Artificial Sweeteners: Gut Microflora, Metabolism, and Diabetes: Potential for significant and unexpected health effects
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Executive Summary
A recent article about the effects of non-caloric artificial sweeteners (NASs) on gut microflora and on gut and nutritional physiology has profound implications for the current epidemic of obesity and diabetes. NAS use appears to alter gut microbiota in a substantial way that has direct effects on glucose tolerance and the utilization of polysaccharides and sugars in the diet. Relationships between short chain fatty acids and insulin regulation and between the composition of gut microflora and obesity have been reported previously. One element previously lacking was a more direct indication of what may initiate changes associated with the diseases. Suez, et al.\(^1\) report a series of experiments using mice and showed substantial changes in gut microflora as well as induction of glucose intolerance in NAS-fed mice. The changes in microflora and the accompanying glucose intolerance appear to be more directly causally related than previously thought. Importantly, results were also presented showing similar events in humans. The work summarized below provides important insight into a potential unexpected association between the frequent consumption of NASs, and the coincidental increase in both obesity and diabetes.

The Experimental Basis – Mice as a Model System for Understanding Gut Microflora
A recent article in Nature raised important issues regarding the use of artificial sweeteners\(^1\). Non-caloric artificial sweeteners (NAS) are some of the most widely used food additives throughout the world. Although there appears to be no overt toxicity associated with the commonly used NASs, the report indicates an important indirect effect, specifically alteration of the populations of bacteria that inhabit the gut. The report contains results from several different lines of investigation important to understanding what the microbial alterations mean to human health. These include alterations in metabolism relevant to diabetes and energy metabolism.

The authors took advantage of recent dramatic improvements in analysis of bacterial flora based on DNA identification using polymerase chain reaction (PCR) of 16S rRNA genes. This allows identification of many species present in the complex microbiological environment of the intestinal tract. In contrast to classical bacterial identification methods requiring culturing of probably only a few of the microbes present, 16S rRNA identification provides a way to characterize microflora in a broad spectrum way that samples even unculturable organisms.

The three NAS tested were saccharin, sucralose and aspartame (chemical structures shown in order, below).

Commercial non-caloric sweeteners contain approximately 5% sweetener and 95% glucose. The sweeteners are hundreds of times sweeter than glucose or sucrose, so their relatively low concentration is organoleptically effective in producing profound enhancement of a sweet taste. Most experiments used the commercial forms of the sweeteners and a control of plain water or water supplemented with glucose or sucrose.

Marked glucose intolerance was noted for all groups of mice fed with an artificial sweetener for 11 weeks but not for groups supplied with plain water or water supplemented with glucose or sucrose. Saccharin produced the most profound effect and was used in many further studies. The initial group of C57Bl/6 mice was lean, but an additional experiment used mice fed with a high fat diet (HFD) supplying 60% of kcal from fat. Pure saccharin was used in the latter experiment, and the quantity fed was equivalent to the FDA acceptable daily intake in humans as adjusted for weight of the animals. The pure saccharin was associated with impaired glucose tolerance after as little as five weeks compared to controls. HFD fed outbred Swiss Webster mice showed a similar effect, i.e. increased glucose intolerance, five weeks after saccharin exposure indicating that the results were not unique to highly inbred and genetically homogenous C57Bl/6 mice.

Another series of measurements was the effect of NAS on metabolic profiles. Liquid and chow consumption, oxygen consumption, walking distance and energy expenditure were measured and were similar among NAS and control mice whether fed a normal or high fat diet. Also, in contrast to the abnormal glucose tolerance noted above, insulin tolerance was normal in both NAS and control mice. This indicates that insulin remained effective in controlling blood glucose, but apparently less insulin was released in NAS-treated mice resulting in elevated glucose. This may be similar to a diabetic state following long-term elevated blood glucose, which is followed by decreased insulin release usually exacerbating diabetes.

To test the relationship between gut microbiota and NAS-induced glucose intolerance, both lean and HFD mice were treated with antibiotics. Ciprofloxacin and metronidazole were used to target Gram-negative bacteria, and vancomycin targeted Gram-positive bacteria in the gut while NAS or control diets were maintained. Glucose intolerance in NAS-treated lean and obese mice was abolished after four weeks of treatment with either Gram-negative or Gram-positive targeted antibiotics. This suggested that commensal microbiota mediated the glucose intolerance and that the types of bacteria involved were diverse. Further testing was carried out by transplanting fecal organisms from NAS-fed mice into chow-fed mice. This resulted in impaired glucose tolerance for mice with the transplanted microbes. Also, microbiota transplanted from control mice to NAS-fed mice resulted in normal glucose tolerance. The results strongly suggest that the intestinal microflora is directly involved in the metabolic derangements.

Changes in the microbes found in the intestinal tract were documented using 16S rRNA sequencing. Finding a change in quantity of a single organism in the gut is not particularly surprising but the results indicate a profound change in the flora with changes in abundance of many species of bacteria. Changes were defined on the basis of operational taxonomic units (OTU), which is the term applied to taxonomic identification data based on DNA sequencing alone. For practical purposes an OTU is equivalent to a species. On this basis, the 16S rRNA data indicated that approximately 40 species were significantly altered in abundance between control mice and NAS-treated mice. The alteration in microflora is referred to as dysbiosis, i.e. substantial alteration of microflora. Bacteria of the genus Bacteroides, very common Gram-negative anaerobic inhabitants of mammalian intestinal tracts, were noted to increase in abundance while other members of the taxonomic order Clostridiales notably declined. In a variety of tests the pattern of change in microbiota was similar indicating similar configurations of dysbiosis following saccharin consumption.
Using current databases containing information about metabolic pathways found in the various bacteria, a gut microbial gene catalogue was constructed to understand the functional effect of changes in gut microflora. That is, many different bacteria metabolize materials in the intestinal tract using a variety of metabolic pathways. The presence and abundance of various pathways can be determined using DNA sequencing. This provides a picture of the metabolism occurring in the gut microflora. The question was did changes in microbes and their abundance in the gut change the metabolism mediated by bacteria?

Specific enzymes associated with particular pathways were quantified. Although there were some differences observed between mice fed glucose compared to plain water, the magnitude of changes in intestinal bacteria in either water or glucose-in-water were much smaller compared to mice treated with saccharin in water. Predominantly, changes in glycan degradation were profoundly affected resulting in considerably greater degradation of polysaccharides in saccharin-fed mice. That is, normally many polysaccharides pass through the gut poorly digested, but when a saccharin-containing diet has been fed for several weeks, the gut microflora are selected for populations that more effectively metabolize these polysaccharides. Consistent with this increased microbe-mediated digestion, short chain fatty acids, e.g. acetate and propionate, increased substantially. In contrast to the polysaccharides which are not absorbed by the gut, the short chain fatty acids are readily transported across the gut making them available as energy sources for the animal. As cited by Suez, et al., the increased energy harvest has been associated with obesity in mice and humans. Five bacterial species were identified as primarily responsible for this metabolic shift. Two of the five species are members of the Bacteroides genus. Both Gram-positive and Gram-negative species are indicated consistent with the lack of specificity for Gram-specific antibiotic treatments mentioned above.

In addition to the glycan metabolic degradation pathway, other enhanced pathways in saccharin-consuming mice included those for starch, sucrose, fructose, and mannose. Glucose transport-dependent pathways decreased in saccharin-consuming mice. The results together strongly indicate that the alterations in the microbiome associated with NAS consumption result in a greater capacity to utilize energy from the diet, which is consistent with tendencies toward obesity.

To determine if the effects of NAS were on the bacteria directly, stool cultures were incubated in a strictly anaerobic environment in the presence of saccharin-containing or control media. As may be expected if the effect is directly on the bacteria, transplant of the cultures into mice resulted in a significant increase in glucose intolerance if in vitro culture included saccharin compared with transplanted cultures from control media. Changes in bacterial species and in expressed metabolic pathways similar to those observed in gut microflora in mice were also observed in the anaerobically cultured cells treated with saccharin. This showed that at least the changes in microflora composition and associated metabolic changes are likely to result from effects by NAS directly on the bacteria.

The protocols and results from the various experiments are outlined in Table 1.

**Observations in Mice Translated Directly to Effects in Humans**

Obviously, manipulation of humans is not as readily accomplished as detailed studies in mice. Nevertheless, Suez et al., using a validated food frequency questionnaire, examined the relationship between long term NAS consumption and clinical parameters in 381 non-diabetic people. The results

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indicated a positive correlation between NAS consumption and increased weight, waist-to-hip ratios, higher fasting blood glucose, glycosylated hemoglobin (HbA1C - used as an indicator of damage to proteins associated with prolonged elevated blood glucose), and impaired glucose tolerance. A high NAS-consuming subgroup of 40 individuals was compared to 236 non-NAS consumers and glycosylated hemoglobin was statistically significantly elevated among the NAS consumers. Using 16S rRNA analysis, 172 people were randomly selected, and statistically significant changes in microflora were detected when comparing NAS consumers with non-NAS consumers. Additionally, microflora changes involving several OTUs were found in NAS consumers compared to non-NAS consumers. However, microflora changes were not necessarily correlated with BMI unless those with a high BMI also had a history of NAS use.

Finally, the effect of NAS on glucose tolerance was tested in seven individual people. These test subjects did not normally use NAS, but NAS-containing foods were consumed over a one week period. Three daily doses of commercial saccharin were administered to achieve levels equivalent to the maximum acceptable daily intake specified by the FDA, 5 mg per kg body weight. Four of the seven individuals developed significantly poorer glycemic responses 5-7 days after NAS consumption compared to their responses during the first four days. Glucose tolerance was neither impaired nor improved among the three NAS non-responders. For the four NAS responders, 16S rRNA analysis indicated pronounced microbiome changes.

Stool samples were collected on day zero and day seven from two NAS non-responders and two NAS responders. Follow-up experiments included transfer of stool from the four humans into normal chow-fed mice. Transfer from the samples taken from NAS-responders seven days after NAS exposure resulted in significant glucose intolerance in the mice, but transfer of stool obtained prior to NAS exposure did not. Regardless of the day after NAS exposure, stool from NAS non-responders did not result in induction of glucose intolerance in the mice into which stool was transferred. Although the sample size for humans is definitely too small to draw general conclusions, the implications are clear and suggest results consistent with what was observed in the extensive experiments with mice.

Results from experiments involving humans are outlined in Table 2.

The primary results described by Suez et al. supplement previous reports indicating correlations between the gut microbiota and obesity. Recent work indicated that modifications of a core microbiome are associated with changes in energy balance and obesity in addition to other recent citations cited above. A major advance in the Suez et al. work is the assignment of a likely cause for the microbiota conversion as being triggered by NAS. This clearly has important clinical and dietary implications, albeit requiring verification and amplification via further testing in humans.

Remaining to be demonstrated is mechanism(s) for the action of NAS in increasing the presence of some bacteria in the gut and decreasing others. The structural differences among the major commercial NAS do not obviously indicate a similar mechanism of action on bacteria considering the benzothiazole structure of saccharin, the frutofuranosyl-galactopyranoside structure of sucralose and the peptide-like structure of aspartame. Indeed, caution in interpretation of results with NAS too broadly is indicated, since the majority of results used saccharin. It appears reasonable to suggest that there will be differences in the way various structurally different NAS act, although some commonality of action is indicated by the present limited tests using sucralose and aspartame.

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The relationship between glucose resistance and NAS also compels further clarification. However, indications that short chain fatty acids in the gut alter insulin secretion date back at least to the 1960s\(^5\). Also, a role for anaerobic bacteria in metabolism in the human gut has been reported for 30 or more years\(^6\). Consistent with ideas of the benefits of dietary fiber, gut bacteria were considered to be significantly responsible for conversion of poorly digestible polysaccharides – sometimes termed non-starch polysaccharides – to short chain fatty acids that were apparently beneficial to health\(^7\).

Gene regulation by fatty acids has been reported and has implications in a number of chronic diseases in addition to obesity and diabetes\(^8\). For present considerations, observations of the importance of short chain fatty acids resulting from activity of gut microbiota may play an important role in regulation of metabolism associated with diabetes\(^9\). The Suez et al. report indicates a negative effect on insulin and glucose regulation by short chain fatty acids produced in the gut. It is clear that many details remain to be determined. Nevertheless, substantial progress in understanding the inherent complexities has recently been demonstrated and it is reasonable to expect that considerable further progress will be forthcoming, perhaps much more rapidly than would have been projected even a few years ago.

**Conclusions and Projections**

It is not surprising to find that a healthy diet requires balances among readily metabolized carbohydrates, short chain fatty acids, difficult to metabolize polysaccharides of starch and non-starch types - metabolism of which are highly dependent on gut bacteria - and physiological parameters including balanced energy intake. However, the complexities of the microbiota and diet and effects by such compounds as synthetic sweeteners have only recently become analyzable in the detail necessary to begin understanding the complexity. New tools using rapid nucleic acid sequencing techniques combined with recently acquired genetic taxonomy and quantification of the tremendous mix of organisms present in the gut have enabled greater understanding of the associated microbe-related biochemistry and physiology. Of course the gut is only one of the microbiomes of interest and importance to further advances in understanding human health and disease.

It is important to appreciate that the Suez et al. results are limited in direct human testing. Few humans were tested in detail. Certainly much less detail was observed with humans than with mice. Of course this is not particularly surprising and, nevertheless, the case for further human testing is compelling. Among many other questions, would treatment with NAS longer than seven days have resulted in more of the humans converting to glucose intolerance? How will testing a much larger human population affect the results? It seems reasonable to speculate that some human populations will respond differently to NAS depending both on genetics and on diet and culture. One of the most compelling questions is how will NAS be shown to have affected and perhaps triggered the current obesity and diabetes epidemics?


Mechanistically, much also remains to be determined. It is possible, although currently only speculative, that NAS transiently induces a substantial insulin release followed by a significant reduction of insulin release leading to glucose resistance on a time scale faster than has previously been considered in development of adult-onset diabetes. Alternatively, no transient insulin increase may be required and NAS may more directly lead to decreased insulin release. The important trigger may occur via increase in gut short chain fatty acids. The mechanism of action on bacteria also remains to be understood. Since the results so far indicate action directly on bacteria, what molecular events lead to increases in some gut bacterial populations and decreases in others? It is clear that many interacting factors are involved, including alteration of bacterial populations in the gut and alteration of nutrients made available for absorption by the gut.

One potentially positive result may be that NAS could be used in situations of low food availability for diets containing high-fiber foods that - without microbiome alterations - typically have low caloric yield. NAS may help to improve efficiency of food utilization for foods containing complex polysaccharides. Furthermore, it would be interesting to determine if a result of NAS-induced microflora change also changes microbial synthesis of vitamins or essential amino acids in addition to short chain fatty acids. Complex polysaccharides present in the diet that are not normally digested to absorbable forms may be metabolized by NAS-induced alterations in gut microflora to short chain fatty acids. The resulting caloric boost may moderate or alleviate energy deficits associated with limited food availability.

As with all good science, the Suez et al. work has induced an important series of questions following from their observations. While the role of NAS cannot provide complete answers to current questions about obesity and diabetes, the likelihood that this work will contribute to those answers is certainly high. This study contributes valuable insight to the growing interest in and understanding of the role of gut microbiota in health and disease.
Table 1. Experiments Conducted in Mice

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Treatment</th>
<th>Treatment Time</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Controls (lean mice): water, water + sucrose, or water with glucose</td>
<td>11 weeks</td>
<td>Normal glucose response, gut microflora, and short-chain fatty acids</td>
</tr>
<tr>
<td></td>
<td>Test (lean mice): water + saccharin, water + sacralose, or water + aspartame</td>
<td>11 weeks</td>
<td>Glucose intolerance &amp; profound changes in gut microflora compared to controls; Increase in short-chain fatty acids</td>
</tr>
<tr>
<td></td>
<td>(Included testing of C57Bl/6 inbred and Swiss-Webster outbred mice)</td>
<td></td>
<td></td>
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<tr>
<td>B</td>
<td>Controls (High fat diet): water, water + sucrose, or water with glucose</td>
<td>11 weeks</td>
<td>Normal glucose tolerance</td>
</tr>
<tr>
<td></td>
<td>Test (High fat diet): water + saccharin</td>
<td>11 weeks</td>
<td>Glucose intolerant (as early as 5 weeks)</td>
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<tr>
<td></td>
<td>(used Swiss-Webster mice)</td>
<td></td>
<td></td>
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<tr>
<td>C</td>
<td>Controls: water, water + sucrose, or water with glucose</td>
<td>11 weeks; 11 wks</td>
<td>Normal glucose tolerance</td>
</tr>
<tr>
<td></td>
<td>Test: water + saccharin, or water + sacralose OR water + aspartame</td>
<td>11 weeks; 11 wks</td>
<td>Normal glucose tolerance</td>
</tr>
<tr>
<td>D</td>
<td>Protocol A NAS-treated, glucose intolerant mice: Transplant gut microflora from protocol A controls</td>
<td></td>
<td>Glucose tolerance restored</td>
</tr>
<tr>
<td></td>
<td>Protocol A control mice: Transplant gut microflora from protocol A NAS-treated</td>
<td></td>
<td>Induced glucose intolerance (determined 6 days after transplant)</td>
</tr>
<tr>
<td>E</td>
<td>Stool from control mice cultured anaerobically</td>
<td></td>
<td>Microflora unchanged</td>
</tr>
<tr>
<td></td>
<td>Stool from control mice cultured anaerobically with saccharin</td>
<td></td>
<td>Microflora changes consistent with NAS-treated mice</td>
</tr>
<tr>
<td>F</td>
<td>Transferred cultured bacteria from Protocol E without saccharin into germ-free mice</td>
<td></td>
<td>Glucose tolerant</td>
</tr>
<tr>
<td></td>
<td>Transferred cultured bacteria from Protocol E + saccharin into germ-free mice</td>
<td></td>
<td>Reduced glucose tolerance</td>
</tr>
</tbody>
</table>
Table 2. Observations and Experiments in Humans

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Survey of NAS use</td>
<td>NAS use correlated with increased weight, higher fasting blood glucose, increased HbA1C</td>
</tr>
</tbody>
</table>
| B        | NAS non-consumers (n = 172)  
NAS consumers (subgroup, n = 40) | Compared NAS non-users with NAS consumers:  
HbA1C increased in NAS consumers  
Microflora profile showed similar changes to those observed in NAS-treated mice  
Microflora changes did not correlate with BMI |
| C        | Seven NAS non-consumers dosed with NAS for seven days | Four of seven → decreased glucose tolerance after 5-7 days (responders)  
Three of seven → no change in glucose response (non-responders) |
| D        | Transfer stool samples after seven days NAS treatment to mice from non-responders in Protocol C  
Transfer stool samples after seven days NAS treatment to mice from responders in Protocol C | Normal glucose tolerance in mice  
Glucose intolerance in mice from seven day samples – normal glucose tolerance from day 0 stool samples from responders |
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Nature paper: Artificial sweeteners