Postprandial Changes in the Function of Digestive Organs in Rats Meal-fed Twice a Day with 40% Casein-based and Protein-free Diets

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Growing rats were meal-fed twice a day in the morning (9:00–11:00) and night (20:00–22:00) with 40% casein-based and protein-free diets in turn, or vice versa. Twenty-five days later, the animals were killed by four in each group at 9:00, 11:00, 14:00, and 18:00, to excise their small intestines and pancreas. Immediately the jejunal mucosa was scraped together and assayed for [3H]leucine incorporation into protein as well as [3H]leucine absorption in epithelial cells. Concurrently with the assay, tissue-specific hydrolase activities were measured. Although small intestinal intrinsic hydrolases did not fluctuate much in activity, the activities of digestive enzymes in the pancreas increased after the intake of the 40% casein-based diet and decreased after the intake of the protein-free diet. This can be accounted for by the supply of synthesis materials for these digestive enzymes. Interestingly a reverse tendency was observed for both the in vitro ‘protein synthesis’ and ‘amino acid transport’ capacities of jejunal mucosal scrapings. Such functional changes are probably under the control of circadian rhythm cued by a feeding schedule.

Much interest was once taken in the nutritional efficiency of segmented protein intake from the viewpoint of a spare diet or protein saving. We also attempted to reevaluate the tolerance limit of repeating protein-unbalanced meals. In the preceding papers, we described how the alternate intake of 40% casein-based and protein-free diets every one or more days was inferior in rat growth to the continuous intake of their fifty-fifty diet, but that such was not the case with meal-feeding (2 h each meal) twice in the morning and night. Namely, the rats alternatively given the high- and non-protein diets were similar in growth to the ones given the 20% casein-based diet at both meals. The restricted meal-time and frequency is a quite usual eating habit in human life. A full meal leads to satiety, thereby interrupting food intake even though a visitation of fasting will be forecast after a while. On that account, meal-feeding is in actuality appropriate to make clear the fluctuations in function before and after eating. When meal-feeding is repeated over a certain period, the circadian rhythm of functions cued by the feeding time become conspicuous in rat digestive organs.

The present paper is concerned with postprandial changes in the function of digestive organs in rats meal-fed alternately with high- and non-protein diets. The in vitro ‘protein synthesis’ and ‘amino acid transport’ capacities of intestinal mucosal scrapings were measured at 4 sets of preprandial and postprandial times, being accompanied by the activity measurement of intrinsically occurring hydrolases not only in the small intestine but also in the pancreas.

Materials and Methods

Animals and feeding schedule. Male Wistar rats aged 4 weeks (purchased from Shimizu Laboratory Supplies Ltd., Kyoto) were individually housed in wire-bottomed cages in an air-conditioned room (temperature 23–25°C; humidity 55–65%) with a half-day light/dark cycle (lighting between 8:00 and 20:00). The animals were divided into 3 groups, which were meal-fed twice a day for 25 days with the 40% casein-based and protein-free diets shown in Table I. After the prescribed period, they were killed four in each group at 9:00, 11:00, 14:00, and 18:00 by blood drawing from the abdominal aorta, and immediately the small intestine (jejunum) and pancreas (whole) were excised. The jejunum was cut open lengthwise on an ice-cold glass plate, where the mucosa was scraped together with a slide glass. The mucosal scrapings were in part used for both ‘protein synthesis’ and ‘amino acid transport’ experiments, the remainder being kept frozen at −80°C together with the pancreas.

Activity measurement about pancreatic and intestinal enzymes. The frozen pancreas was thawed, defatted, and homogenized with 9 volumes of cold saline in a Potter-Elvehjem homogenizer. The supernatant after centrifugation at 12,000 × g for 20 min was divided into two portions; one was used as such for the activity measurement of amylase and lipase in the presence of 10 mm benzamidine and 1 mm PMSF, the other being used for the activity measurement of trypsin and chymotrypsin after activation by porcine enterokinase. The activities of amylase and lipase were measured according to the dinitrosocycclate method and the Cu-diphenylcarbazide method, respectively, and those of trypsin and chymotrypsin were estimated by the use of their specific synthetic substrates, i.e., N-benzoyl-L-arginyl-p-nitroanilide and N-benzoyl-L-tyrosyl-p-nitroanilide, as previously described. The jejunal mucosal scrapings that had been kept frozen were also thawed and homogenized thoroughly. Dilute solutions of the homogenate were used to measure the activities of

Table I. Time Schedule of Meal-feeding and Composition of Experimental Diets

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet supplied at</th>
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<tbody>
<tr>
<td></td>
<td>9:00–11:00</td>
</tr>
<tr>
<td>A</td>
<td>40% Casein-based</td>
</tr>
<tr>
<td>B</td>
<td>Protein-free</td>
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<tr>
<td>C</td>
<td>20% Casein-based</td>
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The composition of the respective diets is as follows (in %): casein (40, 20, 0), α-corn starch (45, 65, 85), oil mix. (5), mineral mix. (5), vitamin mix. (1), and cellulose powder (4). These ingredients were all obtained from Oriental Yeast Co.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; MEM, Eagle’s minimum essential medium; PCA, phosphoric acid; TCA, trichloroacetic acid; EGF, epidermal growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor.

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villus-specific enzymes such as sucrase, leucine aminopeptidase, and alkaline phosphatase. In the sucrase assay, glucose was measured with a commercially available assay kit (Wako Pure Chemical Industry Ltd., Osaka). In the aminopeptidase assay, the activity was due to the absorbance measurement of p-nitroaniline released from L-leucine-p-nitroanilide. Alkaline phosphatase was colorimetrically estimated using p-nitrophenyl phosphoric acid disodium salt as a substrate. Protein was measured by the Bradford method, and a unit was defined as the amount of enzyme capable of releasing one μmol of product per min under the routine assay conditions.

**In vitro incorporation of [3H]leucine into mucosal protein fraction.** Mucosal scrapings corresponding to a jejunal 3-cm segment were thoroughly vortexed in a 50-ml Ehrenmeyer flask containing 2 ml of leucine-free MEM supplemented with 111 KBq of [3H]leucine (du Pont/NEN Research Products, Boston), and incubated at 37°C for 10 min while being bubbled with O₂–CO₂ (95:5) mixture gas. Then, 2 ml of 0.4 N PCA containing 10 mm L-leucine was added to each flask and the precipitate was separated by centrifugation at 1000 × g for 10 min. The pellet was twice washed with 2 ml of 0.2 N PCA, suspended in 0.3 N KOH, and incubated at 37°C for 90 min with gentle stirring. The suspension was chilled in an ice bath, to which a half volume of 10% PCA was added. The resulting pellet was suspended at 37°C for 30 min in 2.25 ml of 10% PCA, and soluble components after centrifugation were discarded. The final pellet was dissolved in 2 ml of 1 N NaOH at 60°C and samples were used for protein and radioactivity measurements in the usual way.

**[3H]Leucine absorption experiment with dispersed mucosal scrapings.** Mucosal scrapings were likewise dispersed in the above-mentioned medium, and incubated for 10 min at 37°C under an O₂–CO₂ (95:5) atmosphere. The dispersed system was separated by squeezing the mixture through a glass fiber filter layered on with Hyflo-super-cel (a filter clay, from Nacalai Tesque Ltd., Kyoto), washed with 5 ml of cold saline in 5 portions, and transferred into a liquid scintillation vial containing 0.5 ml of Protosol (a tissue solubilizer, from New England Nuclear). The vial was kept at 50°C for 3 h with occasional shaking and 50 μl of hydrogen peroxide was poured in to minimize color-quenching. [14C]Inulin was used for correcting unabsorbed [3H]leucine, hence its extracellular retention was assessed as less than 1% of initial amount.

**Analysis of free amino acids in jejunal mucosa.** Several pieces of frozen mucosal scrapings to which was added 5-carboxymethylcysteine (0.1 μmol) as an internal standard were homogenized with 9 volumes of 5% TCA in a Polytron disperm. Denatured protein and insoluble materials were separated by centrifugation and washed with 5% TCA. The supernatant and washings were combined and repeatedly extracted with ethyl ether to remove TCA. The residue after lyophilization of the aqueous layer was dissolved in pH 3.25 citrate buffer, and the amino acids were analyzed with a Hitachi 835 analyzer.

**Plasma corticosterone measurement.** The blood collected from the abdominal aorta with a syringe was centrifuged to separate the plasma. A sample (100 μl), 0.1 N NaOH (200 μl), and methylene chloride (5 ml) were mixed by stirring. The methylene chloride layer after centrifugation was again washed with 2 ml of 0.1 N NaOH containing 10% Na₂SO₄ to extract water-soluble materials and blended with an ethanol-sulfuric acid (3:7) mixture. The fluorescence of the acid (lower) layer after centrifugation was measured (emission at 520 nm; excitation at 468 nm) and the plasma corticosterone level was estimated from a calibration curve for various concentrations of the authentic compound (a product of Sigma Chemical Co., St. Louis).

**Statistical analysis.** Data were obtained as the means ± SE for 4 animals except for growth-related parameters (n = 16), which were compared by analysis of variance according to the Newman–Keuls test or Student's t-test.

**Results**

Figure 1 summarizes the growth curves, food intakes, and protein efficiency ratios of three groups of rats meal-fed alternately with 40% casein-based and protein-free diets twice a day (group A, B) or successively with a 20% casein-based diet at both meals (group C). Group A or B seemed a little inferior to group C in growth, but no significant differences were observed among the three groups. With respect to the daily average food intake every
Fig. 2. Activity Levels of Pancreatic Digestive Enzymes in Meal-fed Rats.

The rats of each group under the meal-feeding conditions were killed by fourts at
stated times on the 25th day. The tissue homogenate was fractionated into RNA,
DNA, and protein in the usual way, which were assayed as previously described.\textsuperscript{9} The
activity levels of these digestive enzymes were expressed as units per mg of
DNA. Each symbol and bar represents the mean \pm SE (n = 4).

morning and night through the feeding period, both groups
A and B ate the 40% casein-based diet still more at
whichever feeding time it was given. Group C provided
with the 20% casein-based diet at both meals ate it somewhat
more in the night. The protein efficiency ratio was C > A ≥ B
in order of its effectiveness.

Figure 2 depicts the activity levels of digestive enzymes
in the pancreas at 4 sets of preprandial and postprandial
times on the 25th day after the start of the experiment. The
postprandial protein/DNA and RNA/DNA ratios tended
to change upward in the group (A or C) provided with the
40% or 20% casein-based diet at the appointed time and
to change downward in the group (B) provided with the
protein-free diet, although their change ranged only to a
limited extent. On the other hand, the trypsin and
chymotrypsin activities just before eating were at low levels
in groups A and C, but at high levels in group B. Conversely,
the amylase and lipase activities at that time were at high
levels in group C as well as in group B. These enzyme
activity levels varied markedly within the postprandial
time in both groups A and B, but to a lesser extent in group
C. Especially, the protease activities became far higher in
group A than in group C beyond postprandial 8 h, despite
their similar level to each other before eating. Figure 3
illustrates the activity levels in the jejunal mucosa of sucrase,
leucine aminopeptidase, and alkaline phosphatase at
pre- and post-prandial times. These intestinal hydrolases did
not fluctuate in activity to such an extent as the pancreatic
digestive enzymes did, so that there were no significant
differences among the three groups at any postprandial time.

Independently of activity measurement for these
hydrolases in the jejunal mucosa, the \textit{in vitro} incorporation of \(^{3}H\)leucine was investigated with adequately
vortexed mucosal scrapings from rat jejunum. The
experimental results are shown in Fig. 4. The rate of
\(^{3}H\)leucine incorporation, being higher in group A than in
group B before a meal, appeared to decrease moderately in
the former provided with the high-protein diet and to
increase by degrees in the latter provided with the
non-protein diet. Group A given the non-protein diet at the
preceding meal (before a half-day) seemed to be eager for
protein at the beginning of the feeding time, while group
B was nutritionally satisfied, as it were, with the high-protein
diet at the preceding meal. Even though this is true, there
remains the possibility of isotope dilution by intracellular
amino acids. For this reason, the free amino acid levels in
the jejunal mucosa were measured with an automated amino
acid analyzer. The analytical results are shown in Fig. 5.
No significant postprandial change was observed between
groups A and B with respect to the cellular levels of branched
chain amino acids such as Leu, Ile, and Val in the mucosa,
although there were considerable fluctuations in Arg, Glu,
and Ala concentrations. In this connection, the leucine transport capacity of mucosal scrapings taken off at pre- and post-prandial times was measured by the flash filtration method usual in the absorption experiment with isolated epithelial cells. Consequently, it was recognized that the leucine transport capacity lessened gradually with time after the high-protein diet intake and varied inversely after the non-protein diet intake as shown in Fig. 6. This tendency was in fair agreement with the observation for the in vitro [3H]leucine incorporation into protein.

Time restriction each meal may bring about such a stress as to affect the plasma corticosterone concentration. Figure 7 compares the plasma corticosterone concentrations at various postprandial times in two groups of rats meal-fed alternately with the 40% casein-based and protein-free diets or vice versa in turn. A decrease in the hormone level was marked at 11:00 in group A, followed by a gradual restoration later on. In this contrast, no significant change was observed at whichever postprandial time in group B. There was a roughly similar directionality in postprandial change between the plasma corticosterone concentration and the mucosal [3H]leucine incorporation into protein or its absorption in epithelial cells.

**Discussion**

It is consistent with the previous observation\(^9\) that the rats of two groups alternately given the 40% casein-based and protein-free diets at two meals according to the meal-feeding schedule grow similarly to the ones of a control given the 20% casein-based diet at both meals, although there is a change in their order of body weight gain to a not significantly different extent from the control. Since the restricted feeding time is an image of usual human practice, two meals of 2 h in the morning and night are regarded as proper to avoid obesity in the animals without exercise loading. If such an eating habit is standardized, the segregated intake of high- and non-protein diets can be said not to impair the animal growth. Even so, it seems more likely that some aftereffects occur in the metabolic functions by the intermittent ingestion of food overabundant in protein or starch.

**Fig. 4.** In Vitro Protein Synthesis Capacity with Jejunal Mucosa in Meal-fed Rats.

Mucosal scrapings were prepared from the respective jejunal mucosae immediately excised after the rats of groups A (●) and B (○) were killed at stated intervals, and examined for the protein or DNA content (upper) and the ability to incorporate [3H]leucine into the protein fraction (lower) under the above-mentioned assay conditions. Two values (means ± SE) with an asterisk in the lower diagram are significantly \((p < 0.05)\) different from that at preprandial time (9:00).

**Fig. 5.** Intracellular Free Amino Acid Levels in Jejunal Mucosae of Meal-fed Rats.

Jejunal mucosae of the same animals as killed at stated intervals in Figs. 2, 3, and 4, were used for amino acid analysis in the usual manner. The intracellular amino acid levels were expressed as nmol per mg of DNA. Closed and open circles represent the means ± SE for 4 animals in groups A and B, respectively.
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Fig. 6. In Vitro Leucine Transport Capacity with Jejunal Mucosa in Meal-fed Rats.

The same jejunal mucosal scrapings as in Fig. 4 were used to assay the intracellular
[^H]leucine uptake. Data were obtained as the radioactivity of[^H]leucine
incorporated into the epithelial cells; the value (mean ± SE) with an asterisk is
significantly (p<0.05) different from that at preprandial time (9:00).

Fig. 7. Plasma Corticosterone Concentrations in Meal-fed Rats.
The plasma from the same animals as in Figs. 2—6 were used for this assay. Values
are the means ± SE (n=4); there is a significant difference at p<0.05 between the
plasma levels obtained at 9:00 and 11:00 in group A.

Digestive enzymes in the pancreas are well-known to
increase with increasing amounts of protein, starch, or fat
consumption. The intake of 40% casein-based diet at a
meal in the morning raised the postprandial levels of
proteolytic enzymes much more 9h after the beginning of
eating than that of 20% casein diet at both meals in the
morning and night did (Fig. 2). The pancreatic levels of
amylase and lipase were also enhanced after the 40%
casein-based diet intake. Interestingly, there are obvious
distinctions in activity level at both preprandial and
postprandial times between the two dietary groups given
the high- or non-protein diet at the appointed time.
Postprandial increases in the pancreatic enzyme levels after
the 40% casein-based diet intake are accounted for by amino
acid supply for protein synthesis and its subsequent
accumulation of zymogen granules exceeding exocytosis,
while the reverse explanation applies to postprandial
decreases in the pancreatic enzyme levels after the
protein-free diet intake. Similarly, the difference just before
eating (at 9:00) between groups A and B is interpreted into
a steady state after the high- or non-protein diet was ingested
last night. Conversely, the range changes of trypsin and
chymotrypsin activities in the pancreas of rats given the
20% casein-based diet at both meals were limited at low
levels; suggesting that these proteolytic enzymes could be
well adapted even to high-protein feeding at either of the
two meals a day. On the other hand, the activities of amylase
and lipase at high levels in the group provided with the
20% casein-based diet fell to half soon after eating and
returned to the initial levels 9h after the beginning of eating.
Although the main food ingredients capable of inducing
amyrase and lipase are starch and fat, respectively, an
appropriate supply of protein is the minimum requirement
for the repair of discharged zymogen granules. It is generally
accepted that cholecytokinin secretion from enterodocrine
cells is stimulated by high-protein feeding, thereby
mediating pancreatic exocytosis. Certainly in our
experiment, the process of exocytosis accompanied by food
intake has been reflected in the temporarily lowered enzyme
levels in the pancreas after the 20% casein-based diet intake.
However, no fall in the pancreatic enzyme levels was
observed after the 40% casein-based diet intake, or rather
the activity levels became progressively higher. The decrease
in activity was conspicuous after the protein-free diet intake.
A plausible explanation for this discrepancy is that the
synthesis of digestive enzymes in the pancreas would have
surpassed their discharge from there after the high-protein
diet intake.

Dietary adaptation has been referred to for terminal
digestive enzymes (i.e., several hydrolyses) in the small
intestine as well. For example, Furuya et al. have reported
that small intestinal disaccharidases, γ-glutamyltransferase,
leucine aminopeptidase, and alkaline phosphatase fluctuate
in activity in response to alternate meal-feeding with high-protein (starch-free) and protein-free (high-starch)
diets. The activity levels of sucrase, leucine aminopeptidase,
and alkaline phosphatase in the intestinal mucosa were of
no significance in postprandial changes not only after the
intake of 40% casein-based diet but also after that of the
protein-free diet, although being offset by the large deviation
in our experiment (Fig. 3). A big difference between our and
their dietary conditions is that our high-protein diet includes
45% starch besides 40% casein relative to their 84%
casein-based diet lacking in starch or sugar. In other words,
the meal-feeding schedule due to the alternation of 40%
casein-based and protein-free diets is not such a drastic
dietary condition as to amplify the circadian rhythm of
small intestinal hydrolyses.

Incidentally, circadian rhythmicity occurs in villus height
and cell number, probably arising from cell proliferation,
migration, and exclusion. Such an influence may possibly
be recognized in the capacity of mucosal epithelial cells for
‘protein synthesis’ or ‘amino acid transport’, no matter how
insignificant the variations of intestinal hydrolyse activities
are before and after eating. The down and up slopes for the 4 sets of data in the ‘protein synthesis’ or ‘amino
acid transport’ capacity (strictly speaking, those in the
[^H]leucine incorporation into protein or its absorption in
cells) were characteristic of the groups given the 40%
casein-based or protein-free diets at the appointed time in
the morning (Fig. 4). These slopes were in the reverse
direction to those obtained by measuring the postprandial activity levels of pancreatic digestive enzymes. This implies
that a rise in the ‘protein synthesis’ or ‘amino acid transport’ capacity is not necessarily dependent on the delivery of
amino acids after eating. In addition, leucine and other branched-chain amino acids have proved not to fluctuate in
cellular concentration irrespective of its being before or
after eating (Fig. 5). Accordingly, it is not strange that the
\[^{3}H\]leucine incorporation into protein (for convenience, defined as the 'protein synthesis' capacity in our experiment) corresponds to the intracellular uptake of the radioactive amino acid. As it is, protein synthesis prevails in crypt cells, from which transport systems for amino acids are originated. At this time, extracellular amino acids are intimately involved in the regulation of neutral amino acid transport System A. For example, starvation for all amino acids causes an increase in the transport capacity for neutral amino acids by incubation in amino acid-free medium.\(^{25}\)

Moreover, certain hormones serve as regulators for transporter induction. As an instance, the action of corticosterone may be cited in relation to the maturation of intestinal functions.\(^{26,27}\) When the plasma corticosterone concentrations before and after eating were compared from this standpoint, the drop in concentration 2 h after the beginning of eating was found to be statistically significant as compared with the level before eating in group A given the 40% casein diet, but not as compared with that in group B given the non-protein diet. If a daring assumption is permitted, this variation is worth notice as one of the reasons for the decreased 'protein synthesis' or 'amino acid transport' capacity in rat mucosa after the 40% casein-based diet intake. Granting that it is so, a variety of intrinsic factors responsible for cell proliferation such as EGF, IGFs, PDGF, TGF\(\alpha\), and oncogene-expression products are familiar to us at present.\(^{28,29}\) It is of much interest in connection with the circadian rhythm of vital functions to what extent some of these factors vary with the meal or its quality.

**References**