A Structure-Activity Study on the Sucrose Taste Antagonist Methyl 4,6-dichloro-4,6-dideoxy- α -D-Galactopyranoside

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In order to assess the effect of the antagonist methyl 4,6-dichloro-4,6-dideoxy- α -D-galactopyranoside (MAD-diCl-Gal) upon the gerbil's chorda tympani sucrose taste response, we tested several concentrations of this compound, as well as single concentrations of closely related derivatives, and found that MAD-diCl-Gal was the most potent inhibitor tested. It appears that the inhibition mechanism is very specific. For example, we have found that 2 chlorine atoms at the C-4 and C-6 positions on the glycopyranoside ring are required for inhibition. In addition, with regard to the orientation of the chlorine atoms, the galacto derivative seems to be more potent than the gluco derivative. We have also found that the methyl glycoside is more potent than the free sugar. With regard to the orientation of the methyl group, MAD-diCl-Gal is more potent than its β -anomer. (Because of this discovery of the methyl group enhancement and orientation effect, we shall discontinue using the acronym diCl-Gal and replace it with the more specific MAD-diCl-Gal.) Of particular significance is the fact that there appears to be a structure-activity relationship between the most active stimulants and inhibitors in that the requirement for an axial orientation at C-1 and the enhancement by the methyl group at that position are the same in both cases. These results suggest that both the stimulator and the antagonist are acting at the same receptor site.

The existence of a mechanism of competitive inhibition is borne out by our concentration experiments in which the antagonist was surmounted by high concentrations of sucrose and each successive concentration of inhibitor depressed the sucrose response to a given degree and in a parallel fashion.

Probably the most definitive piece of evidence for multiple specific sweetener receptor sites was obtained by Zawulich (1973) using alloxan to inhibit the rat's whole nerve electrophysiological taste response to sweeteners. He observed that alloxan inhibited its neural responses to sugars but not to sodium saccharin, glycine, or sodium cyclamate. The alloxan experiment is very important because most other chemical treatments of taste receptors will significantly decrease all sweet taste responses (e.g., Borg et al., 1967; Diamant et al., 1965; Hagstrom, 1957; Hiji and Ito, 1977; Yamamoto and Kawamura, 1971), which leads to the conclusion that there is only a single sweetener receptor site. The rat alloxan inhibition may be a species-related effect because this compound does not inhibit the electrophysiological responses of the gerbil to sucrose.

The failure of alloxan as well as gymnemic acid (Jakinovich

and Oakley, 1975) to inhibit the gerbil's sucrose taste response has encouraged us to search among the nonstimulating sweetener derivatives for possible antagonists (Jakinovich, 1981, 1982). Recently (Jakinovich, 1983) we discovered such an antagonist of the gerbil's sucrose taste response, methyl 4,6-dichloro-4,6-dideoxy- α -D-galactopyranoside (MAD-diCl-Gal), a compound that resembles part of the sweetener molecule chloro-sucrose (4,6-dichloro-4,6-dideoxy- α -D-galactopyranosyl-1',6'-dichloro-1',6'-dideoxy- β -D-fructofuranoside). MAD-diCl-Gal is a specific antagonist of the gerbil's sucrose taste response in that it does not suppress the taste responses to sodium chloride or hydrochloric acid. Moreover, the sucrose antagonism of this compound is overcome by high concentrations of sucrose, suggesting that it is a competitive inhibitor.

As a model compound to be used in further studies of sweet taste, MAD-diCl-Gal has some shortcomings. We have found that it is such a weak inhibitor that the very high concentrations (0.1 M) that must be used in the electrophysiology experiments may prove to be toxic to animals in behavioral experiments. In addition, the strong, bitter taste could cause interpretation problems and add limits to the behavioral studies.

Therefore, we have been synthesizing a number of other chlorinated monosaccharides but have discovered that MAD-diCl-Gal was still the most potent of these compounds.

Materials and Methods

Materials

Animals. Mongolian gerbils, *Meriones unguiculatus*, were obtained from Tumblebrook Farm, West Brookfield, MA, and were less than 1 year old, weighing 50-70 gm.

Compounds. The compounds listed below were synthesized in our laboratory by the methods of the authors cited. Methyl 4,6-O-benzylidene- α -D-galactopyranoside was a gift from Dr. I. J. Goldstein, University of Michigan; the rest of the compounds were purchased from Sigma Chemical Company, St. Louis, MO.

4,6-Dichloro-4,6-dideoxy- α -D-galactose, mp = 183°C, $[\alpha]_D^{25}$ = +129.7° (C = 1.2 in methanol) (Paulsen et al., 1976).

Methyl 4,6-O-benzylidene- α -D-glucopyranoside, mp = 165-166°C, $[\alpha]_D^{25}$ = +109° (C = 1.1, chloroform) (Van Cleve, 1971).

Methyl 4-chloro-4-deoxy- α -D-galactopyranoside, mp = 119-120°C, $[\alpha]_D^{25}$ = +202° (C = 2.0, H₂O), prepared by deacetylating methyl 4-chloro-4-deoxy-2,3,6-tri-O-acetyl- α -D-galactopyranoside (Arita et al., 1972).

Methyl 6-chloro-6-deoxy- α -D-glucopyranoside, mp = 110-112°C, $[\alpha]_D^{25}$ = +136.7° (C = 1.0, H₂O) (Evans and Parrish, 1972).

Methyl 3,6-dichloro-3,6-dideoxy- β -D-allopyranoside, mp = 165°C, $[\alpha]_D^{25}$ = -33.3° (C = 0.6, chloroform) (Dean et al., 1974).

Methyl 4,6-dichloro-4,6-dideoxy- α -D-galactopyranoside, mp = 154°C, $[\alpha]_D^{25}$ = +178° (C = 2.0, H₂O) (Jennings and Jones, 1963).

Methyl 4,6-dichloro-4,6-dideoxy- β -D-galactopyranoside, mp = 153°C, $[\alpha]_D^{25}$ = +12.5° (C = 0.8, H₂O) (Dean et al., 1974).

Methyl 4,6-dichloro-4,6-dideoxy- α -D-glucopyranoside, mp = 125°C, $[\alpha]_D^{25}$ = +122.4° (C = 1.25, H₂O) (Jennings and Jones, 1965).

Melting points were determined on a Fisher-Johns melting-point apparatus and are uncorrected. Optical rotations were done on a O. C.

Received Nov. 1, 1985; revised Feb. 24, 1986; accepted Mar. 18, 1986.

We thank D. Sugarman for manuscript suggestions, A. Ward for typing, and P. Blochiavak for doing the rotations. This work was supported by Grant ROINS 16022 from the NIH and PSC-CUNY.

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Rudolph and Sons polarimeter, Model 564, with a 10 cm cell and a sodium lamp. All evaporations were conducted *in vacuo* at 35–40°C on a rotary evaporator. Purity of the glycosides was evaluated by thin-layer chromatography carried out on activated Silica Gel-G glass plates using various solvents. The sugars were visualized by spraying with 15% H_2SO_4 (vol/vol, in ethanol) and heating for 5–10 min at 150°C on a large, hot plate.

Recovery of MAD-diCl-Gal from used taste solutions. Because synthesis of large quantities of MAD-diCl-Gal is a long and tedious process, the compound was recovered when possible by chloroform extraction from used taste solutions. In these cases, pooled aqueous taste solutions were placed in a separatory funnel and MAD-diCl-Gal was extracted with hot chloroform (4–5 $\times$). The chloroform solutions were concentrated by rotary evaporation and the dried crystalline product collected. It was dissolved in a minimum amount of hot ethanol (absolute) and, when it cooled, petroleum ether (bp = 30–60°C) at twice the alcohol volume was added. The resulting crystal's melting points and optical rotations were found to be in agreement with the literature (Jennings and Jones, 1963).

Taste solutions. All compounds were dissolved in deionized water and, when not used immediately, stored at 2°C for 7 d or frozen at –10°C for later use, at which time they were brought to room temperature.

Methods

Electrophysiology. The method for exposing and recording from the intact chorda tympani nerve has been reported (Jakinovich and Oakley, 1975). The recording was done by touching the nerve with a nichrome electrode (100 μm diameter) that was connected to a differential amplifier (Grass P-511). The neural activity was displayed on an oscilloscope and could be monitored by means of a loudspeaker. The integrated discharge of the nerve was used to characterize the gustatory system since it represents a summation of activity from many receptor cells (Kimura and Beidler, 1961). A response in this study was defined as the difference between the spontaneous activity and the greatest integrated potential elicited by a solution applied to the tongue. The integrator's (Grass 73PA) time constant was set at 0.5 sec, with full-wave rectification. The indifferent electrode was placed on a nearby piece of moist tissue within the auditory bulla.

Stimulation. Chemical stimulation of the tongue was effected by a gravity-flow funnel-tubing system through which deionized water flowed continuously (0.13–0.17 ml/sec). Test solutions (2–4 ml) were alternated with water without interruption of the flow. The temperatures of the water and the taste solutions were identical: $25 \pm 1^\circ\text{C}$. Each compound (and its mixture with sucrose, salt, or acid) was tested twice with a standard solution (0.03 M sucrose) in between each pair. Whenever the standard solution elicited responses differing by more than 15%, all interjacent responses were rejected.

Results

The gerbil's taste nerve's response to these sugars (Fig. 1) was variable. For example, at the concentration tested, 0.1 M, the nerve responded only to compounds I, II, III, IV and VI. Except for the response to compound I, the responses to the other compounds—II–IV and VI—have already been reported (Jakinovich, 1985; Jakinovich and Goldstein, 1976). Those latter compounds were used for comparative purposes. None of the other sugars stimulated the nerve to any significant degree. Only once, using a sample of MAD-diCl-Gal (XII) that contained some impurities, did we see significant responses. Often, we saw very little response or, as we have reported before, no response at all (Jakinovich, 1983). Sometimes we saw a small depression of the summated nerve response when these compounds were applied to the tongue, as shown in Figure 2A.

Structure–activity study

In order to assess the inhibitory nature of MAD-diCl-Gal, these sugars were tested in mixtures with 0.03 M sucrose. We observed the following:

1. Of all compounds tested at 0.1 M, MAD-diCl-Gal (XII) was the most effective inhibitor, suppressing the 0.03 M sucrose response around 80% (Table 1).

Figure 1. Compound structures from Table 1 used in the mixture experiment. (Roman numerals indicate compound structure.) A, α -anomers: I. $\text{R}_1 = \text{H}$; $\text{R}_2 = \text{Cl}$; $\text{R}_3 = \text{OH}$; II. $\text{R}_1 = \text{OH}$; $\text{R}_2 = \text{H}$; $\text{R}_3 = \text{Cl}$; III. $\text{R}_1 = \text{OH}$; $\text{R}_2 = \text{H}$; $\text{R}_3 = \text{OH}$; IV. $\text{R}_1 = \text{H}$; $\text{R}_2 = \text{OH}$; $\text{R}_3 = \text{OH}$; XI. $\text{R}_1 = \text{Cl}$; $\text{R}_2 = \text{H}$; $\text{R}_3 = \text{Cl}$; XII. $\text{R}_1 = \text{H}$; $\text{R}_2 = \text{Cl}$; $\text{R}_3 = \text{Cl}$. B, β -anomers: VI. $\text{R}_1 = \text{H}$; $\text{R}_2 = \text{OH}$; $\text{R}_3 = \text{H}$; $\text{R}_4 = \text{OH}$; $\text{R}_5 = \text{OH}$; VII. $\text{R}_1 = \text{Cl}$; $\text{R}_2 = \text{OH}$; $\text{R}_3 = \text{OH}$; $\text{R}_4 = \text{H}$; $\text{R}_5 = \text{Cl}$; X. $\text{R}_1 = \text{H}$; $\text{R}_2 = \text{OH}$; $\text{R}_3 = \text{H}$; $\text{R}_4 = \text{Cl}$; $\text{R}_5 = \text{Cl}$. C, Compound V; D, Compound VIII; E, Compound IX.

2. Our results do not support the notion that the reasons for the inhibitory nature of MAD-diCl-Gal are that it is poorly soluble and a poor stimulant. For example, MAD-Gal, which did not inhibit the sucrose response at 0.1 M (Fig. 2B), was a

Figure 2. The effect of MAD-diCl-Gal (A) and methyl α -D-galactopyranoside (MAD-Gal) (B) on the gerbil's sucrose response (S). Bars indicate when the solution is applied to the flow system. R is the measure of the response used in these experiments. S+ is the mixture of sucrose (0.03 M) and 0.1 M A or B.

A. MAD-DiCl-Gal

B. MAD-Gal

poor stimulant at that concentration but is known to stimulate well at higher concentrations (Jakinovich and Goldstein, 1976).

3. Neither one of the mono-chloro sugars tested (I and II) inhibited the sucrose response.

4. Merely possessing 2 chlorine atoms does not result in a compound that inhibits the sucrose response; for example, compound VII did not inhibit the sucrose response.

5. The orientation of the substituent group at C-1 appears to be important; the α anomer (XII) was more effective than the β anomer (X).

6. The orientation of the chlorine atom group at C-4 is important. The gluco derivative (XI) is less effective than the galacto derivative (XII).

7. It appears that the methyl group at C-1 enhances inhibition. For example, if you remove this group from MAD-diCl-Gal, the resulting compound (IX) is a less effective inhibitor.

8. Addition of the large hydrophobic benzylidene group, encompassing C-4 and C-6 (compounds V and VIII), does not result in inhibition.

Effect of MAD-diCl-Gal concentration on the sucrose response

As the concentration of MAD-diCl-Gal is varied, a uniform effect is observed on the entire sucrose response curve (Fig. 3). When increasing concentrations of MAD-diCl-Gal are mixed with 0.03 M sucrose, the first noticeable suppression of the sucrose response occurs at 0.01 M (Fig. 4). In addition, the concentration of MAD-diCl-Gal that inhibited the 0.03 M sucrose response by 50% (the IC_{50}) was 0.048 M. The maximum solubility of MAD-diCl-Gal (0.1 M) prevented testing higher concentrations.

Previously (Jakinovich, 1983), we reported that MAD-diCl-Gal had an inhibition constant (K_i) of 0.051 M and was acting

Table 1. Gerbil's chorda tympani nerve responses to various methyl glycopyranosides (0.1 M) and their effect in mixtures with 0.03 M sucrose, ranked according to the mixture response

Compound	Response	Mixture response ± 2 SE	Change (%)	N ^b
Sucrose control	1.00	1.00	—	—
I. Methyl 4-chloro-4-deoxy- α -D-galactopyranoside	0.48 ± 0.16	1.38 ± 0.24	38	4
II. Methyl 6-chloro-6-deoxy- α -D-glucopyranoside	0.46 ± 0.22	1.38 ± 0.30	38	4
III. Methyl α -D-glucopyranoside	0.75 ± 0.13	1.34 ± 0.17	34	6
IV. Methyl α -D-galactopyranoside	0.32 ± 0.12	1.26 ± 0.13	26	5
V. Methyl 4,6-O-benzylidene- α -D-galactopyranoside ^a	0.02 ± 0.04	1.25 ± 0.34	25	5
VI. Methyl β -D-galactopyranoside	0.23 ± 0.06	1.08 ± 0.09	8	5
VII. Methyl 3,6-dichloro-3,6-dideoxy- β -D-allopyranoside	0.04 ± 0.06	0.90 ± 0.07	-10	8
VIII. Methyl 4,6-O-benzylidene- α -D-glucopyranoside ^a	0.015 ± 0.07	0.87 ± 0.12	-13	6
IX. 4,6-dichloro-4,6-dideoxy-D-galactose	0.13 ± 0.10	0.68 ± 0.29	-32	6
X. Methyl 4,6-dichloro-4,6-dideoxy- β -D-galactopyranoside	0.05 ± 0.15	0.56 ± 0.12	-44	6
XI. Methyl 4,6-dichloro-4,6-dideoxy- α -D-glucopyranoside	0.13 ± 0.09	0.54 ± 0.11	-46	6
XII. Methyl 4,6-dichloro-4,6-dideoxy- α -D-galactopyranoside	0.05 ± 0.04	0.20 ± 0.06	-80	16

^a 0.03 M.

^b Number of experiments.

Figure 3. Effect of various concentrations of MAD-diCl-Gal on the sucrose response: ●, sucrose alone; ▲, sucrose plus 0.01 M MAD-diCl-Gal; ■, sucrose plus 0.03 M MAD-diCl-Gal; ◆, sucrose plus 0.1 M MAD-diCl-Gal. $n = 5$ animals; bars, ± 2 SE.

as a competitive inhibitor. Since the graphic representation, together with calculations for deriving the K_i , were not published at that time, we have included these data now (Fig. 4B). The K_i was derived from a modified Dixon and Webb (1964) formula for competitive inhibition, as follows:

$$K_i = \frac{i}{(K_d/K_p) - 1}$$

where i is the concentration of inhibitor; K_d is the dissociation constant of the sucrose response curve in the absence of the inhibitor; and K_p is the dissociation constant of the sucrose response curve in the presence of the inhibitor. Following Figure 4B, the $K_d = 0.027$ M; the $K_p = 0.08$ M; and $i = 0.1$ M. Therefore, $K_i = 0.051$ M.

Optical rotations of mixtures of sucrose with MAD-diCl-Gal

To assess the possibility that the above inhibitors are reacting with sucrose, thereby reducing its effective concentration, we compared the optical rotations of the various mixtures and found that the rotations were simply additive, indicating that there were no interactions (Table 2).

Other compounds screened as possible inhibitors

The results with MAD-diCl-Gal have stimulated our interest in examining the possible inhibitory effects of other compounds on the gerbil's sucrose taste response (Table 3). The criteria for compounds selected include (1) those reported to suppress the sweet or sugar responses in mammals, (2) those that inhibit metabolic processes involving sugars, and (3) sugar derivatives that do not stimulate the gerbil's taste nerve. This latter group of compounds includes both bitter and tasteless compounds. Unlike the case with MAD-diCl-Gal or any of its derivatives, the gerbils' taste response to sucrose was unaffected by these compounds.

Discussion

In a previous paper (Vlahopoulos and Jakinovich, 1984), we published some preliminary results indicating that MAD-diCl-Gal was a potentially useful inhibitor of the sucrose taste response in the gerbil. At that time, though, we were not sure

Figure 4. A, Effect of various concentrations of MAD-diCl-Gal on the 0.03 M sucrose response. The IC_{50} (the concentration of MAD-diCl-Gal which inhibited the sucrose response by 50%), was 0.048 M. $n = 9$ animals; bars, ± 2 SE. B, Beidler plot (1954) of summated nerve responses to sucrose (●) and mixtures of sucrose and 0.1 M MAD-diCl-Gal (■). $n = 5$. C, Concentration; R, response.

whether MAD-diCl-Gal was a competitive, allosteric, or a non-competitive inhibitor. Kinetic plots, although useful tools, can be misleading (Lehninger, 1975). The most definitive evidence for competitive inhibition might have been achieved from binding experiments using radioactive compounds. However, since Cagan (1971) found that sugars bound weakly to gustatory tissues, we feel that binding experiments with MAD-diCl-Gal may not be feasible.

In an attempt to better understand this inhibitory mechanism, we have examined and ruled out the following as possible reasons for suppression of the sucrose response:

Table 2. Analysis of optical rotations of sucrose, MAD-diCl-Gal, and mixtures^a

Solution (M)	Rotation $[\alpha]_D^{25}$
Sucrose (0.03)	+0.72
MAD-diCl-Gal (0.1)	+4.02
Mixture of sucrose and MAD-diCl-Gal	+4.82

^a $n = 5$.

Table 3. Compounds screened as potential inhibitors of the 0.03 M sucrose response

Compound	Concentration ^a	Application method	Reason	Source
1. Acarbose	40 mg/100 ml	Mixture	Sucrase inhibitor and sugar derivative (Schmidt et al., 1982)	Dr. A. Scalafani, Brooklyn College, NY
2. Alloxan	0.3 M	Pretreatment, 3 min	Suppresses rat's taste response (Zawalich, 1973)	Sigma Chemical Co.
3. Gluconic acid	0.1 M	Mixture	Tasteless, nonstimulating sugar derivative	Sigma
4. Glucuronic acid	0.1 M	Mixture	Tasteless, nonstimulating sugar derivative	Sigma
5. Gymnemic acid (purified 80% A ₁ , 20% A _{2,3,4})	5 mg/ml	Pretreatment, 3 min	Inhibits sweet response in humans (Diamant et al., 1965)	Dr. van der Wel, Unilever Research, Netherlands
6. Sucrose octaacetate	0.001 M	Mixture	Bitter, nonstimulating sugar derivative	Sigma

^a The concentrations used were those recommended in the literature or very close to the maximum solubility.

Figure 5. Proposed model of the sucrose site in the membrane of the gerbil's gustatory cell. This model takes into account those inhibitory regions which interact with the chlorine atoms at C-4 and C-6. *Top*, The site is occupied by methyl α -D-glucopyranoside, and this activates the receptor cell. *Bottom*, The site is occupied with MAD-diCl-Gal, which does not activate the cell.

1. *pH Effect*. One possible explanation of the depressed response to the mixture of MAD-diCl-Gal and sucrose is that MAD-diCl-Gal might be acting as an acid, or contain an acidic impurity, since it is known that low pH depresses the sugar taste response in humans (Stone et al., 1969), gerbils (Jakinovich, 1982), hamsters (Hyman and Frank, 1980), and rats (Ogawa, 1969a, b). However, our 0.1 M MAD-diCl-Gal, by itself, has a pH of 5.8, which is more or less the same as the 5.6 of the air-equilibrated deionized water that has been stored in plastic carboys and in which the MAD-diCl-Gal has been dissolved. To check this further, we also conducted experiments using a pH buffer of 7.0 and found that MAD-diCl-Gal still inhibited the sucrose response, all of which tends to rule out any pH effects.

2. *Anesthetic effect*. Our structure-activity study, together with the specificity of taste quality inhibition, i.e., the failure to suppress the NaCl or HCl response, as well as the failure of pretreatment to affect the sucrose response (Jakinovich, 1983), tend to rule out the possibility that MAD-diCl-Gal is behaving as a nonspecific anesthetic, as reported by Von Skramlik (1963).

3. *Compound interaction*. To test the possibility that MAD-diCl-Gal was reacting with sucrose, thus reducing its effective concentration, we examined the mixture by polarimetry and found no evidence of reaction. However, it is still possible that the polarimeter may not be sufficiently sensitive to pick up small reactions.

4. *Bitter effect*. One possibility for MAD-diCl-Gal's suppression of the sucrose response is its intense bitterness, since the behavioral suppression of sweet taste by a bitter substance is well known (Bartoshuck, 1975). This explanation does not seem to be supported because the addition of sucrose octacetate, an intensely bitter sugar, did not suppress the sucrose response. Furthermore, the gerbil's sucrose response is not suppressed by potassium phosphate solutions, which are also bitter (Jakinovich, 1982).

On the other hand, we also believe that the following pieces of evidence support the idea of a specific receptor mechanism and explain the inhibition of the gerbil's sucrose response by MAD-diCl-Gal:

1. *Surmountability*. A crucial piece of evidence for competitive interaction (Ariens, 1964) is the fact that inhibition was surmounted by high concentrations of sucrose (see Fig. 3).

2. *Application methods*. In addition, as was found previously (Jakinovich, 1983), MAD-diCl-Gal must be mixed in solution with sucrose to be inhibitory. Moreover, a 2 min pretreatment

of the gerbil's tongue with 0.1 M MAD-diCl-Gal did not affect the response, also lending credibility to the competitive mechanism.

3. *Parallel curves.* Two additional important pieces of evidence for competitive inhibition are found in Figures 3 and 4B. Figure 3 demonstrates that each successive concentration of the inhibitor will depress the sucrose response to a given degree and in a parallel fashion. Figure 4B, using a different type of analysis (the Beidler plot; Beidler, 1954), with the highest concentrations of MAD-diCl-Gal (0.1 M), also demonstrates a parallel shift. Both treatments of the data lend support to the idea of the mechanism of competitive inhibition.

4. *Inhibition constants.* The fact that measurements of the 2 inhibition constants ($K_i = 0.051$ M and $IC_{50} = 0.048$ M) resulted in nearly identical values suggests that we are possibly measuring common events. In one instance (Fig. 4B), we varied the concentration of sucrose with a single concentration of the inhibitor, while in the other case (Fig. 4A), we used a single concentration of sucrose, adding varied concentrations of the inhibitor. Further supporting competitive inhibition, Figure 4B entails the theoretical representation of the data according to Beidler (1954), whereas the data in Figure 4A are not bound by any theoretical assumptions. It should also be pointed out that the parallel shift in Figure 4B is small, indicating that MAD-diCl-Gal is a weak inhibitor.

5. *Structure-activity of glycosides.* Of all the glycopyranosides tested, the most potent inhibitor proved to be methyl 4,6-dichloro-4,6-dideoxy- α -D-galactopyranoside, which was much more potent than its β - counterpart (see Table 1, compound X). In the same vein, methyl α -D-glucopyranoside and methyl α -D-galactopyranoside were more potent stimuli than their β - counterparts (compounds III and IV, Table 1) (Jakimovich and Goldstein, 1976). This would also support the idea that inhibitors and stimuli are binding at the same receptor but does not rule out an allosteric receptor site.

6. *Enhancement by methyl group.* We have also discovered that the methyl group at the C-1 position of the 4,6-dichloro-4,6-dideoxy- α -galactose molecule enhances the inhibition thereof. In a similar manner, excitation is enhanced if there is a methyl group at the C-1 position of α -D-glucopyranose (Jakimovich and Goldstein, 1976). This suggests 3 possibilities: that there is a specific bonding site for that methyl group in the receptor; that the pyranoside ring is stabilized by the methyl group; or that the methyl group enhances the lipid solubility of the sugar molecule by favoring its entrance into the lipid layer of the membrane. Again, the fact that presence of a methyl group at C-1 enhances both the inhibition and the stimulation seems to indicate that both compounds are interacting at the same receptor site. However, as was mentioned before, this similarity in action does not rule out an allosteric mechanism.

In view of the above, and assuming that the MAD-diCl-Gal molecule is fitting into the sucrose receptor site, we feel that the mechanism of inhibition involves binding of the chlorine atoms to inhibitory regions in the vicinity of the C-4 and C-6 carbons of the glucopyranosyl portion of the sucrose site (Fig. 5). A clue to this mechanism was found in some structure-activity studies conducted in our lab with methyl glycopyranosides (Jakimovich, 1985). We found that the CH_2OH group at C-5 (which also contains the C-6 hydroxyl group) is not required for sugar stimulation. For example, methyl α -D-xylopyranoside and methyl 6-deoxy- α -D-glucopyranoside are as effective as a methyl α -D-glucopyranoside. However, we also found that substitution of a chlorine atom for the C-6 hydroxyl group dramatically reduced the effectiveness of the stimulus, as in the case of methyl 6-chloro-6-deoxy- α -D-glucopyranoside. At that time we thought that possibly the large chlorine atom was causing steric hindrance. However, we now feel that there must be specific inhibitory regions within the site, and that the mono-chloro sugar may be binding

to both an activation region (involving hydrogen bonding of the OH groups at C-1, C-2, and C-4), as well as to inactivation regions of the site (at C-4 and C-6), the reduced response being due to a balance between the 2 interactions. The inactivation process may involve some undetermined weak forces or possibly hydrogen bonding with the chlorine atoms acting as proton acceptors.

With regard to the type of bonding, Ogawa (1969a), using *p*-chloromercuric benzoate (PCMB), *N*-ethylmaleimide (NEM), and iodoacetic acid as inhibitors of the rat's sucrose taste response, demonstrated that a 20 min pretreatment of the tongue was necessary to inactivate the response. Therefore, it is logical to assume that MAD-diCl-Gal, which is a fast-acting and reversible inhibitor, is *not* inhibiting the sucrose response in a manner similar to that proposed by Ogawa.

Our present experiments seem to reaffirm our model, in that methyl 4-chloro-4-deoxy- α -D-galactopyranoside was as poor a stimulant as methyl 6-chloro-6-deoxy- α -D-glucopyranoside. Moreover, MAD-diCl-Gal was a better inhibitor than methyl 4,6-dichloro-4,6-dideoxy- α -D-glucopyranoside, possibly because the chlorine atoms of the galactose molecule are more suitably oriented to bind to the inhibitory region. It would appear from our work that the most potent inhibitors involve derivatives that are themselves poor stimulants. For example, methyl α -D-galactopyranoside is a poorer stimulant than methyl α -D-glucopyranoside and, likewise, MAD-diCl-Gal is a better inhibitor than methyl 4,6-dichloro-4,6-dideoxy- α -D-glucopyranoside.

In view of the above—particularly since the inhibition by the solution of sucrose and MAD-diCl-Gal was overcome by high concentrations of sucrose—together with the many structural similarities between the agonists and antagonists (stimulants and inhibitors), we feel that we now have stronger evidence for the idea of a unique competitive inhibitor, MAD-diCl-Gal.

Thus, mainly on the basis of the surmountability of the sucrose inhibition, which we feel is crucial to competitive inhibition, we scoured the literature in the field of inhibition of mammalian sweet taste and have concluded from the published findings that most of the inhibitors are not competitive. A few researchers, such as Hagstrom (1957), working with gymnemic acid effects on hamsters, claimed competitive inhibition. Faull et al. (1970; quoted by Meiselman and Halpern, 1970), also suggested competitive inhibition in human psychophysical responses to gymnemic acid, basing their conclusions on kinetic plots of the data of Bartoshuk et al. (1969). Ogawa (1969a) claimed that PCMB and NEM both competitively inhibited the sucrose response in electrophysiological tests on rats, using only kinetic plots as evidence. Similarly, Yamamoto and Kawamura (1971) reported that copper acted as a competitive inhibitor of sucrose in electrophysiological rat experiments, again using kinetic plots only. However, since none of the above studies showed that the inhibition by gymnemic acid, as well as by these other inhibitors, was surmountable by high concentrations of sucrose, we can only conclude that the mechanism of competitive inhibition was not strongly supported. Moreover, many of these investigators relied solely on kinetic plots, which we feel may be misleading.

It would appear that a preponderance of our evidence points to a competitive and/or allosteric mechanism of inhibition. MAD-diCl-Gal has provided us with some useful information about the receptor mechanism. It also poses new questions, such as, How can chloro-sucrose, possessing 4 chlorine atoms (2 on the galactopyranosyl portion and 2 on the fructofuranosyl portion) be such a powerful stimulant? (Hough and Phadnis, 1976). However, MAD-diCl-Gal has its limitations, in that its maximum solubility is 0.1 M, it is a weak inhibitor, it has a bitter taste, and, in high enough concentrations, it may prove toxic. Therefore, we are developing other, more suitable, inhibitors

on which preliminary work has been reported (Vlahopoulos and Jakinovich, 1984, 1986).

References

- Ariens, E. J. (1964) *Molecular Pharmacology*, vol. 1, E. J. Ariens, ed., p. 363, Academic, New York.
- Arita, H., N. Veda, and Y. Matsushima (1972) The reduction of chlorodeoxy sugars by tributyltin hydride. *Bull. Chem. Soc. Jpn.* 45: 567-569.
- Bartoshuk, L. M. (1975) Taste mixtures: Is mixture suppression related to compression? *Physiol. Behav.* 14: 643-649.
- Bartoshuk, L. M., G. P. Dateo, D. J. Vandenbelt, R. L. Buttrick, and L. Long, Jr. (1969) Effects of *Gymnema sylvestra* and *Synsepalum dulcificum* on taste in man. In *Olfaction and Taste, Proceedings of the Third International Symposium*, C. Pfaffmann, ed., pp. 436-444, Rockefeller U. P., New York.
- Beidler, L. M. (1954) A theory of taste stimulation. *J. Gen. Physiol.* 38: 133-139.
- Borg, G., H. Diamant, L. Strom, and Y. Zotterman (1967) The relation between neural and perceptual intensity: A comparative study on the neural and psychophysical response to taste stimuli. *J. Physiol. (Lond.)* 192: 13-20.
- Cagan, R. H. (1971) Biochemical studies of taste sensation. I. Binding of ^{14}C -labeled sugar to bovine taste papillae. *Biochim. Biophys. Acta* 252: 199-206.
- Dean, D. M., W. A. Szarek, and J. K. N. Jones (1974) A reinvestigation of the reaction of methyl β -D-glucopyranoside with sulfur chloride. *Carbohydr. Res.* 33: 383-386.
- Diamant, H., B. Oakley, L. Strom, C. Wells, and Y. Zotterman (1965) A comparison of neural and psychophysical responses to taste stimuli in man. *Acta Physiol. Scand.* 64: 67-74.
- Dixon, M., and E. C. Webb (1964) *Enzymes*, 2nd Ed., Academic, New York.
- Evans, M. E., and F. W. Parrish (1972) [30] 6-chloro-6-deoxy- α -D-glucose from methyl α -D-glucopyranoside by reaction with methane-sulfonyl chloride in *N,N*-dimethylformamide. In *Methods in Carbohydrate Chemistry*, vol. 6, R. L. Whistler and J. N. Bemiller, eds., pp. 193-196, Academic, New York.
- Faull, J. R., P. D. Reilly, and B. P. Halpern (1970) The mechanism of action of gymnemic acid. Paper presented at Eastern Psychological Association Program, 41st Annual Meeting, p. 90.
- Hagstrom, E. C. (1957) Nature of taste stimulation by sugar. Ph.D. thesis, Brown University.
- Hiji, Y., and H. Ito (1977) Removal of "sweetness" by proteases and its recovery mechanism in rat taste cell. *Comp. Biochem. Physiol. [A]* 58: 109-113.
- Hough, L., and S. P. Phadnis (1976) Enhancement in the sweetness of sucrose. *Nature* 263: 800.
- Hyman, A. M., and M. E. Frank (1980) Effects of binary taste stimuli on the neural activity of the hamster chorda tympani. *J. Gen. Physiol.* 76: 125-142.
- Jakinovich, W., Jr. (1981) Stimulation of the gerbil's gustatory receptors by artificial sweeteners. *Brain Res.* 210: 69-81.
- Jakinovich, W., Jr. (1982) Stimulation of the gerbil's gustatory receptors by saccharin. *J. Neurosci.* 2: 49-56.
- Jakinovich, W., Jr. (1983) Methyl 4,6-dichloro-4,6-dideoxy- α -D-galactopyranoside: An inhibitor of sweet taste responses in gerbils. *Science* 219: 408-410.
- Jakinovich, W., Jr. (1985) Stimulation of the gerbil's gustatory receptors by methyl glucopyranosides. *Chem. Senses* 10: 591-604.
- Jakinovich, W., Jr., and I. J. Goldstein (1976) Stimulation of the gerbil's gustatory receptors by monosaccharides. *Brain Res.* 110: 491-504.
- Jakinovich, W., Jr., and B. Oakley (1975) Comparative gustatory response in four species of gerbilline rodents. *J. Comp. Physiol.* 99: 89-101.
- Jennings, H. J., and J. K. N. Jones (1963) The reaction of chlorosulphate esters of sugars with pyridine. *Can. J. Chem.* 41: 1151-1159.
- Jennings, H. J., and J. K. N. Jones (1965) Reactions of sugar chlorosulfates. Part V. The synthesis of chlorodeoxy sugars. *Can. J. Chem.* 43: 2372-2386.
- Kimura, K., and L. M. Beidler (1961) Microelectrode study of taste receptors of rat and hamster. *J. Cell Comp. Physiol.* 58: 131-139.
- Lehninger, A. L. (1975) *Biochemistry*, p. 238, Worth, New York.
- Meiselman, H. L., and B. P. Halpern (1970) Effect of *Gymnema sylvestra* on complex tastes elicited by amino acids and sucrose. *Physiol. Behav.* 5: 1379-1384.
- Ogawa, H. (1969a) Effects of metabolic inhibitors on taste nerve responses in the chorda tympani nerve of rats. *Jpn. J. Physiol.* 19: 652-662.
- Ogawa, H. (1969b) Effects of pH on taste responses in the chorda tympani nerve of rats. *Jpn. J. Physiol.* 19: 670-681.
- Paulsen, H., H. Salzburg, and H. Redlich (1976) Selective Blockierung von Sacchariden durch Umacetalisierung mit Cyclohexanon-athylenacetal. Eine Synthese der 4-desoxy-D-xylo-hexopyranose. *Chem. Ber.* 109: 3598-3605.
- Schmidt, D. D., W. Frommer, B. Junge, L. Muller, W. Wingender, and E. Truscheit (1982) α -Glucosidase inhibitors of microbial origin. In *Proceedings of the First International Symposium on Acarbose. Effects on Carbohydrate and Fat Metabolism*, W. Creutzfeldt, ed., pp. 5-15, Excerpta Medica, Amsterdam.
- Stone, H., S. Oliver, and J. Kloeheim (1969) Temperature and pH effects on the relative sweetness of suprathreshold mixtures of dextrose fructose. *Percept. Psychophys.* 5: 257-260.
- Van Cleve, W. (1971) Improved preparation of methyl 4,6-O-benzylidene- α -D-glucopyranoside. *Carbohydr. Res.* 17: 461-464.
- Vlahopoulos, V., and W. Jakinovich, Jr. (1984) Inhibition of the gerbil's electrophysiological sucrose taste response by para-nitrophenyl- α -D-glucopyranoside and chloramphenicol. In *Sixth Annual Meeting of the Association for Chemoreception Sciences*, abstract 66.
- Vlahopoulos, V., and W. Jakinovich, Jr. (1986) Antagonism of the gerbil's sucrose taste response by p-nitrophenyl α -D-glucopyranoside and chloramphenicol. *J. Neurosci.* 6: 2611-2615.
- Von Skramlik, E. (1963) The fundamental substrates of taste. In *Proceedings of the First International Symposium on Olfaction and Taste*, Y. Zotterman, ed., pp. 125-132, Pergamon, Oxford.
- Yamamoto, T., and Y. Kawamura (1971) Inhibitory effect of cupric and zinc ions on sweet taste response in the rat. *J. Osaka Univ. Dental Sch.* 11: 99-104.
- Zawalich, W. S. (1973) Depression of gustatory sweet response by alloxan. *Comp. Biochem. Physiol. [A]* 44: 903-909.