The Interactive Effect of Dietary Fat-Soluble Vitamin Levels on the Depression of Gonadal Development in Growing Male Rats Kept under Disturbed Daily Rhythm—Investigations Based on the L_{16}(2^{15}) Type Orthogonal Array—

Miho HANAI and Takatoshi ESASHI

Department of Nutrition and Life Science, Kanagawa Institute of Technology, 1030 Shimo-Ogino, Atsugi, Kanagawa 243–292, Japan
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Summary The purpose of this study was to clarify the effects of nutrients on the gonadal development of male rats kept under constant darkness as a model of disturbed daily rhythm. In the present study we examined fat-soluble vitamins and their interactions in this test population. Four fat-soluble vitamins (vitamin A (V.A), vitamin D (V.D), vitamin E (V.E) and vitamin K (V.K)) were selected as experimental factors, and the dietary content of these vitamins was normal (AIN-93G) or three times the normal content. Lighting conditions (constant darkness or normal lighting) were also added as a factor. Four-week-old rats (Fischer 344 strain) were kept under constant darkness or normal lighting (12-h light/dark cycle) for 4 wk. The lighting condition and V.E, and the interactions between the lighting condition and V.E and between V.A and V.D were observed to affect the testes and epididymis weights. There was an influence of the lighting condition only on the seminal vesicles and prostate weights and the serum testosterone concentration. Among the constant darkness groups (D-groups), the highest value for testes weight was observed under the normal-V.A, normal-V.D and high-V.E diet. The interaction between lighting condition and V.E showed the testes weight increased slightly in response to changing to a high-V.E diet from a normal-V.E diet under normal lighting (N-group) but was greatly increased in response to this change in the D-group. It became clear that the amount of dietary V.E necessary for the gonadal development of rats increases when rats are kept under constant darkness.

Key Words constant darkness, disturbed daily rhythm, gonad, fat soluble vitamin, orthogonal array

The number of people who are living under disturbed daily rhythm has been increasing due to the globalization of business and social activities, and diversification of the forms of labor. Such irregularities in daily rhythm adversely affect bio-regulatory mechanisms, resulting in an abnormal diurnal rhythm that can impede biological activities structurally and functionally. The process by which such disorders are induced is, in theory, dependent upon the nutritional condition of the individual. However, there are no basic data on the nutritional aspects of maintaining or promoting health under the condition of disturbed daily rhythm. Metabolic function changes under a condition of disturbed daily rhythm such as constant darkness, and it can be presumed that nutritional requirements in such an environment change, as well. The perspective behind this research is as follows: as basic data on the relationship between nutritional status and gonadal development in rats with disturbed daily rhythm accumulate, these data can be used for human research, and finally, dietary reference intakes can be compiled for persons living under disturbed daily rhythm.

Lighting is one of the key external factors for the formation of daily rhythm. Rats kept under constant darkness develop disturbances in their feeding and motor-activity rhythms, and suffer from altered rhythms of hormone secretion and enzyme activity (1, 2). Esashi et al. (3, 4) have reported that rats kept in constant darkness showed depressed gonadal development and a decreased delivering rate. The depression of gonadal development in these rats was accelerated by a low-protein diet (3, 4). These findings indicate that the gonads have high sensitivity to constant darkness and nutrients. Hence, we focused on gonadal development in our efforts to accumulate basic nutritional data on rats kept under constant darkness as a model of disturbed daily rhythm.

In previous papers, we have reported the effects on rat gonadal development of protein, methionine, vitamins, minerals and oil (5), various minerals (6), various amino acids, an AIN-76 diet and an AIN-93G diet (7), the interaction between protein and vitamins (8), and water- and fat-soluble vitamins (9). We have demonstrated that a normal level of water-soluble vitamins and high level of fat-soluble vitamins diet, or a high level of water-soluble vitamins and low level of fat-solu-
Table vitamins diet mitigated the depression of gonadal development in rats kept under constant darkness (9). Therefore, as a next step, we attempted to clarify the effects on gonadal development of four kinds of fat-soluble vitamins under a normal level of water-soluble vitamins diet. The present study was carried out using an orthogonal array, which is the experimental design we applied in previous studies (5–8, 10, 11).

METHODS

1. Animals. Forty-eight Fischer strain (F344) male rats (purchased from Charles River Japan, Inc., Kanagawa, Japan, at 3 wk of age) were preliminarily maintained for a week on the AIN-93G purified diet (12) and then divided into sixteen experimental groups of three rats each. No differences were found among the mean body weights of rats from any of the sixteen groups. The rats were kept under constant darkness (D-groups) or normal lighting (12-h light/dark cycle, N-groups) for 4 wk. Food intake and body weight were recorded every other day. The care of rats kept under constant darkness included lighting a red lamp for about 2 h; the lamp was for photographs and would not cause a phase variation of circadian rhythms. The rats were housed in individual, stainless steel, wire-mesh-bottomed cages at 22 ± 1˚C and 55 ± 5% humidity, in a room free from specific pathogens. Food and distilled water were provided to all rats ad libitum.

Animals were maintained in accordance with the Guideline for the Care and Use of Laboratory Animals (Notification of the Prime Minister’s Office in Japan).

2. Diets. Four fat-soluble vitamins (V.A, V.D, V.E and V.K) and the lighting condition were selected as factors, and the experimental protocol and diet composition were designed based on the L96(215)-type orthogonal array, which can examine five factors (10, 11). The examined factors and their levels are shown in Table 1.

Eight types of experimental diet were prepared based on the L96(215)-type orthogonal array (Table 2). The fat-soluble vitamin contents were normal (AIN-93G diet) or high. The high content, three times the normal content, was determined by the results of a previous study, which had shown that a diet containing three times the amount of vitamin affected the gonadal development (9). The normal and high levels of vitamins are shown as level 1 and level 2, respectively. The dietary protein level was low, 9% casein and 0.135% cystine. Other components were based on the AIN-93G diet.

The eight diets were given to rats kept under constant darkness or normal lighting conditions; i.e., there were eight D-groups and eight N-groups for a total of 16 groups.

3. Analysis. After 4 wk of treatment, the rats were

Table 1. Estimated factors and their levels in the L96(215)-type orthogonal array.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Row</th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Lighting condition</td>
<td>1</td>
<td>Normal lighting</td>
<td>Constant darkness</td>
</tr>
<tr>
<td>B: Vitamin A</td>
<td>2</td>
<td>Normal</td>
<td>High (×3)</td>
</tr>
<tr>
<td>C: Vitamin D</td>
<td>4</td>
<td>Normal</td>
<td>High (×3)</td>
</tr>
<tr>
<td>D: Vitamin E</td>
<td>8</td>
<td>Normal</td>
<td>High (×3)</td>
</tr>
<tr>
<td>E: Vitamin K</td>
<td>14</td>
<td>Normal</td>
<td>High (×3)</td>
</tr>
</tbody>
</table>

Table 2. Composition of the diets based on the L96(215)-type orthogonal array. (g/100 g Diet)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Diet groups</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk casein</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.135</td>
<td>0.135</td>
<td>0.135</td>
<td>0.135</td>
<td>0.135</td>
<td>0.135</td>
<td>0.135</td>
<td>0.135</td>
<td>0.135</td>
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<tr>
<td>α-Corn starch</td>
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<td>62.56</td>
<td>62.66</td>
<td>62.06</td>
<td>62.36</td>
<td>61.76</td>
<td>61.86</td>
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<tr>
<td>Sucrose</td>
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<td>10</td>
<td>10</td>
<td>10</td>
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<td>10</td>
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<td>Fiber</td>
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<td>5</td>
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<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Soybean oil</td>
<td>6.63</td>
<td>5.88</td>
<td>5.88</td>
<td>6.63</td>
<td>5.88</td>
<td>6.63</td>
<td>6.63</td>
<td>5.88</td>
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<tr>
<td>Mineral mix.</td>
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<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
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<tr>
<td>Tert-butylhydroquinone</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
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<tr>
<td>Water-soluble vitamin mix.</td>
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<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
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<td>0.25</td>
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<tr>
<td>Vitamin A</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
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<tr>
<td>Vitamin D</td>
<td>0.25</td>
<td>0.25</td>
<td>0.75</td>
<td>0.75</td>
<td>0.25</td>
<td>0.25</td>
<td>0.75</td>
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<tr>
<td>Vitamin E</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.90</td>
<td>0.30</td>
<td>0.90</td>
<td>0.30</td>
<td>0.90</td>
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<tr>
<td>Vitamin K</td>
<td>0.375</td>
<td>1.125</td>
<td>1.125</td>
<td>0.375</td>
<td>1.125</td>
<td>0.375</td>
<td>0.375</td>
<td>1.125</td>
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</tr>
</tbody>
</table>

1 Vitamin-free milk casein.
2 AIN-93G mineral mixture.
3 AIN-93 water-soluble vitamin mixture.
4 Retinyl palmitate, 1,000 IU/g.
5 Colecalciferol, 400,000 IU/g.
6 DL-α-Tocopherol Acetate, 25 IU/g.
7 Soluble phylloquinone in soybean oil (0.2 mg/g soybean oil).
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decapitated, and the blood and gonadal organs (testes, epididymides, seminal vesicles and prostate) were collected. The gonadal organs were weighed. Blood was centrifuged (3,000 rpm, 15 min, 4˚C), and the serum obtained was stored at −20˚C until analysis, when serum testosterone concentration was measured by a radioimmunoassay kit (CIS Diagnostic Co., Tokyo, Japan).

4. Statistical analysis. Statistical analysis followed the original method for the orthogonal array table (10, 11). For analysis, we used the software developed by the Japan Technology Training Institute Co., Ltd. (Tokyo, Japan; JUSE-QCAS/V6.0).

The effects of factors and interactions between factors on gonadal organ weights and serum testosterone concentrations were determined by ANOVA. The estimated values of gonadal organ weight and serum testosterone concentrations were calculated only in accordance with the factors that showed significant differences (p<0.05). Therefore, the values in the tables are estimated values and have a statistical difference.

RESULTS

The functioning of the gonadal organ during the growing period is in direct proportion to the weight of the organ (13). Therefore, in this study, the weights of the gonadal organs were compared by absolute value, not by the value per 100 g body weight. The highest recorded weight of gonadal organs was evaluated as a suitable indicator of normal development of the rats.

1. Results of ANOVA

The effects of dietary fat-soluble vitamin content on gonadal organ weight, serum testosterone concentration, body weight, total food intake and food efficiency were determined by ANOVA. The estimated values of gonadal organ weight and serum testosterone concentrations were calculated only in accordance with the factors that showed significant differences (p<0.05). Therefore, the values in the tables are estimated values and have a statistical difference.

Table 3. Effects of fat-soluble vitamins on reproductive organ weight, serum testosterone level and body weight: results of analysis of variance.

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<tbody>
<tr>
<td>Testes (g)</td>
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<td>(g/100 g BW)</td>
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<td>Epididymides (g)</td>
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<td>Seminal vesicles (mg)</td>
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<td>(mg/100 g BW)</td>
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<tr>
<td>Prostate (mg)</td>
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<tr>
<td>(mg/100 g BW)</td>
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<tr>
<td>Testosterone (ng/mL)</td>
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<tr>
<td>Body weight (g)</td>
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<tr>
<td>Food intake (g)</td>
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<td>Food efficiency</td>
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</tr>
</tbody>
</table>

1 Lighting condition: normal lighting or constant darkness.
2 *p<0.05, **p<0.01.

Table 4. The estimated value of the combinational effect of fat-soluble vitamins on body weight.

<table>
<thead>
<tr>
<th>Factor level AD</th>
<th>Estimated value (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>160.6</td>
</tr>
<tr>
<td>12</td>
<td>163.8 maximum</td>
</tr>
<tr>
<td>21</td>
<td>148.2 minimum</td>
</tr>
<tr>
<td>22</td>
<td>151.4</td>
</tr>
</tbody>
</table>

1: Normal lighting or normal level. 2: constant darkness or high level.

4. Statistical analysis. Statistical analysis followed the original method for the orthogonal array table (10, 11). For analysis, we used the software developed by the Japan Technology Training Institute Co., Ltd. (Tokyo, Japan; JUSE-QCAS/V6.0). The estimated values of the factors (lighting condition and four kinds of fat-soluble vitamin) that had a significant effect on body weight, total food intake, food efficiency, gonadal organ weight and serum testosterone concentration were determined by ANOVA. The estimated values of gonadal organ weight and serum testosterone concentrations were calculated only in accordance with the factors that showed significant differences (p<0.05). Therefore, the values in the tables are estimated values and have a statistical difference.

2-1. Body weight, total food intake and food efficiency

The lighting condition and VE were observed to affect the body weight (Table 3, Table 4). The highest value for body weight (163.8 g) was observed in rats maintained under normal lighting and a high-VE diet, and the lowest value (148.2 g) was observed in rats maintained under constant darkness and a normal-VE diet.

On the other hand, the lighting condition was observed to affect the total food intake and food efficiency (Table 3). The highest values for total food intake and food efficiency were observed in rats maintained under normal lighting condition (340.6 g, 0.287,
respectively), and the lowest values for total food intake and food efficiency were observed in rats maintained under constant darkness (315.6 g, 0.271, respectively). There was no effect of fat-soluble vitamins on food intake or food efficiency.

As an addition, the change of body weight and the amount of food intake of the D-group and the N-group are shown in Fig. 1. In the D-group, the body weight started to decrease gradually compared to the N-group in the third week, and the food intake started to decrease gradually on the 11th or 12th day.

2-2. Testes weight

The lighting condition and VE, and the interactions between the lighting condition and VE and between VA and VD were observed to affect the testes weight (Table 3, Table 5). The highest value for testes weight (2.213 g) was observed in rats maintained under normal lighting and a normal-VA, normal-VD and high-VE diet, and the lowest value (1.370 g) was observed in rats maintained under constant darkness and a normal-VA, high-VD and normal-VE diet. In the D-groups, the highest value for testes weight (1.868 g) was observed in rats on the normal-VA, normal-VD and high-VE diet (Table 5).

The interaction between the lighting condition and VE showed that the effects of VE differed according to lighting condition. In the N-groups, the testes weight increased slightly when changing to a high-VE diet from a normal-VE diet (0.045 g increase, Table 5) but increased greatly following this change in the D-groups (0.335 g increase, Table 5). In addition, the interaction of VA and VD showed that the testes weight decreased with the change to a high-VD diet from a normal-VD diet when the diet included normal VA (0.163 g reduction, Table 5), but increased following this change when the diet included high VA (0.029 g increase, Table 5).

2-3. Epididymides weight

The lighting condition and VE, and the interactions between the lighting condition and VE and between VA and VD were observed to affect the epididymides weight (Table 3, Table 5). The highest value for epididymides weight (268.2 mg) was observed in rats maintained under normal lighting and a normal-VA, normal-VD and normal-VE diet, and the lowest value (118.3 mg) was observed in rats maintained under constant darkness and a normal-VA, high-VD and normal-VE diet. In the D-groups, the highest value for epididymides weight (218.2 mg) was observed in rats on the normal-VA, normal-VD and high-VE diet (Table 5).

The interaction of the lighting condition and VE showed that the effects of VE differed according to lighting condition. In the N-groups, the epididymides weight
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decreased when changing to a high-VE diet from a normal-VE diet (11.1 mg reduction, Table 5) but increased in response to this change in the D-groups (66.4 mg increase, Table 5). In addition, the interaction of VA and VD showed that the epididymides weight decreased with the change to a high-VD diet from a normal-VD diet when the diet included normal VA (33.5 mg reduction, Table 5) but increased following this change when the diet included a high level of VA (8.6 mg increase, Table 5).

2-4. Seminal vesicle weight
The effect of lighting condition was observed on seminal vesicle weight. There was no effect of fat-soluble vitamins on seminal vesicle weight (Table 3). The maximum value was 119.8 mg in rats kept under normal lighting condition, and the minimum value was 58.2 mg in rats kept under constant darkness.

2-5. Prostate weight
The effect of lighting condition was observed on prostate weight. There was no effect of fat-soluble vitamins on prostate weight (Table 3). The maximum value was 96.3 mg in rats kept under normal lighting, and the minimum value was 59.4 mg in rats kept under constant darkness.

2-6. Serum testosterone concentrations
The effect of lighting condition was observed on serum testosterone concentration. There was no effect of fat-soluble vitamins on serum testosterone concentration (Table 3). The maximum value was 1.603 ng/mL in rats kept under normal lighting, and the minimum value was 0.943 ng/mL in rats kept under constant darkness.

DISCUSSION
Previous studies have demonstrated that body weight gain decreased due to reduced food intake and that food efficiency was lower when rats were kept under constant darkness for 4 wk (6, 8, 9). As in previous studies, the body weight gain decreased when rats were kept under constant darkness in the present study. These results indicate that the decrease of body weight gain in the D-groups was the result of the reduction of food intake and the disturbing of the food intake rhythm.

As shown in Fig. 1, in the D-group, the amount of food intake began to decrease compared with that of the N-group on the 11th or 12th day from the experiment start, and the body weight also began to decrease in the 3rd week from the experiment start. In our other experiment, we have data showing that, in the rats kept under constant darkness, the food intake rhythm maintained the same pattern as that of rats kept under normal lighting condition for 2 wk, with the peak of the food intake rhythm occurring during the dark phase in the rats kept under the normal lighting condition. After 3 wk, however, this peak in food intake rhythm leveled off and then fell gradually in the rats kept under constant darkness. The decline also leveled off eventually, and the rats began to eat continuously (unpublished original data). These changes in the rats’ food intake pattern led to decreased food intake and food efficiency. Further study is needed to clarify the reasons for these changes.

In addition, the effect of VE was shown in body weight gain. The high-VE diet, with 3 times the amount of VE as that of the normal diet, increased the body weight gain in both lighting condition groups. The food intake and food efficiency tended to increase in the high-VE diet (p<0.053 and p<0.085, respectively, data not shown), too. It was thought that the increasing tendency of food intake and food efficiency contribute to the significant increase in body weight in the high-VE diet. Further studies are needed to find the cause which leads to the food intake and food efficiency increase in the high-VE diet. The suitable quantity of VE in the AIN-93G diet was determined by using the index of antioxidant function for the dietary and tissue concentration peroxides (12). Therefore, we believe that the amount of VE in the AIN-93G diet is inadequate to attain the maximum body weight of growing rats, for example, from 4 to 8 wk old.

In the testes and epididymides weights, effects of lighting condition and VE, and the interactions between lighting condition and VE and between VA and VD were shown. There also was an effect of lighting condition only on seminal vesicles and prostate weights. The effects of fat-soluble vitamins were different with each gonadal organ.

Vitamin E is known as an essential vitamin for maintaining reproductive function, and this is thought to originate in the antioxidant action of VE (14). In this study, the testes weight had high values when rats were fed a high-VE diet in both lighting condition groups, and the effect of VE was especially enhanced in the D-groups. The result of testes weight per 100 g BW was similar (data not shown); this indicates that the VE effect was characteristic in the testes.

It was reported that the circadian rhythm disturbance was related to SOD and catalase activity (15). The SOD activity of organs including reproductive organs was not measured in this experiment. But it was thought that the peroxidation of organs progressed in the D-group, which is a model of disturbed daily rhythm, and the requirement of VE with an antioxidant action increased. Further studies to confirm the details of this mechanism are required.

Previous studies demonstrated that there is an interaction between VA and VD, and there is a theory that VA and VD have an antagonistic action during a stage of gene transcription regulation (16–18). A VD receptor with VD forms the heterodimer with a retinoid X receptor (RXR) coupled with retinoic acid, and this heterodimer mediates transcriptional control by acting as the gene promoter. The ratio of VA and VD appears to be important for the formation of this heterodimer.

In this study, there was interaction of VA and VD, which showed that the testes and epididymides weights decreased in response to changing to a high-VD diet from a normal-VD diet when the VA level was normal, but these weights were slightly increased when the VA level was high. This interaction was observed in both
lighting condition groups, indicating that this interaction is not the effect of disturbed lighting condition. The interaction of VA and VD was antagonistic, and it has already been reported that this antagonism affects bone formation (19) and the serum calcium level (20). To date, this effect has not been reported with respect to gonadal development. Because it has been revealed that the VD receptor exists in testicular cells (21), further study will be needed to confirm the antagonist action of VA and VD in a stage of gene transcription regulation in the testicular cells.

In our previous study of the effect of water- and fat-soluble vitamins, we clarified that in the D-groups, gonadal weight decreased in response to changing from a normal to a high level of fat-soluble vitamins when the level of water-soluble vitamins was low or high, but the rat gonadal weight increased in response to this change when water-soluble vitamins were at a normal level (9). In the present study, water-soluble vitamins were at a normal level, and it became clear that, of the fat-soluble vitamins, V.E was involved in the results of the previous study.

In this study, there was no significant effect of fat-soluble vitamins on seminal vesicles or prostate weight, but the prostate weight tended to increase given a high-V.E diet (p<0.08, data not shown). The previous study showed that the prostate weight increased under a high fat-soluble vitamin diet. We believe that the result of the previous study was related to the amount of VE in the diet.

As shown in Table 5, the maximum and minimum values of testes weight in the N-groups were 2.213 g and 2.005 g, respectively, but these values of testes weight in the D-groups were 1.863 g and 1.370 g, respectively. Namely, in the N-groups, the testes weight was decreased by only about 10% by the unsuitable fat-soluble vitamins diet, but in the D-groups, the weight was decreased by more than 25% by the unsuitable fat-soluble vitamin diet. This means that the gonads of rats kept under constant darkness are more sensitive to dietary fat-soluble vitamins than are the gonads of rats under normal lighting conditions.

We have already reported that serum testosterone concentrations were decreased by keeping rats under constant darkness (5–8) and also by feeding them a low-protein diet (5, 8), and the reduction of gonadal organ weight appeared to be caused by the decreased serum testosterone concentration. The dietary vitamin level, when the level was from 1/3 of normal to 3 times the normal level, had no effect on the serum testosterone level, when the level was from 1/3 of normal to 3 times the normal. Therefore, our result is not contradictory to the result of the previous report (24).

In our previous study, when water-soluble vitamins were at a normal level, the normal fat-soluble vitamins diet promoted the depression of gonadal development in rats kept under constant darkness as a model of disturbed daily rhythm, and the high fat-soluble vitamins diet suppressed that depression (9). Therefore, in this study we attempted to clarify the effect on gonadal development of four kinds of fat-soluble vitamins. The results of the present study showed that, in the rats kept under constant darkness, the normal-VA, high-VD and normal-V.E diet promoted the depression of gonadal development, the normal-VA, normal-V.D and high-V.E diet suppressed the depression of gonadal development, and the VE requirement of rats kept under constant darkness increased more than that of rats kept under normal lighting condition. We were able to obtain more detailed results about the effects of fat-soluble vitamins in this study than in our previous studies (9).

This experiment is part of a series of studies, the results of which will reveal the effects of all nutrients and their interaction on gonadal development under the condition of disturbed daily rhythm. From a basic nutritional standpoint, we are accumulating data about the effects of nutrients at the organ level and analyzing and confirming these data. Our goal is to provide information about the most suitable quantities and ratios of nutrients in the case of disturbed daily rhythm. We also want to clarify the mechanism that induces this phenomenon at the cellular level or genomic level.

REFERENCES