

INFLUENCE OF LOW TRYPTOPHAN DIET ON SURVIVAL AND ORGAN GROWTH IN MICE

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SUMMARY

Greater survival and reduced growth were found to characterize mice on a tryptophan deficient diet as compared to fully fed control mice. The 50% survival point was reached by the tryptophan restricted group at 683 days, and by the control group at 616 days. Measurements of body weight, organ weight, and DNA level were made at 8, 12, 24, 36, 52 and 78 weeks of age. Both whole body weight and organ weight of liver, kidney, heart and spleen were about 30% lower in the tryptophan restricted group as compared to the controls, so that the ratio of organ weight to body weight remained at a constant value for both groups. There was no significant change in cell number as determined by DNA measurements, as a result of the tryptophan restriction.

Key words: Tryptophan restriction; Lifespan; Growth; DNA content; Cell number

INTRODUCTION

Dietary restriction has been of particular interest in aging research because it is the most effective means known of increasing lifespan in rodents. The restriction may be carried out by reducing food intake, reducing protein intake or by reducing tryptophan intake. In the first study of dietary restriction and lifespan, McCay *et al.* [1,2] were able to increase the lifespan of rats by 30% through restriction of their caloric intake by 54%. Since then, other dietary manipulations involving caloric restriction [3–5] or protein restriction [6,7] have been shown to increase the lifespan of rats and mice.

Diets which are low in tryptophan have also been shown to increase the lifespan of rats under certain conditions [8–10]. Rats placed on a tryptophan restricted diet which survived the first 23 months of life attained a mean and maximum lifespan of 36 months

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and 45 months, respectively, as compared to controls which had a mean lifespan of 30 months and a maximum lifespan of 41 months [10].

Tryptophan restriction has also been shown to delay physiological aging. Segall and Timiras [10] showed that 67% of female rats which were restricted in tryptophan starting at 3 weeks of age were still able to bear litters between 17 and 28 months of age, while no female in the control group was able to do so. Segall and Timiras [11] later found that tryptophan-restricted female rats could bear litters as late as 36 months of age. In another study [12], the degree of aging in rats on a tryptophan restricted diet was observed by measuring thermoregulatory competence in terms of the time it takes for recovery of normal body temperature following whole body immersion in ice water for 3 min. These studies were done at 3 ages: 7–8 months (adult), 13–15 months (middle-aged), and 24–26 months (old). The results showed that the tryptophan restricted animals returned to normal body temperature more quickly than their control counterparts. Also the middle-aged tryptophan restricted animals behaved like the adult controls in terms of the amount of time it took to attain normal body temperature.

The fact that a tryptophan restricted diet causes delayed growth has been documented in rats, but not in other rodent species. Furthermore, the effect of tryptophan restriction on growth has never been measured in terms of DNA content, so that growth alteration could be expressed in terms of cell number.

The objective for this study was to compare the lifespan and growth rate of mice on tryptophan restricted diets with that of mice on control diets. Since total organ DNA is an index of cell number [13], the DNA content of liver, spleen, heart and kidneys was measured at increasing age intervals to monitor the growth rate of these organs. Since the liver contains polyploid cells, and since polyploidy increases with age and is influenced by diet [14] the DNA measurements in the liver will not reflect cell number. They will, however, provide a relative measure by which growth of the liver may be compared in the control *vs.* the tryptophan restricted group.

The questions that we asked were as follows: (1) What is the effect of tryptophan restriction on survival?; (2) What is the effect of tryptophan restriction on organ growth?; (3) Does the growth of all organs respond in a similar manner to the tryptophan restricted diet?

MATERIALS AND METHODS

Animals and diets

All animals used were male Swiss albino mice obtained from Canadian Breeding Farms, St. Constant, Quebec at 3 weeks of age. The mice were fed Purina rat chow until they had reached an average body weight of 20 g, that is, by 4 weeks of age. At this point the animals were divided randomly into two groups and were fed special diets. The average initial weight of the animals in the three groups was similar. There were no significant differences in their weight as determined by the *F*-test. The fully-fed control group was

fed Teklad diet TD-78071 containing 26% protein. The content of this diet is described by Leto *et al.* [6,7]. The experimental group was restricted with respect to only one key amino acid, tryptophan. Teklad diet TD-78464 is a tryptophan deficient basal diet containing 15% casein hydrolysate and has been described by Segall and Timiras [10]. All diets were obtained from Teklad Co., Madison, WI. The control diet contained 0.47% tryptophan and the tryptophan restricted diet contained 0.08% tryptophan according to Teklad specifications. Another independent report cited in [10] indicates that the tryptophan restricted diet contained 0.62 g/kg tryptophan, or 0.062%. The minimum requirement for tryptophan in mice is 0.10% [15,16].

Animals were housed 2 or 3 per cage except during food and water consumption studies when they were housed singly. Food and water were available *ad libitum*. Animals received 12 h of light/12 h of darkness on a uniform schedule. The temperature of the animal room was maintained at 21°C (70°F).

Lifespan studies

Forty mice divided into two equal groups were used in this study. Group 1 served as control and was fed the 26% protein diet. Group 2 was fed the tryptophan deficient diet. The animals were weighed in the morning, weekly during their growth period and then monthly after their weight had stabilized.

Food and water consumption studies

These studies were done at 8, 24, 36, 52 and 78 weeks of age for animals on each diet. Five animals selected randomly from each diet group were housed singly and used for these experiments. The food was weighed (to the nearest 0.01 g) using a Sartorius balance at the start and finish of each test period which lasted 2–3 days, and was repeated twice consecutively. A known volume of water was measured out at the beginning of each test period using a graduated cylinder and approximated to the nearest milliliter. The amount remaining at the end of the test period was measured by the same means. Care was taken to weigh the food and measure the water at approximately the same time of day throughout the experimental period.

DNA determinations

For all biochemical experiments, groups of 5 mice for each diet were sacrificed by cervical dislocation at 8, 12, 24, 36, 52 and 78 weeks of age. The DNA content of liver, spleen, kidneys and heart was determined. Once the tissues were removed, they were frozen at -10°C until ready for the assay. The procedure followed the method described by Enesco and Leblond [13] with minor modifications. Whole tissues were weighed and then homogenized with 20 ml of 10% TCA in a Waring microblender at maximum speed for 1 min. The homogenates were transferred to 50-ml centrifuge tubes using a Pasteur pipette and kept on ice for 30 min. The homogenates were then centrifuged at 1000 rev./min for 10 min in an IEC centrifuge at 4°C. Supernatants were discarded and

the pellets were washed in 10 ml cold 10% TCA. The samples were centrifuged again at 1000 rev./min for 10 min. The pellets were then washed with 10 ml cold deionized-distilled water and centrifuged again at the same speed. The final wash was with 10 ml cold absolute ethanol. After centrifuging the supernatants were discarded and lipids were removed by extracting three times with 10 ml of ethanol/ether (3:1) at 70°C for 3 min. After each extraction the samples were centrifuged at 1500 rev./min for 15 min. DNA was extracted by suspending the pellets in 5% TCA (5 ml heart, 10 ml other tissues) at 85–90°C for 15 min. Samples were centrifuged at 1500 rev./min for 15 min. This extraction was repeated and pooled supernatants were assayed for DNA by the standard diphenylamine test. To each 1 ml of sample, 2 ml of diphenylamine reagent was added. The diphenylamine reagent was made by dissolving 1 g of diphenylamine in 100 ml of glacial acetic acid with the addition of 2.75 ml of concentrated sulfuric acid. The tubes were incubated in a boiling water bath for 10 min. They were cooled to room temperature and then read using a Bausch & Lomb Spectronic 70 at 595 nm wavelength. A standard curve of DNA concentrations was prepared prior to each assay using powdered DNA (sodium salt, obtained from Eastman Kodak, Rochester, NY). A 1 mg/ml DNA stock solution was used in successive dilutions for the standard curve, which routinely contained concentrations of DNA ranging from 50 to 500 µg/ml.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed to compare dietary groups. When a significant *F* value was obtained, the *a posteriori* least significance difference (LSD) test was used [17]. A 95% confidence level was used. Because of biological variation or variations in measurement, a trend was not inferred unless statistically significant differences between control and experimental groups were observed for all or most of the age groups studied.

RESULTS

The survival curves for the two groups are shown in Fig. 1, which demonstrates that the longest 50% survival and the longest lifespan was attained by the tryptophan restricted mice. The tryptophan restricted group reached 50% survival at 683 days and the control group (26% protein) at 616 days. The maximum lifespan for the tryptophan restricted group was 1097 days, as compared to 1038 days for the control mice. Table I shows the percent survival with age. By 598 days, the control group was at 55% survival and the tryptophan restricted group at 65% survival. The survival curves were clearly separated except toward the end of the lifespan, at the 15–5% survival points.

Figure 2 shows the whole body weight profile with age for the 2 groups of mice studied (*N* = 20/group). The control animals were the heaviest at all times and attained a maximum weight of 49 g. The maximum weight that the tryptophan restricted mice attained was 34 g. Both groups demonstrated a gain in weight until 24 weeks of age and then the body weights plateaued. Body weight is shown in Table II.

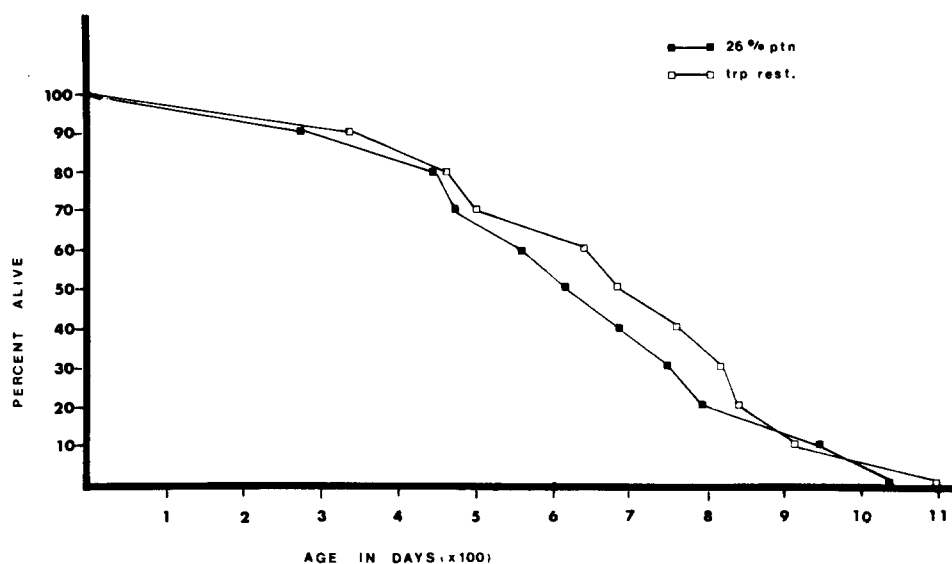


Fig. 1. The survival curves for mice on the control and tryptophan restricted diet are compared.

TABLE I
LIFESPAN DATA

The age in days at which each dietary group reached 95% to 0% survival is shown. $N = 20$ for each group.

% Survival	Age (days) 26% protein control	Tryptophan restricted
95	89	332
90	277	336
85	317	364
80	448	463
75	464	476
70	476	504
65	478	575
60	560	639
55	585	676
50	616	683
45	626	738
40	686	759
35	739	816
30	744	816
25	788	886
20	798	890
15	841	898
10	949	913
5	1008	971
0	1038	1097

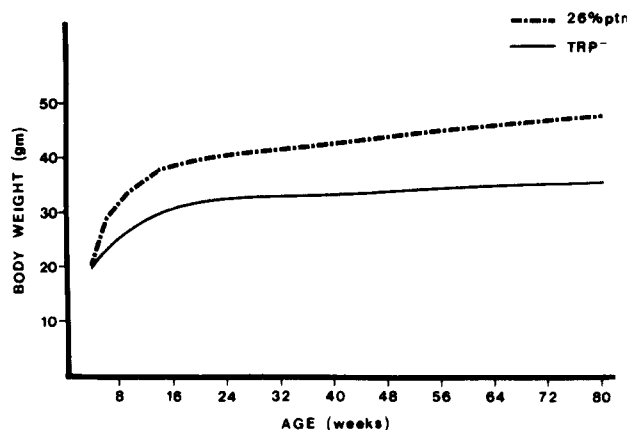


Fig. 2. The whole body weight of control mice on a 26% protein diet is compared with tryptophan restricted mice ($N = 20$).

There were no significant differences in food consumption (g/day) with increasing age either for controls or the tryptophan restricted animals. Although the tryptophan restricted animals consumed slightly higher levels of food than the controls, there were no significant differences between these groups. Water consumption was measured as ml/day. Again, there were no significant differences between the amount of water consumed by the control and experimental groups. This data has been reported in full by de Marte [18].

In Table I, whole body weight and organ weight are compared for mice on the control and on the tryptophan restricted diet. Animals on the tryptophan restricted diet weigh significantly less than animals on the control. Table I shows that the weight of liver and kidney follow the pattern of whole body weight: in the tryptophan restricted mice these organs weigh significantly less than control liver and kidney. In marked contrast, the weight of the spleen and of the heart are not significantly different in control and tryptophan restricted animals except in the very youngest animals at 8 weeks of age.

In Table II, the DNA content of organs from mice on the control and tryptophan restricted diets is compared. The DNA content of liver and kidney of mice on the control diet tended to be greater than that of mice on the tryptophan restricted diet. Because of inherent variability of DNA measurements, we would accept a trend only if it occurred in all age groups measured. In spite of some significant differences in weight seen in the liver and kidney of mice on the control as compared to mice on the tryptophan restricted diet, there was no consistently significant differences in DNA content of these organs as a result of diet. For spleen and heart, there were no significant differences in either weight or DNA content as a result of the tryptophan restricted diet.

In Table III, the ratio of organ weight to body weight is presented for mice on the two diets. This ratio is maintained as a fairly constant value regardless of diet.

At the time of sacrifice at 78 weeks, tumors of the liver were observed in two of the

TABLE II
WHOLE BODY WEIGHT AND FRESH ORGAN WEIGHT ARE COMPARED FOR MICE ON THE CONTROL AND ON THE TRYPTOPHAN RESTRICTED DIET FOR EACH AGE ($N = 5$).

Diet	Age (weeks)	Whole body (g) mean \pm S.D. (g)	Liver wt mean \pm S.D. (g)	Kidney wt mean \pm S.D. (g)	Spleen wt mean \pm S.D. (g)	Heart wt mean \pm S.D. (g)
Tryp rest.	8	23.62 \pm 1.41**	1.12 \pm 0.15**	0.37 \pm 0.01**	0.06 \pm 0.00**	0.11 \pm 0.01**
	12	33.16 \pm 3.56**	1.40 \pm 0.15**	0.46 \pm 0.06*	0.08 \pm 0.01	0.16 \pm 0.01
	24	34.63 \pm 4.35**	1.68 \pm 0.38	0.58 \pm 0.15*	0.09 \pm 0.02	0.19 \pm 0.04
	36	32.35 \pm 7.91**	1.59 \pm 0.39*	0.55 \pm 0.19	0.07 \pm 0.03	0.17 \pm 0.05
	52	33.86 \pm 2.92**	1.46 \pm 0.17**	0.57 \pm 0.09*	0.09 \pm 0.02	0.18 \pm 0.03
	78	30.74 \pm 4.22**	1.38 \pm 0.30**	0.57 \pm 0.09**	0.08 \pm 0.05	0.18 \pm 0.04
Control	8	33.97 \pm 1.55	1.94 \pm 0.27	0.64 \pm 0.11	0.10 \pm 0.01	0.17 \pm 0.01
	12	35.40 \pm 3.65	1.76 \pm 0.27	0.58 \pm 0.07	0.10 \pm 0.02	0.19 \pm 0.05
	24	41.76 \pm 3.51	1.94 \pm 0.30	0.77 \pm 0.17	0.11 \pm 0.02	0.19 \pm 0.03
	36	43.83 \pm 8.26	2.06 \pm 0.26	0.70 \pm 0.12	0.09 \pm 0.03	0.18 \pm 0.03
	52	49.93 \pm 5.78	2.09 \pm 0.27	0.70 \pm 0.09	0.08 \pm 0.02	0.20 \pm 0.02
	78	47.93 \pm 10.22	2.01 \pm 0.30	0.77 \pm 0.09	0.11 \pm 0.03	0.23 \pm 0.06

*Significantly different from control at $P < 0.05$.

**Significantly different from control at $P < 0.01$.

TABLE III

TOTAL ORGAN DNA IS COMPARED FOR MICE ON THE TRYPTOPHAN RESTRICTED DIET AS COMPARED TO CONTROLS FOR EACH AGE ($N = 5$).

<i>Diet</i>	<i>Age (weeks)</i>	<i>Liver DNA ± S.D. (mg)</i>	<i>Kidney DNA ± S.D. (mg)</i>	<i>Spleen DNA ± S.D. (mg)</i>	<i>Heart DNA ± S.D. (mg)</i>
Tryp. rest.	8	2.52 ± 0.39*	1.71 ± 0.78	0.98 ± 0.27**	0.21 ± 0.04
	12	2.32 ± 0.37*	1.99 ± 0.33	1.47 ± 0.32	0.14 ± 0.07
	24	3.15 ± 0.21	1.80 ± 0.70	1.58 ± 0.43	0.14 ± 0.07
	36	2.43 ± 0.62	1.52 ± 0.46	1.09 ± 0.46	0.09 ± 0.03*
	52	2.89 ± 0.63**	1.90 ± 0.53	1.12 ± 0.38	0.10 ± 0.04
	78	3.10 ± 0.82	2.11 ± 0.48	1.39 ± 0.78	0.12 ± 0.06
Control	8	3.71 ± 0.40	2.43 ± 0.45	2.09 ± 0.43	0.23 ± 0.08
	12	2.95 ± 0.14	2.38 ± 0.14	1.52 ± 0.37	0.13 ± 0.06
	24	3.54 ± 0.13	1.90 ± 0.49	1.59 ± 0.59	0.19 ± 0.03
	36	2.65 ± 0.45	2.15 ± 0.56	1.11 ± 0.24	0.14 ± 0.02
	52	1.57 ± 0.25	1.71 ± 0.29	0.99 ± 0.24	0.08 ± 0.03
	78	2.35 ± 0.62	2.56 ± 0.66	1.34 ± 0.61	0.20 ± 0.09

*Significantly different from control at $P < 0.05$.

**Significantly different from control at $P < 0.01$.

control mice on the 26% protein diet. Because of the increased liver weight (3.50 g and 4.15 g) data from these animals was not included in calculating average liver weight for the 78 week control group. No tumors were observed in any mice on the tryptophan restricted diet.

TABLE IV

RATIO OF (ORGAN WEIGHT/BODY WEIGHT) $\times 10^{-2}$ FOR THE ORGANS STUDIED FOR MICE ON THE TRYPTOPHAN RESTRICTED AND CONTROL DIETS AT THE VARIOUS AGES STUDIED

<i>Diet</i>	<i>Age</i>	<i>Liver</i>	<i>Kidney</i>	<i>Spleen</i>	<i>Heart</i>
Tryp. rest.	8	4.74	1.57	0.25	0.47
	12	4.22	1.39	0.24	0.48
	24	4.94	1.70	0.26	0.56
	36	4.92	1.70	0.22	0.53
	52	4.31	1.68	0.27	0.53
	78	4.49	1.85	0.26	0.59
Control	8	5.48	1.88	0.29	0.50
	12	4.22	1.64	0.28	0.54
	24	4.65	1.84	0.26	0.46
	36	4.70	1.58	0.21	0.41
	52	4.19	1.40	0.16	0.46
	78	4.20	1.61	0.23	0.48

DISCUSSION

The results presented here show that mice on the tryptophan restricted diet had a longer 50% survival and maximum lifespan than did control mice on the 26% protein diet. This is the first such study to be conducted with mice. These results are in basic agreement with earlier studies carried out on rats [8–11]. Our findings, that there are fewer tumors in mice on the tryptophan restricted diet, are also in agreement with the findings of these investigators.

With respect to growth, the results presented here show that mice on the tryptophan restricted diet are about 30% smaller than fully fed control mice. Organ weight of the four organs studied – liver, kidney, spleen and heart – was also about 30% less in mice on the tryptophan restricted diet. Because of this proportionate reduction in body weight and organ weight, the ratio of organ weight to body weight remains at a constant value in both experimental and control mice. There is no change in cell number as a result of diet. The smaller tryptophan-restricted animal is presumably constructed of smaller cells. In contrast, dietary restriction imposed by means of a 4% protein diet produces a marked decrease in both cell size and cell number in expanding cell populations of liver and kidney [19]. Since the liver is a polyploid organ, and polyploidy is influenced by age and by diet [14] one can consider growth only in terms of DNA and weight increase, not in terms of cell number, on the basis of the data presented here.

It is interesting to note that Segall and Timiras [10] have demonstrated that the growth inhibition produced by a tryptophan restricted diet is not permanent in rats. When rats were switched from a tryptophan restricted diet to a Purina Rat Chow diet, even at 22 months of age, they resumed growth and reached the weight of age-matched control animals which had received the Purina Rat Chow on a continuous basis. Thus the growth restriction appears to be readily reversible. It would be interesting to determine whether this growth is characterized by increase in cell number, or by increase in cell size only.

The question of why tryptophan restriction delays growth and extends survival and reproductive potential is an intriguing one. The tryptophan restricted diet markedly lowers plasma tryptophan and brain serotonin levels in mice; In an earlier study [20] we have shown that the tryptophan restricted diet used here reduces plasma tryptophan levels by 34–54%, and reduces brain serotonin levels to 52–82% of those values found in control mice.

Does the low level of available plasma tryptophan explain the growth retardation observed here? The reduction in the level of an essential amino acid could reduce the level of protein synthesis required for growth. It is known that tryptophan produces an increase in protein synthesis and polyribosomal aggregation in the liver, and increases DNA-dependent RNA polymerase activity as well as polyribosomal RNA and nuclear RNA synthesis [21].

Alternatively, does the low level of brain serotonin observed in the tryptophan restricted mice explain the growth reduction and extended survival observed in these animals?; Tryptophan is a precursor of serotonin. The tryptophan restricted diet em-

ployed here greatly reduced brain serotonin levels [9,20,22]. Another means of reducing serotonin is to treat rats chronically with d,l-*para*-chlorophenylamine, an inhibitor of brain serotonin synthesis. Segall and Timiras [10] report that this treatment also inhibited growth and maturation in rats. According to Segall and Timiras [10] this evidence favors the hypothesis that the tryptophan restricted diet alters growth and survival due to its lowering of brain serotonin levels, and its subsequent effect on neurotransmitter and hormonal systems. These investigators advance the view that low tryptophan feeding may act by reducing serotonin levels in critical tissues such as the pineal gland [23–27] which would postpone reproductive aging.

Hormonal alterations result from tryptophan deficiency [28]. Carew *et al.* [29] have demonstrated that tryptophan deficiency in chickens results in an elevation of T_3 , but produces no change in thyroid weights or thyroid follicle diameter. Based on the low plasma T_3 levels and on the lower feed conversion efficiencies and lower weight gain observed in the tryptophan deficient chicks, Carew *et al.* [29] suggest that energy intake is being converted to heat rather than to tissue synthesis. This energy wastage could help to explain reduced growth in the tryptophan deficient animals. Carew *et al.* [29] also report that tryptophan deficiency produces alterations in growth hormone level in chicks, but these relationships have not been examined in mammals.

The overall growth reduction observed in the present study clearly implies that the tryptophan restricted diet causes an inhibition or delay in growth operating to an equal extent on many organ systems. This modulation could clearly be controlled at the hormonal or neurotransmitter level.

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