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Dietary Restriction Alone and in Combination with Oral Ethoxyquin/2-Mercaptoethylamine in Mice

Steven B. Harris[1], Richard Weindruch[2], George S. Smith[1], M. Ray Mickey[3], and Roy L. Walford[1]

[1]Department of Pathology, Center for the Health Sciences, University of California at Los Angeles.
[2]Geriatrics Branch, National Institute on Aging.
[3]Department of Biomathematics, University of California at Los Angeles.

To investigate effects of dietary caloric restriction (DR) combined with antioxidant feeding, long-lived hybrid mice were divided into four dietary groups at weaning, and followed until natural death. Groups "C" and "R" received control (97 kcal/wk) and restricted (56 kcal/wk) diets respectively. Groups "C + αox" and "R + αox" received C or R diets supplemented with an antioxidant mixture (2-mercaptoethylamine plus ethoxyquin). R mice (mean life span 41 months) significantly outlived the other three groups (mean life span 30–34 months). Hepatic degeneration and increased hepatoma in the R + αox group suggested unusual hepatotoxicity of this regimen. Antioxidants had little effect on splenic cell mitogen response in similarly fed mice sacrificed at 12–15 months. Gompertz analysis suggests that the beneficial effect of DR may be due to reductions in initial vulnerability or rate-of-aging parameters, or both, and that the relative influence of each factor may vary with animal strain and DR protocol used.

DIETARY caloric restriction (DR) is the most effective known strategy for prolongation of life span (LS) in mammals (Weindruch and Walford, 1988; Masoro, 1988). Although not yet tested in primates,* DR decreases the total incidence, and retards the age-specific peak incidences of a variety of diseases in laboratory rodents. The positive effects of DR on not only average LS, but also maximum LS of many animal species suggests that DR may modify some basic process(es) responsible for aging (Walford et al., 1987). DR is able to increase maximum LS even in normally long-lived strains of rodents. In a previous study, for example, we observed prolongation of maximum LS from 35 months in ad libitum fed controls, to 53 months in restricted mice (Weindruch et al., 1986).

Another laboratory dietary strategy tested with moderate success in LS extension has been antioxidant supplementation. Ethoxyquin and 2-mercaptoethylamine (2-MEA) have increased the *average* LS of mice (particularly in short-lived strains with early incidences of diseases), but not *maximum* species-specific LS (Balin, 1986). The differential effect of DR and antioxidant supplementation on survival curves for long-lived strains of animals suggests that the beneficial mechanism(s) of action of these two treatments differs. Deliberate combinations of the two treatments have not been studied. To investigate the combined effects of DR and antioxidant supplementation, we measured LS, disease incidence, lymphocyte mitogen response, and natural killer cell activity in long-lived hybrid mice subjected to mild or severe DR, or mild or severe DR plus treatment with antioxidants (ethoxyquin plus 2-MEA). As in previous work, we employed purified diets in which dietary carbohydrate intake alone was severely restricted, but not dietary protein, fats, vitamins, or minerals.

**Editor's Note* — True, but see next article.

METHODS

Mice. — The same long-lived F_1 hybrid strain ("C3B10RF$_1$") used in previous work in this laboratory was studied (Weindruch et al., 1986). C3H.SW/Sn females were mated with C57B10.RIII/Sn males in our animal facility. Female progeny were weaned at 21–28 days of age, and individually caged in plastic cages on wood chip bedding under nonbarrier conditions (Weindruch et al., 1986). To monitor for infections, sentinel mice kept in the same room as experimental mice were screened every 6 months for antibody titers against 11 common pathogens. Positive titers were not found during this study.

Basal diets. — At weaning, mice were assigned to one of four dietary regimens. Mice on regimens C and C + αox ate control diet C (Table 1), a 20% casein purified diet. These mice were mildly restricted to about 80% of the ad libitum intake, receiving 97 kcal/wk (406 kj/wk), as 27 g per week of diet C. Mice on regimens R and R + αox ate restricted diet R, a 35% casein, vitamin- and mineral-enriched purified diet. These animals were restricted to 56 kcal/wk, as 16 g per week of diet R. The composition of diets C and R was chosen so that all animals ate very nearly the same weekly amounts of protein, fat, vitamins, and minerals, with differences only in carbohydrate (energy) and fiber intake (Table 1).

Four cohorts of 32 to 42 animals each were assigned to each of the four dietary regimens (Table 2) and studied for longevity and disease patterns. Four additional cohorts of 7–8 animals each were assigned to the same four regimens and studied for immunologic function. The four dietary regimens were: (1) C regimen mice received diet C at 97 kcal/wk; (2) R regimen mice, diet R at 56 kcal per week; (3) C + αox regimen mice, 97 kcal/wk of diet C to which had

Table 1. Composition of Diets

Ingredient[a]	Control Diet (C)[b]		Restricted Diet (R)[c]	
	g/kg of diet	g/mouse/wk	g/kg of diet	g/mouse/wk
Casein, vitamin-free test	200.0	5.4	350.0	5.6
Cornstarch	313.5	8.5	199.0	3.2
Dextrose	313.5	8.5	199.0	3.2
Corn oil	30.0	0.8	52.5	0.8
Mineral mixture, USP XIV[d]	60.0	1.6	110.0	1.8
Fiber	56.0	1.5	39.4	0.6
Vitamin mixture[d]	23.0	0.62	42.5	0.68
Brewer's yeast[d]	4.0	0.11	7.5	0.12
Zinc oxide	0.05	1.4×10^{-3}	0.1	1.6×10^{-3}

[a]These ingredients were purchased from ICN Pharmaceuticals (Cleveland, OH): casein (95.5–97.1% crude protein, catalog #904520), dextrose (#901521), mineral mixture (#902850), fiber (cellulose, #900453), vitamin mixture (#904654), and brewer's yeast (#103312). Cornstarch and Mazola corn oil were purchased locally. Zinc oxide was purchased from Sigma (St. Louis). Diets were mixed about once monthly in 20 kg batches using a Blakeslee CC80 mixer (Chicago, IL) and stored at 4°C until fed.

[b]Fed to group C mice at 27g/wk (4.0g daily on Mon. through Thurs. and 11 g on Friday providing ~97 kcal per week. Composition is given as g ingredient/kg diet. The per week intake of each ingredient for each mouse is also given. Feeding occurred between 7–9 a.m. All food was regularly consumed. The energy density of diet C was 3.6 kcal/gram.

[c]A purified diet enriched in casein, mineral and vitamin mixtures, brewer's yeast, and zinc oxide fed to group R mice as 16g/wk (3.5g daily on Mon. and Weds., 9g on Fri.) providing ~56 kcal/wk. Feeding occurred between 7–9 a.m. and all food was regularly consumed. The energy density of diet R was 3.5 kcal/gram.

[d]The compositions of the USP XIV mineral mixture, the ICN Vitamin Diet Fortification Mixture, and of ICN's brewer's yeast were previously reported (Weindruch et al., 1986).

Table 2. Intakes of Calories and Food Additives

Diet Group	kcals/wk	Additive	% of Diet	Dose/wk
C	97	—	—	—
R	57	—	—	—
C + αox	97	Ethoxyquin	0.25	68 mg
		2-MEA	0.60	162 mg
R + αox				
(0–72 weeks)	56	Ethoxyquin	0.40	64 mg
		2-MEA	1.00	160 mg
(72 weeks to end)	76	Ethoxyquin	0.40	86 mg
		2-MEA	1.00	216 mg

been added two antioxidants: 2.5 g/(kg diet C) of ethoxyquin (1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline [Santoquin™] Monsanto, St. Louis), and 6.0 g/(kg diet C) of 2-Mercaptoethylamine (2-MEA) hydrochloride (2-Aminoethanethiol HCl, Sigma, St. Louis); and (4) R + αox regimen mice, 56 kcal/wk of diet R to which had been added 4.1 g/(kg diet R) of ethoxyquin, and 10.1 g/(kg diet R) of 2-MEA. C + αox and R + αox mice initially ate the same weekly total dose of antioxidants. However, because of a number of early deaths in the R + αox groups, the food intake of these animals was liberalized to 76 kcal/wk, with Mon./Wed./Fri. feedings increased to 4.2/4.2/13.5 g. This diet change began at about 17 months of age, when 80% of the R + αox animals were alive, and resulted in a 35% increase in total antioxidant dose for this group over that for C + αox animals. This diet change also resulted in a 35% greater protein, vitamin, mineral, and fat intake for the R + αox group over the other three groups after 17 months.

Autopsy and histopathology. — All mice were checked daily for deaths. Dead mice were stored immediately at −15 to −20 °C until examined for tumors via gross autopsy. Abnormal appearing tissues were examined microscopically. A few animals (less than 10%) showed too much autolysis to be evaluated.

Immunological studies. — The smaller cohorts of 7–8 mice from each of the four diet regimens were killed at 12–15 months of age for immunologic studies. Splenocyte proliferative responses to the T-cell mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) were measured as previously described (Weindruch et al., 1986). Natural killer (NK) cytolysis of the YAC-1 lymphoma was measured by a standard ^{51}Cr release assay (Weindruch et al., 1983).

Statistical and Gompertz analysis. — Data were analyzed using a commercial statistics package ("CRUNCH Statistical Package," CRUNCH Software Corp., Oakland, CA) or programs written by us for the purpose. The particular statistical test used is given in each table. Significant differences shown by superscript denote those with $p < .05$. Both uncorrected and Bonferroni corrected p values were used in comparisons between groups whenever the correction makes a difference in the statistical significance of the result. Corrected p values are given in the text. Note that when an experiment involves comparisons between diet regimens that have been traditionally compared (i.e., C vs R, and C vs C + αox comparisons), the Bonferroni correction could be argued as being unnecessary. Thus, application of the full correction probably underestimates the significance of the results overall.

Gompertz analysis of life tables was performed using computer programs written by us for this purpose. The Gompertz equation for mortality may be expressed in linear form (Sacher, 1977):

$$\log f(t) = \alpha t + \log q_0 \quad (1)$$

where f(t) is the "fractional" mortality rate at time t, defined as the absolute mortality rate (negative derivative of the number of animals alive with respect to time, -dN(t)/dt) evaluated at time t, divided by the number of animals alive at time t, N(t). The constants α and q_0 are empirically derived mortality parameters which vary from population to population. Analysis of the life tables of the four study groups was carried out by dividing the first 90% of animal deaths in each group into five or six chronological cohorts of uniform size, then calculating an average fractional mortality rate at the time represented by the average LS of each cohort. (The longest-lived 10% of animals were omitted, since the Gompertz equation usually does not describe this part of the mortality curve well.) Logarithmic fractional mortality rates and associated times were then fit to the linear form of the Gompertz equation by least squares analysis.

RESULTS

Body weight. — Body weights are graphed in Figure 1. Control mice peaked in weight at 36 grams at about 18 months. Restricted mice reached a peak weight of 27 grams at about the same age. Groups fed control diets significantly outweighed groups fed restricted diets ($p < .001$) throughout most of life. Antioxidant-supplemented groups were significantly lighter ($p < .001$) than the corresponding nonsupplemented groups during the second year of life in the C groups, and until the third year of life in the R groups (numerical data not shown).

Tumor and other pathology incidence. — Influence of diet on overall tumor incidence is shown in Table 3. Tumor incidence was highest in the C + αox group (90%), but not significantly more so than in the C group. Both R and R + αox groups had a significantly lower tumor incidence than C + αox mice ($p < .001$ and $p < .01$, respectively). R mice dying with a tumor significantly outlived the other groups dying with tumor (C, $p < .02$; C + αox, $p < .05$; R + αox, $p < .10$). R mice dying without a tumor significantly outlived C and R + αox mice ($p < .03$ and $p < .001$, respectively).

Differential effects of diet on the incidence of some common tumor types, and survival of animals expressing them, are shown in Table 4. Tumors were categorized as lymphomas, hepatomas, or miscellaneous other soft tissue neoplasms (including hamartomas and hemangiomas). Lymphoma incidence was lowest (10–13%) in the R and R + αox groups, and for both groups differed significantly ($p < .001$) from that in group C + αox, which displayed the highest incidence (52%). The lymphoma incidence was not significantly different between C and R groups ($p < .15$). Hepatoma incidence was lowest in group C animals (10%), and was highest in group R + αox (42%) with the difference significant ($p < .03$). Conversely, the incidence of soft tissue tumors was significantly lower in group R + αox than in group C ($p < .03$). There was no statistically significant difference between groups in the survival times of animals with hepatoma or lymphoma. Group R mice with soft tissue tumors survived significantly longer than C ($p < .03$) or C + αox ($p < .06$) mice with this pathology.

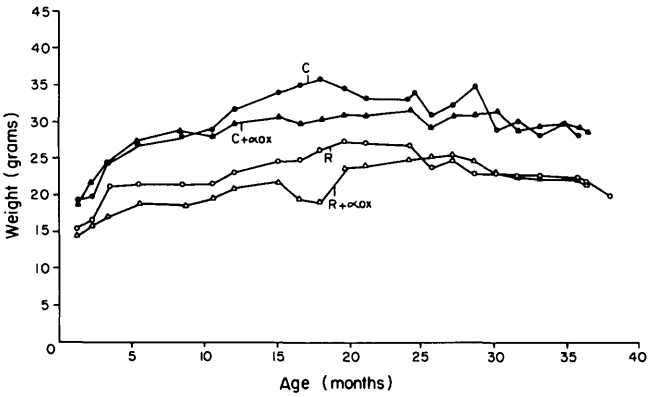

Figure 1. Influences of diet on body weight. Values shown represent average body weight in grams for all animals alive in each group at the indicated age. SEMs (not shown) were typically < 0.5 gram for R and R + αox mice, and < 1.0 gram for C and C + αox mice. The sharp increase in weights in the R + αox group at about 17 months follows an increase in R + αox ration from 56 to 76 kcal/week at that time.

Table 3. Influence of Dietary Restriction/Antioxidant Supplementation on Tumor Incidence and Mean Life Span (LS)

		Mice With a Tumor		Mice With No Tumor	
		Incidence[b]	Mean LS[c]	Incidence[d]	Mean LS[c]
Group	n[a]	%	mo	%	mo
C	35	$^{B}74^{AB}$	32.0 ± 1.1^{A}	26	35.1 ± 1.9^{A}
R	42	$^{A}45^{A}$	$39.0 \pm 1.8^{B*}$	55	41.9 ± 1.3^{B}
C + αox	31	$^{B}90^{B}$	33.7 ± 1.0^{A}	10	40.4 ± 2.0^{AB}
R + αox	31	$^{A}51^{A}$	$33.1 \pm 2.1^{AB*}$	49	32.5 ± 2.2^{A}

[a]Number of animals in life span cohorts, excluding animals not suitable for autopsy (< 10%).

[b]Significance of differences in tumor incidence was evaluated by Yates-corrected chi square (or Fisher's exact test for expected frequencies < 5) testing between each pair of groups (6 tests). Values in each column not sharing superscripts (upper case letter) are significantly different ($p < .05$). Superscripts preceding values are uncorrected. Superscripts following values are based on Bonferroni corrected p values. The minimum detectable % difference in tumor incidence (at corrected $p < .05$) under these conditions is approximately 35%.

[c]Values are means ± *SEM* in months. Statistical significance of differences in mean life span between groups was evaluated by Mann-Whitney rank sums testing between each pair of groups (total of 6 comparisons within 4 groups). Means in each column not sharing superscripts are significantly different (Bonferroni corrected $p < .05$).

[d]Statistical comparison results for this column are identical to those for tumor incidence.

*Significant at $p < .10$.

In addition to the high incidence of hepatoma, hepatocellular damage was noted in the R + αox group, with focal areas of hepatic necrosis and regeneration often seen. No evidence of hepatocellular damage or degeneration was noted in the other groups.

Immunological results. — Splenic lymphocyte proliferation induced by PHA or ConA, and NK cytolytic capacity, were measured in midadult (12–15 month) mice from the four diet groups (Table 5). For both mitogens, response was

Table 4. Influence of Dietary Restriction/Antioxidant Supplementation on Tumor Incidence and Mean Life Span (LS) of Mice with a Particular Tumor Type

		Lymphoma		Hepatoma		Soft Tissue	
Group	n[a]	Incidence[b] %	Mean LS[c] mo	Incidence[b] %	Mean LS[c] mo	Incidence[b] %	Mean LS[c] mo
C	35	*^{A}33^{BC}	^{A}28.6 ± 1.5^{A}	^{A}10^{A}	^{A}33.8 ± 2.5^{A}	*^{A}38^{A}	^{A}33.3 ± 1.4^{A}
R	42	^{B}10^{A}	^{B}38.8 ± 3.9^{A}	^{AB}21^{AB}	^{A}36.4 ± 2.8^{A}	*^{AB}19^{AB}	^{B}42.9 ± 2.7^{B}*
C + αox	31	^{A}52^{B}	^{AB}33.0 ± 1.5^{A}	^{A}16^{AB}	^{A}36.6 ± 1.3^{A}	^{A}26^{A}	^{A}34.2 ± 1.5^{AB}*
R + αox	31	*^{B}13^{AC}	^{AB}22.8 ± 6.2^{A}	^{B}42^{B}	^{A}36.1 ± 1.0^{A}	^{B}6^{B}	^{AB}38.1 ± 3.9^{AB}

[a]Number of animals in life span cohorts, excluding animals not suitable for autopsy (< 10%).

[b]Significance of differences in tumor incidence was evaluated by Yates-corrected chi square (or Fisher's exact test for expected frequencies < 5) testing between each pair of groups (6 tests). Values in each column not sharing superscripts (upper case letters) are significantly different ($p < .05$). Superscripts preceding values are based on uncorrected p values. Superscripts following values are based on Bonferroni corrected p values. The minimum detectable % difference in tumor incidence (at corrected $p < .05$) under these conditions is approximately 35%.

[c]Mean life span (± *SEM* in months) for mice presenting with the indicated tumor at autopsy. Statistical significance of differences in mean life span between groups was evaluated by Mann-Whitney rank sums testing between each pair of groups (total of 6 tests). Means in each column not sharing superscripts are significantly different ($p < .05$). Superscripts preceding values are based on uncorrected p values. Superscripts following values are based on Bonferroni corrected p values.

*Significant at $p < .10$.

Table 5. Influence of Dietary Restriction/Antioxidant Supplementation on Splenic T-Cell Proliferation and Natural Killer Cell Activity in 12–15 Month Old Mice.[a,b]

Group	Cells/spl. × 10^6	PHA[c] cpm	ConA[c] cpm	NK[d] %
C	93 ± 4	^{AD}23,000 ± 5,550^{A}	^{A}71,600 ± 13,600^{A}*	^{A}28 ± 4^{AC}
R	50 ± 8	^{BC}60,300 ± 12,600^{AB}	^{B}128,000 ± 22,600^{A}	^{B}15 ± 3^{BC}*
C + αox	68 ± 6	^{BD}38,900 ± 8,290^{AB}	^{AB}110,000 ± 22,000^{A}	^{A}32 ± 4^{A}*
R + αox	26 ± 5	^{C}73,900 ± 16,800^{B}	^{B}185,000 ± 48,300^{A}*	^{B}11 ± 2^{B}

[a]Values are mean ± *SEM* for immunological cohorts, n = 7–8 mice.

[b]Statistical significance of differences in between mean values for each group was evaluated by Mann-Whitney rank sums testing between each pair of groups (total of 6 tests). Means in each column not sharing superscripts (upper case letters) are significantly different ($p < .05$, unless otherwise indicated). Superscripts preceding values are uncorrected. Superscripts following values are based on Bonferroni corrected p values.

[c]Mitogen stimulation was measured as cpm of ^{3}H-thymidine uptake, corrected for unstimulated uptake.

[d]NK activity data are expressed as percent specific lysis of YAC-1 targets in a 4-hr ^{51}Cr release assay at an effector:target ratio of 100:1.

*Significant at $p < .10$.

greater in R + αox mice than C mice (PHA $p < .05$; for ConA $p < .06$). There was no significant difference between C and R mice, although $p = .12$ for the PHA response. NK activity was significantly decreased by DR in several comparisons (R + αox vs C, $p < .03$; R + αox vs C + αox, $p < .02$; R vs C + αox, $p < .10$), but addition of antioxidants had no clearcut effect.

Longevity. — The longevity of mice in the four diet regimens is shown in Table 6 and Figure 2. Mortality was high in group R + αox initially, but this was corrected by increasing the food intake of this group after 17 months of feeding. The mean LS of R animals (40.5 months) was significantly longer than that of any other group ($p < .001$ for all comparisons with other groups), but mean LS did not differ significantly among the other groups. The maximum LS was greatest for group R at 47.8 mo ($p < 0.1$ for all comparisons with other groups).

Gompertz analysis of C and R life tables from the present study (Figure 3) shows that a linear model is a relatively poor one for the increase in mortality seen with aging in the R group ($r = .84$). In this group, mortality rates rise toward the end of the LS much faster than the Gompertz equation would predict.

Discussion

Average life spans and overall tumor incidences of C and R animals were similar to those of corresponding groups of a previous study with the same strain of mice (Weindruch et al., 1986), although C and R animals in the present study received slightly more calories than in the earlier study, along with moderate compositional changes in the purified diet. However, maximum LS (10th decile) for corresponding groups was significantly longer in the earlier study, and body weights were lower (data not shown). The present study attempted to balance the fat intakes of control and restricted mice (5.25% fat for C diets, 3.0% for R) and used glucose as the dietary sugar. By contrast, Weindruch et al. (1986) fed higher fat content diets (13.5% corn oil) utilizing sucrose as the dietary sugar. Fiber contents of past and present diets also differ slightly. As in our earlier study, DR tended to lower the overall incidence of tumors, with the least effect being on hepatoma. However, in the present study the beneficial effect of DR on total tumor incidence in each group did not reach significance except in comparison with group C + αox, which had a very high incidence (90%) of tumors.

The antioxidants used in this study have each been reported to extend average LS in mice. Earlier tests of the free

Table 6. Influence of Dietary Restriction/Antioxidant Supplementation on Longevity[a,b]

Group	n[c]	Range	Mean Life Span[d]	Maximum Life Span 10th Decile[e]	Median Life Span[f]
C	42	18.7–43.1	33.1 ± 1.0[A]	42.1 ± 0.6[A]	32.4
R	39	21.9–48.6	40.5 ± 1.1[B]	47.8 ± 0.4[B]	43.9
C + αox	32	16.2–42.6	33.9 ± 1.1[A]	41.4 ± 0.6[A]	35.7
R + αox	35	0.7–43.0	29.9 ± 2.0[A]	41.5 ± 0.6[A]	34.3

[a]Values are in months. Means are followed by *SEM*.

[b]Statistical significance of differences between groups was evaluated by Mann-Whitney rank sums testing of each pair of groups (total of 6 comparisons within 4 groups).

[c]Number of animals in life span cohorts.

[d]Mean life span values not sharing a common superscript (upper case letters) were significantly different ($p < .05$) after Bonferroni correction of p values for the number of comparisons. The minimum detectable difference (corrected $p < .05$) for mean life span (under these conditions of group size and distribution) is approximately 6 months in comparing R + αox to other groups, and approximately 4 months in comparing C, R, and C + αox to each other.

[e]Maximum life span values not sharing a common superscript were significantly different at the level of $p < .10$ after correction for the number of tests. The minimum detectable difference for maximum life span (corrected $p < .10$) in groups of this size and distribution is approximately 3 months.

[f]Equal to 50th percentile of survival.

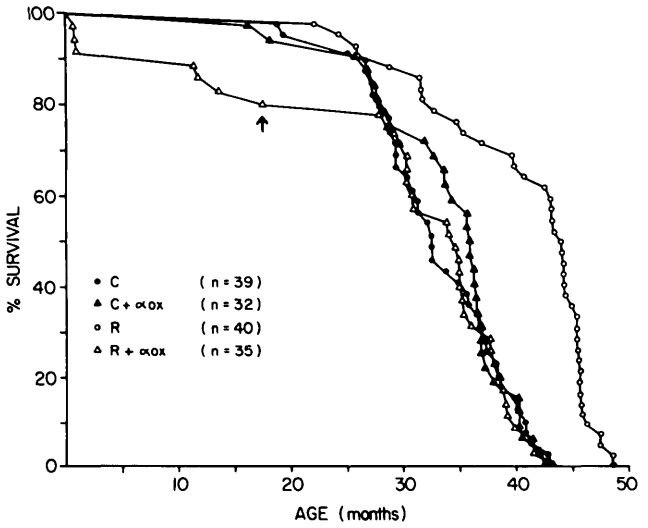

Figure 2. Influences of diet on mouse survival. Each symbol represents a single death. The arrow indicates the time at which caloric intake in the R + αox group was increased from 56 to 76 kcal/wk.

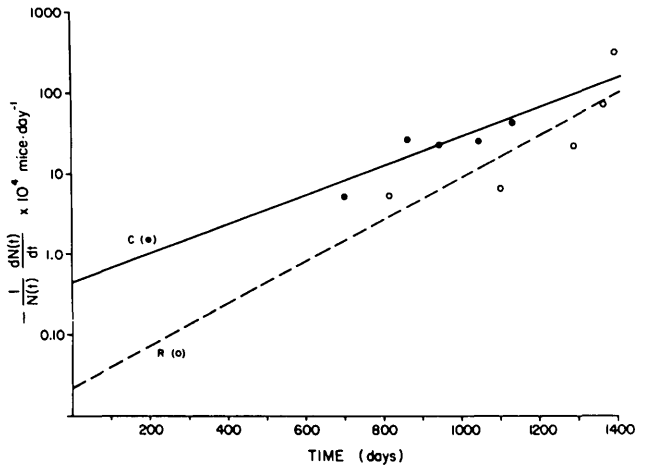

Figure 3. Gompertz analysis of natural mortality in groups C and R. The average fractional decrease (per day) in the size of the groups due to mortality is plotted on the ordinate. Fractional mortalities at the highest ages in the R group rise much more sharply than for C, as indicated by the more rectangular survival curve for the R group in Figure 2.

radical theory of aging by Harman (1957; 1961; 1968a; 1968b) fed 2-MEA in doses of 0.5% or 1.0% of the diet, and the 1.0% regimen was found to sometimes increase average LS (but not maximum LS) in short-lived C3H, AKR, and LAF_1 mice, but not in longer-lived Swiss mice. Kohn (1971) detected no effect of 1.0% dietary 2-MEA on longevity in the relatively long-lived strain C57BL/6J.

The synthetic antioxidant ethoxyquin has been tested as a potential life extension agent only in relatively short-lived strains of mice. Comfort et al. (1971) observed an increase both in maximum LS (from 24 to 30 months) and average LS in C3H mice fed 0.5% ethoxyquin, and Harman (1968b) reported increased average LS in LAF_1 mice fed 0.25% ethoxyquin. We observed no statistically significant increase in average or maximum LS in hybrid mice simultaneously given 0.6% 2-MEA and 0.25% ethoxyquin in a diet restricted to 80% of ad libitum ($p = .5$). (However, a trend toward rectangularization may be indicated by the fact that a much bigger difference between C and C + αox median LS is seen than in mean LS.) Previous "tests" of the free radical theory of aging by feeding of dietary antioxidants have emphasized positive results in modification of LS. In contrast, negative results have resulted in modifications of the basic theory that now potentially make it nearly "data proof" at the level of simple animal feeding and mortality studies. For example, Cutler (1984) has proposed that dietary antioxidants may disrupt natural antioxidant defenses, with a net zero (or even deleterious) effect on aging.

To our knowledge, the effect of deliberate combination of energy restriction and antioxidant supplementation upon mammalian LS has not been reported, although some workers, e.g., Comfort et al. (1971), observed concomitant weight loss in antioxidant-fed ad libitum animals (reviewed by Schneider and Reed, 1985). Pair-feeding studies have suggested that for ethoxyquin, this weight loss is due to reduced food intake (Rudra et al., 1974). We observed weight loss with antioxidant feeding in the present study, despite the use of restricted regimens in which test animals appeared to consume all food given them. (Formal food wastage studies were not done, however.)

In our hands, despite lower doses of both additives than had been used in many previous studies, a combination of

DR and ethoxyquin/2-MEA proved detrimental compared with DR alone, especially during an initial study period where the total weekly dose of antioxidant was not different between R + αox and C + αox groups. During this initial high mortality period, the dose of antioxidant per unit body weight was 50–70% higher in the R + αox group than the C + αox group, due to the smaller size of the former animals. Excess mortality in the R + αox group decreased when the food ration (and therefore weekly antioxidant dose) for this group was increased during the latter part of the study, suggesting that the untoward effect of these antioxidants was influenced more by energy restriction than antioxidant dose.

Although R + αox mice exhibited mortality rates nearly identical to the C group during the latter part of the study, their pathology was quite different, with soft tissue tumors and lymphoma in the C group being replaced by hepatoma in the R + αox group. For hepatoma and soft tissue tumors, these differences were statistically significant ($p < .03$).

Both the unique finding of hepatocellular degeneration and the increased incidence of hepatoma in the R + αox group are consistent with hepatic toxicity of this regimen. The R + αox findings are unexpected from previous toxicologic experience with these dietary additives. Ethoxyquin is concentrated and metabolized in liver and kidney (Skaare and Nafstad, 1979), and although a few hepatic lesions have been noted in two chronic toxicological studies of this compound (Wilson and DeEbs, 1959; Vettorazzi, 1977), most often ethoxyquin-associated lesions are limited to the kidney (Manson et al., 1987; Rudra et al., 1974; Takahashi et al., 1986; Tsuda et al., 1984). Ethoxyquin is not generally held to be hepatocarcinogenic, however, and actually inhibits hepatoma formation in a number of chemical carcinogenesis models (Kensler et al., 1986; Rao et al., 1984). Complete chronic toxicological data are lacking for 2-MEA. Although it has been associated with aortic aneurism and gastrointestinal ulcer in animals (Szabo and Pihan, 1987; Jayaraj, 1983), hepatotoxicity has not been reported. Thus, although the combination of dietary antioxidants and DR used in this study led to liver damage and liver tumor, the pathophysiology remains obscure. The specific compound(s) used in this study might be toxic under DR, or may exhibit hepatotoxicity only at the modestly increased doses fed to the R + αox mice (Table 2). It is possible that toxicity under DR may be a generic property of antioxidants. Studies with other antioxidants combined with DR are needed to clarify this issue.

Our immunologic results agree with prior ones on DR in this strain, where underfeeding raised the lymphocyte proliferative response (Weindruch et al., 1983; 1986) and lowered basal levels of NK activity (Weindruch et al., 1983). Antioxidant-fed mice tended to show higher mitogen-induced proliferation than other groups, but the effect did not reach significance. There was no effect of antioxidants on NK response. In a previous study by Harman et al. (1977), long-term ad libitum feeding of 0.25% ethoxyquin increased splenic ConA response in C3HeB/FeJ mice, and short-term feeding of 0.5% 2-MEA raised humoral immunity.

Gompertz analysis of the C and R life tables gave results somewhat less clear-cut than analysis of data from a previous study with this same mouse strain, where roughly similar amounts of energy restriction (from 85 kcal/wk down to 50 kcal/wk) decreased "rate of aging" mortality ("α slope") but increased the initial vulnerability, q_0. A further decrease to 40 kcal/wk lowered mortality rates in middle age but not at extreme age, giving a greater α slope, a lower q_0 (Figure 4), and thus a "squarer" mortality curve (Weindruch et al., 1986). In this study and the present study, however, sample sizes were too small for a definitive assignment of the decreased mortality in restricted animals to decreases in α slope, q_0, or to both. Two additional studies at this laboratory using C57BL/6J (Cheney et al., 1980) and C3B10RF$_1$ mice (Cheney et al., 1983) found that the effect of DR on late (presumably age-related) mortality may be assigned statistically solely to decreases in q_0.

Sacher (1977) proposed that, for rats, DR decreases mortality by decreasing the α slope age-dependent rise in mortality, an effect that was proposed to override a DR-mediated increase in q_0. Merry and Holehan (1985), in analyzing their own data and data from studies of rats by Ross (1959) and Yu et al. (1982), confirmed Sacher's proposal, although statistical analyses were not shown. In contrast, Lang et al. (1989) found a delay in onset of mortality, but no change in the rate of age-related mortality increase in DR rats, corresponding to maximum LS increase via a decrease in q_0 alone. Studies of hybrid mice at our laboratory have invariably shown greater "squaring" of mortality curves of DR mice, suggesting that here also DR may be favorably influencing Gompertz q_0. Harrison and Archer (1987) have found suggestions of differential effects of strain upon Gompertz parameters during DR in mice.

Although the postulated relationship of Gompertz parameters to physiologic capacity varies with the aging theory under consideration (Strehler, 1977), most theories relate the Gompertz α slope to the age-dependent rate of decline of

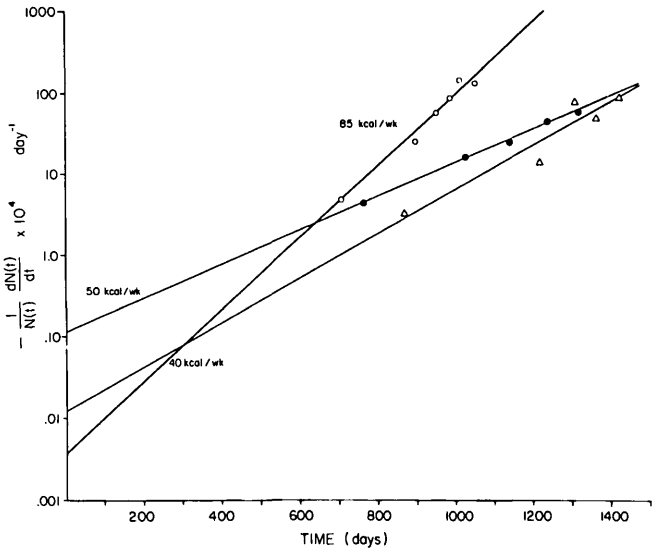

Figure 4. Gompertz analysis of natural mortality in three groups of mice from a previous study (Weindruch et al., 1986). The average fractional decrease (per day) in the size of the groups due to mortality is plotted on the ordinate. The increased life span of animals fed 40 kcal/wk, as opposed to those fed 50 kcal/wk, is a result of lower fractional mortality at earlier ages only. This is opposed to the effect of lowering intake from 85 kcal/wk to 50 kcal/wk, which decreases fractional death rate at all ages.

physiologic capacity of the animal. Both time-dependent physiologic decline and increasing mortality are considered measures of aging. Thus, a consistent finding of life extension in some models of DR without alteration of Gompertz α might be interpreted to mean that in these cases the rate of age-related physiologic decline is not slowed, but instead delayed in time of onset by DR. Another possibility is that the rate of physiologic decline with age may indeed be slowed by DR, but that this happens somehow primarily at younger ages where it does not affect immediate mortality (because of the higher physiologic capacity of young organisms), and therefore has little or no effect on the classical Gompertz α slope.

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Address correspondence to Dr. Steven B. Harris, Department of Pathology, Center for the Health Sciences, UCLA, Los Angeles, CA 90024.

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