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Effects of Sodium Saccharin on the Activity of Trypsin, Chymotrypsin, and Amylase and upon Bacteria in Small Intestinal Contents of Rats[1] (42022)

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Abstract. Rats fed a diet containing 2.5% sodium saccharin (NaSacc) displayed a rapid (24–36 hr) increase in tryptic and chymotryptic activity in the lower half of the small intestine and the cecum compared with control animals. Cecal pH of rats fed NaSacc was lower than controls. The effect of NaSacc on enzymatic activity of intestinal contents and on indigenous bacterial microflora was studied further *in vitro*. Intestinal contents incubated anaerobically with or without NaSacc revealed that the presence of NaSacc led to higher tryptic and chymotryptic activity and higher final pH. Changes in pH do not appear, however, to be important for the increased proteolytic activity induced by NaSacc since autodigestion of trypsin and chymotrypsin in filter-sterilized samples was only slightly affected by pH during *in vitro* incubation. Amylolytic activity, on the other hand, was stabilized by higher pH values. Saccharin stabilized chymotryptic and led to almost complete loss of amylolytic activity during incubation of filter-sterilized samples maintained at adjusted pH values. The amount of reducing sugars remaining in the NaSacc-containing contents from either cecum (*in vivo*) or from *in vitro* incubation of unsterilized small intestinal samples was greater than controls not containing NaSacc. The growth of six bacterial strains isolated from small intestinal contents and incubated in laboratory media was inhibited by NaSacc. Extracellular proteolytic activity from bacterial sources was undetectable after incubation of intestinal bacteria in laboratory media. The present results suggest that the effect of NaSacc upon digestive enzyme composition in the small intestine of rats is not mediated through a direct physiological effect of NaSacc on pancreatic exocrine secretion. It is hypothesized that an inhibition of enzymatic activity by NaSacc in the small intestine and the bacteriostatic effect of NaSacc on bacteria may be responsible for the increased proteolytic activity observed *in vivo* in the cecum following the feeding of a NaSacc-containing diet to rats. © 1985 Society for Experimental Biology and Medicine.

Among non-sugar sweeteners, sodium saccharin (NaSacc) is the only known appealing stimulus to rats, and therefore is commonly used as a taste stimulus for these animals. Other sweeteners are either tasteless or aversive (1–3). Diets adulterated with 0.3–0.7% NaSacc may be appealing to rats (4) whereas the 2.5% level is aversive (5). We noted previously (6) that feeding rats a balanced (17% protein) diet containing 2.5% NaSacc resulted in an increase in proteolytic enzyme activity in the intestinal contents and feces, and a decrease in cecal pH. Although taste stimulation may initiate the cephalic phase of pancreatic digestive enzyme output (7–9), feeding diets containing 2.5% NaSacc to rats causes an increase in the content of proteolytic activity in the intestine to a degree greater than that expected for the cephalic–pancreatic stimulation alone (6). Recent *in vitro* studies have concluded that NaSacc inhibited some proteolytic enzymes (10, 11). Since cecal microbial populations are affected by feeding NaSacc (12) the observed change in cecal pH following NaSacc feeding (6) could be the result of a shift in the ratios of

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types of cecal bacteria. The mechanisms whereby NaSacc induces an increase in intestinal proteolytic enzymatic activity have not been determined. The increased enzyme activity may result from either indirect effects, such as pH changes (13) that may arise from NaSacc–bacterial interaction, from enzymatic activity inhibition (10, 11), or from an intestinal bacterial source (14). The present study was designed to further explore the nature of the effects of NaSacc upon enzymatic activity, pH of intestinal contents, and bacterial activity during *in vivo* and *in vitro* experiments.

Materials and Methods. *Experiment 1: In vivo study.* This experiment determined the effect of feeding NaSacc on the level of activity of amylase, trypsin, and chymotrypsin and the pH in the small intestine and cecum of rats. Sixty male Wistar weanling rats (Charles River, Wilmington, Mass.) were housed individually in cages 30 × 25 × 15 cm at a temperature of 23 ± 2°C with a 12 hr light/dark cycle (lights out at 1900 hr). Rats were acclimated to our laboratory for 2 days and then divided into two groups of 30 each. One group was fed a 17% protein (casein) diet (6), containing (g/kg diet): casein—200; DL-methionine—3; corn oil—80; cellulose—20; cornstarch—643; salt mix (AIN-76, ICN, Cleveland, Ohio)—50; vitamin mix (equivalent to levels in AIN-76 but containing no added sucrose)—2; choline chloride—2. The other group was fed the same diet but adulterated with 2.5% NaSacc w/w at the expense of cornstarch. Each group of rats was further divided into three equal subgroups depending on the day in which rats were sacrificed. During the experiment, food intake was measured daily and body weight every 48 hr. Rats were sacrificed by decapitation with no prior fasting on Days 2, 5, and 9 between 0800 and 1100 hr. The small intestine and the cecum were removed. Contents (chyme) of the upper half and lower half of the small intestine and of the cecum were collected into vessels by compressing the intestine successively with tissue forceps, forcing the contents out into the vessel (6). Care was taken not to damage the tissue and to collect contents without visceral contamination.

The pH of intestinal contents was measured after chyme was diluted by a factor of 3 (w/w) with 0.85% NaCl solution and homogenized with a Polytron homogenizer. The suspension thus obtained was centrifuged at 38,000*g* for 20 min at 2°C. The supernatant fraction was frozen at −15°C until analyzed for enzymatic activity. Trypsin activity was assayed spectrophotometrically at 410 nm using *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) as a substrate (15). Chymotrypsin activity was assayed titrimetrically with the substrate *N*-acetyl-L-tryosine ethylester (ATEE) at pH 8 (16). One unit of trypsin or chymotrypsin was defined as 1 μmole of substrate hydrolyzed per minute at 30°C. To verify that the above activity is not confounded by possible esterases resulting from bacterial activity, homogenates from both *in vivo* and *in vitro* experiments (*in vitro* experiments described ahead) were tested for proteolytic activity using casein as a substrate (17). Amylolytic activity was determined according to Bernfeld (18) with modifications (16). One unit of activity for amylase was defined as 1 mg of reducing sugar released per 3 min at 37°C. The total free reducing sugar was determined by the same procedure but preventing any amylolytic activity by adding the Sumner reagent to the homogenate prior to adding the substrate.

Experiment 2: In vitro study. The purpose of this set of experiments was twofold: (1) to assess the effects of NaSacc on enzymatic and bacterial activity during the *in vitro* incubation of small intestinal contents removed from rats fed a control-unadulterated diet; (2) to determine the effect of pH and NaSacc on enzymatic activity after incubation of intestinal contents sterilized by filtration.

Forty male Wistar rats, 200–220 g, were housed as in Experiment 1. They were fed a 17% casein diet unadulterated by NaSacc for a period of 11 days. On Day 9, a pilot experiment was performed. Ten of the 40 rats were sacrificed and their intestinal contents were incubated *in vitro* for either 17 or 20 hr under various treatment regimens (see below) in order to determine the pH reached by each sample after incubation. Based on these results, pH of some selected samples of intestinal contents was later adjusted during the major experiment. The same treatment procedures were used for both the pilot and the major incubation experiments except that

in the pilot study, no pH adjustments were made. On Day 11, the remaining animals were sacrificed by decapitation. To minimize oxygen toxicity (19), contents of the entire small intestine, including duodenum, jejunum, and ileum, were collected (6) into a glass beaker placed under a hood flushed with nitrogen. Aliquots of 2.5 g were removed from the pooled contents to 20-ml sterile cotton-plugged glass vials.

Glass vials containing the 2.5 g of small intestinal contents were treated in one of the following manners: Condition I—normal samples: Total of 7.5 ml of sterile 0.85% NaCl solution (saline) was added to each vial. Condition II—NaSacc samples: Total of 7.5 ml of saline containing NaSacc was added to achieve a suspension with a level of 2.5% NaSacc. Condition Ia—normal samples pH adjusted: 4 ml of saline were added. pH adjustment to either 4.7, 5.2, or 5.9 (based upon results of pilot experiment performed 2 days earlier, see Fig. 2) was carried out with saline containing 0.01 *N* HCl using an automatic titrator (Radiometer). Total volume was made up to 7.5 ml with saline. Condition IIA—NaSacc, pH adjusted: Treated as described for Ia but with saline containing NaSacc. Condition Ib—normal samples, filter sterilized, pH adjusted: 4 ml of saline were added. Suspension was centrifuged at 38,000*g* for 20 min at 2°C. Supernatant of each vial was passed through a 0.45-μm membrane filter (Millipore Corp.). pH was then adjusted and volume was made up as described for Ia. Condition IIb—NaSacc, filter sterilized, pH adjusted: Treated as described for Ib but with saline containing NaSacc. The sterility of filter-sterilized samples was checked by spreading 0.1 ml of the filtrate over the surface of heart infusion agar containing 0.2% glucose (HIG agar) and incubating the plates aerobically for 3 days at 37°C.[4]

The contents of all vials from each condition were divided into 4 or 5 smaller sterile vials (1.5 ml each) and these either frozen at −15°C, incubated under aerobic conditions at 37°C for 17 hr, or incubated under anaerobic conditions at 37°C for 20 hr. Anaerobic conditions were achieved by the Gas Pak system (BBL Laboratories, Md.). After incubation, bacterial counts were performed and pH of all samples was measured. Samples originating from conditions I, Ia, II, and IIa were centrifuged as above and their supernatant fractions frozen at −15°C until analysis for enzymatic activity or reducing sugars as in Experiment 1. The filter sterilized samples (Ib, IIb) were simply frozen (−15°C) without centrifugation.

Bacterial numbers were determined by diluting samples from individual vials in sterile 0.03 *M* KH_2PO_4 (pH 7.2). Total bacterial numbers were determined by placing 0.05-ml portions of these dilutions onto the surface of HIG agar, and MacConkey's agar was used to selectively enumerate gram-negative bacteria (20). Duplicate counts on these media were prepared from each sample, and one set was incubated aerobically and one set anaerobically at 37°C. The numbers, types, and distributions of bacterial colonies were recorded after 3 days aerobic or 7 days anaerobic incubation. The results are presented as the average of the counts obtained from duplicate samples of amended (i.e., containing NaSacc) or unamended intestinal contents. Before and after incubations, intestinal contents were routinely examined microscopically by Gram staining heat-fixed smears and viewing at 1000×.

Experiment 3. This experiment was designed to determine the effect of NaSacc on the growth and enzymatic activity of isolated bacterial strains from small intestinal contents when grown on laboratory media. Pure cultures of six bacterial strains representing the predominant colonial types observed in Experiment 2 were obtained by repeated streaking on HIG agar. These isolates were identified by microscopic observations and by results from the API-20A bacterial identification system (Analytab Products, Plainview, N.Y.).

Inoculum cells were prepared by growing each strain for 2 days at 37°C on trypticase soy agar (TSA). Growth from these plates was removed with a sterile glass rod and the cells were washed twice in sterile tap water by centrifugation at room temperature. After

[4] Sterilization by antibiotics in combination was attempted but found to be only partially effective. Antibiotics used and their concentration were neomycin, 130 μg/ml; gentamycin, 70 μg/ml; dihydrostreptomycin, 70 μg/ml; and carbenicillin, 70 μg/ml.

suspending the final pellets in 5 ml of deionized water, an equal volume of each strain was inoculated into each of four 18 × 100-mm capped test tubes containing 6 ml 1% peptone–0.05% glucose–0.01% yeast extract medium (PGY) or 6 ml PGY amended with 2.5% NaSacc (PGYS). A low level of glucose (0.05%) was used in PGY since protease synthesis in a variety of bacteria is glucose repressible (21). PGY was adjusted to pH 6.5 before autoclaving. A NaSacc solution was filter sterilized and added after autoclaving to achieve a 2.5% NaSacc concentration. Cell inocula were added to media to provide barely turbid cultures. Bacterial growth was estimated after 6, 24, and 48 hr incubation at 37°C by measuring absorbance at 650 nm in a Spectronic 20 (22). The culture pH was determined, and the presence of trypsin and chymotrypsin activity was assessed using synthetic substrates and casein as described in Experiment 1. TSA was a product of BBL Laboratories, and all other medium components were from Difco Laboratories (Detroit, Mich.).

Statistical evaluation. Analyses of variance were performed for the collected data. When F value was significant (at a 5% level or less), Duncan's post hoc test was applied for comparisons among the means.

Results. *Experiment 1: In vivo study.* Total food intake and body weight gain did not differ between rats fed the NaSacc diet and controls fed the unadulterated diet during the 9-day feeding phase of this experiment. Rats fed the NaSacc diet consumed a total of 107.0 ± 1.8 g (mean ± SEM) and gained 67.7 ± 1.5 g, whereas control rats consumed 108.4 ± 3.4 g and gained 64.9 ± 1.9 g. The results of Fig. 1 confirm those of a previous study (6) indicating that rats fed a diet containing 2.5% NaSacc have higher tryptic and chymotryptic activities in the lower part of the small intestine and in the cecum than control rats. The present results indicate that these enzymatic changes occur by Day 2, and in the cecum this difference in proteolytic activity between rats fed NaSacc and control animals increases with time. On Day 5 amylolytic activity from rats fed NaSacc was lower in the lower part of the small intestine, and on Day 9 higher in cecum compared to control rats. The reasons for the difference

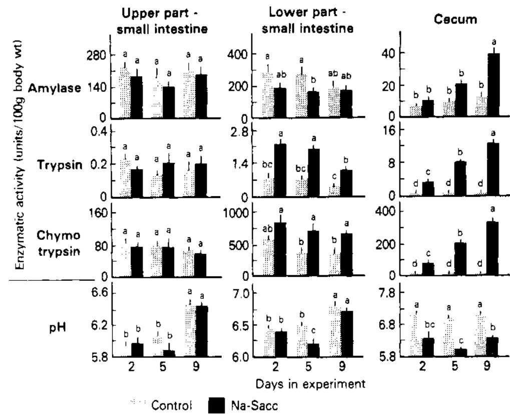

FIG. 1. Effects of a diet containing 2.5% sodium saccharin on pH and the activity of three digestive enzymes along the intestinal tract. Ten animals from each group (NaSacc-fed or control) were sacrificed on Days 2, 5, and 9, and analyses performed as indicated under Materials and Methods. Bars are the mean and SEM of 10 rats per group. Values not designated by the same letter are different at least at $P < 0.05$.

in cecal amylolytic activity between the groups of rats fed the NaSacc-adulterated diet and those fed the control diet are not clear. These may be related to the large difference in cecal pH between the two groups. Feeding NaSacc did not affect enzymatic activity in the upper part of the small intestine. Cecal pH values during the entire experiment were lower in rats fed NaSacc than in controls. In the lower part of the small intestine from rats fed NaSacc, the pH was lower than controls only on Day 5. Feeding NaSacc did not affect the pH in the upper part of the small intestine. A rise in pH of upper and lower small intestinal contents was noted in both groups of rats on Day 9, likely due to an increase in intestinal pH with age of weanling rats.

Experiment 2: In vitro study. No significant differences were found between the aerobically or anaerobically incubated samples. The following data are from anaerobically incubated samples. Samples of small intestinal contents incubated *in vitro* in the presence of 2.5% NaSacc contained higher tryptic and chymotryptic activity than control samples incubated without NaSacc (Fig. 2). Amylolytic activity was almost abolished after incubation *in vitro* whether or not NaSacc was

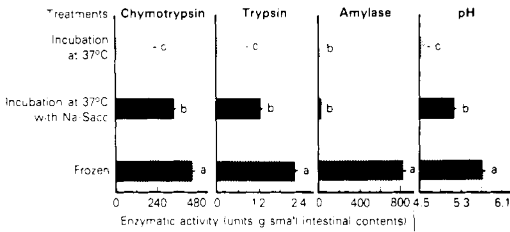

FIG. 2. Enzymatic activity and pH of small intestinal contents incubated *in vitro* at 37°C for 20 hr with and without sodium saccharin. Intestinal samples were unsterilized. The bars labeled "frozen" are values from unsterilized saccharin-free samples that were frozen at −15°C instead of being incubated at 37°C. Bars are the mean and SEM of five samples per treatment. Values not designated by the same letter are different at least at $P < 0.05$.

present in these preparations. The pH before incubation was about 5.8 (see frozen samples, Fig. 2), and was reduced after incubation at 37°C in all samples. Note, however, that samples containing NaSacc registered higher pH values after incubation than did control samples incubated without NaSacc.

In order to determine if pH per se was partly responsible for the differences in enzymatic activity seen in the result of experiments displayed in Figs. 1 and 2, pH adjustments were made to samples that were filter sterilized and incubated in the absence of NaSacc (Fig. 3). Control vials were treated identically except they were frozen at −15°C until analysis rather than being incubated. Incubation after sterilization did not result in pH changes of more than 0.2 units. In general, there was more loss of amylolytic and chymotryptic activity than tryptic activity during *in vitro* incubation at 37°C. Amylolytic activity was reduced concommitantly with pH whether or not samples were incubated or frozen. On the other hand, tryptic and chymotryptic activities were only slightly affected by changes of pH in this range (Fig. 3). Activity of chymotrypsin was reduced during incubation at pH 5.9 compared with incubation at pH 5.4 and 4.9 and also compared with frozen samples at all pH values.

The effects of NaSacc on enzymatic activity during incubation of filter-sterilized samples at 37°C and during freezing of unsterilized samples are shown in Fig. 4. The pH of all samples was adjusted to 5.2–5.4 before incubation or freezing. In both types of treatments, the presence of NaSacc resulted in an almost complete loss of amylolytic activity, but there was an apparent protective effect due to NaSacc upon chymotryptic activity. No effect of NaSacc on tryptic activity was found under these conditions.

Table I shows the results from an *in vitro* experiment on the effect of NaSacc on the bacterial flora of intestinal contents. The types and numbers of bacteria present before incubation were in agreement with those found by others (23). Three *Lactobacillus* species comprised nearly the total bacterial microflora of intestinal contents. *L. acidophilus* was the most common species, but *L. jensenii* and *L. fermentum* were also present. *Escherichia coli* was the only gram-negative species isolated and comprised approximately 1% of the total counts. The results of the viable counts agreed with microscopic examinations of intestinal contents in which

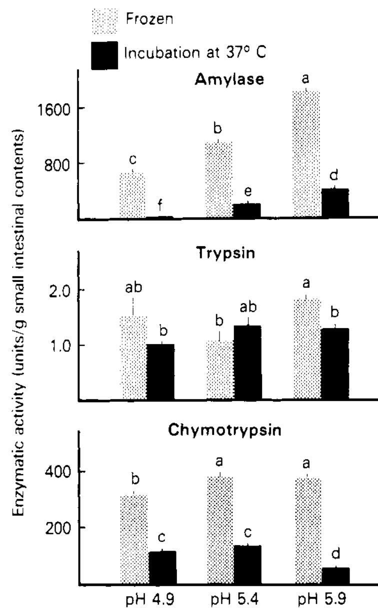

FIG. 3. Effect of pH on enzymatic activity during incubation *in vitro* of filter-sterilized samples of small intestinal contents. The pH of each sample was adjusted prior to incubation or before being frozen. Incubation was then carried out after which enzymatic analyses were performed (see Materials and Methods). Bars are the mean and SEM of five samples per treatment. Values not designated by the same letter are different at least at $P < 0.05$.

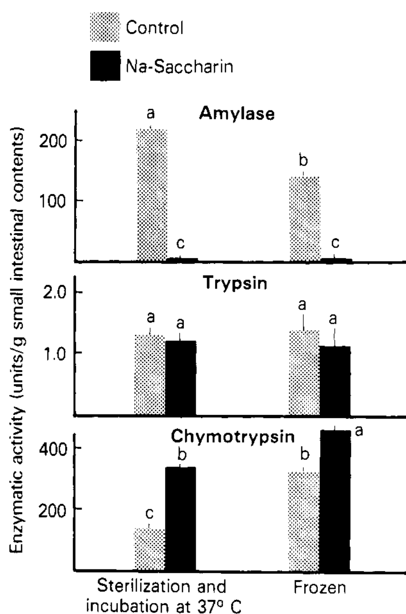

FIG. 4. Effect of sodium saccharin on enzymatic activity during *in vitro* incubation of filter-sterilized samples derived from small intestinal contents and on unsterilized samples frozen at −15°C. All samples were pH adjusted to 5.15. The pH value after incubations was 5.1–5.3. Bars are the mean and SEM of four to five samples per treatment. Values not designated by the same letter are different at least at $P < 0.05$.

three types of gram-positive rods, but almost no gram-negative cells were observed. Compared to counts at time zero, before incubation, large fluctuations in bacterial types and numbers did not occur for samples incubated in the presence or absence of NaSacc. A consistent trend, however, was apparent. The numbers of lactobacilli and *E. coli* both increased during the incubation of unamended samples, but these species both decreased in numbers during incubation of samples amended with NaSacc. This suggests that 2.5% NaSacc has an inhibitory effect on bacterial growth. The inhibitory effect of NaSacc on bacterial growth is consistent with the observations that after incubation at 37°C, those contents amended with NaSacc did not experience as great a drop in pH as did those not treated with NaSacc (Fig. 2).

The total amount (units/g contents) of reducing sugar in the cecum of rats fed NaSacc is contrasted with that found in samples in the *in vitro* study in Fig. 5. In the *in vivo* study (Experiment 1), rats fed NaSacc showed a higher content of cecal reducing sugar than rats fed a control diet. Under *in vitro* conditions (Experiment 2), contents from the small intestine (unsterilized) incubated in the presence of NaSacc also showed higher levels of reducing sugar than control samples. In fact in this *in vitro* study, the reducing sugar level of intestinal contents incubated in the presence of NaSacc was similar to that found in filter-sterilized samples incubated in the presence or absence of NaSacc. These results imply that NaSacc inhibits bacterial growth giving rise to a higher level of reducing sugars.

Experiment 3: Effect of NaSacc on bacterial strains. The results of Table I indicate that during the *in vitro* incubation of small intestinal contents at 37°C, the administration of NaSacc resulted in reduced total counts of lactobacilli and *E. coli.* The presence of NaSacc also resulted in inhibition of the

TABLE I. EFFECT OF SODIUM SACCHARIN ON BACTERIAL COUNTS DURING *IN VITRO* INCUBATION OF RAT SMALL INTESTINAL CONTENTS[a]

Amendment	Incubation (hr)	Bacterial counts CFU/g contents[b]	
		Lactobacillus spp.	*Escherichia coli*
None	0	7.2×10^8	2.4×10^6
	20	12×10^8	8×10^6
Saccharin	0	4.8×10^8	8×10^6
	20	2×10^8	2×10^6

[a] Values for bacterial counts are the mean of two samples from each incubation time. A maximum variation of 20% was observed.
[b] CFU, colony forming units.

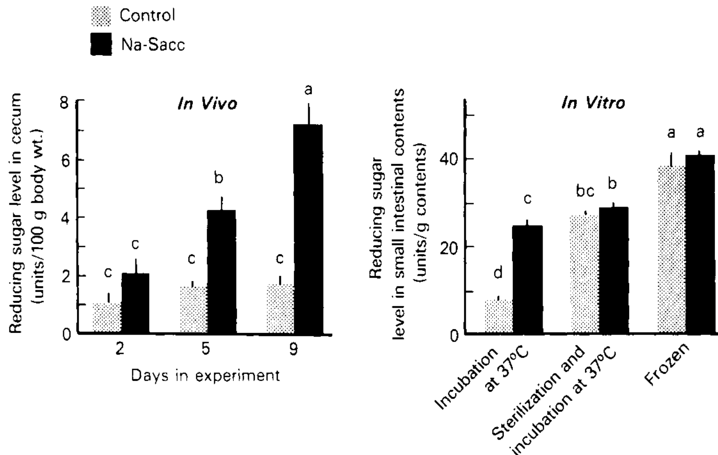

FIG. 5. Total reducing sugar in cecal contents of rats fed a diet containing sodium saccharin (*in vivo*), in normal intestinal contents and in filter-sterilized samples incubated with and without sodium saccharin (*in vitro*). Bars are the mean and SEM of 10 rats per group for the *in vivo* experiment and five samples per treatment for the *in vitro* study. Values not designated by the same letter are different at least at $P < 0.05$.

growth of three *Lactobacillus* species and three *E. coli* strains after their isolation from intestinal contents and incubation in laboratory medium. After 24 hr incubation at 37°C, all bacterial strains incubated in PGYS exhibited between 25 and 42% of the absorbancies obtained by strains incubated in PGY (Table II). This inhibitory effect of NaSacc on growth was still evident after 48 hr. As a consequence of NaSacc growth inhibition, PGYS cultures did not show the same pattern of pH changes as did PGY cultures (Table II). Acid production by *L. acidophilus* and *L. jensenii* was inhibited in PGYS cultures. *L. fermentum* was not highly acidogenic in PGY medium and an inhibitory effect of NaSacc on acid production was not noted. Acid production by *E. coli* incubated in PGY reached a maximum at 6 hr where values of 5.7–5.8 were observed. Due to alkaline reversion, the pH of *E. coli* cultures at 24 hr was 6.1–6.4 and a further increase in pH to

TABLE II. EFFECTS OF SODIUM SACCHARIN ON BACTERIAL STRAINS ISOLATED FROM SMALL INTESTINAL CONTENTS[a]

Bacterium	Growth in NaSacc medium as a % of control[b]	Medium pH after 24 hr incubation		Presence of extracellular proteases[c]
		Control	NaSacc	
L. acidophilus	42	5.1	5.7	None
L. jensenii	25	5.3	6.0	None
L. fermentum	33	6.2	6.1	None
E. coli				
Strain 1	27	6.6	6.0	None
Strain 2	30	6.1	5.9	None
Strain 3	39	6.4	6.0	None

[a] Determined after 24 hr incubation in peptone–glucose–yeast extract medium.
[b] Determined by measuring optical absorbance of each sample. Values are expressed as a percent relative to the absorbance obtained from control media (i.e., no NaSacc) incubation. Values are calculated from absorbances of a single sample from each bacterial strain.
[c] Determined in control and NaSacc media.

7.4–7.6 was observed after 48 hr. In contrast, after 24 hr the pH of *E. coli* cultures in PGYS had dropped to 5.9–6.0, and it was only after 48 hr that these cultures reached a pH of 5.7–5.8.

Extracellular proteolytic activity was not detected in any bacterial culture during or after incubation for up to 48 hr in PGY or PGYS. Failure to detect such activity was not due to insufficient bacterial numbers, as the total counts of bacteria in PGY media after incubation were comparable to those found in intestinal contents (approximately 10^8 CFU/g). The bacterial strains had no action on gelatin or milk and were, therefore, nonproteolytic in standard bacteriological test media.

Discussion. The present results confirm our previous observations (6) that feeding rats a diet containing 2.5% NaSacc induces higher activities of trypsin and chymotrypsin in the lower half of the small intestine and in the cecum compared to control rats fed an unadulterated diet. Further, as found before (6, 12), cecal pH was lower in rats fed NaSacc. The present results suggest that NaSacc prevents the normal elevation in cecal pH rather than inducing a decline in pH. On the other hand, during *in vitro* incubation of small intestinal contents (Fig. 2), pH of samples incubated in the presence of NaSacc was higher than in control samples. This discrepancy can be explained by noting that NaSacc inhibits bacterial growth (Tables I and II), and therefore inhibits pH changes (Figs. 1 and 2) and use of reducing sugar (Fig. 5) which would normally occur when bacteria are present. Further, NaSacc may act in a buffering capacity also inhibiting changes in pH.

The *in vitro* experiments performed with chyme from the small intestine produced qualitatively the same alterations in proteolytic activity as those observed *in vivo* for the lower part of the small intestine. This finding most likely rules out the possibility that the high proteolytic activity found in the intestine of rats fed NaSacc was due to NaSacc stimulated enzyme output from the pancreas by an increase in intestinal acidity (24). Furthermore, pH changes do not seem to be a major contributing factor for the NaSacc-induced high proteolytic enzyme content. The effect of pH on the stability of trypsin and chymotrypsin is known (13). However, in the lower part of the small intestine tryptic and chymotryptic activities were much higher in rats fed NaSacc than in controls under circumstances where either minor or no change in pH values occurred (Fig. 1). In addition, *in vitro* incubation of filter-sterilized samples displayed no major effect of pH on tryptic or chymotryptic activity (Fig. 3).

The *in vitro* experiments (Fig. 4) suggest that NaSacc may reduce amylolytic and enhance chymotryptic activity under conditions where pH is controlled and where bacteria are absent. The nature of this specific effect of NaSacc has not been fully determined. Recent *in vitro* studies (10, 11), suggested that NaSacc inhibited peptic, thermolytic, and tryptic activity. The results from studies on trypsin suggested that NaSacc complexed with the substrate making it unavailable for reaction with the enzyme. If so, then NaSacc, through its inhibition of proteolytic activity, could increase the survival of active enzymes (i.e., trypsin and chrymotrypsin) in the lower portions of the intestine. Further, when the high level of 7.5% NaSacc was fed to rats (11), an increase in cecal protein content was observed, suggesting that protein digestibility in the intestine was reduced. The observed increase in total weight of the cecal contents due to NaSacc (6, 25) might also be due to the antibacterial effects (Tables I and II) of NaSacc, as was proposed previously (11). Administration of penicillin to the diet of mice produced similar results (26). Since the presence of bacteria was claimed to inactivate proteolytic enzymes in the intestine of rats (27), the inhibition of bacterial activity by NaSacc could further contribute to the stability of this proteolytic activity.

It should be noted that the inhibitory effect of NaSacc upon bacterial growth in pooled small intestinal contents *in vitro* and in pure cultures (Tables I and II) is in contrast with the effect of dietary NaSacc on cecal bacteria *in vivo* (12). However, our study differs from that of Kirkland and Anderson (12) in several respects. The results presented in Table I represent the short-term exposure (18–24 hr) of bacteria in pooled small intestinal and cecal contents to 2.5% NaSacc. The dietary administration of 7.5% NaSacc over a 10-

day period, as used in (12), allows opportunity for selection and growth of a bacterial subpopulation resistant to NaSacc or the growth of a new bacterial group whose activity is normally suppressed. This possibility is not excluded by the data of Anderson and Kirkland (12) as their results are presented in the form of total counts. It is also known that the cecum and small intestine are inhabited by distinct bacterial groups (23), and different responses to NaSacc are therefore possible. Differential sensitivity to NaSacc by cecal isolates was, in fact, noted in (12).

It is almost certain that the increased proteolytic activity was not of bacterial origin. No extracellular proteolytic activity was detected in the media of the isolated bacterial strains even though normal growth occurred in control media incubated without NaSacc. The lactobacilli identified in this study have been shown to comprise the dominant microflora of the rat small intestine (23). These bacteria are not proteolytic; instead, they ferment carbohydrates to a variety of acidic products, especially lactic acid (28, 29). The present results therefore do not support the hypothesis (14) that intestinal bacteria may contribute a significant amount of proteases to their host. The fact that there were higher levels of reducing sugars in small intestinal contents containing added NaSacc than in control samples during the *in vitro* incubation (Fig. 5) and that filter sterilization abolished this difference (Fig. 5) strongly suggests that the antibacterial effect of NaSacc (Tables I and II) was responsible for the decrease in the use of reducing sugars by the bacteria. An increase in reducing sugar content was also seen *in vivo* with feeding of NaSacc (Fig. 5). We observed an inhibition of amylase activity by NaSacc *in vitro,* but not *in vivo,* when NaSacc was added to the food. Any inhibition of amylase activity by NaSacc at these levels (25) may not be sufficient to interfere with carbohydrate digestion, since an increase in levels of reducing sugars is observed *in vivo* in the cecum. Amylase activity was inactivated by NaSacc in filter-sterilized samples and even during freezing (Fig. 4). This implies that the reduced amylase activity noted here *in vitro* may not be related to bacterial activity nor may it be simply related to the demonstrated inhibition of amylase activity by NaSacc (25). The nature of the effect of NaSacc upon reduced amylase activity in the *in vitro* experiments should, therefore, be further characterized.

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