Ontogeny of intestinal safety factors: lactase capacities and lactose loads

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O'Connor, Timothy P., and Jared Diamond. Ontogeny of intestinal safety factors: lactase capacities and lactose loads. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R753–R765, 1999.—We measured intestinal safety factors (ratio of a physiological capacity to the load on it) for lactose digestion in developing rat pups. Specifically, we assessed the quantitative relationships between lactose load and the series capacities of lactase and the Na⁺-glucose cotransporter (SGLT-1). Both capacities increased significantly with age in suckling pups as a result of increasing intestinal mass and maintenance of mass-specific activities. The youngest pups examined (5 days) had surprisingly high safety factors of 8–13 for both lactase and SGLT-1, possibly because milk contains lactase substrates other than lactose; it also, however, suggests that their intestinal capacities were being prepared to meet future demands rather than just current ones. By day 10 (and also at day 15), increased lactose loads resulted in lower safety factors of 4–6, values more typical of adult intestines. The safety factor of SGLT-1 in day 30 (weanling) and day 100 (adult) rats was only ~1.0. This was initially unexpected, because most adult intestines maintain a modest reserve capacity beyond nutrient load values, but postweaning rats appear to use hindgut fermentation, as a built-in reserve capacity. The series capacities of lactase and SGLT-1 varied in concert with each other over ontogeny and as lactose load was manipulated by experimental variation in litter size.

carbohydrate digestion; fermentation; glucose transport; rat; weaning

IT HAS BEEN APPRECIATED for a long time that the physiological, morphological, and biochemical capacities of animals have evolved to be qualitatively adapted to the functional loads on them. A related question now attracting widespread interest is: are capacities also quantitatively adapted to their loads? That is, by how large a reserve capacity, if any, do capacities exceed the loads on them?

Pioneering comparative studies by Taylor and colleagues (42, 47) on the respiratory system have shown that many physiological parameters are quantitatively matched to their prevailing peak loads with modest reserve capacities. Studies from our laboratory (7, 27, 29) examined capacity-load relations of the intestinal brush border in many animal species under various conditions and reached a similar conclusion. The evolutionary explanation for this pattern is that biological molecules and structures cost biosynthetic energy and occupy space, but animals have finite quantities of both energy and space. Hence excessive reserve capacities at one step come ultimately at the expense of limited capacities at other steps, with the result that uneco-nomic animals tend to be outcompeted by more economi-cally designed animals. That is, one expects physiological capacities to be characterized by the phrase "enough, but not too much" (15).

Most biological systems consist of a load imposed not on a single capacity, but instead on multiple capacities in series. For example, most biochemical pathways comprise a chain of enzymes or else hydrolases in series with a transporter of their product. Biochemists continue to debate whether the capacities of successive enzymes in a pathway are matched to each other or whether one or a few enzymes in a pathway have much lower capacities and thus represent rate-limiting steps (10, 35, 41). At the heart of this controversy is the problem of extrapolating in vitro measurements of maximal rate (V_{max}) values to in vivo conditions. In particular, when the ionic strength, pH, and composition of experimental solutions used for in vitro studies do not closely duplicate intracellular conditions (as is often the case), and when the structure of the intact tissue has been destroyed by homogenization, extrapolation of conclusions to physiological conditions becomes debatable. The intestinal brush-border offers a decisive advantage for such studies because its hydrolytic enzymes and transport proteins reside in a cell membrane directly exposed to an external solution, the intestinal lumen. Thus brush-border enzyme and transporter capacities can be straightforwardly measured in an intact tissue exposed to an external solution without the uncertainties introduced by homogenization and by replacement of the internal milieu with a solution of possibly quite different composition.

The enzyme lactase and the Na⁺-glucose cotransporter (SGLT-1) of the intestinal brush border constitute an ideal system for testing quantitative principles of animal design. Lactase hydrolyzes the disaccharide lactose into its monosaccharide constituents, glucose and galactose. Both glucose and galactose are then transported across the brush-border membrane by SGLT-1. Because SGLT-1 transports the products of lactase, the two capacities are in series. Both capacities can be measured under physiological conditions in an intact intestinal preparation for comparison with each other and with the dietary lactose load. Some information about reserve capacity was already available for both capacities (8, 22, 36, 44), but the two capacities were measured in separate studies using different experimental preparations, conditions, and dietary lac-
LACTASE SAFETY FACTORS

Measure dietary lactose intake, intestinal lactase capacity, and intestinal SGLT-1 capacity in individual rat pups of both sexes that were 5 (n = 7), 10 (n = 5), or 15 (n = 6) days old. Dietary sucrose intake and SGLT-1 capacity were also measured in weaning (30 days) and adult (100 days) female rats (n = 4 for each). All pups used in these time course experiments were raised in litters of 10 pups each. To minimize any circadian effects, all intestinal capacity measurements were conducted between 8:00 AM and 1:00 PM.

The relationship between a physiological capacity and the load on it can be described by the ratio of capacity to load, also known as the safety factor (2). Determining the quantitative relationship between dietary lactose load and lactase capacity is relatively straightforward: if a pup consumes 1 mmol/day of lactose, then the load on lactase is 1 mmol/day; if the intestinal lactase capacity in this hypothetical example is 3 mmol/day, then the lactase safety factor equals 3 (3 divided by 1). The relationship between dietary lactose load and SGLT-1 capacity, however, requires further explanation. Because the disaccharide lactose is composed of the monosaccharides glucose and galactose, hydrolysis of 1 mmol of lactose yields 1 mmol of glucose and 1 mmol of galactose. SGLT-1 transports both glucose and galactose with approximately equal affinities for both during the suckling period (43). Therefore, a dietary lactose load of 1 mmol/day represents a load of 1 mmol/day on lactase, but of 2 mmol/day on SGLT-1. If SGLT-1 capacity was 3 mmol/day, then the SGLT-1 safety factor would equal 1.5 (3 divided by 2). In other words, in sucking pups consuming lactose, the SGLT-1 capacity must be double the lactase capacity for the two series capacities to possess equal safety factors.

Capacity-load relationships are more straightforward after rats have been weaned. Sucrose, the form of sugar in the adult diet in our experiments, consists of glucose and fructose. Fructose is transported by a separate transport protein distinct from SGLT-1. Hence the load on SGLT-1 after weaning is identical to the dietary sucrose load (because a dietary sucrose load of 1 mmol/day yields a glucose load of 1 mmol/day on SGLT-1).

To assess the effect of altered loads, three 4-pup litters and three 16-pup litters were examined at 15 days of age and compared with each other and with individuals from 10-pup litters. Altered litter size affects milk intake per pup, because a dam's total milk output increases substantially with the number of pups in her litter. Therefore, each pup in a large litter tends to receive less milk per day than a pup in a small litter (22).

Measurement of Milk Intake and Sugar Load

Dietary lactose load was calculated as the product of daily milk intake times milk lactose concentration. As the first step, milk intake was determined by a method similar to that of Coward et al. (11), Knight et al. (31), and Fiorotto et al. (22).

The method involves injecting pups with $^3$H$_2$O and then determining the rate of dilution in the homogenate over time. The underlying principle is that the rate of isotope dilution is proportional to the rate of milk intake. But because the rate of isotope dilution is actually a measure of water turnover, calculating milk intake rate requires also accounting for pup body water content, metabolic water production, and the water content of milk. Because dams regularly consume the urine and feces of their pups, measures of water turnover must also account for the recycling of $^3$H$_2$O from a $^3$H$_2$O-injected pup to the dam and then back to pups (4).

Milk intake determinations required urine samples over the course of 40 h, so measurements for day 5 pups took place from days 3 to 5, those for day 10 pups from days 8 to 10, and...
those for day 15 pups from days 13 to 15. At least two pups from each litter were injected with 50 µl of physiological saline that contained 2 µCi of $^{3}H_{2}O$. To correct for recycling of $^{3}H_{2}O$ through the dam by the method of Baeverstock and Green (4), at least two pups from each litter were sham injected with 50 µl of physiological saline that contained no $^{3}H_{2}O$. At four collection times, −5, 20, 30, and 45 h after injection, 25–100 µl urine samples were collected from injected pups and sham-injected pups and immediately placed in scintillation vials containing scintillation cocktail (3a70, Research Products International, Mt. Prospect, IL). Pups were stimulated to urinate by gently tickling their genital area with a pipette tip. Urine was then collected directly into the pipette tip as the pups urinated. Volume of urine collected, elapsed time since injection, and pup mass were recorded. Samples were counted for radioactivity by liquid scintillation spectrometry. For each sample collection period, the average specific radioactivity of samples from sham-injected pups was subtracted from the values of their injected littermates to correct for recycling. The natural logarithm of recorded. Samples were counted for radioactivity by liquid scintillation spectrometry. For each sample collection period, the average specific radioactivity of samples from sham-injected pups was subtracted from the values of their injected littermates to correct for recycling. The natural logarithm of record, and the slope of the regression line was determined by least squares regression, and the slope of the regression line was determined by least squares regression, and the slope of the regression line was multiplied by the body water content of the pup, by 24, and by 1.1, for the following reasons. Body water content was determined by multiplying pup mass times percent body water, the latter having been measured in separate pups (of each age and litter size used in this study) by drying carcasses to constant weight at 70°C. Multiplying the slope by 24 converted intake rates from hourly to daily. The factor of 1.1 accounts for the water content of rodent milk and for the production of metabolic water from oxidation of milk (30). Fiorotto et al. (22) used a more elaborate method for converting water intake rates into milk intake values but, our method yields values within 5% of theirs for all values of water intake that we measured (M. L. Fiorotto, personal communication).

The second step in determining lactose load was to measure milk lactose concentration. Milk was collected from dams 2 h after pups had been separated from their pups. Dams were lightly anesthetized by inhalation of halothane, then injected intramuscularly with 1.0 IU oxytocin (Sigma, St. Louis, MO) in 200 µl saline. A teat pump was fashioned by drawing a slight vacuum through a vacutainer and collecting milk through polyethylene tubing with a pipette tip at the end. Each mammary gland was evacuated as completely as possible, and 3–6 ml of milk was collected within 25 min. Milk samples were kept frozen at −20°C until analyzed.

Lactose content was determined on duplicate or triplicate samples of milk by a spectrophotometric assay kit from Boehringer-Mannheim (catalog no. 176303, Indianapolis, IN). A frozen sample of human milk from a single source was run in duplicate with each assay as a control for interassay variability. The coefficient of variation (CV) of our lactose assay, based on results from the human control samples, was 4%. All human milk lactose values fell within 5% of each other, so it was not necessary to discard any results or repeat any assays.

Food intake and glucose load for rats 30 and 100 days old were measured each day for 3 days before death, as previously described (25). Briefly, rats were maintained in cages with wire bottoms that allowed feces and oris to drop to a tray below. Feces and oris were collected, separated, and weighed daily. Daily food intake was calculated as the mass of food disappearing from the food dispenser each day, minus the mass of the collected oris. Glucose load is the mass of food eaten times the sucrose content of the food (because 1 mol of sucrose on hydrolysis yields 1 mol of glucose).

Everted Sleeve Preparation and Gut Morphology

Lactase activity was measured in an everted sleeve preparation by a modification of the sucrase assay of Lee et al. (32). Activity of SGLT-1 was measured in the same everted sleeve preparation, as described briefly below and in more detail previously (16, 30). Briefly, rat pups were anesthetized with an intraperitoneal injection of 50–100 µl Nembutal, a laparotomy was performed, and the entire small intestine from pylorus to ileocecal junction was perfused with ice-cold Ringer and then dissected out. The small intestine was divided into equal-length thirds, each of which was lightly blotted dry and weighed. The wet mass of the small intestine was taken as the sum of these three regional wet masses. Each of the three intestinal regions (proximal, middle, and distal) was then everted over a stainless steel rod. From the middle of each region we used five sleeves immediately adjacent to each other: the first and fifth sleeves to assay SGLT-1, the second and fourth sleeves to assay lactase, and the third sleeve as a tissue blank for lactase assays (see Lactase Assay). Segments 1.5–2.0 cm in length were mounted on steel rods and maintained in ice-cold, oxygenated Ringer for 30–60 min before the assays were begun.

Thus we obtained $V_{\text{max}}$ values for lactase and SGLT-1 from adjacent pieces of intestine in the same rat pup, prepared as the same intact intestinal preparation with good control of unstirred layers. Of course, as is the case for all in vitro assays, there may be differences between our in vitro measures of SGLT-1 and lactase activities and the in vivo activities. Two particular concerns are effects of unstirred layers and maintenance of tissue integrity. For example, Levitt et al. (33) showed that unstirred layer effects could persist even with high rates of stirring (we used 1,200 rpm), but they also found that these effects were significantly reduced at substrate concentrations such as the one we used. As for tissue integrity, the low measured values of passive glucose flux across the epithelium (see Appendix) indicate the functional integrity of tissues used in our experiments.

Wet and dry mass values for the stomach, small intestine, cecum, and large intestine were measured as described previously (25) in 5-, 10-, 15-, 30-, and 100-day rats.

Lactase Assay

The everted sleeve lactase assay, like classical homogenate assays (12, 13), is based on the rate of glucose production resulting from the action of lactase on lactose. The assay conditions that we shall now describe were selected on the basis of validation experiments similar to those described previously for sucrase (32) and described in more detail in the Appendix.

The principle of the assay is to determine lactase activity by incubating an everted sleeve preparation in a lactose-containing solution and measuring the rate of glucose production in the incubation solution. Because the glucose normally would be taken up into the tissue by the action of SGLT-1, we inhibited the transporter by preincubating the sleeve for 20 min at 37°C in Ringer solution containing 0.5 mM phlorizin, a transport inhibitor. Validation experiments (see Appendix) showed that this treatment inhibited glucose transport by 99.5 ± 0.2% ($n = 3$) at a glucose concentration of 0.1 mM (the maximum glucose concentration produced in our lactase assays). After the 20-min preincubation period, tissues were incubated for 16 min at 37°C and pH 7.3 in 10 ml Ringer...
containing 50 mM lactose (isosmotically replacing a corresponding amount of NaCl) and 0.5 mM phlorizin and stirred at 1,200 rpm. At the end of the incubation period, two 250-μl samples of the incubation solution were collected for determination of glucose concentration. The 250-μl samples were added to tubes containing 750 μl of lactose solution (1 mM Tris buffer, 200 U/l horseradish peroxidase, 10 mM p-hydrobenzoic acid, 0.2 mM aminothiopyrine, and 4,442 U/l glucose oxidase; Sigma). The Tris halts lactase hydrolysis immediately (12). The lactostat tubes were incubated at 37°C for 30 min, and then absorbance at 500 nm was read by a Beckman DU-640 spectrophotometer. Lactase activity, like glucose uptake activity, was normalized to milligrams wet mass of the intestinal sleeve.

Two slight corrections were subtracted from the measured absorbance. One correction (reagent blank), to account for impurities in the lactose solution, was the absorbance of a 250-μl aliquot of the 50 mM lactose Ringer incubation solution treated identically (i.e., incubated for 30 min in glucostat) except that no intestinal sleeve had previously been incubated in it. The other correction (tissue blank), to account for the presence of impurities in the intestinal sleeve, was the absorbance of a 250-μl aliquot from a Ringer solution that contained 0.5 mM phlorizin and no lactose, but in which an intestinal sleeve had been incubated for 16 min. The aliquot was then incubated for 30 min in glucostat and its absorbance read. The tissue blank absorbance was divided by the tissue’s wet mass in milligrams, and this value was multiplied by the absorbance. One correction (reagent blank), to account for the presence of impurities in the lactose solution, was the absorbance of a 250-μl aliquot of the 50 mM lactose Ringer incubation solution treated identically (i.e., incubated for 30 min in glucostat) except that no intestinal sleeve had previously been incubated in it. The other correction (tissue blank), to account for the presence of impurities in the intestinal sleeve, was the absorbance of a 250-μl aliquot from a Ringer solution that contained 0.5 mM phlorizin and no lactose, but in which an intestinal sleeve had been incubated for 16 min. The aliquot was then incubated for 30 min in glucostat and its absorbance read. The tissue blank absorbance was divided by the tissue’s wet mass in milligrams, and this value was multiplied by the absorbance. One correction (reagent blank), to account for the presence of impurities in the lactose solution, was the absorbance of a 250-μl aliquot of the 50 mM lactose Ringer incubation solution treated identically (i.e., incubated for 30 min in glucostat) except that no intestinal sleeve had previously been incubated in it. The other correction (tissue blank), to account for the presence of impurities in the intestinal sleeve, was the absorbance of a 250-μl aliquot from a Ringer solution that contained 0.5 mM phlorizin and no lactose, but in which an intestinal sleeve had been incubated for 16 min. The aliquot was then incubated for 30 min in glucostat and its absorbance read. The tissue blank absorbance was divided by the tissue’s wet mass in milligrams, and this value was multiplied by the absorbance.

Two further corrections were applied to this preliminary activity: it was multiplied by 1.35 to account for the slight inhibition of lactase by 0.5 mM phlorizin (see APPENDIX), and it was multiplied by 1.36 to account for the Michaelis-Menten constant (K_{m}) of lactase (18 mM; see APPENDIX) by converting activity measured at 50 mM lactose into a V_{max} value. Finally, we calculated lactase capacity of the entire small intestine by calculating the product, for each small intestinal region, of lactase activity per milligram of tissue wet mass times regional wet mass and summing these products over the three regions (25).

SGLT-1 Assay

The activity of SGLT-1 was measured by the everted sleeve method described in detail previously (16, 30). Briefly, the everted sleeves were preincubated at 37°C for 5 min in Ringer solution at pH 7.3 and aerated with 95% O_2-5% CO_2. The 11 mg/g body wt. Lactulose solutions were prepared as 0.7 mM L-[3H]glucose, which is not subject to carrier-mediated transport. Uptake was normalized to milligrams wet mass of the tissue. Preliminary experiments showed that the K_{m} of the lactose transporter using this preparation was <1 mM, so activity in the 50 mM incubation solution was taken to approximate V_{max}. Passive uptake of glucose in suckling rats (see APPENDIX) was <1% of carrier-mediated transport under our assay conditions. Thus this method yields SGLT-1 activity in an intact intestinal preparation with good control of unstirred layers. Just as with lactase, total intestinal SGLT-1 capacity was taken as the sum of regional capacities. As described earlier, because SGLT-1 transports both glucose and galactose and because 1 mol of each results from hydrolysis of 1 mol of lactose, daily lactose loads were multiplied by two to calculate the load on SGLT-1 in suckling pups.

Hydrogen Breath Tests

Adult rats are hindgut fermenters whose cecum and large intestine can break down nutrients not absorbed in the small intestine (1, 46). The extent to which suckling rat pups are capable of fermenting unabsorbed nutrients is unknown. Hence we assessed fermentation activity in rat pups by measuring H_2 production. H_2 gas is expired as a by-product of fermentation, and its production has been measured in many studies of fermentation by adult rats (e.g., 9, 34). We measured H_2 production in 5-, 15-, and 30-day-old rat pups by means of a closed-circuit metabolism system. Rats were placed into sealed Plexiglas metabolism chambers for various lengths of time according to the following protocol: 5-day-old rats in a 596-ml chamber for 15 min, 15-day-old rats in a 4-liter chamber for 15 min, and 30-day-old rats in a 4-liter chamber for 15 min. This protocol was designed to standardize the assay so that rats at each age consumed the same proportion of chamber O_2 during their H_2 production determinations. That is, by following the above protocol, the O_2 content in the chamber declined from 20.9 to 19.9% for rats of all ages. This effort was validated by also measuring chamber O_2 levels after H_2 production determinations in a subset of rats at each age (O_2 content 19.84 ± 0.03, 19.90 ± 0.03, and 19.87 ± 0.03% in 5-, 15-, and 30-day-old rats, respectively; n = 3 for each age; P > 0.38). After the rats had been in the sealed chamber for the stated time, a 50-ml air sample was drawn out through a valve into a plastic syringe, and its H_2 level was then determined by gas chromatography (Carle Instruments AGC-111, Anaheim, CA). The CV for our H_2 production assay was 38%.

H_2 production was determined just before, and each hour (on the hour) for 5 h after, oral gavage of lactulose at a dose of 11 mg/g body wt. Lactulose solutions were prepared as 0.7 g/ml in water, and animals were gavage with 16 μl/g body wt. Lactulose is a sugar that is not absorbed in the small intestine but is known to be fermented in the cecum and large intestine of adult rats (9).

Statistics

Results are presented as means ± SE. Because individual pups within a litter cannot be regarded as statistically independent and the use of multiple pups from a given litter would constitute pseudoreplication, we used only one pup from each litter and dam for data collection. However, over...
the course of many pilot studies and validation experiments, we found that the intralitter CV for our dependent variables rarely exceeded 8–10%. We used ANOVA to assess the effect of age or litter size (the independent variables) on numerous dependent variables (e.g., body mass, nutrient load, gut morphology, intestinal capacities). For some variables we also used analysis of covariance with body mass as the covariate to assess the effect of ontogeny independent of body mass effects. Planned pairwise comparisons were made by Tukey’s test. Paired t-tests were used to compare lactase and glucose transporter capacities to one another, because these data were collected from the same individual rats. All P values presented in the text are from ANOVA unless otherwise specified, and the level P < 0.05 is considered significant. All statistical analyses were conducted with the program Systat 7.0 (SPSS, Evanston, IL).

RESULTS

We shall present our results in the following sequence: body mass and gut morphology, carbohydrate loads and intestinal capacities, H₂ breath tests, and effects of altered litter size. All results except those in the last section (on the effects of altered litter size) refer to pups reared in 10-pup litters.

Body Mass and Gut Morphology

Body mass and wet and dry masses of the stomach, small intestine, cecum, large intestine, and total gut (sum of these four organs) increased significantly with age (P < 0.001 for all quantities; Fig. 1). When expressed relative to total gut wet mass, the cecum and large intestine grew relatively more than the stomach and small intestine. That is, the large intestine and especially the cecum represented an increasing proportion of the total gut mass with increasing age (P < 0.001 for each; Fig. 2). The same trend was evident when tissue masses were standardized to body mass and compared. In fact, between days 15 and 30, cecum and large intestine wet mass as a percentage of total body mass increased by factors of 4.6 and 3.4, respectively, whereas stomach and small intestine wet mass increased only by factors of 1.4 and 1.6, respectively. The same pattern was evident when organ dry masses were analyzed. Small intestine mass increased most dramatically between days 15 and 30 (Fig. 1), coincident with the time of weaning onto a high-carbohydrate diet.

Carbohydrate Load and Intestinal Capacities

Daily milk intake per pup (P < 0.01) and the dam’s milk lactose concentration (P < 0.001) increased significantly with age (Fig. 3, A and B), as has been found previously for rats (22). As a result, the dietary lactose load (product of intake times concentration) increased throughout the suckling period and at 15 days was 3.4 times that at 5 days (Fig. 3C). The increase in carbohydrate load during the suckling period (through day 15) is modest compared with the enormous increase occurring during weaning (days 15–23). From day 15 to 30, dietary sugar load (lactose at day 15, sucrose at day 30) increased from 1 to 17 mmol/day (Fig. 4). This large increase in dietary sugar load during weaning parallels the rapid growth of the small intestine during this same period (Fig. 1).

Regional lactase activities show the well-established (17, 37) positional effect of higher activities in the proximal and middle regions than in the distal region (Fig. 5A). Lactase activity in the middle and distal segments of the small intestine did not vary significantly with age between 5 and 15 days (P > 0.17 and P > 0.74, respectively) but did decline modestly but significantly with age in the proximal segment (P < 0.02). We did not assay lactase after 15 days, when lactase is known from previous studies to disappear.

As found previously for rat intestine (44), SGLT-1 activities showed a proximal-to-distal gradient, with the difference between proximal and middle activities declining with age (Fig. 5B). Among suckling pups (days 5, 10, and 15), there was significant variation in SGLT-1 activity with age in the middle segment (P <
0.04) but not in the proximal (P > 0.87) or distal (P > 0.67) segments. As expected from results of most previous studies of rats (e.g., Refs. 23, 38, and 44) and of other mammals (6, 7), all three segments showed a significant decline in SGLT-1 activity with age when all ages (5, 10, 15, 30, and 100 days) were included in the analysis (P < 0.001 for each segment). In contrast, Faltova et al. (19) and Younoszai and Lynch (49) reported an increase in glucose uptake rates with age in rats. However, Faltova et al. (19) normalized glucose uptake activity to body mass. Because the ratio of intestine mass to body mass increases three- to fivefold (Fig. 1; Ref. 44) during the age range that they examined (days 10–30), recalculation of their results normalized to intestine mass would yield activities that declined with age, as we found. It is difficult to compare the results of Younoszai and Lynch (49) with other studies, because they measured uptake at increasing sugar concentrations in rats of increasing age.

The average of the three regional activities was calculated for each animal (dashed lines in Fig. 5), and it was also used for assessing the effect of ontogeny. Neither average lactase activity (P > 0.5) nor average SGLT-1 activity (P > 0.3) varied significantly with age among 5-, 10-, and 15-day-old pups (Fig. 5). Both lactase and SGLT-1 capacities, the integrated products of regional activities times intestinal mass over the whole length of the small intestine, increased significantly over this time (Fig. 4; P < 0.001 in both cases). This is due entirely to increased small intestine mass, because average activities do not change with age in sucklings.

During the suckling period, lactase and SGLT-1 capacities increase with age, but not by as high a factor
as that by which load increases. Therefore, the safety factors (capacity-load ratios) for both lactase and SGLT-1 are significantly lower at 15 days than at 5 days (Fig. 6). The safety factors measured in day 5 pups are among the highest ever found for intestinal capacities, whereas those at days 10 and 15 are more typical of previous measures of intestinal capacities (25, 27). Lactase safety factors were significantly greater than those for glucose transport, although the difference after 5 days is very modest (paired t-tests; \( P < 0.01 \) at each age). Recall that, even though SGLT-1 capacities are greater than lactase capacities, SGLT-1 safety factors are lower in sucklings due to the double load that lactose places on SGLT-1 (see previous discussion in Experimental Design). At 30 and 100 days, the dietary glucose load (in the form of sucrose) was within 15% of SGLT-1 capacity (Fig. 4), and hence SGLT-1 safety factors were \( \approx 1.0 \) at each age (Fig. 6). In fact, at 30 days the SGLT-1 safety factor was not significantly different from 1.0 (\( P > 0.3 \)), whereas at 100 days the difference did prove to be statistically significant (\( P < 0.04 \)).

Hydrogen Breath Tests

Hydrogen production in untreated rats (i.e., before oral lactulose gavage) varied significantly with age (\( P < 0.001; \) Fig. 7), which suggests that baseline fermentation activity was greater in weaned rats than in sucklings. Planned pairwise comparisons showed that \( \overline{H}_2 \) production was higher in day 30 weanlings than in either day 15 (\( P < 0.01 \)) or day 5 (\( P < 0.02 \)) pups. There was no significant difference between values at day 5 and day 15 (\( P > 0.9 \)). The increased fermentation activity in 30-day-old weanlings is associated not only with the enormous increase in dietary disaccharide load (Fig. 4) but also with the introduction of fiber into their diet.

After oral gavage of lactulose, \( \overline{H}_2 \) production increased in 30- and 15-day-old rats, but there was no change in \( \overline{H}_2 \) production over time in 5-day-old rats (Fig. 8). When \( \overline{H}_2 \) production was analyzed over the complete 5-h time course, there was a significant effect of age (\( P < 0.001 \)), but not of time (\( P > 0.7 \)); nor was there a time \( \times \) age interaction (\( P > 0.8 \)). There was, however, a significant time \( \times \) age interaction effect on \( \overline{H}_2 \) production in the first 2 h after gavage (\( P < 0.01 \)).

Effects of Altered Litter Size

To determine the effects of altered dietary loads on intestinal safety factors, pups were raised in three litter sizes beginning within 24 h of birth and then examined on day 15. Our rationale for varying litter size was that pups in large litters receive less milk per pup than pups in small litters (22). Thus daily lactose load would be greater for pups in small litters than in large litters.

Litter size did prove to have a significant effect on milk intake per pup (\( P < 0.001 \)), lactose content of the milk (\( P < 0.001 \)), and daily lactose load per pup (\( P < 0.02, \) Fig. 9). As a consequence of the effect of litter size on daily milk intake and lactose concentration, individuals from a 4-pup litter had a 66% greater daily lactose load on day 15 than those from a 16-pup litter (Fig. 9C).
Similarly, small intestine mass and body mass were 70 and 20% greater, respectively, in pups from a 4-pup litter than in those from a 16-pup litter (Fig. 9A). That is, pups in large litters received less milk and consequently grew more slowly. Many previous studies have demonstrated that small intestine mass is proportional to body mass (e.g., 26), so the stunted growth of the small intestine was predictable.

Neither average lactase activity (P > 0.09) nor average SGLT-1 activity (P > 0.15) varied significantly with litter size (Fig. 10A), but, as a result of the inverse relationship between litter size and small intestine mass, lactase (P < 0.03) and SGLT-1 (P < 0.05) capacities did vary significantly with litter size (Fig. 10B). Pairwise comparisons revealed that neither lactase (P > 0.79) nor SGLT-1 (P > 0.97) capacities differed significantly between 4- and 10-pup litters. However, pups in 16-pup litters showed significantly lower lactase (P < 0.02) and SGLT-1 (P < 0.05) capacities than pups in 10-pup litters. Safety factors for lactase and SGLT-1 were all between 3 and 6 (Fig. 10C) and did not vary significantly with litter size (P > 0.16 and P > 0.38, respectively), because both small intestine mass (hence lactase and SGLT-1 capacities) and load decreased in parallel with litter size (compare Fig. 9, A and C). Safety factors for lactase were slightly but significantly greater than those for SGLT-1 at all ages (paired t-tests; P < 0.01 at each age). Recall again that, even though SGLT-1 capacities are greater than lactase capacities, SGLT-1 safety factors are lower due to the double load of dietary lactose on SGLT-1.

DISCUSSION
Evolution of Safety Factors

The developing mammalian gut experiences two major shifts in the functional demands placed upon it: at birth, when the gut suddenly assumes full responsibility for nutrient acquisition from the placenta; and at weaning, when the nutrient input changes from milk to an adult diet of different composition. Corresponding to the changes in functional demands, the gut undergoes developmental changes in many physiological and anatomical properties (7).

We shall discuss the ontogenetic relationships between functional demands and physiological capacities in the context of safety factors. The safety factor of a physiological parameter is the ratio of its capacity to the load placed on it. Related terms are “reserve capacity” and “safety margin” (defined as capacity minus load) (25). Although the term “safety factor” is...
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originally from the field of engineering (2), biologists have found the concept very useful in examining quantitative aspects of animal design.

Quantitative studies of mammalian limb bones (2, 5) and respiration (42, 47) have shown that morphological and physiological capacities meet the loads placed on them with modest reserve capacities. That is, safety factors fall in the range of 1–5. Hammond et al. (27) assessed intestinal brush-border safety factors in mice under various energetic demands. They showed that safety factors for glucose transport, proline transport, and sucrase fell in the range of 3–5 in control mice and decreased to 1–2 when the mice faced energetic challenges such as lactation and cold temperature. These safety factors evolved as a result of cost-benefit trade-offs between the penalties for possessing insufficient capacities and the costs of maintaining excessive capacities. Although insufficient capacities may result in broken limb bones or unabsorbed nutrients, excessive capacities require bioenergetic costs to build and maintain and occupy valuable space (15). The costs associated with excessive capacities result in their elimination by natural selection. For example, animals that evolve in dark caves lose their functional eyes, birds that evolve on predator-free islands lose their ability to fly, and bacteria lose their ability to synthesize nutrients provided to them in the growth medium (18).

Safety Factors Over Ontogeny: Preweaning

The first question that we posed in the introduction concerned the safety factors for lactase and glucose/galactose transport in developing rats. The safety factors of 4–5 that we measured at days 10 and 15 are typical of other biological systems that have been examined. But 5-day-old pups possess high factors (8–13) for both lactase and glucose/galactose transport.

Two major reasons and two secondary reasons suggest themselves as explanations. One likely major reason is that lactase substrates present in milk include not only lactose itself but also glycoproteins plus glycolipids such as lactosylceramide. The latter substrates have a much higher affinity for lactase than does lactose; hence they inhibit lactase hydrolysis by lactase (8). That is, the activity and capacity of lactase for lactose are somewhat lower under physiological conditions than under our experimental conditions, and the total load on lactase is higher than we calculate from the lactose load alone, so that both effects tend to make our calculated safety factors (ratios of capacity to load) overestimates.

A second major likely reason is that, although lactose loads double approximately every 4 days as suckling rats grow and require more milk, there is some unpredictability in milk intake and pup growth rates, so that a safety factor that appears high in an average pup could be normal in an especially rapidly growing pup. A similar example is the prenatal rise in intestinal lactase activity in most mammals (17, 28), even though this capacity is not used at all until after birth. The timing of birth, however, is also somewhat unpredictable.

A further minor consideration is that if lactose concentrations in the intestinal lumen during digestion are not considerably above the $K_m$ of lactase, lactose hydrolysis would operate at a rate below $V_{max}$. In that case, an apparently high safety factor calculated on the basis of the $V_{max}$ of lactose would correspond to a more modest safety factor at physiological lactose concentrations. This consideration probably makes a real but not major contribution to the high safety factors of sucklings: milk lactose concentrations (Fig. 3) are 59–107 mM, well above the lactase $K_m$ of 18 mM, but lactose concentration must decline as milk traverses the intestine and is diluted with secretions and as lactose becomes hydrolyzed. Finally, our calculation ignores considerations of transit time by assuming that the measured daily lactose load is delivered to the intestine at a steady rate over 24 h. In adult animals, the metering action of the pyloric sphincter ensures that this is approximately true (21), but it is unknown whether it is also true for suckling animals. If lactose delivery from the stomach to the small intestine (or lactose transit through the intestine) were strongly pulsed, instantaneous loads would be higher than the time-averaged loads that we have calculated, and safety factors would be correspondingly lower.

Safety Factors Over Ontogeny: Postweaning

Our other initially surprising result was that safety factors for the glucose transporter were only 1 in 30- and 100-day-old rats. Recall that intestinal safety factors for the glucose transporter and for sucrase in mice are normally 3–4 and only decrease to 1–2 when increased energetic demands such as lactation and cold temperatures result in dramatically increased nutrient loads (27). The rats' safety factor of 1 under nondemanding conditions would seem to imply that brush-border carrier-mediated glucose uptake capacity is close to rate-limiting, with almost no small intestinal reserve capacity available for absorbing additional glucose. Although passive glucose uptake could in principle provide additional uptake capacity, our measurements (APPENDIX) showed passive glucose uptake to be $<1\%$ of carrier-mediated uptake, in agreement with measurements made on intestines of unanesthetized rats in vivo (45).

However, adult rats are accomplished hindgut fermenters that can recover energy from nutrients not absorbed in the small intestine (46). Such nutrients are fermented into volatile fatty acids by the bacterial flora of the cecum and large intestine and then absorbed and used as an energy source. This process thereby serves as a built-in reserve capacity over and above the glucose uptake capacity of the small intestine itself. The increased relative masses of the cecum and large intestine on weaning (Fig. 2) support the interpretation that fermentative capacity increases at the age at which the small intestinal SGLT-1 safety factor decreases (Fig. 6). Our measurements of $H_2$ production (Figs. 7 and 8) add functional support to the morphological data and suggest the following: that 5-day-old pups do not possess the cecal flora required for fermentation;
that 15-day-old pups are capable of fermentation when challenged with an excessive nutrient load, but do not normally use fermentation; and that 30-day-old weanlings use fermentation as an intestinal reserve capacity to digest dietary fiber and any simpler nutrients not absorbed in the small intestine. Like rabbits (7), rats ferment not only dietary fiber but also any simple carbohydrates reaching the cecum (9). Thus although the uptake capacity of the small intestine provides adult rats with no reserve capacity for sugar digestion, the hindgut provides considerable reserve capacity.

Series Capacities

The second question that we posed in the introduction concerned the relationship between series capacities. Because most biological systems consist of multiple capacities in series, failure of any individual step can result in failure of the system. In other words, a chain is only as strong as its weakest link (3). For example, flux through a metabolic pathway can occur only at the rate of the slowest (hence rate-limiting) step. Considerations of economy would tend to lead to series capacities being approximately matched to one another, because excessively low capacities would impose a bottleneck on the entire system. Similarly, excessively high capacities at individual steps could never be used and would represent wasted energy and/or space (3, 15). Previous studies of series capacities for carbohydrate digestion in mice showed that safety factors for sucrose hydrolysis and for glucose transport are matched to each other over a range of dietary sucrose intakes (27, 48).

In our study of rates, the safety factor for lactase was somewhat higher than that for SGLT-1 at each age examined (Fig. 6). However, the ontogenetic pattern of declining safety factors with increasing age was the same for both proteins, and the absolute difference between the two safety factors was only 22% on days 10 and 15. This difference is minor when one reflects that the two capacities and the loads on them are on such different ontogenetic trajectories: in the 10 days after day 15 (when weaning begins), daily lactose load declines from its peak value to zero, whereas daily glucose load (in the form of sucrose) increases by a factor of ~15.

Effect of Altered Load

The third question that we addressed in this study concerned the effect of altered loads on intestinal safety factors. Unlike most engineered systems (such as elevators), many biological systems regulate their capacities in response to variations in load. In particular, intestinal transporter capacities are regulated in adult animals by two different mechanisms: transporter induction and repression, resulting in changes in mass-specific activities; and growth and atrophy of intestinal mass. For example, mass-specific uptake activities of intestinal transporters for glucose, fructose, and amino acids increase specifically in response to increased dietary levels of their substrate (20). When an adult animal is faced with energetically demanding conditions such as cold temperature or lactation (which result in increased dietary intake of all nutrients), intestinal capacities of all transporters increase in parallel as a result of increased intestinal mass (27).

We examined whether, in suckling rats, intestinal capacities adapted to changes in nutrient load, and (if so) whether series capacities adapted in parallel. By varying litter size as a means of altering daily lactose load per pup, we were able to achieve a 66% increase in load in 15-day-old individuals raised in 4-pup litters compared with those raised in 16-pup litters. Yet there was no significant change in safety factor for either lactase or glucose transport (Fig. 10). That is, both capacities increased in direct proportion to load as a result of increases in gut mass without change in mass-specific activities. This particular case exemplifies a general pattern: intestinal adaptation tends to preserve safety factors. When energetic demands (hence dietary nutrient loads) increase, intestinal mass and/or digestive activities also tend to increase, resulting in safety factors being maintained at values ≥1 (25, 27).

In other words, intestinal adaptation to variation in nutrient load is characterized by the phrase “enough, but not too much” (15).

Perspectives

We conclude by mentioning two promising experiments for understanding more deeply the quantitative design of carbohydrate digestion in rats.

First, an obvious complement to this study of lactose digestion in rat pups is a more complete examination of sucrose digestion in rat weanlings and adults. We have already examined their glucose uptake, but we have not yet assessed the first step in sucrose digestion, its hydrolysis by sucrase. Sucrese activities can be readily measured by the same everted sleeve preparation that we used for lactase (32). Adult mice maintain considerable small intestinal reserve capacity of sucrase (48), yet mice may not use hindgut fermentation to the same extent as rats (the cecum and large intestine comprise 25% of the total gut mass in rats, but only 15% in mice). Do adult rats dispense with sucrase reserve capacity (just as we found for their glucose transporter), relying instead on hindgut fermentation?

Second, a more refined assay of H₂ production than the one that we used (e.g., Ref. 34) may permit a quantitative assessment of nutrient fermentation. One could thereby partition total sugar digestion into the contributions of 1) small intestinal hydrolysis and uptake and 2) cecal and large intestinal fermentation.

APPENDIX

A previous paper from this laboratory (32) worked out optimal conditions for assaying mouse intestinal sucrase in an everted sleeve preparation and validated the method by comparing it with the classical Dahlqvist assay (12, 13) in intestinal homogenates. This appendix very briefly describes the corresponding experiments for rat pup intestinal lactase, using pups 15 days old from 10-pup litters and omitting description of procedural details identical to those already described for mouse intestinal sucrase (32).
Selection of Optimal Conditions

Inhibiting the glucose transporter. Because the everted sleeve assay is based on measuring the production of glucose resulting from the action of lactase on lactose and released into the incubation solution, we needed to inhibit glucose uptake from the incubation solution into the tissue. To do so, we added phlorizin, an inhibitor of SGLT-1, to both the preincubation Ringer solution and the lactose-containing incubation. Phlorizin inhibition of SGLT-1 was measured in the presence of 0.1 mM glucose solutions, because that was the maximum glucose concentration produced in our assays. Preincubating sleeves for 20 min in a Ringer solution containing 0.5 mM phlorizin inhibited SGLT-1 activity (compared with adjacent control sleeves from the same rat, preincubated without phlorizin) by 99.5 ± 0.2% (n = 3). SGLT-1 inhibition was identical (99.5 ± 0.2%) when we used a phlorizin concentration of 1.0 mM and a 20-min preincubation period. We also found that the inhibition was lower when the preincubation time was reduced to 10 min for both 0.5-mM (98.7 ± 0.2% inhibition) and 1.0-mM (98.8 ± 0.2% inhibition) phlorizin concentrations. Hence we chose a 20-min preincubation period with 0.5 mM phlorizin.

Effect of phlorizin on lactase activity. In addition to inhibiting SGLT-1 almost completely, phlorizin also slightly inhibits lactase itself (40). Because the everted sleeve lactase assay requires the use of phlorizin (preventing us from comparing lactase activity in the presence and absence of phlorizin), we used homogenate assays to measure phlorizin inhibition of lactase. Our homogenate assay was modified from Dahlqvist's (12, 13) classical homogenate protocol and was identical to the homogenate protocol of Lee et al. (32) except that we used a lactose substrate rather than sucrose. A 20-min preincubation without phlorizin had no effect (compared with no preincubation), hence lactase did not deteriorate during the extra 20 min required for preincubation. However, a 20-min preincubation in 0.5 mM phlorizin inhibited lactase activity by 26 ± 1% (n = 3). Hence we multiplied measured lactase activities by 1.0 − 0.74 = 1.35 to correct for phlorizin inhibition of lactase itself.

Passive glucose uptake. Passive glucose uptake, just like carrier-mediated glucose uptake by SGLT-1, would tend to cause our assay to yield underestimated lactase activity. To assess the magnitude of this potential error, we measured passive uptake coefficients for glucose as described previously (30) and then calculated the amount of glucose absorbed passively under our assay conditions. Using a 16-min incubation and assuming a final glucose concentration <0.1 mM in the incubation bath solution (the maximum glucose concentration produced in our assays), we calculated that <0.03% of the glucose in the incubation solution would be taken up passively by the tissue. This introduces a negligible error into our lactase activity measurements.

Incubation time. The incubation time for everted sleeves in lactose-containing solutions should be long enough that the amount of released glucose is large and accurately measurable, but not so long that the lactase substrate is becoming exhausted and hence that the curve of the glucose production against time is becoming sublinear. We incubated two sleeves from each of four rat pups, and from each incubation solution we drew two duplicate samples of 250 µl each at 4, 8, 16, and 20 min. We found that glucose production increased linearly with time (r² = 1.00 for linear regression) up to 20 min, with a y-intercept near zero. This linearity implies that lactase properties are not changing over the incubation time, lactose substrate is not becoming exhausted, and inhibition of lactase by liberated glucose is negligible under our assay conditions. Hence we settled on a 16-min incubation, well within the linear phase of glucose production.

Incubation concentration. We could not use the everted sleeve assay to measure lactase activity as a function of lactose concentration and thereby calculate the lactase Km value, because the assay requires phlorizin, which is a competitive inhibitor of lactase (40). Instead, we determined the lactase Km by the homogenate assay. Lactase activity was measured at lactose concentrations ranging from 1 to 100 mM, and the data were fitted to the Michaelis-Menten equation, yielding a calculated Km value of 18 mM. This is close to the previously reported Km values of 21 mM for 10- to 15-day-old rat pups (39) and 16 mM for rat pups and adults (8). Hence we used an incubation concentration (C) of 50 mM lactose and multiplied the resulting activities by 1.36 (the factor calculated from the Michaelis-Menten equation with Km = 18 mM and C = 50 mM) to obtain the Vmax value. We decided not to use an incubation concentration >50 mM because that would have required excessive reduction in NaCl concentration to maintain osmolality constant.

Validation of Everted Sleeve Assay

Reproducibility. The apparent CV of lactase activity for six adjacent midintestinal sleeves from the same rat pup was 13%. We refer to this CV as apparent because part of the variation represents a real proximal-to-distal gradient in lactase activity along the intestine (Fig. 5); actual reproducibility is evidently better than 13%. When we calculated the mean lactase activity for midintestinal sleeves of each of three rat pups, the interanimal CV of those three mean values was 9%.

Effect of intestinal position. Lactase activity assayed in sleeves along the length of the small intestine exhibited peak values in the middle region and lowest values in the distal region (Fig. 5). The same positional effect has been observed previously for lactase activity in intestinal homogenates from suckling rats (17, 37).

Comparison of sleeve and homogenate assays. We measured reproducibility of the classical Dahlqvist assay of lactase. Our experimental design was essentially the same as the design described above for measuring reproducibility in sleeves. Lactase assays in homogenates of six midintestinal tissues from each of three rat pups yielded intradividual and interindividual CVs of 25 and 16%, respectively. Both of these values are higher than the corresponding CVs reported above for sleeves (13 and 9%, respectively).

We then compared results of lactase activity determinations by the sleeve and homogenate methods in adjacent midintestinal tissues of the same rat pup. Both determinations were at the same lactose concentration (50 mM) and the same incubation temperature (37°C). However, incubation solution pH, buffer, ionic strength, and ionic composition differed. The homogenate determinations were made under the classical Dahlqvist conditions (pH 6.0, 0.1 M Na+ maleate buffer), whereas the sleeve determinations were under a slight modification of our usual conditions (pH 7.4, balanced salt solution of ionic strength equivalent to 0.16 M NaCl, 20 mM HEPES buffer). These conditions differ from our usual sleeve conditions only in the substitution of 20 mM HEPES buffer (which does not need to be aerated) for our usual aerated 20 mM HCO3− buffer to facilitate direct comparisons between sleeves and homogenates, because homogenates cannot be aerated. A separate experiment established that HEPES and HCO3− buffers at pH 7.4 yield lactase values not significantly different from one another (P > 0.15, n = 3). Under these conditions, sleeve assays yielded lactase activities 41 ± 3% (n = 3) of the values from homogenate assays.
Three factors could contribute to these differing values resulting from the two methods: 1) the differing preparations themselves (homogenate vs. sleeve); 2) the differences in solution composition, including choice of buffer, ionic strength, and ionic composition (0.1 M Na+ maleate vs. 0.15 M Ringer with HEPES buffer); and 3) the differing pH values (6.0 vs. 7.4). We partitioned the contributions of these three factors as follows. Under identical solution conditions (pH 7.4 Ringer with HEPES) the ratio of homogenate to sleeve lactase activity was 0.66 ± 0.03 (n = 3); this represents the contribution of different preparations alone. For homogenates studied at pH 7.0, lactase activity in 0.1 M Na+ maleate homogenates at pH 6.0 was 1.51 ± 0.06 (n = 3) times the activity in Ringer with HEPES; this represents the contribution of solution composition alone. As for the contribution of pH alone, lactase activity in 0.1 M Na+ maleate homogenates at pH 6.0 was 1.15 ± 0.09 (n = 4) times the activity in Ringer with HEPES; this represents the contribution of solution composition alone. Hence activity at pH 6.0 is more than three times the activity under identical conditions.

For our purposes in this study, the advantage of the sleeve assay is that it permits quantitative comparison of lactase activity in different preparations. Partly offsetting these two effects, the sleeve assay yields activities 41% of that measured in homogenates at pH 6.0 in 0.1 M Na+ maleate buffer. The major reason is that the Dahlqvist assay is conducted at a lower pH; a minor reason is that the solution composition used in the Dahlqvist assay yields slightly higher activity than the composition used in the sleeve assay. Partly offsetting these two effects, the sleeve assay yields activities 50% higher than the Dahlqvist assay under identical conditions of pH and solution composition.

For our purposes in this study, the advantage of the sleeve assay is that it permits quantitative comparison of lactase activity with SGLT-1 activity, measured in the same preparation under the same conditions.

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