The effects of difructose anhydride III (DFAIII) on stimulating calcium absorption was investigated in humans. We studied changes in the time-course of characteristics urinary calcium excretion in 12 healthy men given 0.3, 1.0 or 3.0 g of DFAIII and 300 mg of calcium as calcium carbonate. In addition, urinary excretion and urine concentrations of creatinine and deoxypyridinoline were determined. Urine calcium excretion every 2 hours after the intake were higher over than that of the control subjects. The total amount of urinary calcium excretion for 10 hours was significantly greater in the subjects given 1.0 g or 3.0 g of DFAIII than that of the control subjects. However, there were no differences in the urine concentrations of creatinine and deoxypyridinoline between the subjects given DFAIII and the control subjects. These findings suggest that low dose of DFAIII had a stimulating effect on calcium absorption in humans.

Key words: difructose anhydride III; calcium; deoxypyridinoline; urine; human

With the objective of increasing calcium absorption in vivo, caseinphosphopeptide and fructooligosaccharide preparations have been developed, and their practical application has been progressing.1,2) It is well known that calcium absorption is generally poor and its current intake by humans is insufficient from the nutritional aspect. The issue of calcium deficiency still exists, and functional foods to increase calcium absorption are being paid particular attention in future.

Difructose anhydride III (DFAIII; di-D-fructofuranose 1,2':2,3 di-anhydride) is a disaccharide produced by inulin fructotransferase, an enzyme of Arthrobacter sp. H65-7.3) The chemical structure of DFAIII is shown in Fig. 1. It has been demonstrated DFAIII increased calcium absorption in vivo balance study with normal and ovariectomized rats and reveals by the Ussing-type chamber technique with the rats isolated intestinal mucosa that DFAIII increased calcium absorption through the paracellular route in the small and large intestine.4–7) However, DFAIII has not been demonstrated as a special food valuable for calcium absorption in humans. We examine here the effects of DFAIII intake on calcium absorption in healthy male adults that were chosen to eliminate any influence of sexual cycle-related hormonal changes.

Materials and Methods

Subjects. The contents, purposes and procedures of this study were fully explained to the subjects by the doctor in charge according to The Helsinki Declaration with the approval of the ethics committee of FANCL Co., Ltd. and that of Osaka Ono Clinic. Informed consent in writing was obtained before the start of this study. Twelve healthy males satisfying the following conditions were selected as the subjects: 1) no history of a daily intake of calcium-enriched food or health food for stimulating of calcium absorption as supplements; 2) no intake of vitamin K and other drugs for treating and preventing osteoporosis, and of any agents mediating bone metabolism, as well as no experience of bone-related disorders, diabetes and other hepatic and renal function disorder; 3) no treatment for alcohol-dependent
Test food and control food. DFAIII used in the study was produced by Nippon Beet Sugar Manufacturing Co., Ltd., by the fermentation method, and its purity was 99%. Three kinds of mixture composed of 0.3, 1.0 or 3.0 g of DFAIII and 750 mg of scallop shell powder as calcium carbonate (corresponding to 300 mg of calcium) folded in with an aluminum-stick were used as the test food. As the control food, only 750 mg of scallop shell powder as calcium carbonate (corresponding to 300 mg of calcium) folded in with the aluminum-stick was used.

Test procedures. The twelve subjects were assigned to two groups (groups A and B). The experiment was carried out in four stages different in the amount of DFAIII taken. The subjects in group A underwent the first stages (750 mg of scallop shell powder as calcium carbonate), the second (0.3 g of DFAIII + 750 mg of scallop shell powder as calcium carbonate), the third (1.0 g of DFAIII + 750 mg of scallop shell powder as calcium carbonate), and the fourth (3.0 g of DFAIII + 750 mg of scallop shell powder as calcium carbonate), the experiment gradually changing from the control food to the test food containing a progressively larger amount of DFAIII. The six subjects of group B underwent the first course (3.0 g of DFAIII + 750 mg of scallop shell powder as calcium carbonate), the second (1.0 g of DFAIII + 750 mg of scallop shell powder as calcium carbonate), the third (0.3 g of DFAIII + 750 mg of scallop shell powder as calcium carbonate) and the fourth (750 mg of scallop shell powder as calcium carbonate alone). Thus, the content of DFAIII in the test food was progressively reduced with each stage, sequential courses involved a wash-out interval of 2 weeks between each. Guidance in normal daily life was given so that the subjects daily routine was consistent all between each. Guidance in normal daily life was given.

On the test day, each subject was let to get up at 7:00 and given 200 ml of distilled water after urination and excretion. Two hours later (at 9:00), urine was collected. Immediately after that, the directed test food and distilled water (200 ml) were given to supplement the defined breakfast. Thereafter, urine was collected at two-hour intervals, the intake of distilled water (200 ml) was repeated at one-hour intervals up to 19:00 with lunch-time inserted at 13:00, adding up to 10 hours after the beginning of the test. During the test period, each subject was instructed to spend time resting in an inpatient room, and any other food apart from except for the defined meals and distilled water was prohibited.

<table>
<thead>
<tr>
<th>Time</th>
<th>Urine</th>
<th>Water</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:00</td>
<td>Discard</td>
<td>200 ml</td>
<td>Breakfast + test food</td>
</tr>
<tr>
<td>9:00</td>
<td>Collect</td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>10:00</td>
<td></td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>11:00</td>
<td>Collect</td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>12:00</td>
<td></td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>13:00</td>
<td>Collect</td>
<td>200 ml</td>
<td>Lunch</td>
</tr>
<tr>
<td>14:00</td>
<td></td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>15:00</td>
<td>Collect</td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>16:00</td>
<td></td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>17:00</td>
<td>Collect</td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>18:00</td>
<td></td>
<td>200 ml</td>
<td></td>
</tr>
<tr>
<td>19:00</td>
<td>Collect</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The schedule for the test day is presented in Table 2.

The volume of each urine sample was determined when collected and each sample was put into a screw-capped glass tube and stored in a dark and cool place. The urine calcium, creatinine and deoxypyridinoline levels were determined immediately after the end of the test. Urinary calcium was measured by the o—CPC method (Calcium-HR; Wako Pure Chemical Industries Ltd., Osaka, Japan), urinary creatinine was measured by the enzymatic method (Pureauto-S CRE-L; Daiichi Pure Chemical Co., Ltd., Tokyo, Japan), and urinary deoxypyridinoline was measured by the enzyme-immunity measurement method (Osteolinks-DPD; Sumitomo Bio-Medicine Co., Ltd., Osaka, Japan).

Statistical analysis. Measurements for each 6 subjects in groups A and B, under the same conditions for the test food were combined into those for 12 subjects and are expressed as the mean ± S.E.M. The significance of differences between the test food group and the control group was analyzed by Student’s T-test (P < 0.05).

Results

Time-course changes in the urine calcium level

Time-course changes in the urine calcium levels (A',
B' and C') and the calcium levels adjusted by the urine creatinine content (A, B and C) determined every 2 hours are shown in Fig. 2. When 1.0 or 3.0 g of DFAIII was given, both the urine calcium level and calcium levels adjusted by urine creatinine had increased 2–4 and 4–6 hours after the intake, this increase being significantly higher than that in the control subjects given the control food. The group given 1.0 or 3.0 g of DFAIII then showed urinary calcium excretion to gradually decrease, resulting in a similar level to that of the control group at a time 8–10 hours after the intake. The group given 0.3 g of DFAIII showed a significant increase in the urine calcium level and calcium level adjusted by urine creatinine 4–6 hours after the intake.
after the intake, this level subsequently dropping to a similar level to that of the control.

Total amount of urinary calcium excretion
The total urinary calcium excretion over 10 hours after the intake of the test food and the control food is presented in Fig. 3. The total urinary calcium excretion by the subjects given 0.3 g of DFAIII was slightly higher than that of the control, although the difference was not statistically significant. When compared with the control, the total urinary calcium excretion was clearly larger by the subjects given 1.0 g of DFAIII (\( p < 0.01 \)), while the difference was statistically significant (\( p < 0.05 \)) by the subjects given 3.0 g of DFAIII.

Amount of urine, and creatinine and deoxypyridinoline levels
The total amount of urine secreted over 10 hours from the intake of the test food or the control food, as well as the urine creatinine and deoxypyridinoline levels are shown in Table 3. There were no significant differences in any of these values between any group given 0.3, 1.0 or 3.0 g of DFAIII, and the control.

Discussion
This study examined the stimulatory effects of DFAIII on calcium absorption in humans. Previous other studies on these effects have used radioisotopes or stable isotopes in clearance and other such tests.\(^8\)\(^{–10}\) We used the urinary calcium excretion as an indicator of calcium absorption. This method is based on the positive correlation between the intestinal calcium absorption and urinary calcium excretion and has often been used.\(^11\)\(^{–19}\) Although the calcium clearance value cannot be obtained, this is a practically valuable method for estimating the ability for calcium absorption. Application of the method, requires no differences in the renal functions and bone absorption between the subject groups, and data are collected by using a marker such as creatinine of deoxypyridinoline. The stimulatory effects of DFAIII on calcium absorption were therefore carefully examined in consideration of those requirements. The group given 3.0 g of DFAIII showed a urine calcium level that significantly increased during the periods 0–2, 2–4 and 4–6 hours after its intake. The group given 1.0 g of DFAIII showed a similar tendency. The total amount of urinary calcium excreted was also increased by the intake of 1.0 or 3.0 g of DFAIII without affecting the urine volume or urine levels of creatinine and deoxypyridinoline. The DFAIII-associated increase in urinary calcium excretion was also apparent after data collection, based on the amount of urinary creatinine excreted. It is thus suggested that the increase in urinary calcium excretion was not attributable to any alteration of renal functions like a change in the renal blood flow. Moreover, the urine deoxypyridinoline level remained unchanged in the two groups given DFAIII and the control food. The triplet collagen fiber secreted from osteoblasts is incorporated in the bone and forms a cross linkage between deoxypyridinoline and pyridinoline. It has been reported that deoxypyridinoline constitutes nearly 22% of the intraosseous tissues and that this compound is excreted to urine in accompaniment to bone absorption. It is important to determine the urine deoxypyridinoline level as a bone absorption marker. Since the urine deoxypyridinoline level did not change in this study, it is assumed that the increase in urine

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total urine (ml/0–10h)</th>
<th>Creatinine (mg/0–10h)</th>
<th>Deoxypyridinoline (nmol/nmol(Cr))/0–10h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1685 ± 12.4</td>
<td>689 ± 41.1</td>
<td>19.8 ± 1.7</td>
</tr>
<tr>
<td>DFAIII (0.3 g)</td>
<td>1718 ± 138.4</td>
<td>684 ± 29.8</td>
<td>19.1 ± 2.1</td>
</tr>
<tr>
<td>DFAIII (1.0 g)</td>
<td>1686 ± 125.3</td>
<td>670 ± 32.0</td>
<td>20.4 ± 1.6</td>
</tr>
<tr>
<td>DFAIII (3.0 g)</td>
<td>1794 ± 81.1</td>
<td>678 ± 29.4</td>
<td>19.7 ± 1.3</td>
</tr>
</tbody>
</table>

(Cr: Creatinine, Mean ± S.E.M. (n = 12).)

Fig. 3. Total Amount of Urinary Calcium Excreted over 10 Hours after Intake of the Test or Control Food. Vertical bars represent S.E.M. (n = 12). Statistical differences: \#p < 0.1, ##p < 0.05 compared with control.
calcium level associated with the intake of DFAIII would not have been attributable to osteolysis. The results of this study therefore suggest that the increase in urinary calcium excretion after DFAIII intake might have been due to stimulated calcium absorption in humans. However, we should emphasize the necessity for further study on changes in the urine hydroxyproline and pyridinoline levels as well as in the glomerular filtration rate.

It has been demonstrated by the Ussing chamber technique with rats that the calcium absorption in all gastrointestinal areas was dose-dependently stimulated by DFAIII.\(^4\) It has also been clarified that the mechanism for such stimulation involved active transport through the tight junction of the enteric canal.\(^4\) However, it is unclear whether the stimulation of calcium absorption in the present study was caused by the same mechanism or not. Meanwhile, we compared the urinary calcium excretion after an intake of DFAIII with that of fructooligosaccharide, whose stimulatory effect on calcium absorption has been demonstrated. It was found that the increase in urinary calcium excretion occurred earlier after the intake of DFAIII than with fructooligosaccharide.\(^20\) Since mineral absorption by fructooligosaccharide is attributable to fermentation in the large intestine, the increase in urinary calcium excretion might reflect the in vitro evidence; i.e., calcium absorption in all regions of the enteric canal was increased by DFAIII. Furthermore, the results of the present study show that the total urinary calcium excreted over 10 hours increased with increasing intake of DFAIII. Dose-dependent enhancement by DFAIII of calcium absorption has also been demonstrated by the Ussing chamber technique.\(^4\) It is therefore intriguing that the results obtained from different types of experiment are very similar in respect of dose-dependent enhancement.

Since hormonal balance changes related to menstruation might affect the results, females seemed not to be suitable for this assessment by the present procedure, so the present study was conducted on male subjects. However, it seems more important to clarify the effects of DFAIII on calcium absorption by females which is prone to influence by dieting, pregnancy and menopause. It also seems very important to clarify the amount of calcium retained in the body.

The results of this study demonstrate enhanced of calcium absorption in humans by an intake of DFAIII. It is therefore possible that DFAIII would be a useful food material for promoting calcium absorption.

References

16) Hosono, A., Ohtsuki, M., Ohta, A., and Adachi, T., The trial experiment of mineral absorption as assessed by urinary excretion of calcium and magnesium in healthy


