Long-Term Effect of Energy Restriction at Different Protein Levels on Several Parameters of Nutritional Assessment

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Summary Experiments were conducted to evaluate long-term effects of energy restricted diets containing different protein levels initiated in adult life on various parameters of nutritional assessment. Rats (male SPF Wistar) were fed 20%-casein diet ad libitum, or 10%, 20%, or 40%-casein diets (group 10R, 20R, and 40R, respectively) under energy-restricted conditions, which corresponded to about 60% ad libitum feeding, from 18 to 55 weeks of age. Energy restriction induced a decrease of body-lipid percentage. Although skeleton weight and tail length increased throughout the experimental period in all the groups, these increases were found to be depressed under energy restriction. On the other hand, protein intake level under the energy restriction did influence plasma concentrations of urea and cholesterol, hematocrit, and systolic blood pressure. The group fed the 40%-casein diet tended to be lower in total cholesterol levels at 55 weeks of age, while the group fed 10%-casein diet tended to be lower in hematocrit and higher in systolic blood pressure levels. When mature rats were fed the 60%-energy-restricted diet for a long-term period, those on the 20% and 40%-casein diets showed somewhat more preferable levels of hematocrit and blood pressure than those on the 10%-casein diet; the 10%-casein diet did not, however, reveal symptoms of malnutrition.

Key Words: long-term effect, energy restriction, protein intake level, biochemical parameters, hematocrit, cholesterol, blood pressure, body composition, skeleton, matured rat

Energy restriction has a beneficial effect on life extension in experimental animals (1-4). This life-prolonging action has been considered to be the result of delaying the onset of some age-related diseases and retarding several physiological aging processes (1, 2). On the other hand, though the relationships between protein...
intake and life span or physiological aging have been examined in some studies (5–10), a definite conclusion has not been obtained. To understand physical fitness throughout a lifetime, it is very important to know the effect of long-term dietary manipulations on the nutritional state as well as on longevity. There are many findings on the relationship between protein intake level and the nutritional state in short-term studies, but very few long-term studies. In particular, there are few studies that have examined the long-term effect of protein intake levels under energy restriction on nutritional or physiological states.

In most long-term studies, including ones on aging and life span, dietary manipulations in experimental animals have been begun in early life or immediately after weaning. The restriction of energy or protein intake have both been observed to induce retardation of growth rate or occasionally raising mortality in early life (11, 12). Thus, these dietary manipulations are unsuitable for application in humans. Considering this point, in this study we used rats fed ad libitum until fully matured and examined the long-term effects of protein intake level under energy restriction on some parameters for nutritional assessment.

**MATERIALS AND METHODS**

Male SPF Wistar rats were kept on a commercial diet until 16 weeks of age and then fed a standard diet containing 20% casein for 2 weeks. Then they were divided into 5 groups of 9 to 12 animals each, and one group of them was sacrificed as age-control at 18 weeks of age (group I). One of the remaining groups continued to receive the standard diet as a control group (group 20A). The other three groups (groups 10R, 20R, and 40R) were fed diets containing 10, 20, or 40% casein under energy-restricted conditions. Compositions of the diets are shown in Table 1. Diets 1, 2, and 3 are 10%-casein, 20%-casein, and 40%-casein diets, respectively. The food intake of these isocaloric groups was reduced gradually at the beginning of the experimental period in order to avoid stress by acute food restriction: ad libitum in the first week, 15 g of the diet the second week, and thereafter 10 g of the diet. The animals were fed experimental diets until 55 weeks of age and then all were sacrificed.

The animals were kept in an air-conditioned room at 22 ± 2°C with a 12-h light period from 0800 to 2000 h. Daily food intake and weekly increments in body weight changes of each rat were recorded. Tail length and blood pressure were measured at the beginning (18 weeks of age) and the end of the experimental period (55 weeks of age). Systolic blood pressure was determined by tail cuff measurements with a photoelectric plethysmograph (MPP-36, Nihon Kohden, Tokyo).

Blood was withdrawn by cardiac puncture after anesthetizing the rats with pentobarbital sodium (5 mg/100 g). Hematocrit (packed cell volume percent = PCV%) was determined in atrial blood collected in heparinized capillary tubes and the PCV% was read directly.

Plasma concentrations of total protein, albumin, uric acid, urea, total cholesterol, HDL-cholesterol, and creatinine were determined by the following methods:

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Table 1. Composition of diet.

| Ingredient                  | Amount (%) |
|-----------------------------|------------|
|                             | Diet 1     | Diet 2     | Diet 3     |
| Casein¹                     | 10.0       | 20.0       | 40.0       |
| Sucrose¹                    | 24.2       | 20.9       | 14.2       |
| α-Cornstarch¹               | 48.5       | 41.8       | 28.5       |
| Corn oil¹                   | 5.0        | 5.0        | 5.0        |
| Cellulose powder¹           | 5.0        | 5.0        | 5.0        |
| Vitamin mixture²            | 2.0        | 2.0        | 2.0        |
| Mineral mixture             |            |            |            |
| Macro-elements³             | 4.8        | 4.8        | 4.8        |
| Micro-elements⁴             | 0.2        | 0.2        | 0.2        |
| L-Methionine⁵               | 0.3        | 0.3        | 0.3        |

¹ From Oriental Yeast, Tokyo. ² From Oriental Yeast, Tokyo. Contents per 100 g of diet: retinyl acetate, 1,000 IU; cholecalciferol, 200 IU; dl-α-tocopheryl acetate, 10.0 mg; menadione, 10.4 mg; thiamine · HCl, 2.4 mg; riboflavin, 8.0 mg; pyridoxine · HCl, 1.6 mg; cyanocobalamin, 1.0 μg; d-biotin, 40 μg; folic acid, 0.4 mg; d-calcium pantothenate, 10.0 mg; nicotinic acid, 12.0 mg (these 11 ingredients are standard vitamins for rats and mice as reported by the American Institute of Nutrition (13)); ascorbic acid, 60 mg; p-aminobenzoic acid, 10.0 mg; inositol, 12.0 mg; and choline chloride, 400 mg. ³ From Oriental Yeast, Tokyo. Content per 100 g of diet: CaHPO₄·H₂O, 699 mg; KH₂PO₄, 1,235 mg; NaH₂PO₄, 449 mg; NaCl, 224 mg; calcium lactate, 1,684 mg; ferric citrate, 153 mg; MgSO₄, 344 mg; ZnCO₃, 5.28 μg; MnSO₄·H₂O, 5.76 μg; CuSO₄·5H₂O, 1.44 μg; and KI, 0.48 μg. ⁴ Composition as reported by Ebihara et al. (14). ⁵ From Wako Pure Chemical Industries, Osaka.

total protein, Biuret method (15); albumin, BCG method (16); urea, Indophenol method (17); uric acid, uricase-peroxidase method (kit: Determiner UA, Kyowa Medex, Tokyo); total cholesterol, enzymatic method (18); HDL-cholesterol, heparin-manganous method (19); creatinine, the method by Folin (20).

Half of the rats in each group were used for determining wet and dry skeleton weights and ash content in skeleton. The skeleton which was isolated from the rest of the carcass by wrapping it with aluminum foil and heating at 120°C for 20 min in an autoclave was weighed before and after being dried at 105°C for 24 h. Ash content in the skeleton was determined by weighing after igniting the sample in a 550°C muffle furnace for 48 h.

The remaining half of the rats in each group were analyzed for body protein, fat, ash, and water contents. Frozen carcasses were chopped up and passed 4 to 5 times through an electric mincer. Portions of the minced carcass were accurately weighed in aliquots ranging from 50 to 100 g and were dried at 105°C for 24 h to calculate the moisture percentage. The dried carcasses were then ground again and the resulting portions were used for determining fat, protein, and ash contents.
Protein content was obtained by multiplying nitrogen coefficient 6.25 by the nitrogen content determined by the Kjeldahl method. Fat content was determined using a lipid-extract apparatus (EX-Fat, Nihon Zeneraru Ltd., Tokyo). Ash content was determined in the same way as for skeleton.

The differences between the groups I and 20A, and the groups 20A and 20R were examined by Student's t-test for evaluating the effect of aging and energy restriction, respectively. Statistical significance was accepted at \( p < 0.05 \). The data of three restricted groups (groups 10R, 20R, and 40R) were subjected to one-way analysis of variance by use of Duncan's multiple range test with the upper level of significance chosen at \( p < 0.05 \), for evaluating the effect of protein intake level under energy restriction. All data have been expressed as means ± SD.

RESULTS

Food intake and body weight

Changes in food intake and body weight are shown in Fig. 1. Food intake of group 20A was nearly constant throughout the experiment and averaging 16 g per day. The energy intake of the dietary-restricted groups corresponded to about 60%...
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of that of group 20A. The body weight of group 20A increased continuously, while that of the three energy-restricted groups decreased until about 30 weeks of age, thereafter recovering gradually, close to what they were at the beginning of the experiment. Protein intake level under energy restriction did not influence body weight.

**Hematocrit**

The hematocrit data are presented in Fig. 2. The value of group 10R, which averaged 44.8%, was significantly lower than those of the other groups. There were no significant differences between the values of groups 20A and I, and the groups 20A and 20R, and the hematocrit range was found to be 48.2 to 50.0%.

**Biochemical parameters of plasma**

Biochemical parameters of plasma are presented in Fig. 3. Values of total protein, uric acid, cholesterol and HDL-cholesterol were slightly lower in the energy-restricted groups than in the *ad libitum* group at 55 weeks of age. However,

![Fig. 2. Long-term effect of energy restriction and protein intake levels on hematocrit. Rats from group I were sacrificed as an initial control at 18 weeks of age. Each of the remaining 4 groups was fed 20%-casein diet *ad libitum* (group 20A) or 10%- , 20%- , or 40%-casein diets under restricted conditions (groups 10R, 20R, and 40R, respectively) until 55 weeks of age. Statistical significance was examined between groups I and 20A, groups 20A and 20R, and groups 10R, 20R, and 40R (*p* < 0.05).](image)
Fig. 3. Long-term effect of energy restriction and protein intake levels on blood biochemical parameters. See Fig. 2 legend for details about the 5 groupings and the statistical significance.
there were no significant differences in any parameters. Under energy restriction, only the values of urea and cholesterol were influenced by protein intake level, the urea level being lowest in group 10R and highest in group 40R, and the cholesterol level being lowest in group 40R.

**Blood pressure**

Systolic blood pressure values of each group are shown in Fig. 4. Blood pressure, except in group 10R, was significantly decreased from 18 to 55 weeks of age. The systolic blood pressure of group 10R, however, averaged 128 mmHg at 55 weeks of age and was significantly higher than those of the other groups.

**Tail length and skeleton weight**

Tail length, skeleton weight, and the ash content are shown in Table 2. Tail length increased from 18 to 55 weeks of age in each group. Those of restricted groups tended to be very slightly shorter than those of group 20A. The skeleton weight, particularly the dried weight, was significantly lower in the restricted group than in the *ad libitum* group.

![Fig. 4](image-url)  
*Fig. 4.* Long-term effect of energy restriction and protein intake levels on systolic blood pressure. See Fig. 2 legend for details about the 5 groupings and the statistical significance.

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Table 2. Tail length and weight and ash content in skeleton.

| Ages     | 18 weeks | 55 weeks |
|----------|----------|----------|
|          | Ad libitum | Restricted |
| Groups   | I | 20A | 10R | 20R | 40R |
| Tail length (cm) | (12) | 21.4±0.4 | 22.6±0.4* | 22.3±0.4 | 22.0±0.5** | 22.1±0.5 |
| Skeleton | (6) | 20.7±0.8 | 26.8±2.8* | 24.3±1.5 | 22.5±2.1** | 23.6±1.3 |
| Wet weight (g) | (6) | 13.0±0.4 | 18.3±1.7* | 15.5±0.6 | 15.4±1.4** | 15.8±0.8 |
| Dried weight (g) | (5) | 7.7±0.3 | 10.0±0.8* | 8.6±0.2 | 8.4±0.7** | 8.5±0.6 |
| Ash (g) | (5) | 37.0±2.1 | 36.7±2.3 | 35.5±2.0 | 37.4±0.4 | 36.2±1.3 |

1 Number of samples. 2 Means ± SD: asterisks (*) indicate significant differences between group I and 20A (p<0.05), (**) indicate significant difference between groups 20A and 20R (p<0.05). 3 Ash weight as a percentage of wet skeleton weight.

Ash weight of skeleton, which also increased for the experimental period, was significantly lower in group 20R than in group 20A. However, there were no significant differences among the three restricted groups fed different protein levels (groups 10R, 20R, and 40R). Additionally, ash weight as percentage of wet skeleton weight, which averaged around 36% in all the groups, were not affected by the differences of age, energy intake, and protein intake level.

Body composition

Table 3 shows body composition, i.e., protein, lipid, water, and ash contents. The percentage of protein in body composition tended to be slightly decreased during the experimental period with the exception of group 40R. The 40%-casein diet suppressed the decrease of protein percentage with aging. Ash percentage was less influenced by the alteration of energy- and protein-intake levels.

Lipid and water percentages were relatively variable with alteration of energy intake. The lipid percentage in ad libitum rats increased from 22 to 32% for the period from 18 to 55 weeks of age, while the water percentage decreased. Of the dietary restriction groups, group 40R tended to have the lowest lipid percentage but without significant difference from the other two restricted groups. On the whole, however, protein intake level under energy restriction did not significantly influence the body composition.

DISCUSSION

Nutritional value of a diet is often evaluated from the observation data on short-term feeding of experimental animals. In living organisms a certain mechanism...
is working toward adaptation functionally and morphologically to various diets containing different amounts of energy and other nutrients, which requires the passage of some time until completion. Therefore, there is probably much difference in evaluation of nutritional states between short-term and long-term feeding protocols. Considering the above, it is very important for nutritional assessment to be made following long-term feeding as well as short-term feeding.

It has been generally recognized that energy-restricted and low-protein diets fed from weaning age in rodents resulted in the decrease of hematocrit and hemoglobin concentration (21, 22). However, there are no reports on whether or not these parameters changed when energy-restricted diet were given to mature rats for several months. We found in the present study that the hematocrit was not influenced by long-term feeding of 60%-energy-restricted diet. However, the data also showed that in such energy-restricted conditions, a 10%-casein diet did reduce the value of hematocrit, but within normal range. Liepa et al. (23) have demonstrated that serum cholesterol concentration increases in ad libitum-fed rats with increasing age, while dietary restriction depresses the age-related increase of the cholesterol. In addition, Masoro et al. (24) have reported that dietary restriction initiated at 6 months of age was as effective as dietary restriction begun at 6 weeks of age in modulating the age-related changes in serum lipids, and than the restriction of protein intake was not as effective as energy restriction. Our data demonstrated that plasma levels of cholesterol as well as HDL-cholesterol were slightly lower in the energy-restricted groups that in the ad libitum group, and were significantly lower in rats fed a high protein diet under energy restriction. There are few data which have made observations concerning plasma nitrogen components in long-term or aging studies. No characteristic result was obtained in regard to the influence of dietary restriction and protein intake level on plasma levels of nitrogen

| Age     | 18 weeks | 55 weeks |
|---------|----------|----------|
|         |          | Ad libitum | Restricted |
| Groups  | I        | 20A       | 10R         | 20R         | 40R         |
| Protein | 19.3±0.6 | 17.8±0.1* | 18.2±0.4    | 18.0±1.5    | 19.2±0.7    |
| Lipid   | 21.9±1.1 | 32.1±3.4* | 26.4±1.4    | 26.8±3.9**  | 24.0±1.1    |
| Water   | 53.6±0.8 | 45.6±2.1* | 51.7±2.7    | 50.1±2.5**  | 51.6±0.7    |
| Ash     | 3.1±0.2  | 3.0±0.2   | 3.4±0.5     | 3.0±0.5     | 3.4±0.4     |

1 Values represent protein, lipid, water, and ash content as a percentage of carcass weight. 2 Number of samples. 3 Means±SD: Asterisks (*) indicate significant differences between group I and 20A (p<0.05), (***) indicate significant difference between groups 20A and 20R (p<0.05).
components, except for the fact that urea level increased with protein intake level. Judging from these blood parameters, no symptoms of malnutrition were observed in any of the groups, even in the group fed the energy-restricted diet containing only 10% casein. These data suggest that alteration of 10 to 40% of protein levels in the diet has not much effect on blood parameters, even under energy restriction.

Baskin et al. (25) have observed changes in blood pressure with aging in male Fisher rats and found that they showed higher blood pressure at 3 months of age as compared to values at other ages. Findings in the present study, in which the control rats were 18 weeks of age (about 4 months), were similar to those reported by Baskin, in spite of different strains of rats used, that is, blood pressure was significantly higher than at 55 weeks of age. However, this discrepancy may be due to changing conditions in measurement occurring over time since the degree of tameness to the handlers is definitely different between 18 and 55 weeks of age. Yu et al. (26) and Wyndham et al. (27) have shown findings that no difference existed in blood pressure between rats fed ad libitum and restricted diets. Our data also demonstrated similar results. However, the fact that blood pressure at 55 weeks of age was higher in group 10R than in other groups suggests that low protein intake under energy restriction might contribute to slightly elevated blood pressure compared to high protein intake.

It has already been demonstrated by the study of human and experimental animals that the proportion of body fat content increases after maturing with aging until a certain age is reached (28). The increase of body fat percentage seems to occur even in animals fed an energy-restricted diet, if this diet contains energy sufficient for maintaining body weight (29). This fact also was recognized in the present study. Nettleton and Hegsted (30) have observed long-term effects of protein intake on body composition and recognized that body protein and lipid contents were scarcely affected by protein intake level under 60%-energy-restricted diet. Our data also showed that protein intake level did not significantly influence body composition.

In this study, energy restriction tended to depress plasma levels of total protein, cholesterol, HDL-cholesterol and uric acid, the increase of skeletal mass and the lipid percentage in body composition, compared to ad libitum feeding. Various protein intake levels under energy restriction did not influence most of these parameters, but did influence plasma urea and cholesterol levels, hematocrit and blood pressure. Although there was no difference in body weight between each of the energy-restricted groups, the rats fed on energy-restricted diet containing 10% casein showed minor differences in hematocrit and blood pressure. However, these values were within the normal range and also the differences in these parameters were not great between each group fed different protein levels of diet. Therefore, it is rather difficult to evaluate how protein intake levels influence the nutritional state under conditions of energy restriction. Simply judging from present data, the 20% and 40% casein diets may be slightly preferred to the 10% casein diet under the conditions in which matured rats were examined on a long-term feeding protocol.
with energy restriction.

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