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Fruit Fly Bioassay To Distinguish "Sweet" Sugar Structures

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Palatable response to dietary sugars plays a significant role in influencing metabolic health. New structures are being explored with beneficial health properties, although consumer acceptance relies heavily on desirable sensory properties. Despite the importance of behavioral responses, the ability to elucidate structure-preference relationships of sugars is lacking. A wild population of *Drosophila melanogaster* was used as a model to perform pairwise comparisons across structural groups to characterize a fruit fly bioassay for assessing sugar preference. Preference was successfully described in structurally relevant terms, particularly through the ability to directly test sugars of related structures in addition to standard sucrose comparisons. The fruit fly bioassay also provided the first report on the relative preference for the β-linked sugar alcohol, gentiobiitol. In making reference to well-known human preferences, the bioassay also raises opportunities for greater understanding of behavioral response to sugar structures in general.

KEYWORDS: Alternative sweeteners; carbohydrate; *Drosophila*; gentiobiitol; gentiobiose; glucobiose; insect behavior; sucrose

INTRODUCTION

With a foundation dating back centuries, and once reserved for the privileged, sweet-tasting carbohydrates (predominantly sucrose) are extensively added to many modern food products. As concerns grow about the health implications of existing sweeteners, efforts are focused on developing alternative sugars with the proviso that suitable properties including desirable taste are maintained. Due to the complexities of performing human sensory trials, published data on structure-taste relationships are fragmentary, not well supported by experimental evidence, or quoted in product information without reference. With a host of new carbohydrate products being developed and entering the market in recent years, an improved ability to determine structures with a favorable response is of interest.

The utility of *Drosophila melanogaster* (fruit fly) as a model for research in chemosensory behavior is well accepted. Although the taste systems of mammals and flies have evolved independently and rely on different anatomical and molecular structures, there is a remarkable degree of similarity in the responses to tastes including "sweetness" and "bitterness" (*1*, *2*). A recent report has also demonstrated similarity in the response of the fruit fly to high-intensity sweeteners recognized by humans (*3*), expanding on earlier work using *Phormia regina* (blow fly) (*4*). This is particularly intriguing considering the variation in response displayed for these compounds in more closely related mammals, such as New World monkeys (*5*, *6*). With this in mind, the current study characterized structural groups of sugars in a fruit fly bioassay by comparing their preference to sugars that are perceived as sweet by humans.

The aim of the study was to derive a measurement of relative sweetness for a potential novel sweetener, gentiobiitol. In addition, comparison of structures containing related glucosidic linkages was intended to identify linkages responsible for increased preference that could contribute to the design of novel sweeteners.

MATERIALS AND METHODS

Materials. Groups of structurally related sugars were used in the assays: (i) α-glucobioses (kojibiose (glc-1,2-glc), nigerose (glc-1,3-glc), maltose (glc-1,4-glc) and isomaltose (glc-1,6-glc)); (ii) β-glucobioses (sophorose (glc-1,2-glc), laminaribiose (glc-1,3-glc), cellobiose (glc-1,4-glc,) and gentiobiose (glc-1,6-glc); (iii) sucrose isomers (sucrose (glc-1,2-fru), turanose (glc-1,3-fru), leucrose (glc-1,5-fru), and palatinose (glc-1,6-fru)); (iv) sugar alcohols (maltitol (reduced maltose), gentiobiitol (reduced gentiobiose), and maltotriitol (reduced maltotriose)) and trisaccharides (melezitose (glc-1,3-fru-1,2-glc), erlose (glc-1,4-glc-1,2-fru), panose (glc-1,6-glc-1,4-glc), and maltotriose (glc-1,4-glc-1,4-glc)). Kojibiose, nigerose, isomaltose, sophorose, gentiobiose, leucrose, and panose were sourced from Carbosynth (Berkshire, U.K.), whereas erlose, maltotriose, and maltotriitol were supplied by Hayashibara Biochemical Laboratories (Okayama, Japan). Laminaribiose was purchased from Megazyme (Co. Wicklow, Ireland), and all remaining carbohydrates were purchased from Sigma-Aldrich (St. Louis, MO). A 200 mM stock solution of each carbohydrate was prepared in distilled water (dH_2O) and stored in aliquots at −20 °C.

Gentiobiitol Synthesis. Synthesis of gentiobiitol via the reduction of gentiobiose was performed by Epichem Ltd. (Murdoch, Australia) and was based on a modification to the method of Abdhel-Akher et al. (*7*). Briefly, 4.8 g of gentiobiose was dissolved in 50 mL of dH_2O and combined with 1.0 g of sodium borohydride in 25 mL of dH_2O. The reaction was

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allowed to proceed at room temperature for 4 h and quenched with acetic acid after confirming the reaction to be complete by thin layer chromatography. The solution was then concentrated and the product precipitated using methanol. Further purification, deacetylation, and concentration were carried out to yield a final solution of 0.70 M gentiobiitol in dH_2O (4.05 g, 99% purity).

Collection and Maintenance of *D. melanogaster* Populations. Laboratory stocks of *Oregon-R* were used in pilot experiments to establish the assay format and equipment. A new population of wild-type flies was established to exclude the possibility that the discriminating ability of the laboratory population had become attenuated due to limited dietary exposure. Wild-type flies were used exclusively in assays of sugar preference.

Ten female *D. melanogaster* were captured on the University of Queensland campus between February 27, 2009, and March 6, 2009. Traps consisted of empty 1 mL pipet tip boxes baited with mashed banana and sprinkled with live yeast (*8*). Standard 1 mL pipet tips with the ends cut off were inserted to create a one-way entrance to the trap. Traps were deployed in the field for 24 h surrounded by Tanglefoot insect barrier (The Tanglefoot Co., Grand Rapids, MI) to prevent ants and other crawling insects from entering. Traps were inspected for flies, and females were transferred to separate vials containing standard corn meal nutrient medium. The individual females were monitored until their offspring eclosed and their sons could be identified. Identification of *D. melanogaster* males was carried out by examining the sex combs, as *D. melanogaster* show distinct differences from other commonly found *Drosophila* species. All flies were reared in 250 mL bottles at 25 °C on a 12:12 h light/dark regimen on standard corn meal medium.

Two-Choice Behavioral Assay. Assays were carried out using a 96-well plate with a layer of Parafilm stretched over it to keep the food and flies on the surface and out of the wells. Test sugars at a uniform concentration of 4 mM were mixed with either brilliant blue (25 mg mL^{-1}) or erythrosine (90 mg mL^{-1}) (New Directions, Marrickville, Australia) in 0.5% agarose. Initially, plates were replicated with the colors inverted for each sugar to test for color bias, but after 12 trials this approach was not continued as it was determined that color had no effect on preference as had been reported previously (*9*).

A minimum of 50 flies aged < 5 days were starved for 24 h on filter paper soaked in dH_2O for each experiment (*3*, *9*−*13*). Both males and females were used as it has been reported that sex does not affect feeding preference (*12*). All feeding experiments were carried out in the morning as circadian rhythm can affect feeding behavior (*14*, *15*). Flies were allowed to feed for 2 h in the dark before being frozen for 48 h. Scoring of abdomen color was carried out visually with a dissecting microscope (Olympus SZ51, Center Valley, PA/Zeiss Stemi 2000, Thornwood, NY), and the flies were grouped into the following categories: red (R), blue (B), purple (P), and none. Red and blue flies were distinct, whereas purple flies varied in shade depending on the quantity of sugar eaten. Preference index (PI) was calculated as the number of red or blue flies plus half the number of purple flies divided by the total number of flies that fed: PI = R or $B + {}^1/_2P/(R + B + P)$. When using the wild-type population, feeding rates of > 50% were consistently observed. Each paired comparison was replicated at least three times with separate plates and flies.

RESULTS AND DISCUSSION

D. melanogaster flies were exposed to traditional sugars, including glucose and sucrose, and a range of potential or current alternative sweeteners. Fruit fly sugar preferences were determined using two-choice behavioral assays based on the method of Tanimura et al. (*16*). Initial optimization was performed to confirm that food dyes did not influence preference and to establish reproducible conditions for fruit fly behavior. In addition to confirming earlier studies, including the importance of aging (*17*, *18*), we found that a consistent time of day for starving and feeding improved the reproducibility of the assays. We suggest this improvement is likely due to the effect of circadian rhythms on feeding behavior (*14*, *15*).

A newly established fruit fly line from a wild population was employed for data on sugar preference as it was generally observed to be more consistent in its behavioral response, displaying higher feeding rates throughout the experiments than inbred laboratory lines. Preference index (PI) was calculated to measure the preference for one carbohydrate in relation to another, determined from the feeding experiments following scoring of abdomen color. PI for each carbohydrate choice was a proportion of 1.00, with a value of 0.50 equating to an equal preference for the two sugars being tested. This approach enabled gustatory responses to sugars to be reported in a defined and sensitive manner. The carbohydrate structures used in the study were selected to represent broad structural groups and to target specific monosaccharide linkages through selection of related di- and trisaccharides. Tests were performed with equimolar (4 mM) solutions, including standard comparisons paired with either sucrose or glucose, which was again based on the work of Tanimura et al. (*16*) in addition to the current optimization (data not shown).

Comparisons focused first on defining the preferences of the fruit fly, relative to commonly held views of human carbohydrate preference (*19*). Initially, four α-glucobioses along with their corresponding β-glucobioses were tested against equimolar sucrose (**Figure 1a**). The results showed that the α-linked sugar was significantly preferred over the β-linked sugar except in the case of isomaltulose and gentiobiose (glc-1,6-glc), where the small observed difference was not significant. This general preference for α-structures over β-structures is similar to the preferences demonstrated in humans (*20*). Whereas the α-glucobiose samples were more preferred than their corresponding β-glucobiose, in each case significant consumption of the β-linked sugar did occur. In the case of the β-1,6-glucobiose (gentiobiose), this was somewhat surprising, as this sugar has previously been reported to have a "bitter" taste in humans (*21*−*23*). Molecular studies of fruit fly taste receptors have shown that the flies perceive, and subsequently avoid, substances such as caffeine that are bitter-tasting to humans (*2*). The consumption of gentiobiose by the fruit flies may indicate that the preferences diverge for this structural group. To explore further the preferences for α-linked and β-linked sugars, experiments were conducted by directly pairing the α- and β-forms as the two opposing choices (**Figure 1b**). Differences were more pronounced using this direct approach, with the preference for α- over β-glucobioses confirmed and all statistically significant. The greatest difference in PI occurred with the α-1,4 and β-1,4 samples (maltose and cellobiose), suggesting a possible strong preference for α-1,4 carbohydrates. Interestingly, the difference between the α-1,6 (isomaltose) and β-1,6 (gentiobiose) sugars remained smallest. This limited difference between gentiobiose and the related α-linked structure may suggest a somewhat elevated preference for gentiobiose by the fruit flies relative to other β-linked structures. The plausibility of this may be strengthened by the presence of β-1,6-glucans in yeast cell walls, an extract of which is included in the laboratory diet of the fruit fly as well as part of their natural diet. Similarly, with such a pairwise test this may also indicate a reduced preference for isomaltose or, more specifically, α-1,6-linked glucose.

To characterize our model system further, we determined preferences for a second structural group of sugars, the sucrose isomers. Sucrose and three isomers (turanose, leucrose, and palatinose) were compared to either sucrose or glucose at equimolar concentrations. The control experiment comparing sucrose to itself resulted in a PI that was not significantly different from 0.5, as expected. The results showed that turanose was a more strongly preferred isomer than leucrose or palatinose (**Figure 1c**). A previous study detecting responses of sugar-sensing neurons in fruit fly has reported similar findings, with sucrose and

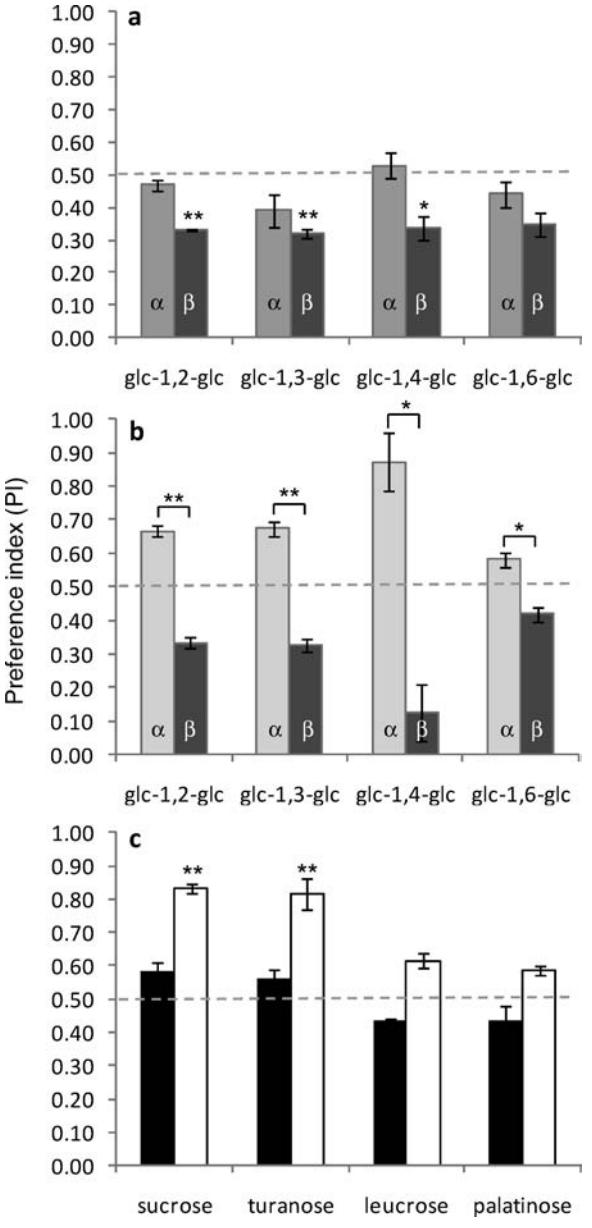

Figure 1. Characterizing sugar preferences of *Drosophila melanogaster* by pairwise preference assays. Results are expressed as preference index (PI), where a PI > 0.5 indicates elevated preference toward the indicated test sugar. Preference for α- over β-glucobioses was demonstrated by comparing each of the eight sugars to sucrose (**a**) as well as by directly comparing the α and β structures to one another (**b**). (**c**) Preference for the sucrose isomers, turanose, leucrose, and palatinose, was measured relative to both sucrose (black bars) and glucose (white bars). Bars indicate ± standard error (n = 3−6). A paired t test was performed, with ∗ corresponding to $p < 0.05$ and ∗∗ to $p < 0.01$.

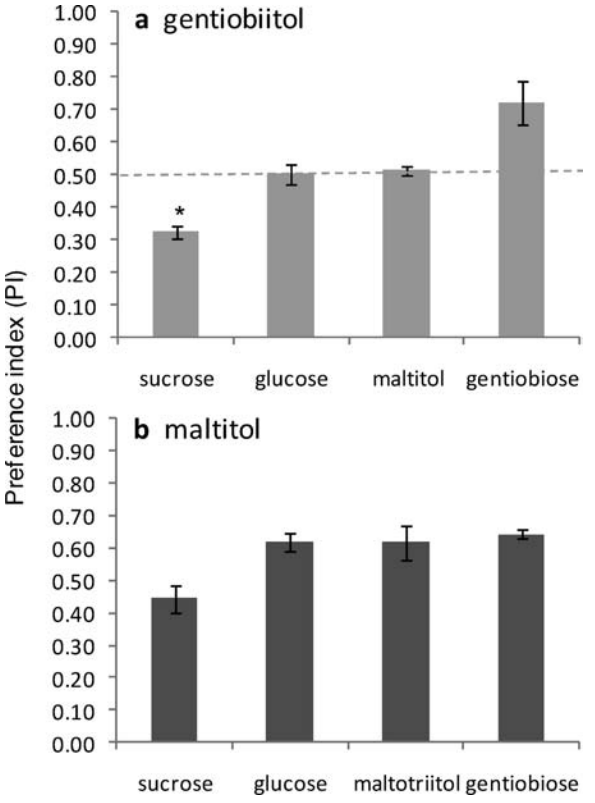

Figure 2. Preference for the sugar alcohols gentiobiitol and maltitol determined by the fruit fly bioassay. Results are expressed as preference index (PI), where a PI > 0.5 indicates elevated preference toward the indicated test sugar. (**a**) Preference for gentiobiitol relative to known "sweet" compounds, including sucrose, glucose, and maltitol, as well as gentiobiose showed that whereas less preferred than sucrose, a PI similar to that of equimolar glucose and maltitol was observed. (**b**) Preference for the alternative sweetener maltitol relative to sucrose, glucose, maltotriitol, and gentiobiose showed only slightly elevated preference for maltitol against sucrose and glucose than the same comparisons for gentiobiitol. The effect of an additional α-1,4-glucose in maltotriitol did not significantly increase its preference when compared to maltitol. Bars indicate ± standard error (n = 3−5). A paired t test was performed, with ∗ corresponding to $p < 0.05$ and ∗∗ to $p < 0.01$.

turanose displaying greater responses over leucrose and palatinose (*12*). These preferences are similar to those suggested for humans, although turanose, despite being poorly studied in humans, has been described anecdotally as half as "sweet" as sucrose (*24*, *25*). *D. melanogaster* may have an elevated response to turanose because of the greater natural abundance over the two other isomers tested, turanose being present at higher levels in nectar and honey, for example (*26*, *27*). In broad terms, these experiments also reinforced the utility of the fruit fly behavioral assay relative to human preferences, as comparisons to glucose resulted in consistently greater PI for the opposing carbohydrate than comparisons to sucrose for all samples. Because glucose is perceived as being approximately 75% as "sweet" as sucrose in humans (*19*), a higher PI would be expected for the opposing sugar when being compared to glucose.

Having characterized a range of linkages with generally accepted information on human preferences, we then applied the assay to a compound with no previous data. The ability to increase perceived sweetness of α-linked disaccharides following conversion to a sugar alcohol is known, and disaccharide alcohols such as maltitol are now commonly used as alternative sweeteners. The effect of reduction on β-linked structures is less studied. Conversion of the β-glucobiose, cellobiose, into a sugar alcohol has been shown to decrease "sweetness" in one study with human subjects (*28*). In the present study, gentiobiitol, the reduced product of the β-1,6-linked glucobiose, gentiobiose, was assessed to determine any effect on preference. Gentiobiitol has recently been identified in transgenic sugar cane plants engineered to produce sorbitol (*29*), and the potential application of this novel sugar as an alternative sweetener is of interest. A comparison to equimolar sucrose was first performed (**Figure 2a**), and gentiobiitol was shown to be ingested with a PI of 0.32 (±0.02), not significantly more than the preference for gentiobiose

compared to sucrose in earlier experiments (**Figure 1a**). Further comparisons were conducted with glucose and maltitol, which revealed PIs for gentiobiitol of close to 0.50, suggesting a perceived sweetness similar to these known sweeteners. Glucose and maltitol are perceived as having similar relative sweetness by humans (*19*), and this was also seen with the fruit fly data. The comparison of gentiobiitol with gentiobiose was also conducted as earlier tests indicated that comparing similar structures directly could resolve preferences between structures more clearly than indirect comparisons of both structures to sucrose or glucose. In this instance, although not statistically significant (at $p < 0.05$), the comparison of gentiobiitol to gentiobiose (**Figure 2a**) suggested a slightly increased preference for the sugar alcohol over the related β-glucobiose, although further testing would be needed to confirm this. A similar analysis of the sweetener malitol was conducted (**Figure 2b**), comparing the α-linked sugar alcohol to sucrose, glucose, maltotriitol, or gentiobiose in feeding assays. Relative to sucrose, maltitol was found to have a PI of 0.44 (±0.02) and relative to glucose, a PI of 0.62 (±0.03). Whereas these values are somewhat higher relative to the same comparisons with gentiobiitol, which may seem to contradict the similar preference for maltitol and gentiobiitol indicated by **Figure 2a**, differentiating changes in PI of approximately 0.1 may be beyond the resolution of the bioassay. It is possible that varying the concentration of sugars could overcome this limitation and allow a more precise determination of relative sweetness.

Further experiments were performed to investigate the effect of chain length on preference through analysis of trisaccharide structures (**Figure 3**). Trisaccharides displayed elevated preferences by the fruit flies when compared to sucrose, in contrast to the accepted carbohydrate preferences of humans. Stronger preference toward erlose and melezitose (**Figure 3a**) over sucrose would not be expected in humans, as it is thought that increased chain length decreases palatability, although these sugars have not been well characterized by detailed studies in humans. For the fruit fly, however, this is not unexpected because of the greater likelihood of being encountered in their natural diet. Melezitose and erlose are present in honeydew, for example, and have been described as a food source for other fruit fly species (*30*−*32*). Additionally, the strong preference for these trisaccharides has been reported in other insects (*5*, *33*).

Comparisons with panose and maltotriose enabled further exploration of the fruit fly preference for starch-related linkages that was suggested by initial analysis of maltose (**Figure 3**). Panose, which contains both an α-1,4 and an α-1,6 linkage, showed higher preference over glucose, although a markedly lower PI when compared to maltose (**Figure 3a**). This was consistent with the suggestion of a reduced preference for isomaltose (α-1,6-glucobiose) mentioned earlier, rather than increased preference for gentiobiose (β-1,6-glucobiose). In the experiments shown in **Figure 3b**, maltotriose was significantly preferred over maltose, with increased chain length of an additional α-1,4-glucose invoking an elevated response in *D. melanogaster*. This provides further examples of the strength of the bioassay in comparing related structures to define preferences structurally, as separate comparisons of maltotriose or maltose each paired to sucrose, for example (**Figure 3b** and **1a**, respectively) revealed only a minimal difference. A separate assessment of maltotriose preference revealed a high PI (0.90 ± 0.02) over panose (**Figure 3b**), further evidence for the preference of α-1,4-glucose over α-1,6-glucose by *D. melanogaster*. This strong preference for α-1,4-glucose also dominated over the elevated response toward sugar alcohols, with maltotriose showing high PI over both maltitol and maltotriitol (**Figure 3b**). In terms of human preference for starch-related structures, we are not aware of any published studies to define the relative preference of α-1,4-glucose over α-1,6-glucose,

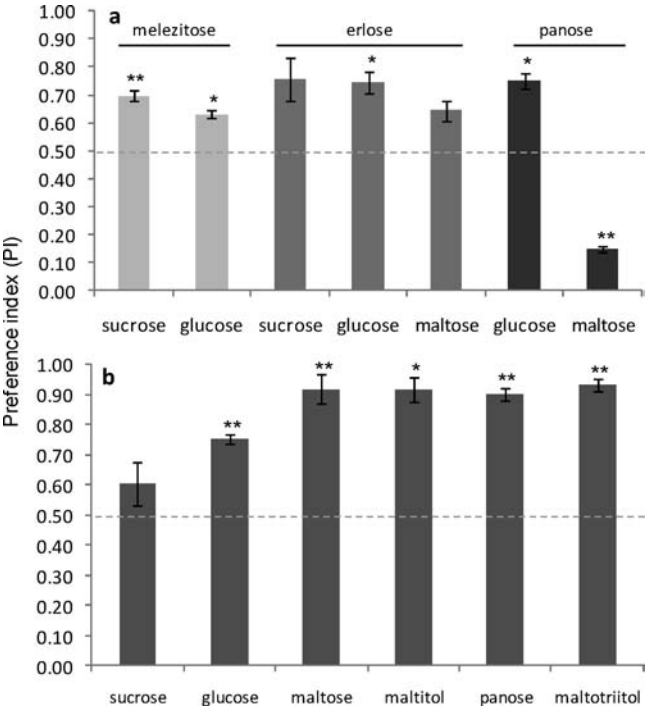

Figure 3. Preference for trisaccharides with an emphasis on starch-related structures. (**a**) The PI was determined for the trisaccharide indicated at the top of the bars, compared to glucose as well as sucrose and/or maltose. Melezitose, erlose, and panose showed higher preference over glucose and constituent disaccharides except for a distinct reduction in PI for panose when paired with maltose. (**b**) Determination of PI for maltotriose when paired with a variety of structures, including sugar alcohols, showed that whereas maltotriose was not significantly preferred over sucrose, the PI against glucose, maltose maltitol, panose, and maltitriitol was significantly higher. Results are expressed as preference index (PI), where a PI > 0.5 indicates elevated preference toward the indicated test sugar. Bars indicate ± standard error ($n = 3$−4). A paired *t* test was performed, with ∗ corresponding to $p < 0.05$ and ∗∗ to $p < 0.01$.

although it has been reported that soluble starch can enhance sucrose "sweetness" in humans (*34*). Whereas human taste may be influenced by starch-related structures, these structures directly invoke strong appetitive behavior in *Drosophila* with a preference for α-1,4-glucose over α-1,6-glucose.

The ability to discriminate between chemical compounds in food by taste receptors is common to both fruit flies and humans. In flies, the sensory receptors are distributed on the labial palps, the legs, and the wings. A family of 68 *gustatory receptor* (*Gr*) genes encodes receptors that operate in combinations to detect sugars and a range of other compounds (*35*). In contrast, the human taste receptors are located in the tongue and palate and are encoded by genes that have no sequence similarity to the fruit fly receptors (*2*). The results described here have reinforced the remarkable similarity in preference for sweet compounds that is mediated by these very different receptor systems. For example, the flies appear to show an enhanced preference for α-linked glucobioses over the corresponding β-glucobioses, similar to human preferences. Sucrose was also preferred over glucose, and the sucrose isomers, leucrose and palatinose, were less attractive than sucrose. Important questions remain about the ability of individual receptor complexes to bind multiple ligands and to sense relative sweetness (*2*). Comparative studies of fly and human responses to closely related compounds may help to illuminate common mechanisms for sensory perception.

In summary, we have demonstrated the strength of a behavioral assay using the model organism *D. melanogaster* that, although seemingly simple in its execution, is reproducible and sensitive and can reveal specific structure-preference relationships of carbohydrates not easily obtained with humans. We extended work on the similarities and differences between the fruit fly and human responses and note the strength in the comparison between these species for disaccharides, with deviation reported in the response toward trisaccharides (particularly those containing α-1,4-glucose). Opportunities for improved understanding of the response to specific carbohydrate structures in humans have been highlighted, including the sucrose isomer turanose, and starch-related glucosidic linkages. The latter may be useful because of the health benefits of altered starch structures being reported. Finally, results suggest a sugar alcohol produced from a β-glucobiose may prove to be sweet-tasting, and we consider it of interest to examine the human response to gentiobiitol in detail, particularly alongside gentiobiose.

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