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The Effects of Diet, *ad Libitum* Feeding, and Moderate and Severe Dietary Restriction on Body Weight, Survival, Clinical Pathology Parameters, and Cause of Death in Control Sprague-Dawley Rats

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A 2-year study was conducted in Sprague-Dawley rats to compare the effects of *ad libitum* (AL) feeding and dietary restriction (DR) on body weight, survival, cause of death, and clinical pathology parameters. Three groups of 120 rats/sex each received the following daily rations of a maintenance rodent diet: *ad libitum* (AL group); 75% of adult AL food consumption (25% DR group); and 45% of adult AL food consumption (55% DR group). Among the 3 groups, there were generally no differences in relative (food intake per gram of body weight) food consumption. Compared to the AL group, decreased body weight gain occurred in DR groups and was associated with an increase in survival proportional to the DR rate. The main cause of death was pituitary adenomas in all groups. Decreases in total leukocyte, segmented neutrophil, lymphocyte, and platelet counts occurred in the 55% DR group. In serum biochemistry, there were decreases in total protein, albumin, total and HDL cholesterol, and total calcium, and increases in alkaline phosphatase activities and chloride in 55% DR females, as well as decreases in triglycerides in the 55% DR group and in 25% DR females. Results of urinalyses showed decreases in urine volume and protein, and increases in urinary pH in both DR groups. In conclusion, a DR rate of approximately 25% appears to be appropriate for Sprague-Dawley rats in toxicity and carcinogenicity assays to improve survival without impairing growth and routine clinical pathology parameters.

Key Words: **Sprague-Dawley rat; dietary restriction; body weight; survival; cause of death; clinical pathology.**

In rat carcinogenicity studies, regulatory agencies generally expect an overall, minimal survival of 50%, or 25 animals/sex/group at the end of the 2-year study period. However, over the past 3 decades, a decline in laboratory rat survival has been seen in most rat strains, including Sprague-Dawley (SD), Fischer 344, Wistar, and Long-Evans rats (for reviews see Keenan *et al.*, 1995a,b; Nohynek *et al.*, 1993), very commonly used strains in toxicity and carcinogenicity studies.

While both genetic and environmental factors are involved, rat survival can be improved by simple dietary restriction (DR) (for reviews see Weindruch, 1996). Indeed, DR is the most efficient and convenient means to intervene in aging and disease outcomes (for reviews see Holehan and Merry, 1986; Masoro *et al.*, 1991a,b). In addition, DR appears to be one logical solution, since the decrease in life span of laboratory rats has coincided with a trend to obesity of the affected strains (for reviews see Rao *et al.*, 1990). However, moderate DR is known to prevent or delay both spontaneous and carcinogen-induced tumors (for review see Keenan *et al.*, 1996). Therefore, it seems necessary for each strain and stock of rats to determine an appropriate rate of DR that will increase survival and will not substantially modify other biological parameters.

With this objective in mind, a 2-year study was conducted in SD rats from Charles River France, given either *ad libitum* (AL) feeding or 2 different dietary-restricted regimens, approximately 75% (25% DR) and 45% (55% DR) of adult AL food consumption. This paper presents data showing the relation between the rate of food intake and the increased survival rate and decreased incidence of causes of death, as well as the effects of food regimens on routine clinical pathology parameters in this stock of SD rats.

MATERIALS AND METHODS

Test Animals

Three-hundred and sixty male and 360 female SD, Crl:CD®(SD)BR (International Standard) rats were obtained from Charles River France (Saint-Aubin-lès-Elbeuf, France). They were received and maintained throughout the study under VAF (virus antibody free) pathogen status. The animals were 35 days old at study initiation, with males weighing 104–195 grams and females 104–180 grams. The rats were housed based on a random allocation scheme, in individual suspended stainless steel wire cages, in air conditioned rooms with a temperature of approximately 22°C, humidity between 30 and 70%, and a 12-h light cycle; they had free access to tap water. The animals were identified by tail tattoos and assigned to the 3 different regimen groups described below in order to get similar initial mean body weights (BW) across study groups.

Diet and Dietary Regimens

The experimental groups contained 120 rats/sex/group. All rats were given certified UAR AO4C rodent diet from UAR (Villemoisson sur Orge, France).

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TABLE 1
Feeding Regimens

Study groups	Feed (g/day)				Physiological energy (kcal/day)
	Total amount	Protein	Fat	Carbohydrate	
AL group					
Males	31.9	5.0	1.0	19.4	106.5
Females	21.9	3.4	0.7	13.3	73.1
25% DR group					
Males	24.0 [75.2%]	3.8	0.7	14.6	80.2
Females	17.0 [77.6%]	2.7	0.5	10.3	56.8
55% DR group					
Males	14.0 [43.9%]	2.2	0.4	8.5	46.8
Females	10.0 [45.7%]	1.6	0.3	6.1	33.4

Note. Amounts are mean values. For AL group, average of mean values for food consumption are given. For DR groups, percent of AL food consumption is given in brackets.

The mean diet composition over the 106 study weeks (SW) was 15.7% protein, 3.1% fat, and 60.7% carbohydrate, and contained 3.34 kilocalories (kcal)/gram of physiological energy (PE), calculated using Atwater physiologic fuel values. The feeding regimens, summarized in Table 1, were as follows:

AL group: rats were fed *ad libitum*.

25% DR group: rats were given daily measured amounts of diet (approximately 75% of adult SD rat AL daily food consumption), approximately 24 g/day for males and 17 g/day for females.

55% DR group: rats were given daily measured amounts of diet (approximately 45% of adult SD rat AL daily food consumption), approximately 14 g/day for males and 10 g/day for females.

Study Design

This was a 2-year study, with 14-, 29-, and 53-week interim necropsies and a 106-week terminal necropsy. Before study initiation, 20 rats/sex/group were selected to be euthanized at each interim necropsy; all remaining surviving rats were euthanized at terminal necropsy. The protocol was approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories (MRL; West Point, PA, USA). To mimic handling and drug administration throughout carcinogenicity studies done by oral gavage in MRL, rats were given daily, by oral gavage, 5 ml/kg BW of 0.5% aqueous methylcellulose, prepared from methylcellulose 400 cps (Methocel A4C Premium; Colorcon, Bougival, France) in deionized water. The parameters we monitored during the study are presented below.

Antemortem observations. All animals were observed daily for general appearance, behavior, signs of morbidity, and mortality. They were weighed pretest, once in SW 1, twice a week from SW 2 to SW 13, and once a week from SW 14 to SW 104. In the AL group, food consumption (FC) was generally measured once a week, on 15 rats/sex, and over a 3-day period, up to SW 102. In DR groups, FC was measured generally once a week, on 15 rats/sex/group, and over a 24-h period, up to SW 14; from SW 15, FC was observed twice a week at the time of dosing of each rat.

Hematological and serum biochemical determinations were performed after an overnight fast, in SW 4, 8, and 11, on the 20 rats/sex/group to be euthanized at the 14-week interim necropsy; in SW 27 on the 20 rats/sex/group to be euthanized at the 29-week interim necropsy; in SW 39 and 51 on all surviving rats to be euthanized at the 53-week interim necropsy; and in SW 79 and 103 on 19–20 rats/sex/group to be euthanized at final necropsy. Blood samples were drawn from the orbital sinus under ether anesthesia into EDTA-treated tubes (hematology) or serum separator tubes (serum biochemistry). Hematological parameters included red blood cell (RBC) count, hemoglobin (Hb) concentration, hematocrit (Hct), mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet count (PLT), and white blood cell count (WBC) (Coulter Counter Model S Plus IV H.D.; Coultronics France, Margency, France). Leukocyte differential counting was obtained from microscopic examination ($\times$ 1000) of blood smears stained using the Hematek staining system (Ames, Puteaux, France), according to a modified Wright's procedure. Serum biochemical parameters included glucose, urea nitrogen, creatinine, total protein, albumin, high density lipoprotein (HDL) cholesterol, total cholesterol, triglycerides, activities for aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase (ALK PHOS), and creatine kinase, and levels of sodium, potassium, chloride, phosphorus, and total calcium (Hitachi 717-6000 04–07; Roche Diagnostics, Meylan, France). Urinalyses were performed at the same time points as listed above for hematological and serum biochemical parameters on half of the corresponding animals, placed in their own cages overnight for sampling. Analyses included quantitative determination of urinary volume (urine weight determination) and specific gravity (Atago refractometer; Bioblock, Illkirch, France), semi-quantitative determination of pH, protein, glucose, bilirubin, occult blood, ketones, and urobilinogen (Urotron RL9 with Combur 9 test RL Teststrips; Boehringer, Meylan, France), and microscopic examination ($\times$ 100 or 400) of sediments.

Postmortem observations. Each interim necropsy was conducted on 20 rats/sex/group; all remaining surviving rats were euthanized at terminal necropsy. Prior to scheduled necropsy, rats were fasted overnight; they were euthanized by exsanguination while under carbon dioxide anesthesia and underwent a complete routine necropsy. A complete necropsy was also done on all rats dying spontaneously or sacrificed moribund. Terminal BW, and weights of major organs and mammary masses were recorded from all rats. Samples of most tissues, including all gross changes, were fixed in neutral, 10% formalin. The testes and epididymides were fixed in Bouin's fixative. Representative sections of most tissues were prepared by routine methods and stained with hematoxylin and eosin for microscopic examination, which included salivary glands, stomach, small intestine, large intestine, liver, pancreas, adrenals, pituitary, thyroids and parathyroids, kidneys, urinary bladder, ovaries, uterus, testes and epididymides, prostate, skin, mammary gland, heart, lung, spleen, lymph nodes, thymus, bone and bone marrow, skeletal muscle, brain, spinal cord, sciatic nerve, eyes with optic nerve, Harderian gland, and any gross lesions. The diet-related histopathological changes will be reported in further publications.

Statistics

Statistical analyses were performed on results from determinations of hematological and serum biochemical parameters, and urinary volume and specific gravity. Data were analyzed for homogeneity of variance by Levene test

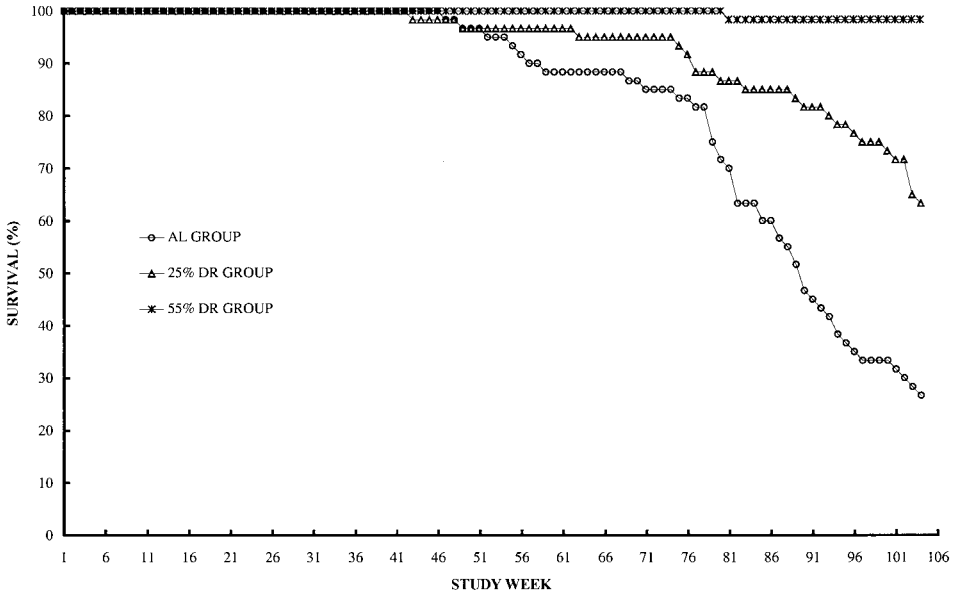

FIG. 1. Mean survival in male SD rats.

(Levene, 1961), normality by Wilk & Shapiro *W* statistics (Shapiro and Wilk, 1965, 1968; Wilk and Shapiro, 1968), and statistical significance at $p \leq 0.05$ was based on an ANOVA, using a trend test. If criteria for homogeneity and/or normality were not met, data were examined after Rankit transformation (Harter, 1961) followed by an ANOVA.

RESULTS

Survival and Cause of Death

Survival in both sexes was directly related to the amount of food consumed (Figs. 1 and 2). At study termination (Table 2), the percent survival in the 55% DR, 25% DR, and AL groups was 98, 57, and 22%, respectively, for males, and 98, 58, and 37%, respectively, for females. In the AL and 25% DR groups, mean survival generally showed a similar decline from SW 47 to SW 80 when mortality dramatically increased in both sexes from the AL group. In this AL group, 50% survival was achieved in SW 88 in males and in SW 91 in females. In the 55% DR group, a single death occurred in males in SW 81 and in females just before final necropsy.

Within the first study year (Table 2), accidental deaths occurred in approximately 7% of the AL and 25% of the DR groups due to failures to recover from ether anesthesia. Causes and incidences of spontaneous deaths are summarized in Table 3. In all groups, the main causes of death were neoplasms that

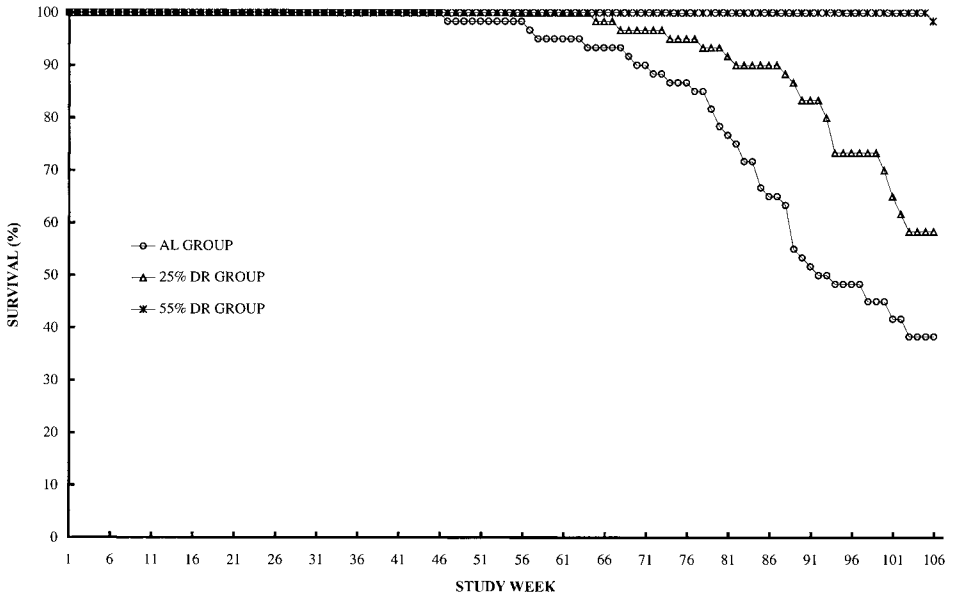

FIG. 2. Mean survival in female SD rats.

TABLE 2
Mortality and Survival Rate at Study Termination

Study groups	Percentage incidence		
	Accidental deaths	Spontaneous deaths	Survival rate
AL group			
Males	5.0	73.3	61.7
Females	1.7	21.7	36.6
25% DR group			
Males	6.7	36.7	56.6
Females	0	41.7	58.3
55% DR group			
Males	0	1.7	98.3
Females	0	1.7	98.3

accounted in the 55% DR, 25% DR, and AL groups for 2, 28, and 48%, respectively, in males and 2, 37, and 57%, respectively, in females, of all deaths. These tumors were benign in approximately 70% of the cases and consisted mainly of pituitary adenomas. Malignant and metastatic tumors were observed only in rats from the AL and 25% DR groups. Besides tumors, renal lesions were lethal in 3% of the AL males and in 2% of all the 25% DR rats. In addition, in the AL group only, a few deaths were attributed to liver necrosis (5%), non-neoplastic pituitary lesions (2%), and heart lesions (2%).

Body Weight and Food Consumption

Throughout the study, rats fed AL had a higher BW than those fed a restricted diet (Figs. 3 and 4). Mean BW values before each necropsy are presented in Table 4. At these time points, compared to AL-fed rats, decreases in mean BW gain were observed in males and females from the 25% DR (–30 to –36% and –39 to –57%, respectively) and 55% DR (–73 to –78% and –83 to –95%, respectively) groups. The general feature of the mean BW curves in males was similar among the groups. A steady weight gain was observed up to a maximum BW in SW 66–67, followed by a plateau up to the end of the study with only a small decline from SW 100–102. The maximum BW mean values ± standard deviation (SD) in males from the AL, 25% DR, and 55% DR groups were 829 ± 112 g, 609 ± 35 g, and 337 ± 21 g, respectively. In females, the mean BW curve followed a different pattern in each group. In the female AL group, there was a steady BW increase up to a maximum (583 ± 111 g) in SW 100, followed by a small decrease. In the female 25% DR group, the maximum mean BW (319 ± 30 g) was reached in SW 91 and plateaued up to SW 100, when a decrease occurred. In the female 55% DR group, an initial mean BW loss was observed up to SW 9, followed by a very slight but steady weight increase up to a maximum (201 ± 12 g) in SW 81, and a plateau up to a very slight decrease in SW 100.

In the AL group, the range of mean absolute FC was 28–35 g/day in males and 20–25 g/day in females; at study termination, the total mean ± SD of average FC values was 31.9 ± 1.7 g/day in males and 21.9 ± 1.3 g/day in females. Animals on restricted diets almost always consumed all the diet offered. Mean FC, relative to BW (gram of food per gram of body weight), is shown in Figures 5 and 6. Compared to the AL group, mean relative FC was decreased in the first 3 months only in the 55% DR group. From SW 13, values remained similar among the groups, up to study termination in males and up to SW 72 in females, when occasional decreases occurred thereafter in AL-fed females.

Clinical Pathology Parameters

Throughout the study, few hematological parameters, reported in Table 5, were affected by 55% DR. Compared to the AL group, there was a decrease in mean WBC values (–30 to –70%) in both sexes from the 55% DR group, due to decreases in mean values for segmented neutrophils (–4 to –70%) and lymphocytes (–24 to –55%). In addition, a very slight, but

TABLE 3
Incidence (%) of Causes of Spontaneous Deaths

	AL group		25% DR group		55% DR group	
	Males	Females	Males	Females	Males	Females
Neoplasms	48.3	56.7	28.3	36.7	1.7	1.7
Benign	33.3	38.3	20.0	25.0	1.7	1.7
Malignant	5.0	13.3	5.0	8.3	0	0
Metastatic	10.0	5.0	3.3	3.3	0	0
Non-neoplastic lesions	11.7	1.7	1.7	1.7	0	0
Liver	5.0	0	0	0	0	0
Kidney	3.3	0	1.7	1.7	0	0
Pituitary	1.7	1.7	0	0	0	0
Heart	1.7	1.7	0	0	0	0
Undetermined causes	13.3	3.3	6.7	3.3	0	0

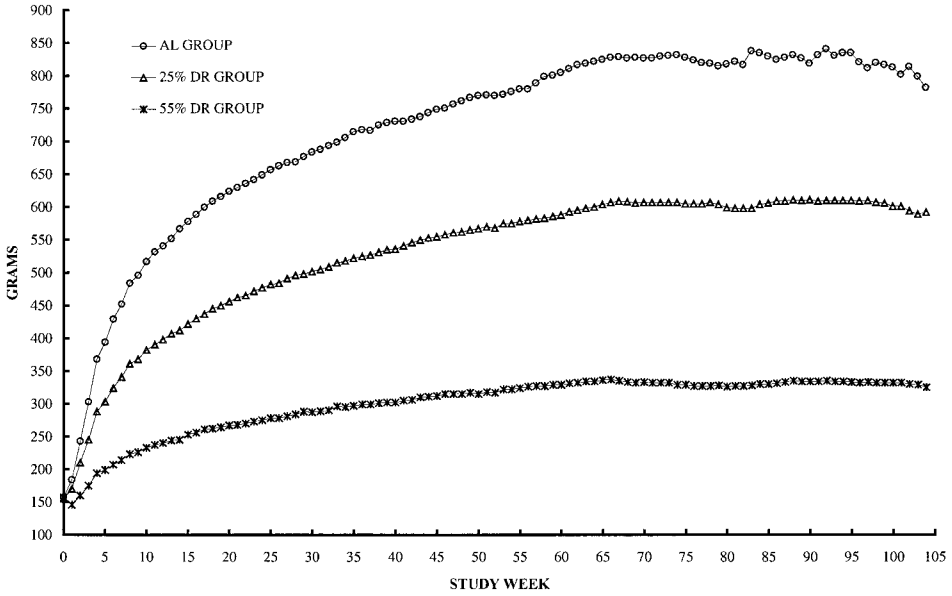

FIG. 3. Mean body weights in male SD rats.

generally statistically significant, decrease in platelets (up to –20%) was also noted for the same rats. In males from the 25% DR group, mean WBC values remained relatively constant throughout the study (see Table 5); however, when compared to males from the AL group, there was a relative, statistically significant decrease of this parameter in SW 79 (–35%) and 103 (–51%), when WBC values were increased in AL-fed males. There were no diet-related changes in hematological parameters of females from the 25% DR group.

Several serum biochemical parameters were affected by DR (Table 6) and especially in females given 55% DR. In these animals, compared to AL-fed females, there were decreases in serum total protein (–9 to –23%), albumin (–8 to –21%), and total calcium (–3 to –11%) throughout the study; decreases in serum triglycerides (–29 to –83%), and HDL (–26 to –37%) and total cholesterol (–19 to –34%) from SW 11 and 27, respectively, as well as increases in serum ALK PHOS activities (+44 to +260%) and chloride (+3 to +7%) throughout the study. Decreases in serum triglycerides were also observed in males given 55% DR (–29 to –80%) throughout the study and in females given 25% DR (–29 to –67%) from SW 27 onwards. All these serum biochemical changes were statistically significant. There were no diet-related changes in serum biochemical parameters of males from the 25% DR group.

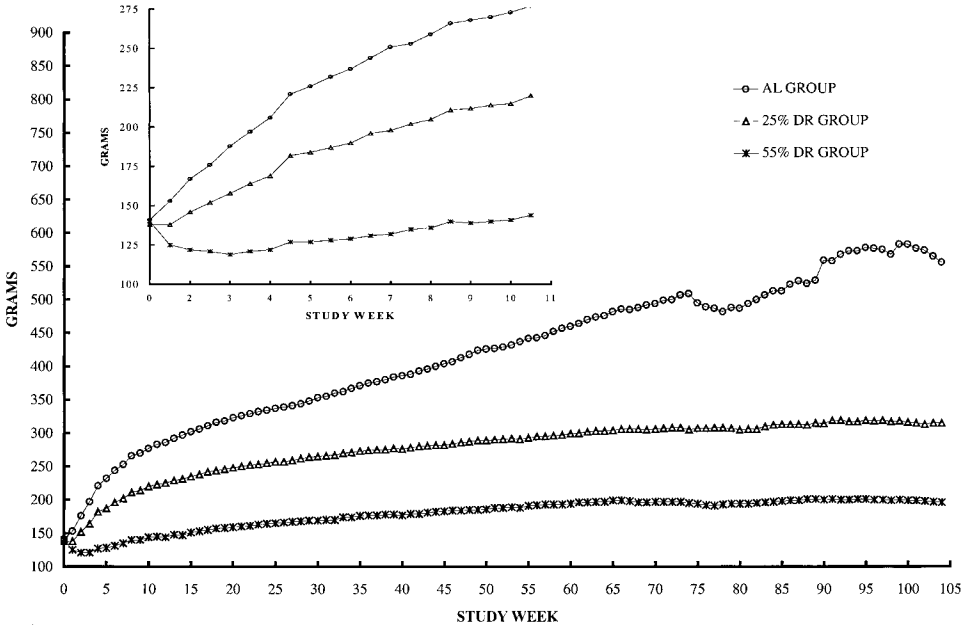

FIG. 4. Mean body weights in female SD rats.

TABLE 4
Mean ± SD Body Weight Values (g)

Study groups	Pretest	Study week 14	29	53	104
AL group					
Males	157 ± 13	567 ± 58	677 ± 78	772 ± 109	782 ± 82
Females	141 ± 11	297 ± 33	348 ± 45	432 ± 76	556 ± 125
25% DR group					
Males	155 ± 14	412 ± 22	498 ± 25	575 ± 28	592 ± 46
Females	138 ± 11	231 ± 14	264 ± 16	292 ± 22	315 ± 32
55% DR group					
Males	157 ± 14	245 ± 15	288 ± 19	322 ± 20	325 ± 22
Females	140 ± 11	147 ± 10	169 ± 13	189 ± 13	196 ± 17

Compared to AL-fed animals, mean urinary volume (Table 7) was decreased in the 55% DR group throughout the study (–50 to –87%) and in the 25% DR group mainly until SW 27 (–50 to –71%); these decreases, however, were not always statistically significant except in males from the 55% DR group. In both DR groups, median values for urinary pH were increased throughout the study and median values for urinary protein were decreased until SW 39 (25% DR females), 51 (25% DR males), or 103 (55% DR males and females) (Table 8).

DISCUSSION

The present data confirm the close correlation between survival and BW: survival of SD rats was inversely related to BW and to the daily amount of ingested food. At study termination, the survival rate of the AL group did not come up to the regulatory agency's expectation in carcinogenicity studies, when that of the DR groups did. In a previous study done under the same experimental conditions in our SD rat population fed either AL or 25% DR, Laroque *et al.* (1997) showed that initial BW did not correlate to 2-year survival rates. Only in AL-fed rats, did the authors find a correlation between 1-year BW and 2-year survival: the highest 1-year BW correlated with the lowest 2-year survival. This absence of a relationship between early BW and 2-year survival was consistent with studies in Fischer-344 rats (Turturro *et al.*, 1995), but was in contrast with reports on Wistar and SD rats, and on other species (Nohynek *et al.*, 1993; Roe *et al.*, 1991; Ross, 1976; Ross and Bras, 1971; Turnbull *et al.*, 1985). As suggested by Laroque *et al.* (1997), an important determining factor of the 2-year survival seems to be the total food intake producing excessive BW gain. Therefore, possible differences in BW gain among laboratories, due to husbandry methods affecting food availability and intake (Laroque *et al.*, 1997), may partially explain these discrepancies and the different ages at which a relationship between BW and 2-year survival is established.

Increases in BW in most of the laboratory rodents have been shown to be associated with increases in the onset and severity

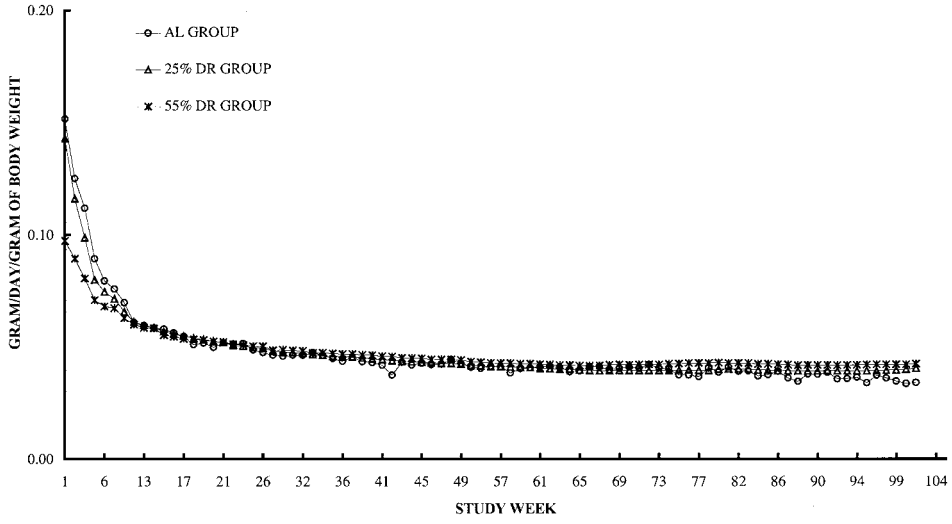

FIG. 5. Mean, relative to body weight food consumption in male SD rats.

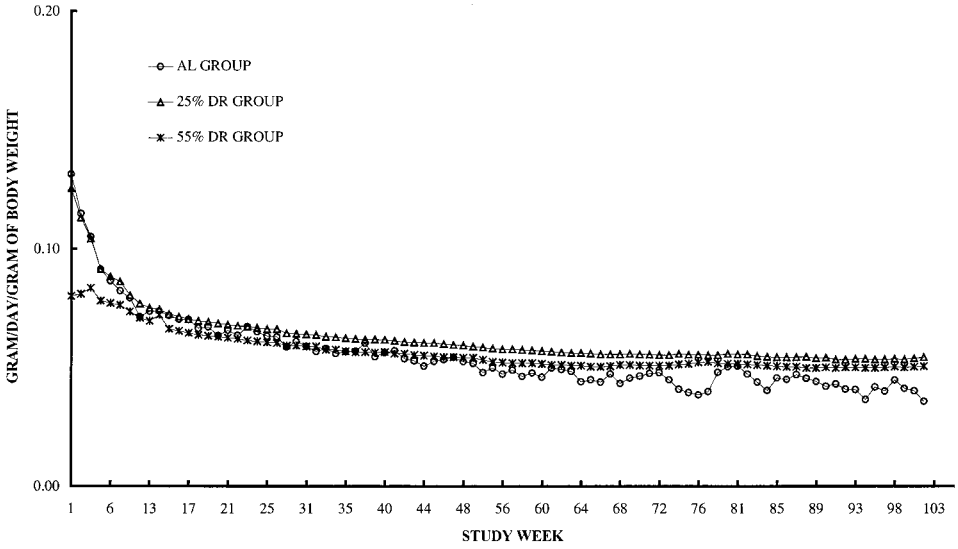

FIG. 6. Mean food consumption in female SD rats, relative to body weight.

of spontaneous degenerative diseases and tumors (Hart *et al*., 1995; Haseman and Rao, 1992; Keenan *et al*., 1992; Lang, 1991; Rao *et al*., 1990; Turturro *et al*., 1995, 1996) resulting in decreased survival (Allaben *et al*., 1991, 1996; Duffy *et al*., 1989; Hart *et al*., 1995; Haseman and Rao, 1992; Keenan *et al*., 1992, 1994a,b, 1995a,b, 1996, 1997; Lang, 1991; Newberne

TABLE 5
Mean Values ± SD for Hematological Parameters Affected by Dietary Restriction

	Study week							
Parameter (unit)	4	8	11	27	39	51	79	103
WBC ($10^3/\mu$l)								
AL males	12.8 ± 2.5	13.0 ± 2.2	11.6 ± 2.3	11.9 ± 2.8	12.5 ± 2.3	11.6 ± 2.1	14.7 ± 5.0	19.8 ± 34.2
25% DR males	13.4 ± 3.5	14.1 ± 8.7	12.4 ± 3.7	11.9 ± 3.8	12.1 ± 2.2	10.7 ± 2.0	9.5 ± 2.3*	9.7 ± 3.7*
55% DR males	7.6 ± 2.3*	8.2 ± 2.5*	5.9 ± 1.6*	6.7 ± 1.7*	6.7 ± 1.9*	6.9 ± 2.3*	6.0 ± 1.6*	6.0 ± 1.5*
AL females	9.0 ± 3.1	7.9 ± 2.4	7.8 ± 2.6	7.5 ± 1.9	7.9 ± 1.8	8.5 ± 2.4	7.1 ± 1.8	7.2 ± 2.1
25% DR females	9.2 ± 2.2	8.2 ± 2.0	8.5 ± 2.2	8.0 ± 1.6	6.9 ± 1.5*	6.8 ± 1.8*	7.3 ± 2.2	6.2 ± 1.3
55% DR females	6.3 ± 2.2*	5.2 ± 1.3*	5.1 ± 1.7*	4.9 ± 1.6*	4.4 ± 1.4*	5.4 ± 1.5*	4.7 ± 1.0*	5.0 ± 1.6*
Seg. Neutro. ($/\mu$l)								
AL males	962 ± 284	965 ± 500	1280 ± 686	1395 ± 616	1607 ± 533	2058 ± 621	4761 ± 3272	3866 ± 2133
25% DR males	1073 ± 484	2297 ± 6646	921 ± 653	1646 ± 2785	1714 ± 1216	1703 ± 743	2140 ± 1007*	2838 ± 1590*
55% DR males	427 ± 120*	927 ± 1093	638 ± 747*	962 ± 605*	1380 ± 788	1369 ± 514*	1445 ± 694*	1780 ± 581*
AL females	1087 ± 744	866 ± 446	827 ± 579	861 ± 624	1229 ± 426	1385 ± 978	1772 ± 822	2407 ± 1245
25% DR females	696 ± 531*	473 ± 314*	676 ± 307	751 ± 440	1023 ± 715*	1135 ± 1046	1885 ± 1832	1669 ± 799*
55% DR females	335 ± 144*	411 ± 227*	382 ± 456*	487 ± 346*	548 ± 243*	827 ± 434*	980 ± 516*	1471 ± 805*
Lympho. ($/\mu$l)								
AL males	11678 ± 2470	11884 ± 2268	10191 ± 2114	10362 ± 2490	10698 ± 2138	9312 ± 1978	9586 ± 2940	8909 ± 5756
25% DR males	12170 ± 3233	11742 ± 3460	11321 ± 3421	10104 ± 2003	10136 ± 1778	8785 ± 1670	7141 ± 2127*	6528 ± 2505*
55% DR males	7166 ± 2274*	7181 ± 2210*	5201 ± 1532*	5613 ± 1561*	5144 ± 1398*	5409 ± 2149*	4481 ± 1397*	4036 ± 1051*
AL females	7775 ± 2789	6970 ± 2075	6893 ± 2251	6582 ± 1880	6601 ± 1662	6976 ± 1689	5235 ± 1401	4599 ± 1334
25% DR females	8399 ± 2097	7691 ± 1865	7791 ± 2165	7216 ± 1496	5792 ± 1325*	5624 ± 1114*	5330 ± 1134	4393 ± 803
55% DR females	5902 ± 2217*	4754 ± 1213*	4621 ± 1535*	4373 ± 1457*	3816 ± 1384*	4546 ± 1394*	3663 ± 846*	3460 ± 1271*
PLT ($10^3/\mu$l)								
AL males	1218 ± 126	1102 ± 117	1099 ± 133	1136 ± 135	1140 ± 168	1112 ± 173	1208 ± 231	1189 ± 263
25% DR males	1148 ± 151	1126 ± 229	1080 ± 128	1154 ± 177	1151 ± 137	1088 ± 142	1106 ± 197	968 ± 188*
55% DR males	1030 ± 156*	964 ± 222*	950 ± 241*	981 ± 111*	1092 ± 122	1025 ± 179	1021 ± 162*	1018 ± 103*
AL females	1194 ± 130	1138 ± 131	1128 ± 133	1110 ± 114	1082 ± 92	1040 ± 97	1140 ± 190	1064 ± 178
25% DR females	1103 ± 111*	1059 ± 108*	988 ± 179*	1091 ± 135	1102 ± 138	1073 ± 119	1040 ± 159	1026 ± 176
55% DR females	985 ± 114*	910 ± 112*	944 ± 124*	1006 ± 103*	980 ± 106*	988 ± 179	991 ± 125*	976 ± 93

* Trend statistically significant through the indicated rate of diet restriction ($p \leq 0.05$).

TABLE 6
Mean Values ± SD for Serum Biochemical Parameters Affected by Dietary Restriction

Parameter (unit)	Study week							
	4	8	11	27	39	51	79	103
Total protein (g/dl)								
AL males	6.4 ± 0.2	6.8 ± 0.2	6.8 ± 0.3	7.3 ± 0.3	7.4 ± 0.3	7.5 ± 0.3	6.7 ± 0.6	7.4 ± 0.4
25% DR males	6.2 ± 0.2*	6.5 ± 0.3*	6.5 ± 0.2*	7.1 ± 0.3*	7.3 ± 0.3	7.2 ± 0.4*	7.0 ± 0.5	7.4 ± 0.7
55% DR males	6.0 ± 0.3*	6.3 ± 0.2*	6.3 ± 0.2*	6.6 ± 0.2*	6.7 ± 0.3*	6.7 ± 0.3*	6.4 ± 0.4*	6.8 ± 0.4*
AL females	6.6 ± 0.4	7.1 ± 0.3	7.5 ± 0.4	8.0 ± 0.5	8.2 ± 0.4	8.2 ± 0.4	7.6 ± 0.6	8.1 ± 0.7
25% DR females	6.4 ± 0.4	6.8 ± 0.4*	7.1 ± 0.5*	7.5 ± 0.6*	8.0 ± 0.4*	8.1 ± 0.6	7.7 ± 0.5	8.1 ± 0.5
55% DR females	6.0 ± 0.2*	6.1 ± 0.2*	6.3 ± 0.3*	6.3 ± 0.3*	6.3 ± 0.3*	6.6 ± 0.4*	6.5 ± 0.3*	71. ± 0.4*
Albumin (g/dl)								
AL males	3.8 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.3 ± 0.4	3.3 ± 0.2
25% DR males	3.7 ± 0.1	3.8 ± 0.2	3.8 ± 0.1*	3.9 ± 0.1*	3.8 ± 0.1	3.7 ± 0.2	3.6 ± 0.2	3.5 ± 0.4
55% DR males	3.7 ± 0.2*	3.8 ± 0.1*	3.8 ± 0.1*	3.8 ± 0.1*	3.7 ± 0.2*	3.6 ± 0.2*	3.5 ± 0.1	3.5 ± 0.2*
AL females	4.0 ± 0.2	4.2 ± 0.2	4.4 ± 0.2	4.7 ± 0.3	4.7 ± 0.2	4.4 ± 0.3	4.0 ± 0.4	3.9 ± 0.4
25% DR females	3.9 ± 0.2	4.1 ± 0.3	4.2 ± 0.3*	4.4 ± 0.4*	4.6 ± 0.2	4.5 ± 0.3	4.1 ± 0.3	4.0 ± 0.3
55% DR females	3.7 ± 0.1*	3.7 ± 0.1*	3.8 ± 0.1*	3.8 ± 0.2*	3.7 ± 0.1*	3.7 ± 0.2*	3.6 ± 0.2*	3.6 ± 0.2*
HDL choles. (mg/dl)								
AL males	46 ± 12	44 ± 13	46 ± 14	59 ± 15	59 ± 24	58 ± 21	52 ± 20	58 ± 21
25% DR males	47 ± 10	47 ± 8	48 ± 9	60 ± 15	61 ± 16	55 ± 12	69 ± 22*	63 ± 31
55% DR males	51 ± 11	54 ± 9*	56 ± 9*	52 ± 12	60 ± 11	59 ± 9	62 ± 11*	59 ± 13
AL females	56 ± 13	57 ± 13	62 ± 14	82 ± 15	89 ± 22	77 ± 25	87 ± 30	86 ± 35
25% DR females	60 ± 8	65 ± 13	66 ± 11	79 ± 19	95 ± 17	88 ± 20	82 ± 20	80 ± 16
55% DR females	58 ± 10	64 ± 12	57 ± 10	58 ± 13*	56 ± 13*	57 ± 13*	55 ± 13*	60 ± 13*
Total choles. (mg/dl)								
AL males	70 ± 16	61 ± 15	63 ± 19	81 ± 18	85 ± 36	87 ± 39	84 ± 27	109 ± 31
25% DR males	73 ± 13	70 ± 11*	66 ± 11	81 ± 14	93 ± 20	81 ± 15	98 ± 27	109 ± 34
55% DR males	84 ± 12*	78 ± 11*	80 ± 12*	74 ± 13	85 ± 14	87 ± 12	90 ± 12	98 ± 21
AL females	75 ± 14	70 ± 12	74 ± 15	95 ± 15	103 ± 22	102 ± 26	122 ± 41	135 ± 72
25% DR females	86 ± 12*	82 ± 14*	82 ± 10	96 ± 20	110 ± 19	108 ± 21	109 ± 21	110 ± 20
55% DR females	85 ± 14*	89 ± 14*	79 ± 15	77 ± 16*	77 ± 16*	82 ± 17*	84 ± 17*	89 ± 17*
Triglycerides (mg/dl)								
AL males	100 ± 31	108 ± 30	114 ± 40	207 ± 85	253 ± 134	248 ± 124	155 ± 70	149 ± 49
25% DR males	112 ± 42	98 ± 41	102 ± 31	130 ± 48*	180 ± 76*	162 ± 44*	143 ± 41	124 ± 45
55% DR males	71 ± 22*	67 ± 22*	60 ± 24*	59 ± 26*	58 ± 23*	50 ± 20*	56 ± 22*	64 ± 28*
AL females	61 ± 26	61 ± 32	66 ± 44	131 ± 59	234 ± 126	252 ± 126	202 ± 141	251 ± 249
25% DR females	63 ± 24	57 ± 14	63 ± 22	93 ± 41*	106 ± 51*	108 ± 56*	107 ± 48*	82 ± 32*
55% DR females	56 ± 18	52 ± 20	47 ± 20*	54 ± 18*	56 ± 26*	44 ± 14*	48 ± 14*	46 ± 15*
ALK PHOS (U/l)								
AL males	268 ± 59	162 ± 35	121 ± 26	105 ± 125	76 ± 18	76 ± 21	84 ± 32	70 ± 21
25% DR males	255 ± 40	168 ± 36	143 ± 29*	80 ± 16	122 ± 238	65 ± 13	67 ± 17*	63 ± 28
55% DR males	272 ± 45	199 ± 38*	174 ± 36*	121 ± 27*	113 ± 19*	99 ± 23*	97 ± 32*	109 ± 34*
AL females	151 ± 31	97 ± 21	73 ± 16	35 ± 9	32 ± 11	33 ± 10	38 ± 19	34 ± 17
25% DR females	169 ± 35	111 ± 22	91 ± 21*	46 ± 14*	34 ± 10	33 ± 11	36 ± 10	43 ± 16*
55% DR females	217 ± 28*	184 ± 35*	172 ± 34*	126 ± 38*	90 ± 24*	78 ± 28*	96 ± 34*	94 ± 35*
Chloride (mEq/l)								
AL males	101 ± 2	101 ± 2	101 ± 2	105 ± 1	106 ± 2	106 ± 2	106 ± 1	102 ± 2
25% DR males	102 ± 2*	102 ± 2	102 ± 1	105 ± 2	106 ± 1	106 ± 1	107 ± 2	103 ± 2
55% DR males	105 ± 1*	105 ± 2*	105 ± 1*	108 ± 2*	109 ± 2*	109 ± 1*	108 ± 1*	105 ± 2*
AL females	100 ± 2	102 ± 2	102 ± 2	104 ± 2	104 ± 2	104 ± 2	102 ± 2	100 ± 3
25% DR females	101 ± 2*	102 ± 2	103 ± 1*	105 ± 1	104 ± 2	105 ± 1*	104 ± 2*	101 ± 2
55% DR females	103 ± 2*	106 ± 2*	106 ± 2*	108 ± 2*	110 ± 1*	110 ± 1*	109 ± 2*	106 ± 1*
Total calcium (mg/dl)								
AL males	10.7 ± 0.3	10.6 ± 0.3	10.4 ± 0.3	10.5 ± 0.4	10.7 ± 0.3	10.9 ± 0.3	10.6 ± 0.5	10.6 ± 0.3
25% DR males	11.0 ± 0.3*	10.7 ± 0.3	10.5 ± 0.3	10.4 ± 0.3	10.6 ± 0.3	10.7 ± 0.3	10.6 ± 0.3	10.6 ± 0.6
55% DR males	10.6 ± 0.3*	10.4 ± 0.3	10.2 ± 0.3*	10.0 ± 0.3*	10.1 ± 0.3*	10.4 ± 0.3*	10.3 ± 0.3*	10.3 ± 0.3*
AL females	10.5 ± 0.4	10.6 ± 0.4	10.6 ± 0.4	10.9 ± 0.4	11.3 ± 0.4	11.1 ± 0.3	10.8 ± 0.5	11.3 ± 0.5
25% DR females	10.5 ± 0.2	10.6 ± 0.4	10.5 ± 0.4	10.6 ± 0.5	11.2 ± 0.4	11.3 ± 0.4	10.9 ± 0.4	11.1 ± 0.4
55% DR females	10.2 ± 0.3*	10.1 ± 0.4*	9.9 ± 0.2*	9.8 ± 0.2*	10.1 ± 0.3*	10.2 ± 0.3*	10.2 ± 0.3*	10.7 ± 0.3*

* Trend statistically significant through the indicated rate of diet restriction ($p \leq 0.05$).

TABLE 7
Mean Values ± SD for Urinary Volume

	Study week							
Parameter (unit)	4	8	11	27	39	51	79	103
Volume (ml)								
AL males	15 ± 5	10 ± 6	11 ± 6	11 ± 8	16 ± 9	15 ± 7	19 ± 7	11 ± 4
25% DR males	6 ± 4*	5 ± 3*	5 ± 4*	6 ± 5	10 ± 10*	10 ± 9	6 ± 3*	11 ± 5
55% DR males	2 ± 2*	3 ± 2*	4 ± 3*	5 ± 6*	3 ± 3*	3 ± 3*	5 ± 4*	3 ± 2*
AL females	7 ± 11	8 ± 9	5 ± 5	8 ± 6	10 ± 6	11 ± 6	11 ± 5	9 ± 5
25% DR females	2 ± 2	3 ± 3	2 ± 3	3 ± 5*	8 ± 8	16 ± 16	12 ± 8	11 ± 9
55% DR females	2 ± 1	3 ± 2	2 ± 3	2 ± 3*	2 ± 2*	3 ± 4*	2 ± 2*	2 ± 2*

* Trend statistically significant through the indicated rate of diet restriction ($p \leq 0.05$).

and Sotnikov, 1996; Rao *et al*., 1990; Roe, 1994; Turturro *et al*., 1995). Many of these adverse effects are the results of increased normal and neoplastic tissue growth by AL overfeeding of calories (for review see Keenan *et al*., 1998). DR in rodents has long been known to extend the life span by retarding the aging processes (for review see Masoro *et al*., 1991a). The main concept, which has emerged from different studies, is that DR enables rodents to utilize nutritional fuel for metabolism in less damaging ways than in AL conditions (for review see K. P. Keenan *et al*., 1998). At study termination, the incidence of deaths caused by both neoplasms and non neoplastic lesions was increased in the AL group, compared to the DR groups, but, pituitary adenoma, which is the most common cause of death in SD rats (Chandra *et al.*, 1992; Chvedoff *et al.*, 1982; Keenan *et al.*, 1995a, 1996; Nohynek *et al.*, 1993; Pettersen *et al.*, 1996), remained the main cause of death among the different groups. Keenan *et al.* (1995a) suggested that moderate DR acts on pituitary tumors by delaying the time of onset, but not the growth rate or progression once these tumors develop. Among the degenerative lesions as causes of spontaneous deaths, chronic renal disease (CRD) was noted only in AL-fed males. It has been shown that AL-fed SD males, compared to SD rats on DR, have the earliest onset, highest incidence, and most severe CRD and that this is a common cause of death in this population (Keenan *et al.*, 1995b). One hypothesis for CRD pathogenesis is that AL feeding induces hemodynamic changes and hyperfiltration at the level of the glomerulus, which results in glomerular hypertrophy leading to CRD; DR is thought to delay the progression of the glomerular diseases and, subsequently, the CRD onset (Keenan *et al.*, 1995b).

In AL-fed males, BW mean values observed throughout the study were similar to those obtained by Nohynek *et al.* (1993) in SD rats from the same stock, given the same diet, housed

TABLE 8
Median Values for pH and Protein from Semi Quantitative Urinalyses

	Study week							
Parameter	4	8	11	27	39	51	79	103
pH								
AL males	7	7	7	8	9	8	7	6
25% DR males	9	9	9	9	9	9	9	7
55% DR males	9	9	9	9	9	9	9	9
AL females	6	7	6	6	6	7	6	6
25% DR females	9	9	9	9	6	7	7	7
55% DR females	9	9	9	9	9	9	9	9
Protein								
AL males	NEG	TRACE	TRACE	TRACE	NEG	±1	±3	±3
25% DR males	TRACE	TRACE	TRACE	TRACE	TRACE	NEG	TRACE	TRACE
55% DR males	NEG	NEG	NEG	NEG	NEG	NEG	TRACE	TRACE
AL females	TRACE	TRACE	NEG	NEG	TRACE	TRACE	TRACE	±3
25% DR females	TRACE	NEG	TRACE	NEG	NEG	NEG	NEG	TRACE
55% DR females	NEG	NEG	NEG	NEG	NEG	NEG	TRACE	TRACE

Note. NEG, negative; TRACE, 30 mg/dl; ±1, 60 mg/dl; ±3, 300–500 mg/dl.

individually, and maintained under similar experimental conditions. The survival rate obtained in this group after exclusion of the accidental deaths was 27% and corresponds to the worst rate given by these authors in studies starting in 1988 and 1989. Since mean BW curves, as functions of time, were similar among AL and 25 and 55% DR male groups, both DR rates can be considered to increase survival without impairing physiological growth in this gender. In AL-fed females, mean values for BW, compared to data from Nohynek *et al*. (1993), were very slightly higher and, subsequently, the survival rate was very slightly lower. As noted in males, survival was improved by both rates of DR. However, BW loss and BW retardation occurred in females given 55% DR during the first 3 months of the study and it is known that in female SD rats, there is a disproportionate loss of body fat with DR (Turturro *et al.*, 1993). Therefore, such BW alterations during early adult life may impair sensitivity of toxicity studies and the rate of 55% DR is considered undesirable in studies conducted with female SD rats. The highest rate of BW gain was observed in AL-fed rats and was associated with the highest absolute daily FC. However, when FC was calculated as grams of ingested food (and subsequently of kilocalories) per gram of BW, there was no significant difference among groups. Therefore, rats fed AL consumed the greatest number of grams and kilocalories per rat but approximately the same number of grams and kilocalories per gram of BW as rats on DR. This has been previously noted in SD rats by Keenan *et al.* (1994b, 1995b). These results rule out hypotheses that DR acts by reducing the intake of calories or other nutrients per unit of body mass. BW apparently adjusts to the reduced food intake in such a way that there is no decrease in the intake of nutrients or calories per gram of BW. This adjustment suggests that the conversion of calories to body mass is similar in rats fed under both AL and DR conditions, and that metabolic rate, oxygen consumption, and food utilization may be similar. The reduction in nutrient intake per rat rather than per unit of body mass is the maneuver that prevents excessive growth, early BW gain, the early onset of spontaneous degenerative diseases and tumors, and poor survival (Keenan *et al.*, 1994a). The antiaging action of DR seems to be a reduction in the total amount of food consumed and thus energy intake per animal (Duffy *et al.*, 1989; Masoro and McCarter, 1991b; Masoro *et al.*, 1982; Weindruch and Walford, 1988).

Most of the changes observed in the clinical pathology parameters were similar to those seen in Charles River SD rats in a companion study with similar design and experimental conditions (Coleman *et al.*, 1997). In the literature, DR has been shown to induce decreases in WBC, neutrophils, and lymphocytes in rodents (Coleman *et al.*, 1997; C. Keenan *et al.*, 1998; Keenan *et al.*, 1994a; Kim and Gilman-Sachs, 1989; Kubo *et al.*, 1984; Prescott-Mathews *et al.*, 1998; Roe *et al.*, 1995; Weindruch and Walford, 1988) and in primates (Walford *et al.*, 1992). These changes are among the hallmarks of the alterations induced in rodents by antiaging low-calorie regimens (Weindruch and Walford, 1988). However, it is important to point out that these decreases were only observed in our study with a 55% rate of DR. Less data are available regarding a possible DR-related effect on PLT. In the Biosure Study (Roe *et al.*, 1995) conducted with Wistar rats, PLT were significantly higher in males on 20% standard maintenance DR at 6, 12, and 18 months, whereas, throughout our study, PLT were lower in both sexes on 55% DR and similar among AL and 25% DR groups. Therefore, this apparent DR effect on PLT remains questionable. No noteworthy changes in RBC parameters were observed in any of the two MRL studies conducted in SD rats, whereas increases in RBC counts, Hb concentration, and Hct have been reported in the literature in Wistar rats (Pickering and Pickering, 1984; Roe *et al.*, 1995). Again, a possible DR effect on RBC parameters remains questionable. The decreases in serum lipids are among the most commonly reported effects of DR in rodents (Coleman *et al.*, 1997; C. Keenan *et al.*, 1998; Keenan *et al.*, 1994a; Liepa *et al.*, 1980; Masoro *et al.*, 1983; Prescott-Mathews *et al.*, 1998; Reaven and Reaven, 1981; Sachan and Das, 1982; Snyder and Towne, 1989; Turturro *et al.*, 1993; Yu *et al.*, 1984). Indeed, DR acts as a modulator of age-related increases in serum lipids (Liepa *et al.*, 1980; Masoro *et al.*, 1983; Reaven and Reaven, 1981; Snyder and Towne, 1989). Sachan and Das (1982) showed that lower levels of serum lipids do not appear to be indicative of essential fatty acid deficiency since, in their 50% DR-fed SD rats, the levels of linolenic acid were elevated in both neutral and polar lipids, and activities of drug-metabolizing enzymes were not decreased, as observed with deficiency of essential fatty acids. In the present study, decreases in total and HDL cholesterol were observed only in the 55% DR females, whereas decreases in triglycerides more clearly corresponded to the level of DR and to the percentage of decreases in BW gain, with both sexes affected in the 55% DR group and the females in the 25% DR group. This better concordance between triglyceride levels (as opposed to cholesterol levels) and the level of DR has already been mentioned by Turturro *et al.* (1993), and lowered triglyceride levels with DR have been consistently found by these authors across a number of genotypes of rodents. This suggests that organismic fat metabolism is significantly modified by DR (Turturro *et al.*, 1993). Reaven and Reaven (1981) have suggested that DR enhances insulin sensitivity and therefore prevents the age-related rise in hepatic triglyceride secretion. DR-related decreases in plasma total protein and albumin have been observed in SD rats by Coleman *et al.* (1997) and in Wistar rats by Roe *et al.* (1995), whereas Snyder and Towne (1989) mentioned decreases in serum total protein and globulin without noteworthy changes in serum albumin. Birchenall-Sparks *et al.* (1985) reported that DR slows the rate of protein synthesis in rats and Snyder and Towne (1989) suggested that the reduction in serum globulin might be due to a decreased antigen stimulus. Therefore, the decreases in serum total protein we observed in 55% DR females may be due to the combination of both effects. In-

creases in serum ALK PHOS activities have been described in DR SD rats by Coleman *et al.* (1997), and in DR Wistar rats by Roe *et al.* (1995) and Snyder and Towne (1989). Ross (1969) found a close correlation between levels of hepatic enzyme (including ALK PHOS) activities in rats and life expectancy, and that long-term caloric restriction led to the persistence into middle and old ages of enzyme profiles seen in young rats. Similarly, the higher serum ALK PHOS activities observed in 55% DR females throughout our study may reflect DR-induced retardation of the age-related decrease in serum ALK PHOS activities (C. Keenan *et al.*, 1998) and, therefore, the persistence of younger profiles of these enzymes in the blood. DR-related decreases in serum total calcium have been described in male Fischer 344 rats (Kalu *et al.*, 1988a,b) and, in the present and companion (Coleman *et al.*, 1997) studies, only in 55% DR female SD rats. The prevention by DR of age-related increases in serum parathyroid hormone and calcitonin, and decreases in 25-hydroxyvitamin D, and, therefore, the inhibition of age-related hyperparathyroidism and senile bone loss could explain these decreases in serum total calcium (Kalu *et al.*, 1984, 1988a,b). DR increases in plasma chloride levels have been reported only in Wistar rats together with increases in RBC parameters, and may have reflected some hemoconcentration (Pickering and Pickering, 1984). Since the increases in chloride in 55% DR females were not observed in the companion study (Coleman *et al.*, 1997) and in the absence of DR-related increases in RBC parameters, this DR change remains questionable. DR-related decreases in glycemia are commonly reported in rats (Coleman *et al.*, 1997; Keenan *et al.*, 1994a,b; Masoro *et al.*, 1989; Prescott-Mathews *et al.*, 1998; Roe *et al.*, 1995; Sachan and Das, 1982; Snyder and Towne, 1989) and support the widely held theory that DR acts by preventing long-term damage of fuel use from the glycation reaction (for review see Keenan *et al.*, 1994b). Surprisingly, there was no DR-induced hypoglycemia in the present study. The increased urinary pH in DR rats has been described in various strains (Coleman *et al.*, 1997; Pickering and Pickering, 1984; Prescott-Mathews *et al.*, 1998; Roe *et al.*, 1995) as has been the decrease in proteinuria (Prescott-Mathews *et al.*, 1998; Roe *et al.*, 1995; Tapp *et al.*, 1989; Wu *et al.*, 1989). Both DR regimens induced these changes in the present study. According to Roe *et al.* (1995), DR-related reduced proteinuria seen in rats correlates with the delay in the onset of CRD, and urinary pH proves to be one of the best early indicators of survival, with high urinary pH associated with high survival. In opposition to the present data, increases in urinary volume have been described in DR rats (Pickering and Pickering, 1984; Roe *et al.*, 1995). However, on at least one occasion, the increase was almost certainly related to the times when the animals were fed and when the urine samples were collected (Roe *et al.*, 1995). Therefore, DR-related changes in absolute and relative (to BW) urinary volume should be further investigated in order to reach a conclusion.

The results of the present study confirm the beneficial effects of DR in rodents. AL-fed rats, with their early and high incidence of tumors and endocrine disturbances, cannot be regarded as appropriate models for detecting carcinogenicity relevant to humans (Abelson, 1992; Roe, 1981, 1988, 1993; Roe and Lee, 1991; Roe *et al.*, 1995). In addition, CRD being generally a common cause of death in AL-fed rats (Keenan *et al.*, 1994b; Roe *et al.*, 1995; Short and Goldstein, 1992), the chronic damage and progressive loss of renal function may either mask a subtle compound-related nephrotoxicity or enhance renal injury to the remaining hypertrophied nephrons (Hard and Alden, 1992; Keenan *et al.*, 1994a; Short and Goldstein, 1992) and thus, compromise toxicity studies. Under moderate DR, BW gain is reduced, but growth remains regular, survival is improved and reaches the regulatory agencies' expectations in carcinogenicity studies, the incidence of tumors and diseases, including CRD, is reduced, and routine clinical pathology parameters are not significantly modified. Therefore, a moderate rate of DR of approximately 25% is recommended in carcinogenicity and toxicity assays conducted with SD rats. The adjustment of the DR rate to the different SD stocks should be made under the experimental conditions described in the present study. The condition of note is the determination of the adult AL food consumption mean values that should be made in single-housed rats given daily AL regular rodent diet.

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