33%) or dl-α-tocopheryl acetate (0.71%), respectively, per 100 g of feed.

One-half of the animals in each of the dietary groups was used for longevity studies; biochemical measurements were performed on the survivors of the other half of the mice at 12-months of age when approximately two-thirds of the controls were dead. Biochemical measurements were also performed for comparative purposes on 3-month and 6-month old mice eating the basal diet ad libitum.

The BHT and restricted mice consumed less feed and were smaller in body size than the controls, the reduction in feed intake being 5 percent and 33 percent, respectively, and the reduction in body weight being 9 percent and 19 percent, respectively, at 30 weeks of age.

The serum cholinesterase activity was significantly higher in 6-month, restricted and BHT mice than in the 12-month old controls. The BHT mice had a significantly higher liver catheptic activity than the control mice, while the values of the 3-month, 6-month and restricted mice were significantly lower than that of the controls. The catheptic activity of mice diagnosed as having leukemia was

significantly higher than that of non-leukemic mice regardless of diet. Kidney succinoxidase activity was not significantly influenced by age or diet.

The percent acid soluble collagen of the skin was significantly higher in the 3-month, 6-month and restricted animals than in control mice. The *in vitro* lipid peroxidation of brain homogenates of only the vitamin E mice was significantly lower than that of the controls.

The mean life span of the mice fed the basal diet *ad libitum* was 336 days. Feed restriction increased the mean life span to 403 days and reduced the incidence of death due to leukemia. Vitamin E did not have a significant effect on either life span or the incidence of leukemia; on the other hand the BHT mice showed a marked tendency towards an extension in mean life span and a reduction in the incidence of death due to leukemia. The latter findings were attributed to the slight degree of self-imposed feed restriction observed in these animals. There was a significant negative regression of life span on growth rate, and when life span was adjusted for growth rate there was no difference in the estimated longevity of the four dietary groups.

Thus, feed restriction increased the mean life span and influenced the biochemical parameters in such a way that, in general, they resembled the values obtained for the chronologically younger animals. On the basis of these observations it was concluded that dietary free radical inhibitors *per se* did not increase life span but their effect was mediated *via* the mechanism of feed restriction.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	3
	Free Radical Damage in Biological Systems	3
	Lipid Peroxidation and the Accumulation of Lipopigments	5
	Changes in the Solubility of Collagen	6
	Changes in Enzyme Activity with Age	8
	Feed Restriction, Longevity and Other Biological Parameters	10
	Free Radical Inhibitors and Longevity	13
III.	EXPERIMENTAL PROCEDURE	15
	General Plan	15
	Diets	17
	Methods	24
	Preparation of tissue samples	24
	Thiobarbituric acid test	27
	Determination of cholinesterase activity	28
	Determination of succinoxidase activity	30
	Determination of catheptic activity	32
	Protein determination	33
	Determination of soluble and insoluble collagen in skin	35
	Statistical methods	38
IV.	RESULTS	39

CHAPTER PAGE

V. DISCUSSION 52

Effect on Longevity. 52

Effect on Enzymes. 55

Effect on *In Vitro* Lipid Peroxidation. 56

Effect on Collagen 57

VI. SUMMARY. 59

REFERENCES. 62

APPENDIX. 70

VITA. 73

LIST OF TABLES

TABLE		PAGE
1.	Experimental Groups.	16
2.	Composition of Diets	23
3.	Composition of the Salt Mixture and the Level of Minerals Provided.	25
4.	Level of Vitamins Provided by Vitamin Mixture.	26
5.	The Effect of Diet on the Mean Daily Feed Intake Per Cage of Five Mice During the Growth Period	40
6.	The Effect of Diet on the Body Weight of AKR Male Mice .	41
7.	The Effect of Diet on the Half Survival Time and Mean Life Span of AKR Male Mice	42
8.	The Effect of Age and Diet on Body and Organ Weights at Time of Sacrifice	45
9.	The Effect of Age and Diet on the Percent Protein in Liver and Kidneys of AKR Male Mice	47
10.	The Effect of Age and Diet on Enzymatic Activities of AKR Male Mice	48
11.	The Effect of Age and Diet on the <u>In Vitro</u> Formation of Malonyldialdehyde by Homogenates of Brains of AKR Male Mice.	50
12.	The Effect of Age and Diet on the Collagen Content of Skin of AKR Male Mice	51

LIST OF FIGURES

FIGURE		PAGE
I.	Normal Mouse With No Apparent Enlargement of the Thymus, Spleen or Lymph Nodes.	18
II.	Mouse With Moderate Enlargement of Thymus and Spleen . .	19
III.	Mouse With Marked Enlargement of Thymus, Spleen and Lymph Nodes.	20
IV.	Normal and Enlarged Spleens.	21
V.	Enlarged Thymus Glands	22

CHAPTER I

INTRODUCTION

It has been suggested that aging may be due in part to the deleterious effect of free radicals produced in the course of normal metabolism. Free radical lipid peroxidation and/or the products of lipid peroxidation have been reported to result in changes in the character of membranes of cellular particulates, inactivation of enzymes and the cross-linking of collagen *in vitro*. The damage to isolated mitochondrial and microsomal fractions induced by free radical lipid peroxidation has been reported to be inhibited by the addition of α-tocopherol and other free radical inhibitors to *in vitro* systems.

Synthetic free radical inhibitors incorporated into the diet have been reported to produce a beneficial effect on the life span of mice of several different strains. Similarly, feed restriction has been reported to have a beneficial effect on life span and also, to reduce the incidence of chronic disease in both rats and mice.

Changes in certain biological parameters are known to occur with age. For example, the activities of selected enzymes may increase or decrease with age, the cellular content of lipopigments increases, and the solubility of collagen decreases with age.

A comparison of the effects of the incorporation of free radical inhibitors in the diet on life span and age associated biochemical parameters with those due to feed restriction should provide an indication as to whether their modes of action are similar.

Thus the present study was undertaken to investigate the following: whether the incorporation of α-tocopherol could cause an extension in life span of AKR male mice similar to that caused by the synthetic free radical inhibitor, butylated hydroxytoluene; whether the incorporation of α-tocopherol and butylated hydroxytoluene in the diet could delay the age associated changes in selected biochemical parameters *in vivo*; and whether the age associated changes are comparable in feed restricted mice and those fed free radical inhibitors.

CHAPTER II

REVIEW OF LITERATURE

Free Radical Damage in Biological Systems

In 1956 Harman (1) advanced the theory that aging may be due in part to the deleterious effects of free radicals produced in the course of metabolism. Polyunsaturated fatty acids (PUFA) are considered to be a primary source of free radicals within the cell, since the autoxidation of lipids is known to be a free radical chain process (2). Cellular and subcellular membranes are rich in polyunsaturated fatty acids (3); therefore, lipid peroxidation could have a very damaging effect on these membranes.

McCay et al. (4) have reported that the activity of a membrane-bound enzyme system, NADPH oxidase in isolated microsomes, which involves the transfer of electrons from substrate to oxygen, promotes the simultaneous formation of peroxides in the polyunsaturated fatty acids of phospholipids in the membrane. The phospholipid peroxides then undergo a chain cleavage reaction producing phospholipids containing a variety of carbonyl moites. This process results in a marked alteration in the membrane structure as observed by measurement of turbidity changes. During this reaction the formation of malonyldialdehyde (MDA) was proportional to the total amount of PUFA utilized. The peroxidative cleavage of PUFA was decreased by dietary α-tocopherol or the addition of free radical inhibitors to the in vitro system.

Robinson (5) has also reported that conditions which cause turbidity changes in microsomes and other subcellular particulates are

accompanied by the formation of lipid peroxides as measured by the thiobarbituric acid test (TBA) and oxygen uptake.

In view of the known protein cross-linking properties of certain difunctional aldehydes, Horten and Packer (6) studied the interactions between MDA and rat liver mitochondria. Electron micrographs of mitochondria after treatment with MDA for 1 to 4 hours revealed that, although mitochondria were mainly intact, they had become swollen to about twice their original size as compared with similar electron micrographs of untreated mitochondria. They suggested that the mechanism of the reaction is probably the formation of an enamine between the enol of MDA and the amino groups of the proteins.

When lysosomes were subjected to induced free radical systems, the lysosomal membrane was found to be extremely labile to such damage as indicated by the release of bound enzymes such as aryl sulfatase, cathepsin, β-glucuronidase, acid phosphatase and ribonuclease (7). Allison and Paton (8) have shown that chromosomal aberrations can be caused by the enzyme deoxyribonuclease (DNase) released from lysosomes and suggest that lysosomal damage may play a part in carcinogenesis since activation of lysosomal enzymes can release cells from mitotic inhibition.

On the other hand, MDA has been shown to inhibit DNA replication, protein synthesis and cell division in human fibroblast cultures. This effect is thought to be due to the direct interaction of MDA with DNA (9).

A number of studies have shown that lipid peroxidation can result in inactivation of mitochondrial and microsomal enzymes. Tappel (10) reported that in heart and liver mitochondria, the NAD-cytochrome C

reductase and the succinoxidase systems were good indices of lipid peroxidation damage. The mechanism of lipid peroxidation damage was studied in a reaction system consisting of linolenic acid, cytochrome C and oxygen. Concomitant with a reduction in the solubility of cytochrome C there was an increase in the incorporation of the peroxidized linolenate into the enzyme (11). Similarly, O'Malley et al. (12) reported that low concentrations of lipid peroxides can cause the inhibition of erythrocyte acetylcholinesterase, in vitro. Such inhibition could be prevented by the addition of α-tocopherol to the system.

Using ribonuclease (RNase) as an experimental model, Chio and Tappel (13) studied the inactivation of enzymes by the oxidation products of PUFA. They showed that loss of RNase activity was accompanied by the appearance of fluorescence in the enzyme-lipid reaction mixture. RNase inactivated by MDA had a fluorescence and a gel filtration pattern similar to those enzymes inactivated by PUFA. It was postulated by these authors that MDA acted as an agent for the intra- and inter-molecular cross-linking of ribonuclease (14).

Lipid Peroxidation and the Accumulation of Lipopigments

The intercellular accumulation of autofluorescent lipopigments has for many years been linked to the aging process because of a positive correlation between the degree of accumulation of the pigment and the age of the animal (15). Reichel et al. (16) reported that the cerebral sections of senescent Wistar rats and C_{57}BL/6J mice contained significantly higher counts of pigment granules than younger animals. The in vitro studies of Chio et al. (17) have suggested that all biological

molecules and structures which contain amino groups could become constituents of lipopigments by cross-linking with MDA and other carbonyl compounds originating from the peroxidation of PUFA.

In view of the role of lipid peroxides in the formation of age pigments, Yoshikawa and Herai (18) investigated the effect of age and diet on the lipid peroxide content of the brain. Rats 12 to 18 months of age gave consistently higher TBA values than one month old animals. The TBA values observed in animals given α-tocopherol by intramuscular injection were consistently lower than those of controls of similar age.

Changes in the Solubility of Collagen

It is well documented that macromolecules which are not renewed in the body undergo age changes. One macromolecule which has been extensively studied in relation to age associated changes is the collagen fiber of connective tissue. The physical-chemical changes produced in collagen during *in vivo* aging are thought to be due to the increase in the number of intra- and inter-molecular cross-linkings which gradually transform the soluble molecules of tropocollagen into insoluble fibers (19). Piez (20) reported that there is strong evidence that the intramolecular cross-linkage involves the condensation of a lysyl residue in one α-chain with a lysyl derived aldehyde group in another and that this is the major cross-linking mechanism *in vivo*.

The longer the time that has elapsed since a collagen molecule was synthesized the more firmly it will be bound into a collagen aggregate. This is a continuous process so that at any given time there will be a spectrum of collagen aggregates of varying degrees of cross-linking. The cross section of these aggregates which is extracted and the age of

their constituent molecules has been shown to depend on the extraction medium used (21). It has been reported that when young rats were injected with ^{14}C glycine the radioactivity first appeared in the salt soluble collagen, some time later in the acid soluble fraction and eventually in the "insoluble" collagen, indicating that the salt soluble fractions were the most recently synthesized (21).

Zika and Klein (22) studied the changes in collagen mass in rat skin from 2 weeks to 26 months and report that the relative content of collagen as percent of skin weight reached a maximum value of 25.3 percent at 4 months after which it declined gradually to 22.6 percent by 26 months.

Wirtschafter and Bentley (23) determined the neutral salt soluble collagen in skin of Long-Evans rats from the age of 1 to 700 days. The maximum concentration of the salt soluble fraction occurred at 35 days of age after which it decreased with age.

The age changes in the solubility of rat tail tendon collagen have been studied by several investigators. Everitt et al. (24) reported a decrease in the acid soluble collagen from 85 percent at the age of 25 days to 15 percent at 600 days of age. No further changes were observed. Similarly, Butzow and Eichhorn (25) reported a decrease in the 0.5 M acetic acid soluble collagen in tail tendon from 80 percent at 12 months to 25 percent at 24 months.

Age changes in the collagen of mouse skin have not been extensively studied. In a study (26) comparing various methods of obtaining acid-soluble collagen fractions, extraction with 0.5 M acetic acid at 40°C was found to be the preferred method for mouse skin. These authors

further reported that the acid soluble fraction in the skin of SM/J male mice decreased from 64 percent of the total collagen at the age of four months to 45 percent at 21 months of age. Leto et al. (27) have also reported a similar change in the acetic acid soluble fraction of skin for C_{57}BL/6J female mice. The percent extractable collagen from skin decreased from 37.2 percent at 10 months of age to 24.1 percent at 28 months of age.

In view of the current hypothesis regarding the part played by aldehydes in the cross-linking of collagen, Milch (28) studied approximately 200 different compounds of biological interest and found that certain aldehydes such as glyceraldehyde, pyruvaldehyde, acetaldehyde and crotonaldehyde, which arise in the course of normal metabolism, were capable of increasing the cross-linking of collagen in vitro. Dialdehydes such as MDA and succinaldehyde have also been shown to act as cross-linking agents (29). Buch and Schneir (30) reported that of the total MDA extracted from collagen, 38 percent appeared in the salt soluble fraction, and 62 percent appeared in the acid soluble fraction. Denaturation of the acid soluble collagen followed by chromatography on carboxymethyl cellulose resulted in a 57 percent recovery of collagen in the form of monomers and dimers which did not contain MDA, suggesting that MDA may be associated with the more aggregated forms of collagen.

Changes in Enzyme Activity with Age

It has been clearly demonstrated that aging is accompanied by a decline in the physiological performance of organs and organ systems (31). To account for such changes, numerous investigators have studied the changes in the enzymatic activity of tissues with age.

Barrows and coworkers (32, 33) observed a 10 to 20 percent age decrement in liver and kidney succinoxidase activity in rats. Leto et al. (27) reported a highly significant decline in kidney succinoxidase activity of C_{57}BL/6J female mice between the ages of 10 and 29 months. A depression in kidney succinic dehydrogenase activity of 30-month old male and female C_{57}BL/Icrf/a^{t} mice as compared to that of the 6-month old mice has also been reported by Wilson and Franks (34).

The effect of age on cholinesterase activity has been studied in both man and laboratory animals. Shanor et al. (35) found the cholinesterase activity in plasma and erythrocytes of old male subjects to be decreased by 24 percent as compared with corresponding values in young subjects. Similarly, Barrows et al. (36) studied human subjects 20 to 90 years of age and found a significant age associated decrease in serum cholinesterase in the male subjects. They also reported that the serum cholinesterase activity of female Wistar rats aged 24 to 27 months was significantly lower than that of 12- to 14-month old animals. Although a decline in liver cholinesterase activity was observed in the old rats, this difference was not significant.

Verkhratsky (37) reported a significant decline in the acetylcholinesterase activity in the heart and gastrocnemius muscle of rats from 1 to 26-28 months of age. Several investigators have reported a decline in the cerebral cholinesterase activity with age in rats and mice (38, 39, 40). Maletta (40) found that the cholinesterase activity of the sensory motor cortex of the mouse brain reached a peak at 44 days and that at 16 months the activity was significantly below the value at 44 days of age.

In contrast to the decline with age in cholinesterase and succinoxidase activity, the activity of the lysosomal enzymes appears to increase with age. Bjorkerund (41) determined the activity of various lysosomal enzymes in human liver tissue from individuals of different ages. A significant increase with age was found in cathepsin, acid phosphatase and aryl sulfatase activity.

Barrows and Roeder (42) reported an increase in liver catheptic activity with age in three different strains of rats. Similarly, Beauchene et al. (43) observed an increase in liver catheptic activity in both Wistar and Sprague-Dawley rats. An increase in the liver catheptic activity with age has also been reported for C_{57}BL/6J mice (27).

A report from a group of Japanese workers (44) showed that there was a change with age in the cellular distribution of acid ribonuclease. Mice and hamsters of both sexes aged 2 months to 20 months were studied. The cytoplasmic/particulate activity of liver acid RNase increased gradually in both mice and hamsters from the age of 4 months to the age of 20 months. The value at 20 months was three times that of 2 month old animals. The authors stated that these results suggest that during the process of aging the lysosomal membrane undergoes permeability changes, so that enzymes, such as RNase, within the particle tend to be released into the cytoplasm.

Feed Restriction, Longevity and Other Biological Parameters

As early as 1935, McCay et al. (45, 46) reported an extension of life span in the rat by extreme underfeeding. Berg and coworkers (47, 48) undertook a study to determine if less severe feed restriction would have the same beneficial effect on life span. Sprague-Dawley rats were

restricted in feed intake to 33 percent or 46 percent of ad libitum fed rats. At 800 days of age, only 48 percent of the unrestricted male rats were alive as compared to more than 80 percent of the restricted males.

In addition, the latter investigators noted a significant difference between the unrestricted and restricted animals in the incidence of cardiac, renal and vascular lesions as well as the incidence of tumors. In the unrestricted males, the frequency of lesions at 800 days of age was 100 percent as compared to 66 percent for the 33 percent restricted rats. All the females surviving to 800 days on the restricted intake were free from disease while 57 percent of the ad libitum fed rats had lesions (48).

Ross (49) reported that regardless of the proportion of the protein and carbohydrate components of the diet, feed restriction resulted in enhanced life expectancies, higher mean life spans and greater 50 percent survival times. More recently Ross and Bras (50) have reported that even a short term restriction early in life has a beneficial effect on the incidence of tumors. Rats subjected to approximately 50 percent feed restriction for seven weeks post weaning showed a 14-83 percent reduction in the incidence of various types of tumors.

The beneficial effects of feed restriction on life span in mice have also been reported. Lee and coworkers (51) subjected C_3H mice to 33 percent caloric restriction and reported a remarkable difference in the survival rates of the ad libitum fed and restricted mice. At 76 weeks of age all the control mice were dead whereas 75 percent of the restricted mice were surviving, and 100 percent of the latter animals

were not dead until 136 weeks of age. These results confirmed an earlier report by King and Visscher (52) for C_3H mice.

Besides life span, feed restriction has also been reported to have an effect on enzyme activity patterns. Ross (53) reported that in rats the hepatic catalase, ATPase and histidase activity increased while alkaline phosphatase decreased in activity until mature weight was reached. After mature weight was attained a reversal in the direction of the activity of all four enzymes took place. The general pattern of enzyme activity in rats restricted in calories or protein was the same as that of the controls, but the point of inflection and the degree of change in enzyme activity could be modified by the feed restriction to resemble those of chronologically younger animals. The longer the enzymatic transition period was delayed, the longer the rats were free of tumors.

Barrows and Roeder (54) have reported that discernible differences in enzymatic activities were seen in the tissue of young growing animals whose life expectancy was increased by feed restriction. However, they stated that increased longevity was not necessarily associated with patterns of enzymatic activities which are similar to those of chronologically younger animals. Fanestil and Barrows (55) showed that undernutrition increased the life span of the rotifer and prolonged the length of the egg laying period. Dietary restriction also delayed the time at which the age associated changes in lactic dehydrogenase and malic dehydrogenase occurred.

Feed restriction has also been shown to slow down the aging of rat tail tendon collagen fibers (56). Everitt (57) reported that the aging of collagen can be retarded by feed restriction. In his studies the greatest retardation occurred in the rats eating 50 to 70 percent

of the mean ad libitum intake. Deyl et al. (58) studied the effect of a 50 percent dietary restriction on the accumulation of connective tissue proteins in heart, liver and kidneys of rats. They reported a distinct accumulation of connective tissue proteins at 20 months of age in the controls which was delayed until 32 months of age in the restricted animals.

Free Radical Inhibitors and Longevity

Free radical reactions have been implicated in the degradation of biological systems. On this basis, increasing the amount and/or degree of unsaturation of dietary fat might be expected to increase the rate of biological degradation. To evaluate this possibility, Harman (59) fed semisynthetic diets containing 5, 10 or 20 percent (w/w) of either lard, olive oil, corn oil, safflower oil or distilled triglycerides of menhaden oil to groups of C_3H female mice, Swiss male mice and male rats of the Charles River strain throughout life. The diets contained α-tocopherol in an amount estimated to be sufficient to prevent vitamin E deficiency in animals on the most readily peroxidized diet. Increasing the amount and/or degree of unsaturation of the dietary fat decreased the mean life span of C_3H female mice in part due to an increased incidence of mammary carcinoma. Similar trends were evident in the rats and Swiss mice. A report by Pearce and Dayton (60) has indicated that replacing the saturated fats with unsaturated lipids in the diet of 174 human subjects was correlated with an increased incidence of death due to carcinoma as compared to subjects on a conventional diet.

Several reports have indicated that the addition of antioxidants to otherwise adequate diets resulted in a prolongation of the normal life span of various strains of mice. In 1957 Harman (61) reported that the addition of several different antioxidants to the diet resulted in a 20 percent increase in the 50 percent survival time of AKR male mice. Using a different strain of mice, LAF_1, Harman (62) later reported that the addition of 0.50 percent (w/w) butylated hydroxytoluene to the diet resulted in a 45 percent increase in the mean life span of these animals as compared to the controls.

Ethoxyquin, another commonly used antioxidant, has also been reported to have a beneficial effect on the life span of C_3H mice (63).

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

Male weanling mice of the AKR strain were obtained from Cumberland View Farms, Clinton, Tennessee, for this study. All animals were housed five to a cage in 8" X 11" X 8" wire mesh cages. The animals were divided into experimental groups as shown in Table 1. Each experimental group, except the 3- and 6-month old groups fed the basal diet, was further divided into two groups, one for longevity studies and the other for biochemical studies. Mice fed the basal diet ad libitum served as controls for both the biochemical and longevity studies.

The animals receiving the diets containing 2, 6-di-tert-butyl hydroxytoluene or dl-α-tocopheryl acetate were adapted to their respective diets gradually. At the age of six weeks, the animals were offered a diet containing 0.5 meq of additional antioxidant per 100 g of feed. The level of BHT and α-tocopheryl acetate was increased by 0.5 meq per 100 g of feed every other week until the mice were receiving a diet containing 3 meq BHT or 3 meq of additional α-tocopheryl acetate per 100 g of feed by 16 weeks of age. The animals were then maintained on these diets throughout the study.

The control, 3-month, 6-month, BHT and vitamin E groups were fed ad libitum throughout the study. The restricted mice were allowed to eat ad libitum for 15 out of every 48 hours (5 PM to 8 AM, every other day). All animals were provided with water ad libitum.

TABLE 1

EXPERIMENTAL GROUPS

Group	Number of Animals		Diet
	Biochemical	Longevity	
3-month	15	--	Basal
6-month	15	--	Basal
12-month[a]	30	28	Basal
Restricted	20	20	Basal, fed 15 hours per 48 hours
BHT	20	20	Basal, plus 3 meq BHT per 100 g feed (0.33%)
Vitamin E	20	20	Basal, plus 3 meq dl-α-tocopheryl acetate per 100 g feed (0.71%)

[a]Referred to as controls.

Daily feed intake per cage of mice was calculated continuously for the first six months. Thereafter daily feed intake was calculated every fourth week. The animals were weighed weekly for the first six months and every other week thereafter.

All cages were examined at least once a day and all deaths recorded. The dead mice were autopsied and examined for the presence of leukemia as evidenced by enlarged lymph nodes, spleens and/or thymus glands (64). Each of the three glands was rated on a scale with zero representing no apparent enlargement and three representing marked enlargement. Leukemia was considered to be the major cause of death of any mouse receiving a combined score of four or more. Examples of leukemic and non-leukemic mice are shown in Figures I, II and III. Spleen and thymus with various degrees of enlargement are shown in Figures IV and V, respectively.

At 12 months of age, when approximately two-thirds of the control animals were dead, the remaining animals in each biochemical group were sacrificed, and biochemical tests were performed. The animals in the longevity groups were continued on their respective diets until they died a natural death.

Diets

The composition of the semipurified diets used in this study is shown in Table 2. Safflower seed oil was used because of its high polyunsaturated fatty acid content (65). The oil used contained no added antioxidants or preservatives.



Figure I. Normal mouse with no apparent enlargement of the thymus, spleen or lymph nodes.



Figure II. Mouse with moderate enlargement of thymus and spleen.



Figure III. Mouse with marked enlargement of thymus, spleen and lymph nodes.



Figure IV. Normal and enlarged spleens (Ratings assigned: from top to bottom: 0, 2, 3).

Rating Scale: 0 = no apparent enlargement
1 = slight enlargement
2 = moderate enlargement
3 = marked enlargement



Figure V. Enlarged thymus glands (Ratings assigned: from top to bottom: 1, 2, 3).

Rating Scale: 0 = no apparent enlargement
1 = slight enlargement
2 = moderate enlargement
3 = marked enlargement

TABLE 2

COMPOSITION OF DIETS

	Basal g/100 g	BHT g/100 g	Vitamin E g/100 g
Casein[a]	20.00	20.00	20.00
Sucrose	31.00	31.00	31.00
Cornstarch	31.00	31.00	31.00
Safflower Seed Oil[b]	10.00	10.00	10.00
Salt Mixture	3.50	3.50	3.50
Vitamin Mixture[a]	2.00	2.00	2.00
"Alphacel"[a] non-nutritive bulk	2.50	2.17	1.79
Butylated Hydroxy-toluene[c]	--	0.33	--
dl-α-Tocopheryl Acetate[a]	--	--	0.71

[a]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[b]Hain Pure Food Company, Inc., Los Angeles, California.

[c]Eastman Kodak Company, Rochester, New York.

The salt mixture used was a modification of the Williams-Briggs salt mixture (66). The modifications were made in order to supply the mineral requirements of the mouse as reported by the NRC Committee on Animal Nutrition (67). Table 3 shows the composition of the salt mixture. The level of vitamins provided by the vitamin mixture is shown in Table 4.

The level of vitamins in the basal diet was adequate according to the NRC Committee on Animal Nutrition (67). The basal diet contained 9.9 mg of α-tocopherol per 100 g of feed; hence the total amount of α-tocopherol in the vitamin E diet was approximately 0.72 g per 100 g feed.

II. METHODS

Preparation of Tissue Samples

All animals were allowed to eat ad libitum the night prior to sacrifice. In the morning the animals were decapitated and blood samples for cholinesterase determinations were collected in small test tubes. The samples were centrifuged[a] for 10 minutes at 510 x g at 4°C. The serum was collected and stored on ice for four to five hours before being assayed.

The liver, brain and kidneys of the animal were removed immediately after decapitation, weighed and homogenized in a tissue grinder with a teflon pestle. The homogenates were made up to a final concentration of 5 percent (w/v) with water and kept on ice until assayed.

The lymph nodes, spleen and thymus glands were examined and the mice diagnosed as leukemic or non-leukemic by the previously described criteria (p. 17).

---

[a]Lourdes Centrifuge; 9 RA Rotor.

TABLE 3

COMPOSITION OF THE SALT MIXTURE AND THE LEVEL OF MINERALS PROVIDED

Component	Percent of Salt Mixture	mg/100 g Feed
$CaHPO_4$	44.940	1573
KCl	18.702	654
Na_2HPO_4	18.600	651
$CaCO_3$	10.000	350
$MgSO_4$	6.571	230
$MnSO_4 \cdot H_2O$	0.440	15
$FeC_6H_5O_7 \cdot 5H_2O$	0.431	15
$ZnCO_3$	0.275	10
$CuSO_4$	0.037	1.2
KIO_3	0.003	0.1
Na_2SeO_4	0.0003	0.01

TABLE 4

LEVEL OF VITAMINS PROVIDED BY VITAMIN MIXTURE

Vitamin	mg/100 g Diet
Vitamin A	0.53
Vitamin D	0.004
α-Tocopherol	10.0
Ascorbic Acid	90.0
Inositol	10.0
Choline Chloride	150.0
Menadione	4.5
p-Aminobenzoate	10.0
Niacin	9.0
Riboflavin	2.0
Pyridoxine HCl	2.0
Thiamine HCl	2.0
Calcium Pantothenate	6.0
Biotin	0.04
Folic Acid	0.18
Vitamin B_{12}	0.0027

The skin on the back of the animal was removed, washed in cold water and blotted dry. The skin was then placed in a stoppered test tube and stored at -20°C for six months until assayed for collagen.

Thiobarbituric Acid Test

The *in vitro* formation of lipid peroxides by brain homogenates was assayed by the thiobarbituric acid (TBA) test as described by Placer *et al.* (68) The heat-induced reaction of one mole of MDA, a secondary product of lipid peroxidation, with two moles of TBA in an acid solution forms a trimethene colored substance which can be measured spectrophotometrically.

Reagents.

1. Thiobarbituric Acid Reagent: 0.8 *g* TBA was dissolved in approximately 20.0 ml of 0.1 N NaOH. The solution was neutralized with 7 percent perchloric acid (w/v) and then made up to 100 ml with water. Two volumes of the TBA solution were mixed with one volume of 7 percent perchloric acid just prior to use.

2. Tris-KCl Buffer: pH 7.4: 24.28 *g* Tris and 11.84 *g* KCl were dissolved in 150-200 ml water; the pH was adjusted to 7.4 with HCl and then made up to a liter with water.

3. Pyridine-Butanol Mixture: Three volumes of pyridine and one volume of n-butanol were mixed together daily.

Procedure. The brain homogenates were centrifuged[a] for ten minutes at 400 x *g* at 4°C. One-tenth ml of the supernatant fluid was

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[a]Lourdes Centrifuge; 9 RA Rotor.

incubated with 1.4 ml buffer for 30 minutes in a 37°C water bath. The tubes were allowed to cool and 1.5 ml TBA reagent was added. The mixture was heated in a boiling water bath for 10 minutes using a marble as a condenser. After cooling to room temperature, 3.0 ml of the pyridine-butanol mixture and 1.0 ml 1 N NaOH were added and the samples mixed. The absorbance was determined at 550 nm using a Beckman B spectrophotometer. The concentration of MDA equivalents present in the sample was estimated using the following formula:

$$\text{Nanamoles MDA/10 mg tissue} = \frac{\text{Volume} \times A_{550}}{0.164} \times 2$$

In order to obtain the constant (0.164), 1, 1, 3, 3 tetraethoxypropane was hydrolyzed in 0.01 N HCl (69), standard curves were run on the resultant MDA solution, and the constant was calculated as described by Placer et al. (68).

Determination of Cholinesterase Activity

The Warburg manometric method (70) was used to assay serum cholinesterase activity.

In this method the enzymatic hydrolysis of the ester proceeds in a bicarbonate-carbonic acid buffer. The carbonic acid concentration is fixed by the pressure of CO_2 in an atmosphere of N_2 and CO_2. The acid liberated reacts with the bicarbonate ion to form carbonic acid which dissociates and CO_2 is given off in amounts equivalent to the acid produced and is estimated manometrically.

Reagents.

1. Bicarbonate Ringers Solution: 100 ml of 0.9 percent NaCl, 30 ml of 1.26 percent $NaHCO_3$ and 2.0 ml of 1.76 percent $MgCl_2 \cdot 6H_2O$ were mixed together and the pH adjusted to 7.4 by bubbling a 95 percent N_2-5 percent CO_2 gas mixture through the solution.

2. Acetylcholine Bromide Solution: 9.35 g acetylcholine bromide was made up to 100 ml with a dilute HCl solution, pH 4. This solution was diluted 1:3 with the buffer just prior to use.

Procedure. One-armed Warburg flasks, without center wells, were placed on ice and 2.65 ml of buffer solution and 50 µl serum were added to each center compartment. Three-tenths ml of diluted acetylcholine bromide solution was placed in each side arm. The thermobarometer contained 3.0 ml water in the center compartment.

The side arm vent was greased and inserted in the open position. The flasks were attached to the manometers and placed in the 25°C water bath. The Brodies solution was raised to 150 mm and the flasks were then gassed for five minutes with 95 percent N_2-5 percent CO_2 mixture. The system was closed immediately, the shaker turned on and the contents of the flasks allowed to equilibrate for five minutes. The zero time reading was recorded using 150 mm as the reference point. At 10-second intervals the substrate in the side arms was dumped into the center compartments. Readings were recorded every five minutes for 30 minutes for each sample.

The microliters of CO_2 produced per milliliter of serum per hour was calculated as follows:

(Reading at 30 minutes - Zero time reading) x (Flask constant + Manometer constant) $\pm$ [Change in thermobarometer x (Flask constant + Manometer constant)] x 40

The flasks and manometers were calibrated using mercury according to the method of Umbreit et al. (71).

Determination of Succinoxidase Activity

Kidney succinoxidase activity was determined by a modification of the method of Schneider and Potter (72). Succinic acid is oxidized by succinic dehydrogenase in the presence of an excess of cytochrome C and cytochrome oxidase. The oxygen consumed in this reaction is proportional to the succinic acid oxidized and is measured manometrically.

Reagents.

1. Sodium Phosphate Buffer, 0.1 M: 14.3 g Na_2HPO_4 was dissolved in approximately 200 ml water, the pH adjusted to 7.4 with 1 N HCl and the solution made up to a liter with water.

2. Sodium Succinate Solution, 0.5 M: 3.3768 g disodium succinate hexahydrate was made up to 25 ml with 0.1 M phosphate buffer. The solution was made up daily.

3. Cytochrome C[a] Solution, 1 x 10^{-4} M: 13 mg cytochrome C was made up to 10 ml with cold water daily.

4. $CaCl_2$ Solution, 0.012 M: 0.1764 g $CaCl_2 \cdot 2H_2O$ was made up to 100 ml with water.

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[a]M.W. 13000 Fe:0.34 Nutritional Biochemical Corp., Cleveland, Ohio.

5. $AlCl_3$ Solution, 0.012 M: 0.2900 g $AlCl_3 \cdot 6H_2O$ was made up to 100 ml with water.

Procedure. One-armed Warburg flasks with center wells were placed on ice, and 0.2 ml 10 percent KOH was added to each center well. The reaction mixture was prepared by mixing together 40.0 ml buffer, 36.0 ml water, 12.0 ml sodium succinate, 16.0 ml cytochrome C, 4.0 ml $AlCl_3$ and 4.0 ml $CaCl_2$. To the center compartment of the flask 3.1 ml of this mixture was added and 0.2 ml of the kidney homogenate was placed in the side arm. A piece of fluted filter paper was placed in the center well. The thermobarometer was prepared with 3.5 ml water in the center compartment.

The side arm vent was greased and inserted in the closed position. The flasks were attached to the manometers and placed in the 37°C water bath. The Brodies solution in the manometers was raised to 250 mm, the shaker turned on, and the contents of the flasks allowed to equilibrate for 10 minutes. The system was then closed and the zero time reading recorded. The homogenates were dumped into the center compartments at 10 second intervals, and readings were recorded every five minutes for 30 minutes.

The microliters of O_2 per 100 mg of kidney (wet wt) per hour was calculated as follows:

(Reading at 30 minutes - Zero time reading) x (Flask constant + Manometer constant $\pm$ [Change in thermobarometer x (Flask constant + Manometer constant)] x 20

The flasks and manometers were calibrated using mercury according to the method of Umbreit et al. (71).

Determination of Catheptic Activity

Liver cathepsin was determined using a modification of the method described by Anson (73). Hemoglobin is used as the substrate and the tyrosine equivalents are estimated spectrophotometrically in the digestion products not precipitable by TCA, using the Folin-Ciocalteau reagent.

Reagents.

1. Hemoglobin (Hb) Solution: 6 g Hb was dissolved in 40 ml of water, allowed to stand overnight in the refrigerator and then made up to a 100 ml with water. The Hb solution was centrifuged[a] for 20 minutes at 13,000 x g at 4°C. The supernatant fluid was stored at -20°C and thawed just prior to use.

2. Sodium Acetate Buffer, pH 3.7: 16.408 g sodium acetate ($C_2H_3NaO_2$) was dissolved in approximately 200 ml of water, the pH was adjusted to 3.7 with 1 N HCl and then made up to one liter with water.

3. "Mixture" for tyrosine standards: 100 ml of 10 percent (w/v) trichloroacetic acid (TCA), 20 ml buffer and 40 ml water were mixed together.

4. Tyrosine Standard Solution: 10 ml of a tyrosine stock solution containing 1 mg tyrosine per ml of 0.1 N HCl was made up to 100 ml with the "Mixture." Working standards containing 10 to 100 μg tyrosine were prepared daily by diluting the tyrosine standard solution with the "Mixture."

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[a]Lourdes Beta-Fuge Model A; 9 RA Rotor.

5. NaOH - Na_2HPO_4 Solution: 0.6 N NaOH and 0.5 M Na_2HPO_4 were mixed together just prior to use in the proportion of 7:1.

6. Phenol Reagent: Folin-Ciocalteau phenol reagent was diluted with two parts water just prior to use.

Procedure: Erlenmeyer flasks were set up containing 0.8 ml Hb solution, 1.0 ml H_2O and 0.9 ml buffer. Following the addition of 0.3 ml of 5 percent liver homogenate, the flasks were incubated for 30 minutes at 37°C in a mechanical shaker moving at the rate of 90 cycles per minute. The reaction was terminated by the addition of 5.0 ml ice cold 10 percent TCA. The flasks were chilled on ice for 30 minutes after which the contents were filtered using Whatman #42 filter paper. Four ml of NaOH-Na_2HPO_4 solution was added to 2.0 ml of filtrate and the contents of the test tube mixed. Next, 1.2 ml of diluted phenol reagent was added and the sample was mixed immediately using a mechanical mixer. The samples were allowed to stand for 30 minutes at room temperature, and the absorbance was determined at 550 nm using a Beckman B spectrophotometer. The tyrosine equivalents per 100 mg of liver (wet wt) per hour were calculated as follows:

$$\mu g \text{ tyrosine}/100 \text{ mg liver}/hr = \frac{A \text{ of Sample} - (A \text{ of Hb Blank} + A \text{ of Homogenate Blank})}{A \text{ of Std}} (\text{Conc of Std}) \, 53.33$$

Protein Determination

The liver and kidney proteins were isolated by a modification of the method of Shibko et al. (74) and assayed with the Biuret reagent (75). The Biuret test depends on the formation of complex ions between

copper and proteins in an alkaline solution to give a violet color which can be measured spectrophotometrically.

Reagents.

1. Biuret Reagent: 3 g $CuSO_4 \cdot 5H_2O$, 9 g $NaKC_4H_4O_6 \cdot 4H_2O$, 8 g NaOH and 5 g KI were dissolved in water and made up to one liter.

2. Bovine Serum Albumin Standard: 1 g bovine serum albumin (BSA) was dissolved in 0.4 N NaOH and made up to 100 ml with the same. Working standards containing 1 to 10 mg BSA per ml were prepared daily by diluting the stock standard with 0.4 N NaOH.

3. Perchloric Acid Solution (PCA) 0.6 percent (w/v): 5 ml of 70 percent perchloric acid was made up to one liter with 95 percent alcohol.

Procedure. One-tenth ml 70 percent PCA was added to 1.0 ml of homogenate, mixed thoroughly and chilled on ice 15 minutes. The samples were then centrifuged[a] at 800 g for five minutes. The supernatant fluid was poured off and the precipitate washed with 3.0 ml of 0.6 percent PCA solution. The samples were centrifuged and the precipitate washed with 3.0 ml of ether. After the third centrifugation, the supernatant fluid was discarded, and the protein pellet was dissolved in 3.0 ml of 0.4 N NaOH. To 1.0 ml of the protein solution, 4.0 ml of Biuret reagent was added. Then the samples were mixed using a mechanical mixer, incubated for 30 minutes in a 45°C water bath, allowed to cool and the absorbance

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[a]International Centrifuge Model SBB #50092 H.

determined at 550 nm using a Beckman B spectrophotometer. The percent protein was calculated as follows:

$$\text{Percent Protein} = \frac{\text{A of Unknown}}{\text{A of Std}} \text{ (Conc of Std) (6)}$$

Determination of Soluble and Insoluble Collagen in Skin

Acid soluble collagen was extracted from mouse skin using a modification of the method described by Leto et al. (27). The hydroxyproline content of both the soluble and insoluble collagen fractions was determined by the method of Woessner (76). The method for the assessment of tissue collagen depends on the determination of its hydroxyproline content. This amino acid comprises a definite proportion of the collagen molecule while being found only in negligible amounts in other proteins.

Acid hydrolysis releases the hydroxyproline of collagen which is then oxidized to a pyrrole by Chloramine T. The pyrrole is then condensed with p-dimethylaminobenzaldehyde to form a red chromagen which can be measured spectrophotometrically.

a. Extraction of Soluble Collagen. The skin was shaved as closely as possible, placed on a block and the rest of the hair removed gently with a scapel. All adhering subcutaneous fat and tissue was removed until the skin was paper thin and translucent. The skin was then dried approximately 48 hours in a dessicator over anhydrous calcium chloride until a constant dry weight was reached. The skin was cut up into fine pieces with scissors, and a weighed sample (approximately 6 mg) was incubated with 5 ml 0.5 M acetic acid at 37°C for 12 hours in a mechanical shaker oscillating at the rate of 90 cycles per minute.

The samples were then centrifuged[a] for 70 minutes at 21,600 x *g* at 4°C. Both the supernatant fluid and residue were assayed for hydroxyproline to determine soluble and insoluble collagen, respectively.

b. Hydroxyproline Determination. The hydroxyproline content of the soluble and insoluble collagen fractions was determined by the method of Woessner (76).

Reagents.

1. Hydroxyproline Stock Solution: 25 mg vacuum dried L-hydroxyproline[b] was made up to 250 ml with 0.001 N HCl. Ten ml of the stock solution was made up to 100 ml with 0.001 N HCl. Working standards containing 1-10 µg hydroxyproline were prepared daily by diluting the latter solution with water.

2. Citrate Buffer, pH 6: 50 *g* citric acid, 120 *g* sodium acetate trihydrate and 34 *g* NaOH were dissolved in 300 ml of water. The pH was adjusted to 6 with HCl and the buffer made up to one liter with water. The buffer was stored in the refrigerator under toluene.

3. Chloramine T (sodium p-Toluenesulfonchloramide) Solution, 0.05 M: 1.41 *g* Chloramine T was dissolved in 20 ml water and 30 ml methylcellosolve[c] and 50 ml buffer were added. The solution was made up daily.

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[a]Lourdes Beta-Fuge Model A; 9 RA Rotor.

[b]Calbiochem, Los Angeles, California.

[c]Fisher Scientific Company, Fair Lawn, New Jersey.

4. p-Dimethylaminobenzaldehyde[a] (PAB) Solution, 20 percent: 20 g PAB was made up to 100 ml with methylcellosolve.

5. Perchloric Acid, 3.15 M: 27 ml of 70 percent perchloric acid was made up to 100 ml with water.

Procedure. The total residue after extraction of soluble collagen was hydrolyzed in 4.0 ml of 6 N glass distilled HCl. To 1.0 ml of the supernatant fluid containing the soluble collagen was added 1.0 ml of 12 N HCl for hydrolysis. The samples were hydrolyzed for three hours at 130°C and then filtered and neutralized. The hydrolyzed residue and the supernatant fluid solutions were diluted with water to 100 ml and 50 ml, respectively.

One ml of the Chloramine T solution was added to 2 ml of the hydrolyzed solution, mixed and allowed to stand at room temperature for 20 minutes. After this 1.0 ml PCA was added to each tube, mixed and the tubes allowed to stand for five minutes. One ml of PAB was added and mixed using a mechanical mixer until no streaks could be seen. The tubes were placed in a 60°C water bath for 20 minutes, cooled in tap water for five minutes and the absorbance determined at 555 nm using a Beckman B spectrophotometer.

The hydroxyproline content was calculated from the standard curve using the following equations:

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[a]Eastman Kodak Company, Rochester, New York.

For Soluble Collagen:

μg hydroxyproline/mg skin =

$$\frac{\frac{\text{A of Sample x Conc of Std}}{\text{A of Std}}\ (25)\ (\text{ml Supernatant})}{\text{Dry Weight of Skin in mg}}$$

For Insoluble Collagen:

$$\text{μg hydroxyproline/mg skin} = \frac{\frac{\text{A of Sample x Conc of Std}}{\text{A of Std}}}{\text{Dry Weight of Skin in mg}} \quad (50)$$

The collagen content was calculated by multiplying the hydroxyproline values by 7 (23).

Statistical Methods

The significance of the differences between means was tested using the Students t test. A complete analysis of covariance was performed on the life span data of mice on the four dietary treatments. The differences in the incidence of death due to leukemia in the four groups were tested for significance using Chi squares. All the above analyses were performed as described by Steel and Torri (77).

A least squares analysis of variance (78) was performed on the life span data for leukemic and non-leukemic mice.

CHAPTER IV

RESULTS

The mean daily feed intake per cage of five mice fed the basal and experimental diets at 10, 20 and 30 weeks of age is shown in Table 5. The feed intakes of the restricted mice at each of these periods were significantly less ($P < 0.001$) than those of control mice (animals eating the basal diet ad libitum) at corresponding ages. The BHT mice ate significantly less ($P < 0.05$) than the control mice only at 10 and 20 weeks of age. The reduction in food intake was 33 percent in the restricted mice and five percent in the BHT mice at 30 weeks of age, as compared to the controls. The feed intake of the vitamin E mice was not significantly different from that of the control mice.

The body weights (Table 6) of the restricted mice were significantly less than those of the controls at 10, 20, 30, 40 and 50 weeks of age ($P < 0.005$). Similarly the BHT mice weighed significantly less than the controls at all ages ($P < 0.05$). The reduction in body weights of the restricted and BHT mice at 30 weeks of age were 19 percent and 9 percent, respectively, as compared to that of the control mice. The vitamin E mice did not differ significantly from the controls with regard to body weight.

Table 7 shows the half survival time and mean life span of the mice in the longevity groups. The longest half survival time of 381 days was observed for the restricted mice, and this was 14.4 percent longer than the half survival time of 333 days obtained for the control mice. The BHT mice had a half survival time of 376 days, and the

TABLE 5

THE EFFECT OF DIET ON THE MEAN DAILY FEED INTAKE PER CAGE OF FIVE MICE DURING THE GROWTH PERIOD

Age Weeks n (Cages)	Control 12	Restricted 8	BHT 8	Vitamin E 8
	g	g	g	g
10	13.2 ± 0.4[a]	8.7 ± 0.3[b]	11.8 ± 0.6[c]	12.4 ± 0.2
20	14.4 ± 0.4	9.6 ± 0.2[b]	13.6 ± 0.5[c]	13.9 ± 0.6
30	13.7 ± 0.2	9.2 ± 0.2[b]	13.0 ± 0.8	13.8 ± 0.3

[a]Mean ± SE.

[b]Significantly different from control, $P < 0.001$.

[c]Significantly different from control, $P < 0.05$.

TABLE 6

THE EFFECT OF DIET ON THE BODY WEIGHT OF AKR MALE MICE

Age Weeks n	Control 58	Restricted 40	BHT 40	Vitamin E 40
	g	g	g	g
10	24.1 ± 0.4[a]	22.5 ± 0.4[b]	23.0 ± 0.4[c]	23.2 ± 0.4
20	31.3 ± 0.4	26.0 ± 0.4[d]	28.7 ± 0.4[d]	31.1 ± 0.6
30	32.8 ± 0.6	26.7 ± 0.3[d]	29.7 ± 0.6[d]	33.0 ± 0.7
40	32.8 ± 0.7	26.3 ± 0.3[d]	29.9 ± 0.7[b]	31.1 ± 0.7
50	31.3 ± 1.0	26.5 ± 0.4[d]	27.1 ± 1.1[b]	29.3 ± 1.0

[a]Mean ± SE.

[b]Significantly different from control, $P < 0.005$.

[c]Significantly different from control, $P < 0.05$.

[d]Significantly different from control, $P < 0.001$.

TABLE 7

THE EFFECT OF DIET ON THE HALF SURVIVAL TIME AND MEAN LIFE SPAN OF AKR MALE MICE

	Control	Restricted	BHT	Vitamin E
n	28	20	20	20
Half Survival Time (days)[a]	333	381	376	310
Mean Life Span (days)	336 ± 16.7[b]	403 ± 23.4[c]	376 ± 24.0	344 ± 15.0

[a]Age at which 50 percent of the animals in the group were dead.

[b]Mean ± SE.

[c]Significantly different from controls, $P < 0.05$.

vitamin E mice 310 days; this was 12.9 percent higher and 6.9 percent lower, respectively, than that of the control mice.

The mean life span of the restricted mice, 403 days, was significantly greater than the mean life span of 336 days for the controls. The BHT mice tended to have a mean life span which was higher than that of the controls, but the difference was not significant. The mean life span of the vitamin E mice did not differ significantly from that of the controls.

There was a significant negative regression ($P < 0.005$) of life span on growth rate for the mice in the longevity groups. The regression equation was $\hat{Y} = 469.9 - 1441.2X$; where $\hat{Y}$ equals estimate of life span in days and X equals the growth rate in grams per day. The analysis of covariance (Table 13, Appendix) using adjusted treatment means did not show any significant differences in life span between the four treatment groups. The adjusted mean life spans were: control, 357 days; restricted, 363 days; BHT, 378 days; vitamin E, 353 days.

The incidence of deaths due to leukemia in the restricted mice in the longevity group was 30 percent (6 of 20) as compared to 60 percent (17 of 28) in the controls; this difference was significant at the $P < 0.05$ level. The BHT and vitamin E mice had corresponding values of 40 percent (8 of 20) and 55 percent (11 of 20), respectively. Neither of these values was significantly lower than that of the controls. The mean life span of the leukemic mice, 333 days, was significantly lower ($P < 0.05$) than the mean life span of 383 days for the non-leukemic mice. However, the interaction between leukemia and diet was not significant with respect to life span (Table 14, Appendix).

The body weights and organ weights of the mice at sacrifice are shown in Table 8. There was no significant difference in the body weights of the vitamin E mice as compared to the control mice. The restricted ($P < 0.05$), BHT ($P < 0.005$) and 3-month mice ($P < 0.005$) weighed significantly less than the control mice. The 6-month mice weighed 33.6 g and were significantly heavier ($P < 0.05$) than the controls.

Although there were no significant differences between the liver weights of the control mice and the mice in the other groups, both the restricted and BHT mice tended to have larger livers, 1.70 g and 1.53 g, respectively, as compared to 1.43 g for the control mice. However, the liver composed a significantly greater ($P < 0.05$) percentage of the body weight of the BHT mice as compared to that of the controls, being 7.2 percent and 5.0 percent, respectively.

There were no significant differences in the kidney weights of the various groups as compared to that of the control mice. The kidney weight, as percent of body weight, of the 6-month mice, was significantly lower ($P < 0.001$) than that of the controls.

The brain weight of the BHT mice was significantly lower ($P < 0.005$) than that of the controls; however, the proportion of body weight composed of brain weight was significantly higher in the BHT mice than in the controls ($P < 0.001$). The brain also composed a significantly larger ($P < 0.05$) percentage of the body weight of the 3-month and the vitamin E mice, whereas the corresponding value for the 6-month mice was significantly lower ($P < 0.05$) than that of the controls.

TABLE 8

THE EFFECT OF AGE AND DIET ON BODY AND ORGAN WEIGHTS AT TIME OF SACRIFICE

	Basal			Restricted	BHT	Vitamin E
	3-Month	6-Month	12-Month[a]	12-Month	12-Month	12-Month
n	14	12	12	12	8	10
Body Weight (g)	24.8 ± 0.6[bc]	33.7 ± 1.0[d]	29.3 ± 1.4	25.6 ± 1.0[d]	21.8 ± 1.7[c]	27.5 ± 0.7
Liver Weight (g)	1.18 ± 0.06	1.32 ± 0.02	1.43 ± 0.24	1.70 ± 0.08	1.53 ± 0.11	1.38 ± 0.09
Liver Wt/Body Wt x 100	4.8 ± 0.2	3.9 ± 0.1	5.0 ± 1.0	6.6 ± 0.2	7.2 ± 0.7[d]	5.2 ± 0.4
Kidney Weight (g)	0.36 ± 0.01	0.39 ± 0.07	0.42 ± 0.01	0.34 ± 0.07	0.31 ± 0.02	0.38 ± 0.02
Kidney Wt/Body Wt x 100	1.4 ± 0.04	1.2 ± 0.20[e]	1.6 ± 0.10	1.4 ± 0.07	1.4 ± 0.07	1.4 ± 0.04
Brain Weight (g)	0.40 ± 0.001	0.42 ± 0.004	0.41 ± 0.006	0.40 ± 0.007	0.39 ± 0.001[c]	0.42 ± 0.004
Brain Wt/Body Wt x 100	1.6 ± 0.04[d]	1.3 ± 0.03[d]	1.4 ± 0.06	1.6 ± 0.09	1.8 ± 0.11[e]	1.6 ± 0.02[d]

[a] Referred to as controls.

[b] Mean ± SE.

[c] Significantly different from control, $P < 0.005$.

[d] Significantly different from control, $P < 0.05$.

[e] Significantly different from control, $P < 0.001$.

The percent protein in the livers and kidneys of the mice is shown in Table 9. The livers of the restricted mice contained 16.5 percent protein, and this was significantly lower ($P < 0.05$) than 19.8 percent protein in the livers of the control mice. The percent protein in the livers of the other groups did not differ significantly from the controls. The content of protein in the kidneys of the 6-month mice, 17.1 percent, was significantly higher than that of the controls, 16.0 percent ($P < 0.05$). The percent protein in the kidneys of the other groups was not significantly different from control values.

The effects of diet and age on liver cathepsin, serum cholinesterase and kidney succinoxidase activity are shown in Table 10. The liver catheptic activity of the 3-month ($P < 0.005$), 6-month ($P < 0.05$) and restricted ($P < 0.05$) mice was 856, 902 and 890 μg tyrosine/100 mg/hour, respectively, and these values were significantly lower than 1157 μg tyrosine/100 mg/hour for the control mice. The BHT mice showed a significantly higher ($P < 0.005$) catheptic activity than the controls.

The liver catheptic activity of the leukemic mice was compared with the activity of non-leukemic mice. Regardless of diet, the activity of the leukemic mice was found to be significantly higher ($P < 0.001$) than that of the non-leukemic mice. The catheptic activity of the non-leukemic mice was 983 μg/100 mg/hour as compared to 1548 μg/100 mg/hour for the leukemic mice.

The serum cholinesterase activity of the control mice was 1579 μl CO_2/ml/hour. The 6-month, restricted and BHT mice had significantly higher activities, 1895, 2036 and 2803 μl CO_2/ml/hour, respectively, than the controls. The differences between the kidney succinoxidase

TABLE 9

THE EFFECT OF AGE AND DIET ON THE PERCENT PROTEIN IN LIVER AND KIDNEYS OF AKR MALE MICE

Organ	Basal			Restricted 12-Month	BHT 12-Month	Vitamin E 12-Month
	3-Month	6-Month	12-Month[a]			
n	14	12	12	12	8	10
Liver (%)	21.3 ± 0.71[b]	19.6 ± 0.74	19.8 ± 0.78	16.5 ± 0.79[c]	19.8 ± 0.85	19.2 ± 0.47
Kidney (%)	16.8 ± 0.20	17.1 ± 0.30[c]	16.0 ± 0.38	16.0 ± 0.22	15.4 ± 0.68	16.5 ± 0.35

[a]Referred to as controls.

[b]Mean ± SE.

[c]Significantly different from control, $P < 0.05$.

TABLE 10

THE EFFECT OF AGE AND DIET ON ENZYMATIC ACTIVITIES OF AKR MALE MICE

	Basal			Restricted	BHT	Vitamin E
	3-Month	6-Month	12-Month[a]	12-Month	12-Month	12-Month
n	14	12	12	12	8	10
Liver Cathepsin (μg tyrosine/ 100 mg wet wt/ hr)	856 ± 36[bc]	902 ± 34[d]	1157 ± 83	890 ± 56[d]	1447 ± 113[d]	1192 ± 116
Serum Cholin-esterase (μl CO_2/ml/hr)	1752 ± 103	1895 ± 61[c]	1579 ± 66	2036 ± 55[e]	2803 ± 295[e]	1770 ± 136
Kidney Succin-oxidase (μl O_2/100 mg wet wt/hr)	3620 ± 54	3691 ± 64	3578 ± 82	3710 ± 82	3728 ± 155	3308 ± 135

[a] Referred to as controls.

[b] Mean ± SE.

[c] Significantly different from control, $P < 0.005$.

[d] Significantly different from control, $P < 0.05$.

[e] Significantly different from control, $P < 0.001$.

activity of the 3-month, 6-month, restricted, BHT and vitamin E mice were not significantly different from those of the controls.

The TBA values expressed as MDA equivalents, for the *in vitro* formation of peroxides in brain homogenates, are shown in Table 11. The brain TBA values for the vitamin E mice were significantly lower ($P < 0.05$) than the controls. No other significant differences were observed in the brain TBA values for the various groups as compared to the controls.

Table 12 shows the effect of diet and age on the collagen content of the mice skins. The total collagen content, expressed as μg/mg skin (dry weight), of the control mice did not differ significantly from that of any other group.

The 3-month ($P < 0.001$), 6-month ($P < 0.001$) and restricted ($P < 0.01$) mice had a significantly higher percentage of soluble collagen than the controls. The percent soluble collagen was 84, 75 and 63 for the 3-month, 6-month and restricted mice, respectively, as compared to 58 percent for the controls. The BHT mice tended to have a higher proportion of soluble collagen, 62 percent, than the controls, but this difference was not significant. The percent soluble collagen in skin of vitamin E mice did not differ significantly from that of the controls.

TABLE 11

THE EFFECT OF AGE AND DIET ON THE IN VITRO FORMATION OF MALONYLDIALDEHYDE BY HOMOGENATES OF BRAINS OF AKR MALE MICE

MDA Equivalents	Basal			Restricted 12-Month	BHT 12-Month	Vitamin E 12-Month
	3-Month	6-Month	12-Month[a]			
n	14	12	12	12	8	10
Nanamoles/10 mg tissue	92 ± 5[b]	92 ± 4	91 ± 6	92 ± 4	89 ± 4	76 ± 3[c]

[a]Referred to as controls.

[b]Mean ± SE.

[c]Significantly different from control, $P < 0.05$.

TABLE 12

THE EFFECT OF AGE AND DIET ON THE COLLAGEN CONTENT OF SKIN OF AKR MALE MICE

	Basal			Restricted	BHT	Vitamin E
	3-Month	6-Month	12-Month[a]	12-Month	12-Month	12-Month
n	14	12	12	12	8	10
Total Collagen (μg/mg skin dry wt)	598 ± 20[b]	600 ± 25	620 ± 28	606 ± 27	582 ± 16	637 ± 27
Soluble Collagen (μg/mg skin dry wt)	499 ± 18[c]	452 ± 20[c]	356 ± 11	382 ± 16	362 ± 22	379 ± 10
(As percent of total)	84 ± 1.0[c]	75 ± 0.9[c]	58 ± 1.4	63 ± 1.0[d]	62 ± 4.0	60 ± 1.4

[a]Referred to as controls.

[b]Mean ± SE.

[c]Significantly different from control, $P < 0.001$.

[d]Significantly different from control, $P < 0.01$.

CHAPTER V

DISCUSSION

Effect on Longevity

The failure of BHT and vitamin E to significantly increase the life span of the mice would seem to be in contrast to the results of Harman (61, 62) and Comfort et al. (63) who reported that inclusion of free radical inhibitors in the diet of mice had a beneficial effect on their life spans. The 50 percent survival time of the control mice in the present study was 333 days as compared to that of only 234 days obtained by Harman (61) for AKR mice. Kohn (79) recently reported that the life span of C_{57}BL/6J mice could not be increased by BHT when the control mice exhibited optimal life spans. In a study on longevity of 22 inbred strains of mice, Storer (80) reported the mean life span of AKR male mice to be 326 days which compares favorably with the mean life span of 336 days for the controls obtained in this study. Thus the present findings are in agreement with those of Kohn.

The tendency for an increase in life span of the BHT mice might be explained by the slight degree of self-imposed feed restriction, 5 percent at 30 weeks of age, and the accompanying reduction in body weight, 9 percent at 30 weeks of age. This observation is strengthened by the finding in the present study that a moderate degree of feed restriction and consequent reduction in body weight, 33 percent and 19 percent, respectively, of the restricted mice at 30 weeks of age resulted in a significantly longer mean life span than that of the controls.

Other investigators (61, 62, 63, 79) have consistently found a depression in the body weight of mice when antioxidants were incorporated in their diets.

The procedure used to adapt the mice to the levels of BHT employed in this study minimized the early weight losses and deaths resulting from the incorporation of BHT in the diet as reported by Kohn (79). In preliminary studies in this laboratory, it was found that mice fed diets containing 0.33 percent BHT, without adaptation, would die within a week. Higher levels of dietary fat increased the toxicity of BHT. It is possible that the depression of feed intake and body weight together with the natural selection of long lived mice as a result of early deaths in other studies (61, 62, 79) could account for the seemingly beneficial effects of antioxidants on life span.

The possibility that feed restriction and a slowing down of growth rate are responsible for the tendency for BHT mice to live longer is strengthened by the facts that there was a significant negative regression of life span on growth rate and that there were no significant differences between the mean life spans of the four dietary groups after they were adjusted for growth rate. This supports the finding of Everitt and Webb (81) who showed that there was a significant negative correlation between the age at which maximum weight was attained and life span. Thus it would seem that the mechanism whereby feed restriction affected life span was by slowing down of the growth process.

Feed restriction also resulted in a significantly lower incidence of deaths due to leukemia as compared to the controls. This observation is in agreement with those of Ross and Bras (50) and Berg and

Simms (47, 48) who reported a decrease in the incidence of tumors in feed restricted rats. However very little work has been done to study the mechanism of this effect. It has been suggested by Ross that the inciting factor which can act as a determinant in the formation of tumors in later life may already be present in early life and the age at which it manifests itself in a target organ depends on the diet-sensitive rate of growth and development of the host. This explanation may very well hold true for the development of leukemia in AKR mice, in view of the recent report by Lowy et al. (82) that the full genome of the murine leukemia virus may be present in an unexpressed form in all cells of the AKR mouse strain.

The developmental stage of carcinogenesis is influenced by the mean mitotic activity of tissue (83). The mitotic activity has been observed to be inhibited by the caloric restriction in proportion to the degree of restriction (84).

The adrenocorticoid hormone, cortisone, has been reported to cause a "dramatic dissolution of lymphocytes" (85). Cortisone has also been shown to inhibit mitotic activity in primitive lymphoid cells. At physiological concentration in the normal animal, the inhibitory effect is thought to balance the local factors tending to stimulate lymphopoesis (86). Upton and Furth (87) confirmed this influence of cortisone on lymphoid leukemia development by showing that the chronic administration of cortisone reduced the incidence of leukemia in AKR mice. In view of the reports (88, 89, 90) that starvation and caloric restriction increase the output of the corticotropin releasing factor, corticotropin and plasma corticosterone in various species, it may be

postulated that the beneficial effect of chronic feed restriction on the development of leukemia, at least in part, is mediated through an increased secretion of cortisone in the feed restricted animals.

Effect on Enzymes

The significantly higher liver catheptic activity observed in the controls as compared to the 3-month and 6-month mice is in agreement with previous reports that catheptic activity increases with age (41, 42, 43). Ross (53) has reported that during aging the activity level of a number of hepatic enzymes such as catalase, histidase and ATPase increases whereas the level of alkaline phosphatase decreases. He further observed that these patterns could be altered by nutritional means to resemble those of a chronologically younger animal. Thus, since liver catheptic activity is known to increase with age the restricted mice in this study had a catheptic activity corresponding to that observed in the chronologically younger animals.

A marked increase in the catheptic activity of leukemic mice as compared to the non-leukemic mice was observed. Once leukemia develops, variable degrees of tumor infiltration may be observed in other organs such as the liver (64). Bowers (91) has reported that rat lymphoid tissue possesses a rather unusual type of lysosome, one rich in cathepsins, but relatively poor in other lysosomal enzymes. The increased catheptic activity in the leukemic mice may be due to the infiltration of the liver by lymphoid tissue in lymphatic leukemia.

Cholinesterase activity has been reported (40) to increase during the early part of life, plateau in adult life and decline during

senescence. The serum cholinesterase of the mice in this study exhibited a similar pattern. Feed restriction again delayed the age associated change in serum cholinesterase activity. The marked increase in both serum cholinesterase activity and the liver catheptic activity in the BHT mice may be a reflection of the reported ability of BHT to induce liver enzymes and the hypertrophy of the liver (92, 93). Although other workers (32, 33) have reported small decrements in kidney succinoxidase activity with age, neither age nor dietary manipulation had any significant effect on the activity of this enzyme in the present study.

Effect on In Vitro Lipid Peroxidation

Only the vitamin E mice had TBA values which were significantly lower than those of the controls. The TBA values are considered to depend on the balance of prooxidants and antioxidants in the tissue being tested (94). Wiss et al. (95) have shown that the concentration of tocopherol in various organs increased with an increase in the level of dietary α-tocopherol. This would indicate that in the vitamin E mice the high concentration of α-tocopherol in the brain was able to decrease the in vitro formation of lipid peroxides which resulted in lower TBA values. A similar effect of dietary α-tocopherol on TBA values for rat brain homogenates has been reported by Yoshikama and Herai (18).

The failure of BHT to cause a similar reduction in the TBA values may be explained in part by the results of Wiss et al. (95). These investigators showed that in rats given an oral dose of radioactive antioxidant (6, ethoxy-1, 2 dihydro-2, 2, 4 trimethyl quinoline) there was a rapid increase in the concentration of the compound in a number of

organs, maximum values being obtained within 30 minutes after dosing, with most of the dose being excreted in 24 hours. On the other hand, the increase in the α-tocopherol content of the various organs was much slower, but the levels remained high up to 96 hours after the dose. Distribution studies in the cell particles revealed that the synthetic antioxidant was predominantly present in the soluble fraction whereas about 80 percent of the α-tocopherol was located in the structural parts of the cell and especially concentrated in the mitochondria and microsomes. If a similar pattern of uptake and tissue distribution holds true for BHT, it is possible that the tissue levels of BHT are not maintained at the same level as α-tocopherol.

Effect on Collagen

The decrease in percentage of soluble collagen in skin with age observed in this study agrees with the observations of other workers (26, 27). Feed restriction delayed the rate at which collagen became insoluble. Everitt (57) has suggested that feed restriction may cause a decrease in the level of some metabolite or enzyme concerned in the cross-linking of collagen. In view of the report by Milch (28) that aldehydes arising in the course of normal metabolism were capable of cross-linking collagen, in vitro, this seems to be a plausible hypothesis.

On the other hand, it has been reported that as long as the animal is growing it will synthesize "young" or soluble collagen, the amount of soluble collagen being proportional to the growth rate rather than the chronological age (23). Therefore it is possible that feed restriction merely results in a slowing down of the growth process so

that the restricted animals are still growing and synthesizing new collagen when growth has virtually ceased in the controls. The tendency for the BHT mice to have a higher percentage of soluble collagen than the controls may also be a reflection of the depressed growth rate of the BHT mice.

The concentration of total collagen in skin did not change significantly with age. This is in agreement with the findings of Zika and Klein (22) for rat skins.

CHAPTER VI

SUMMARY

The effects of the addition of the free radical inhibitors α-tocopherol and butylated hydroxytoluene to the diet, and of feed restriction on selected age associated biochemical parameters and life span of AKR male mice were investigated. The 3- and 6-month old animals fed the basal diet ad libitum had a significantly lower liver catheptic activity and the 6-month old mice had a significantly higher serum cholinesterase activity as compared to the 12-month old controls. The percentages of acid soluble collagen in the skins of 3-month and 6-month old animals were markedly higher than that of the controls. No age associated changes were observed in the kidney succinoxidase activity nor in the in vitro formation of lipid peroxides by brain homogenates.

The mean life span of the mice fed the basal diet ad libitum was 336 days. Feed restriction increased the mean life span to 403 days and also decreased the incidence of deaths due to leukemia. The levels of liver catheptic activity and serum cholinesterase activity of the 12-month old restricted mice resembled those of the 3-month and 6-month old mice fed the basal diet ad libitum. Similarly the percentage of acid soluble collagen in the skin of restricted animals was comparable to that of chronologically younger animals.

The mice fed diets containing BHT exhibited a marked trend towards an increase in life span, a decreased incidence of leukemia and a higher percentage of acid soluble collagen as compared to the 12-month old mice fed the basal diet ad libitum. These non-significant

differences are considered to be due to the slight degree of self-imposed feed restriction and the concomitant lower body weights of these animals. The liver cathepsin and serum cholinesterase activities of the BHT mice were significantly higher than those of the controls.

Addition of vitamin E to the diet did not influence the feed intake nor the growth rate of the mice. The vitamin E mice resembled the controls in all parameters studied with the exception of the *in vitro* lipid peroxidation of brain homogenates. Significantly lower thiobarbituric acid values were observed for the brain homogenates of the vitamin E mice as compared to the controls.

The kidney succinoxidase activity was not influenced either by the feed restriction or by the addition of antioxidants to the diet. Regardless of diet the liver catheptic activity of leukemic mice was significantly higher than that of non-leukemic mice.

The BHT and restricted mice consumed less feed and were smaller in body size than the controls, the reduction in feed intake being 5 percent and 33 percent, respectively, and the reduction in body weight being 9 per cent and 19 percent, respectively, at 30 weeks of age. The life span of the mice was significantly influenced by the growth rate. In general a rapid growth rate was associated with a short life span. When adjusted for growth rates, the mean life spans of the controls, restricted, BHT and vitamin E mice did not differ significantly from each other.

Thus, dietary free radical inhibitors, *per se*, did not result in an increase in life span nor did they prevent the age associated changes in the biochemical parameters studied. Feed restriction increased the

mean life span, decreased the incidence of deaths due to leukemia and influenced the level of enzyme activity and the solubility of collagen such that they resembled the values obtained for the chronologically younger animals. On the basis of these observations it was concluded that dietary free radical inhibitors mediated their effect on life span *via* the mechanisms of feed restriction.

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APPENDIX

TABLE 13

ANALYSIS OF COVARIANCE FOR GROWTH RATE AND LIFE SPAN OF AKR MALE MICE

Source	d.f.	M.S.	F	P
Total	87			
Among treatments using adjusted mean life spans	3	2,397	1.00	N.S.
Regression life span on growth rate	1	127,773	17.83	0.005
Residual	83	7,165		

TABLE 14

LEAST SQUARES ANALYSIS OF VARIANCE FOR MEAN LIFE SPANS OF LEUKEMIC AND NON-LEUKEMIC AKR MALE MICE

Source	d. f.	M.S.	F	P
Total	85			
Leukemic vs. non-leukemic	1	48,981	6.19	0.01
Diets	3	9,866	1.25	N.S.
Interaction	3	269	1.00	N.S.
Residual	78	7,911		