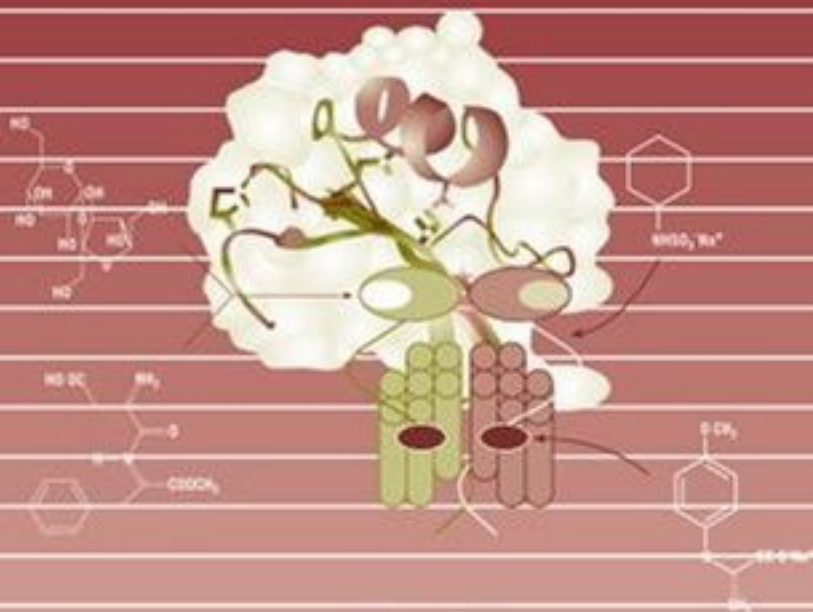# Sweetness and Sweeteners

Biology, Chemistry, and Psychophysics

EDITED BY

Deepthi K. Weerasinghe and Grant E. DuBois

ACS Symposium Series 979

Sweetness and Sweeteners: Biology, Chemistry and Psychophysics

Deepthi K. Weerasinghe, Editor
The Coca-Cola Company

Grant E. DuBois, Editor
The Coca-Cola Company

Based on a symposium at the 231st ACS National Meeting, Atlanta, Georgia, March 26–30, 2006

Sponsored by

ACS Division of Agricultural and Food Chemistry

ACS Symposium Series 979

Sweetness and Sweeteners: Biology, Chemistry and Psychophysics

Deepthi K. Weerasinghe, Editor
The Coca-Cola Company

Grant E. DuBois, Editor
The Coca-Cola Company

Based on a symposium at the 231st ACS National
Meeting, Atlanta, Georgia, March 26–30, 2006

Sponsored by

ACS Division of Agricultural and Food Chemistry

Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

ACS Books Department

Preface

Most living beings aspire for satiety and in acceptance of food products sweet taste plays a major role. Sugar, which is one of the most commonly used materials to impart sweet taste, has the disadvantage of being associated with a number of adverse health related issues. As a result, many research hours are spent in trying to produce low and non- caloric sweeteners and to modify the temporal profiles of these to mimic that of sugar.

Since the discovery of taste receptors and the recent breakthrough in the culturing of primary taste bud cells, substantial progress has been made in the field of Sweeteners and their perception. Utilizing this knowledge, some research groups have directed their efforts toward enhancing sweetness, by way of additives that result in a net reduction of calories. The symposium was organized to highlight the current state of the science and to report recent significant findings in Biology, Chemistry, and Psychophysics of sweeteners and sweetness.

The symposium was opened with an excellent overview by my coorganizer, who has spent his entire scientific career in understanding sweetness.

The book will follow the seven sessions of the symposium as separate chapters:

(1) Structural Studies of the Sweetener Receptor, (2) Modeling of the Sweetener Receptor, (3) Sweet Taste Transduction, (4) Quantifying the Responses of Sweet-Sensitive Taste Bud Cells, (5) Modulation of Sweet-Sensitive Taste Bud Cell Signaling, (6) Advances in the Discovery and Commercial Development of Synthetic Non-Caloric Sweeteners, and (7) Advances in the Discovery and Commercial Development of Natural Non-Caloric Sweeteners

Deepthi K Weerasinghe
Flavor Creation Department
The Coca-Cola Company
P.O.Box 1734
Atlanta, GA 30301

Grant E DuBois
Strategic Research Department
The Coca-Cola Company
P.O.Box 1734
Atlanta, GA 30301

Table of Contents
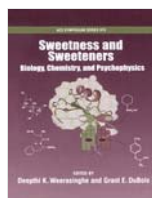
Sweetness and Sweeteners
Biology, Chemistry, and Psychophysics

Editor(s): Deepthi K. Weerasinghe [1] , Grant E. DuBois [2]
Volume 979
Publication Date (Print): March 04, 2008
Copyright © 2008 American Chemical Society

ISBN13: 9780841274327
eISBN: 9780841221116
DOI: 10.1021/bk-2008-0979

[1] The Coca-Cola Company
[2] The Coca-Cola Company

Sponsoring Divisions: Division of Agricultural & Food Chemistry

☐ Select All   For Selected Articles:

T1R2, T1R3, and the Detection of Sweet Stimuli

Stephan Vigues, Jeanette R. Hobbs, Yiling Nie, Graeme L. Conn, and Steven D. Munger

Chapter 4, pp 65-75
DOI: 10.1021/bk-2008-0979.ch004

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [955K]
PDF w/ Links [973K]

Sponsored Access

In Vitro Models to Study Taste: Can Sweetness Be Modeled in a Dish?

Nancy E. Rawson and M. Hakan Ozdener

Chapter 5, pp 76-93
DOI: 10.1021/bk-2008-0979.ch005

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1993K]
PDF w/ Links [2001K]

Sponsored Access

MODELING OF THE SWEETENER RECEPTOR

Structure-Activity Relationship and AH-B after 40 Years

Terry E. Acree and Michael Lindley

Chapter 6, pp 96-108
DOI: 10.1021/bk-2008-0979.ch006

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1243K]
PDF w/ Links [1248K]

Sponsored Access

Crystal Structures of the Sweet Protein MNEI: Insights into Sweet Protein-Receptor Interactions

Jeanette R. Hobbs, Steven D. Munger, and Graeme L. Conn

Chapter 7, pp 109-116
DOI: 10.1021/bk-2008-0979.ch007

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [919K]
PDF w/ Links [933K]

Sponsored Access

Molecular Models of Sweet Taste Receptors Provide Insights into Function

Meng Cui, Peihua Jiang, Emeline Maillet, Marianna Max, Robert F. Margolskee, and Roman Osman

Chapter 8, pp 117-132
DOI: 10.1021/bk-2008-0979.ch008

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1835K]
PDF w/ Links [1861K]

Sponsored Access

Pharmacophore Modeling of Sweet and Bitter Tasting Molecules

Nicolas Froloff

Chapter 9, pp 133-146
DOI: 10.1021/bk-2008-0979.ch009

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1316K]
PDF w/ Links [1327K]

Sponsored Access

Multiple Receptors or Multiple Sites? Modeling the Human T1R2-T1R3 Sweet Taste Receptor

Gabriella Morini, Angela Bassoli, and Piero A. Temussi

Chapter 10, pp 147-161
DOI: 10.1021/bk-2008-0979.ch010

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1533K]
PDF w/ Links [1564K]

Sponsored Access

Computational Docking to Sweet Taste Receptor Models

D. Eric Walters

Chapter 11, pp 162-167
DOI: 10.1021/bk-2008-0979.ch011

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [659K]
PDF w/ Links [675K]

Sponsored Access

SWEET TASTE TRANSDUCTION

What Can Psychophysical Studies with Sweetness Inhibitors Teach Us about Taste?

Veronica Galindo-Cuspinera and Paul A. S. Breslin

Chapter 12, pp 170-184
DOI: 10.1021/bk-2008-0979.ch012

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1557K]
PDF w/ Links [1565K]

Sponsored Access

Abstract

Hi-Res PDF [1671K]

A Method to Measure Taste Qualities, Taste Intensity, and Temporal Profile of Compounds Aimed at Human Consumption by Taste Nerve Recordings in Monkeys

Göran Hellekant and Yiwen Wang

Chapter 13, pp 185-201
DOI: 10.1021/bk-2008-0979.ch013

Peer-Reviewed Book Chapter

PDF w/ Links [1683K]

Sponsored Access

The Bittersweet Search for Bitter-Sweet Interactions: Cell to Cell Communication in the Taste Bud

M. Scott Herness, Fang-li Zhao, and Yu Cao

Chapter 14, pp 202-219
DOI: 10.1021/bk-2008-0979.ch014

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1979K]

PDF w/ Links [1983K]

Sponsored Access

---

cAMP: A Role in Sweet Taste Adaptation

Nirupa Chaudhari and Sue C. Kinnamon

Chapter 15, pp 220-229
DOI: 10.1021/bk-2008-0979.ch015

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [847K]

PDF w/ Links [863K]

Sponsored Access

Saccharin: Artificial Sweetener, Bitter Tastant, and Sweet Taste Inhibitor

Marcel Winnig, Christina Kuhn, Oliver Frank, Bernd Bufe, Maik Behrens, Thomas Hofmann, and Wolfgang Meyerhof

Chapter 16, pp 230-240
DOI: 10.1021/bk-2008-0979.ch016

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1150K]

PDF w/ Links [1161K]

Sponsored Access

Permeation of Amphipathic Sweeteners into Taste-Bud Cells and Their Interactions with Post-Receptor Signaling Components: Possible Implications for Sweet-Taste Quality

M. Naim, M. E. Shaul, A. I. Spielman, L. Huang, and I. Peri

Chapter 17, pp 241-255
DOI: 10.1021/bk-2008-0979.ch017

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1708K]

PDF w/ Links [1735K]

Sponsored Access

QUANTIFYING THE RESPONSES OF SWEET-SENSITIVE TASTE BUD CELLS

We Are What We Eat, but Why? Relationships between Oral Sensation, Genetics, Pathology, and Diet

Derek J. Snyder, Valerie B. Duffy, Susan E. Marino, and Linda M. Bartoshuk

Chapter 18, pp 258-284
DOI: 10.1021/bk-2008-0979.ch018

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [2670K]

PDF w/ Links [2684K]

Sponsored Access

Perception and Acceptance of Sweeteners

Jeannine F. Delwiche and Amanda R. Warnock

Chapter 19, pp 285-295
DOI: 10.1021/bk-2008-0979.ch019

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1199K]

PDF w/ Links [1215K]

Sponsored Access

Interindividual Differences of Taste Sensitivity in Humans and Hamsters: Multiple Receptor Sites for Single Organic Molecules

Annick Faurion

Chapter 20, pp 296-334
DOI: 10.1021/bk-2008-0979.ch020

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [3694K]

PDF w/ Links [3708K]

Sponsored Access

The "Bitter-Sweet" Truth of Artificial Sweeteners

C. T. Simons, C. Adam, G. LeCourt, C. Crawford, C. Ward, W. Meyerhof, and J. P. Slack

Chapter 21, pp 335-354
DOI: 10.1021/bk-2008-0979.ch021

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1844K]

PDF w/ Links [1848K]

Sponsored Access

Abstract

The Use of Rodent Models to Link Neurobiological Processes with the Psychophysics of Sweet Taste

A. C. Spector, S. Eylam, and C. D. Dotson

Chapter 22, pp 355-366
DOI: 10.1021/bk-2008-0979.ch022

Peer-Reviewed Book Chapter

Hi-Res PDF [1192K]
PDF w/ Links [1202K]

Sponsored Access

MODULATION OF SWEET-SENSITIVE TASTE BUD CELL SIGNALING

Functional Characterization of the Human Sweet Taste Receptor: High-Throughput Screening Assay Development and Structural Function Relation

Xiaodong Li and Guy Servant

Chapter 23, pp 368-385
DOI: 10.1021/bk-2008-0979.ch023

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1767K]
PDF w/ Links [1768K]

Sponsored Access

Development of Transient Receptor Potential Melanostatin 5 Modulators for Sweetness Enhancement

R. W. Bryant, K. S. Atwal, I. Bakaj, M. T. Buber, S. Carlucci, R. Cerne, R. Cortés, H. R. Devantier, C. J. Hendrix, S. P. Lee, R. K. Palmer, C. Wilson, Q. Yang, and F. R. Salemme

Chapter 24, pp 386-399
DOI: 10.1021/bk-2008-0979.ch024

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1729K]
PDF w/ Links [1743K]

Sponsored Access

Enhancers for Sweet Taste from the World of Non-Volatiles: Polyphenols as Taste Modifiers

Jakob P. Ley, Maria Blings, Susanne Paetz, Günter Kindel, Kathrin Freiherr, Gerhard E. Krammer, and Heinz-Jürgen Bertram

Chapter 25, pp 400-409
DOI: 10.1021/bk-2008-0979.ch025

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1059K]
PDF w/ Links [1068K]

Sponsored Access

Evaluation of High-Intensity Sweetener Modulators

Cheryl R. Mitchell, Richard Ray, and Marian Schwartz

Chapter 26, pp 410-419
DOI: 10.1021/bk-2008-0979.ch026

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1264K]
PDF w/ Links [1265K]

Sponsored Access

Improving the Taste of Artificial Sweeteners Using Flavors

Masashi Ishikawa, Akio Nakamura, Ayano Fujiki, Junichi Ide, and Kensaku Mori

Chapter 27, pp 420-429
DOI: 10.1021/bk-2008-0979.ch027

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1392K]
PDF w/ Links [1395K]

Sponsored Access

ADVANCES IN THE DISCOVERY AND COMMERCIAL DEVELOPMENT OF SYNTHETIC NON-CALORIC SWEETENERS

Why Should an Organic Chemist Study Sweet Taste?

Angela Bassoli, Gigliola Borgonovo, Gilberto Busnelli, and Gabriella Morini

Chapter 28, pp 432-443
DOI: 10.1021/bk-2008-0979.ch028

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1728K]
PDF w/ Links [1733K]

Sponsored Access

Sweeteners and Sweetness Modulators: Requirements for Commercial Viability

Grant E. DuBois

Chapter 29, pp 444-462
DOI: 10.1021/bk-2008-0979.ch029

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [2910K]

Sponsored Access

Development of New, Low Calorie Sweetener: New Aspartame Derivative

Yusuke Amino, Kenich Mori, Yasuyuki Tomiyama, Hiroyuki Sakata, and Takeshi Fujieda

Chapter 30, pp 463-480
DOI: 10.1021/bk-2008-0979.ch030

Peer-Reviewed Book Chapter

Abstract

Hi-Res PDF [1650K]
PDF w/ Links [1655K]

Sponsored Access

Challenges to Reducing Sugar in Foods

D. Kilcast, C. den Ridder, and C. Narain

Abstract

Sponsored Access

Color Figure Inserts

pp 1-20
DOI: 10.1021/bk-2008-0979.ap001

First Page

Hi-Res PDF [2286K]
PDF w/ Links [2287K]

Sponsored Access

Color Figure Inserts

pp 1-20
DOI: 10.1021/bk-2008-0979.ap001

First Page

Hi-Res PDF [2286K]
PDF w/ Links [2287K]

Chapter 1

Sweetness and Sweeteners: What Is All the Excitement About?

Grant E. DuBois

Strategic Research Department, The Coca-Cola Company, One Coca-Cola Plaza, Atlanta, GA 30301

Sweetness is a very important sensation and has been throughout human history. Sugar is the prototypical sweet stimulus and, as evidence of its historical importance, one need only consider the wars fought and people enslaved over it, as has been reviewed by Mintz.[1] More sweeteners, principally sugar, but also syrups derived from starch, as well as at least 10 non-caloric sweeteners, are added to foods and beverages than any other ingredient type. The human attraction to sweetness is innate as has been demonstrated by Steiner in the study of newborns who clearly exhibit strong liking for sweet-tasting stimuli.[2]

© 2008 American Chemical Society

1

Sweetness and Sweeteners

The Role of the Chemist

As a consequence of the importance of sweet taste in affecting human behavior as well as in the human diet, chemists have been active in elucidating the structures of naturally occurring sweeteners as well as in the discovery of synthetic sweeteners since early in the 19th century. And this work continues today as the ever-elusive goal of accurate reproduction of sugar taste in a non-caloric sweetener system has not yet been achieved. A summary of some of the major contributions by chemists in elucidation of structures of natural sweeteners and in discovery of synthetic sweeteners is presented in Figure 1. Shown here are 10 sweet-tasting organic compounds, where the latter 9 are all used in foods and beverages today. Only the first compound, m-nitro-aniline (1), reported in 1846 by Muspratt and Hofmann,[3] is not a commercial sweetener. It is included here since it is the first sweet-tasting organic compound of defined structure which I have been able to find in the scientific literature. Each of the latter 9 compounds is used in sweetening foods and beverages today and some information on each of them is as follows:

• Saccharin (2): The sweetness of saccharin was discovered by Fahlbergin the laboratory of Remsen at Johns Hopkins University in 1879 and was commercialized in the U.S. as the first product of the Monsanto Chemical Company. Saccharin continues today to be an important sweetener in many foods and beverages. The discovery and development of saccharin have recently been reviewed by the author.[4]

• Glucose (3): Glucose is a carbohydrate present in many fruits and has always been a significant component of the human diet. It is also a key nutrient although most of the glucose ingested is in the form of starch. The chemical structure of glucose was reported by Fischer in 1891.[5] Today, the diet of most people contains substantial glucose present in syrups derived from corn starch as well as other starch sources.

• Sucrose (4): Sucrose has been known since antiquity as the sweet crystalline component of sugar cane. However, the structure elucidation of sucrose was not completed until 1926 by Haworth and Hirst.[6] Sucrose today is produced on a very large scale with 2005 world production at ca. 145 million MT. Sucrose (*aka* sugar) is the "consumer's standard" as relates to sweet taste quality.

• Cyclamate (5): The discovery of cyclamate as a sweet-tasting compound was made by Sveda in the laboratory of Professor Audrieth at the University of Illinois.[7] Cyclamate is available for food use in the sodium and calcium salt forms. Cyclamates have been widely used in foods and beverages

2: Saccharin 1879: (Fahlberg & Remsen)

3: Glucose 1891: (Fischer)

4: Sucrose 1926: (Haworth & Hirst)

1 1846: (Muspratt & Hofmann)

5: Cyclamate 1944: (Audrieth & Sveda)

10: Neotame 1992: (Nofre & Tinti)

6: Stevioside 1955: (Fletcher & Mosettig)

9: Sucralose 1976: (Hough & Phadnis)

8: Acesulfame 1973: (Klaus & Jensen)

7: Aspartame 1965: (Schlatter & Mazur)
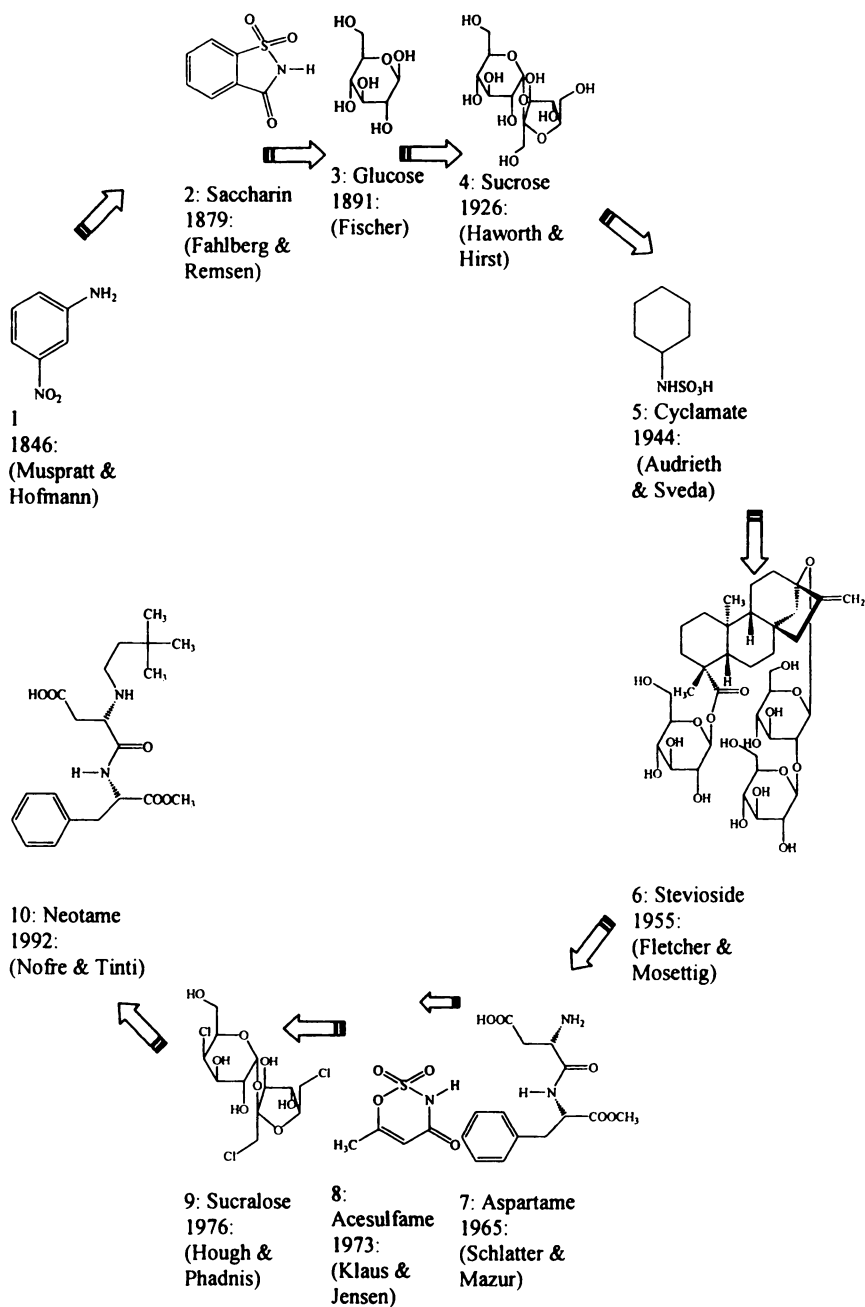
Figure 1. Progress in the discovery of synthetic and natural non-caloric sweeteners.

in blends with other non-caloric sweeteners. The blend of cyclamate with saccharin was *the enabler* of the diet food and beverage industry in the 1960s. Up until that time, saccharin was the only approved non-caloric sweetener and diet foods and beverages with good taste quality were not possible.

- Stevioside (6): A total of 8 sweet-tasting glycosides of an entkaurene-type diterpenoid known as steviol have been isolated from the plant *stevia rebaudiana* (Bertoni), indigenous to Paraguay. In the common variety of the plant, stevioside is the most abundant steviol glycoside and several groups participated in elucidation of its structure. This work was completed in 1950s and 1960s by the groups of Fletcher and Mossettig of the National Institutes of Health. The history of the structure elucidation of stevioside has been reviewed by Phillips.[8]

- Aspartame (7): Aspartame is the non-caloric sweetener of greatest commercial significance up until this time. The sweet taste of aspartame was serendipitously discovered in 1965 by Schlatter in the laboratory of Mazur of Searle Pharmaceutical Company.[9] Aspartame is unique among non-caloric sweeteners as its metabolism leads only to natural amino acids and methanol, all of which are provided in much higher amounts on consumption of common foods. And, just as cyclamate enabled the beginning of the diet food and beverage industry in the 1960s, aspartame was *the enabler* of a rebirth of this industry in the 1980s, following the 1970 FDA removal of cyclamates from the US food supply and restrictions on its usage in other countries.

- Acesulfame (8): Acesulfame, a sweetener related in structure to saccharin, was reported in 1973 Clauss and Jensen of Hoechst AG.[10] Acesulfame, as its potassium salt, is commonly blended with other non-caloric sweeteners in foods and beverages.

- Sucralose (9): Discovery of the substantial elevation in sweetness potency of sucrose by halogen substitution of sugar hydroxyl groups was reported in 1976 by Hough and Phadnis of the University of London.[11] The most well known member of this structural class is sucralose.

- Neotame (10): Neotame, a structural analogue of aspartame with substantially increased potency, was reported in 2000 by Nofre and Tinti of the Université Claude Bernard.[12] It is most commonly used today in blends with carbohydrate sweeteners.

Since the beginning of organic chemistry, chemists have discovered hundreds of synthetic and natural sweeteners with the result that today, at least 50 structural classes of sweet-tasting organic compounds are known. And since early in the 20th century, chemists have appreciated that the chemical compounds that exhibit sweet taste activity are of very diverse structure. In an effort to make sense of this diversity, chemists began to make models for the common pharmacophore that was assumed present in sweet-tasting compounds. Several of the models developed over the 20th century are illustrated in Figure 2. The earliest pharmacophore model was reported by Cohn, in 1914.[13] He argued

that all sweet-tasting compounds possess a common *glucophore*. Shortly thereafter, Oertly and Myers (Stanford University), in effort to explain the substantially increased potencies of some sweeteners argued that, in addition to a *glucophore*, such compounds must also contain an *auxogluc*.[14] Much later, in 1967, Shallenberger and Acree (Cornell University) reported their well-known *A-H/B model*.[15] They hypothesized that all sweeteners contain H-bond donor and H-bond acceptor groups separated by not <2.5 or >4.0Å. The Shallenberger/Acree model was subsequently enhanced, first, in 1972, by Kier (Northeastern University),[16] then, in 1991, by Rohse and Belitz (Institut für Lebensmittelchemie der TU München)[17] and, also in 1991, by Nofre and Tinti (Université Claude Bernard),[18] to better explain the elevated sweetness potencies of many sweeteners. The Cohn, Oertly/Myers, Shallenberger/Acree, Kier, Rohse/Belitz and Nofre/Tinti models are illustrated in Figure 2. Many other chemists have been active in sweetener model development including groups led by van der Heijden (Unilever),[19,20] Walters (The NutraSweet Company),[21] Goodman (University of California, San Diego),[22] Temussi (University of Naples)[23] and Bassoli (University of Milan).[24]

An assumption implicit in nearly all of the models referenced above, is that sweetness is initiated following the binding of a sweetener to a single site (i.e., orthosteric site) on a single receptor. However, recent receptor/sweetener mapping studies by Li and associates (Senomyx)[25] and by the collaborative team of the Margolskee, Max and Osman groups (Mt Sinai School of Medicine)[26,27], demonstrate that the human sweetener receptor contains at least 3 orthosteric sites and thus the A-H/B model as well as all of the common pharmacophore models, are substantial oversimplifications. And therefore at least 3, and probably more, pharmacophore models are required to characterize the relationship between chemical structure and sweet taste. The first model consistent with this logic was the Walters/Culberson Model developed for the aspartame orthosteric site. In the development of this model, only sweeteners for which there was evidence of a common binding locus were included in model development. Later, in 1995, D'Angelo and Iacobucci (The Coca-Cola Company) employed Comparative Molecular Field Analysis to enhance the Walters/Culberson Model to provide quantitative predictive power.[28]

In summary, over nearly two centuries, chemists have discovered many, many sweet-tasting organic compounds. And, over the last century, they have developed increasingly useful models that may be applied to the design of new sweeteners which better replicate the sweet taste quality of sugar than ever before.

Sweetness and Sweeteners: The Role of the Biologist

The science of sweet taste, and the molecules that initiate it, is no longer the exclusive domain of the chemist. Over the last two decades, biologists have
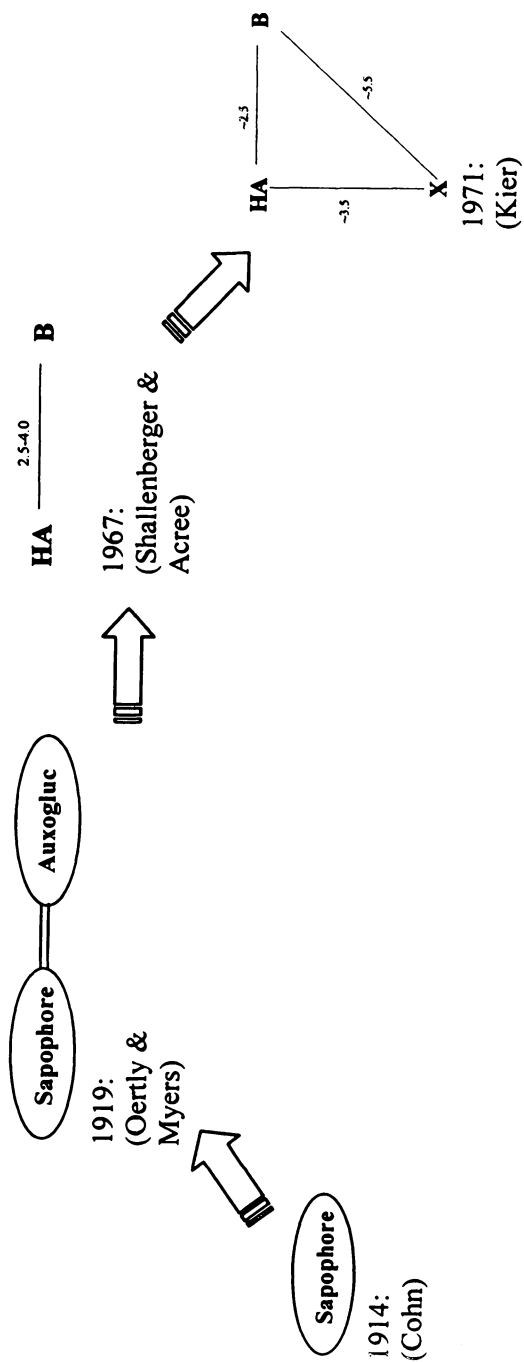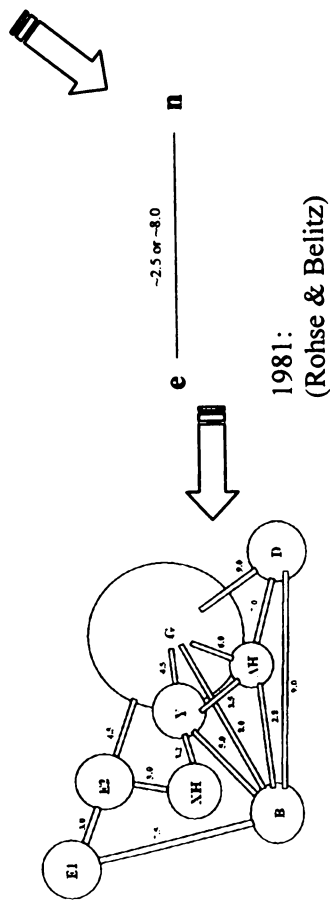
Figure 2. Progress in the development of sweetener pharmacophore models.

played an ever-increasing role in understanding sweetness and how the molecules responsible for sweetness initiate their effects. And their efforts have led to some fantastic advances in understanding the pathways whereby sweeteners excite sweet-sensitive taste bud cells. Some of the most significant scientific breakthroughs are summarized in Figure 3. And in the following discussion, comment is made on these breakthroughs, nearly all of which have occurred within the last decade and with the most significant occurring just within the last 5 years.

Until the late 1980s, the biochemistry of sweet taste was largely unknown. Then, evidence began to accumulate that sweetness must be G Protein-Coupled Receptor (GPCR) mediated. The discovery of gustducin, a G protein, in the Margolskee laboratory, was generally accepted as strong support for GPCR involvement in sweet taste.[29] And throughout the 1990s, sweet taste was thought to result from activation of several GPCRs since the findings of biochemical, electrophysiological and psychophysical experiments could most easily be explained by a plurality of receptors, a topic reviewed by Faurion in 1987[30] and by the author in 1997.[31] Then a breakthrough occurred dramatically improving our understanding of sweet taste. In 2001, a collaborative team from the laboratories of Zuker (University of California, San Diego) and Ryba (National Institutes of Health) reported the discovery of the rat sweetener receptor.[32] In a functional assay, they showed that responses to many of the substances that rats generalize to sucrose taste appear to be mediated by a receptor which required the co-expression of two 7-transmembrane domain (TMD) proteins. They named these proteins T1R2 and T1R3 and speculated that they combine to form a heterodimer which is the sweetener receptor. This receptor is now commonly referred to as T1R2/T1R3. Both T1R2 and T1R3 are members of the small family of Class C GPCRs. The most studied members of the Class C GPCRs are the 8 metabotrophic glutamate (mGluR), 1 γ-aminobutyric acid type B (GABA$_B$R) and 1 extracellular calcium (ECR) receptors, which have been recently reviewed by Pin.[33] The mGluRs and the ECR are believed to be homodimers and the GABA$_B$R, a heterodimer. The rat sweetener receptor discovery was quickly followed by the report in 2002 by Li and coworkers at Senomyx of parallel findings for the human system.[34] As in the rodent, the results were most consistent with human sweet taste initiation by the single heterodimeric receptor T1R2/T1R3. Heterologous cells (i.e., HEK-293 cells), in which both human T1R2 and human T1R3 were expressed, responded to all structural types of sweeteners tested in a manner consistent with expectation from sensory experiments. Thus, at the present time, there is a general consensus that the heterodimer T1R2/T1R3 is the sweetener receptor. Thus, all initial evidence on rat and human sweet taste was consistent with sweet taste emanating from activation of a single receptor. In 2003, however, evidence was reported by the Zuker and Ryba laboratories for a second sweetener receptor in the mouse. Their results suggested that a T1R3-only

receptor, perhaps a homodimer, is functional in the mouse as a carbohydrate-only sweetener receptor.[35] However, no evidence has yet been reported for such a receptor in humans or other animals. At about the same time as the pioneering work by the Zuker and Ryba laboratories, several other laboratories also identified the T1R3 component of the sweetener receptor including teams led by Alexander Bachmanov (Monell Chemical Senses Center),[36] Marianna Max and Robert Margolskee (Mt. Sinai School of Medicine),[37] Linda Buck (Harvard Medical School),[38] and James Battey (National Institutes of Health).[39]

Class C GPCRs are unique in that they possess very large N-terminal Venus flytrap-like domains (VFDs). For the case of the metabotrophic glutamate receptor mGluR1, in 2000, Kunishima and coworkers reported that its VFD closes on binding glutamate, hence the analogy to a Venus flytrap.[40] This precedent and the fact that the sweetener receptor and the umami receptor, shown in parallel work by Li and coworkers to be the GPCR heterodimer T1R1/T1R3, contain the common subunit T1R3, lead to the expectation that sweeteners likely bind in the VFD of T1R2. Subsequent work by Li and coworkers probed the fundamental question of sweetener binding locus with the finding that, while some sweeteners do bind in the VFD of T1R2 (e.g., aspartame and neotame), at least one sweetener (i.e., cyclamate) does not, but rather binds within the 7-TMD of T1R3.[41] The binding of cyclamate to the TMD of T1R3 was corroborated by site-directed-mutagenesis studies in the Margolskee laboratory which provided significant detail on the interactions of cyclamate with T1R3.[25] Other work in the Margolskee laboratory on brazzein, a natural protein sweetener, showed that its locus of binding is in the cysteine-rich domain (CRD) of T1R3, a subunit of the protein which connects the VFD and TMD domains.[42] The human sweetener receptor is the first Class C GPCR demonstrated to have multiple agonist binding loci (orthosteric sites).

A topic of considerable controversy in the field of taste research has been that of taste quality coding. Taste bud cells (TBCs) are known to be innervated by nerve fibers of three gustatory nerves, the chorda tympani, the glossopharyngeal and the suprapetrosal nerves. Each of these nerves is a bundle of many individual fibers and some have argued that taste quality is coded by a cross-fiber pattern of activity and others have argued that individual fibers are taste modality specific. Evidence for taste-modality-specific coding was first provided by electrophysiological studies in chimpanzees by Hellekant and Ninomiya. They carried out single fiber recordings and reported that some fibers responded only to sweeteners,[43] while others responded only to bitterants[44] leading them to conclude that taste quality is coded at the level of the TBC. In other words, they argued that individual TBCs are specific sensors for sweet, bitter, umami, sour or salty. Further convincing evidence for taste-modality-specific coding comes from recent work from the Zuker and Ryba laboratories. Early in 2003, working with PLC$_{\beta 2}$ knockout mice, they engineered mice in which they selectively rescued PLC$_{\beta 2}$ function in bitter-receptor expressing cells and found that these mice responded normally to

Sweetener
Receptor:

A <u>Single</u> Receptor
(Heterodimeric
GPCR
T1R2/T1R3)

2001: Zuker &
Ryba (Rat)
2002: Li (Human)

Sweet-Taste
Transduction:

Effector Enzyme
(PLC$_{\beta2}$) and Ion
Channel (TRPm5)

2002: Margolskee
2003: Zuker &
Ryba

Sweet-Taste
Transduction:

G-Protein
(Gustducin)

1992:
Margolskee

Sweetener
Receptor
Mapping:

Multiple Loci of
Sweetener Binding
on T1R2/T1R3

2004: Li
(Aspartame and
Cyclamate)
2004: Margolskee
(Cyclamate and
Brazzein)
2005: Munger
(Sucrose)

Sweet Taste
Coding:

Labeled Line to
CNS

1991/1994:
Hellekant
& Ninomiya
2003: Zuker &
Ryba

Figure 3. Progress in elucidation of the biochemistry and pharmacology of sweet taste.

bitterants but still exhibited no responses to sweet or umami stimuli.[45] Later, working with T1R knockout mice that gave no responses to sweet or umami stimuli, they engineered mice in which human T1R2 function was added to sweet-sensitive TBCs and found that these mice responded to aspartame, a compound sweet to humans but inactive in mice.[46] They also selectively introduced an opioid receptor into sweet-sensing TBCs and observed that these mice now responded with attraction to opioid agonists. In summary, the evidence is now convincing that taste quality is coded at the level of the TBC with a labeled line communication pathway to the CNS.

The recent identification of the sweetener receptor and elucidation of the mechanism of sweet taste coding was preceded by the discovery, in the Margolskee laboratory, of gustducin, a specific G protein that mediates sweet taste, as has already been discussed. Other key elements of the sweet taste transduction cascade were also identified in the Margolskee laboratory[47] as well as by the Zuker/Ryba team.[48] Both groups found that phospholipase Cβ2 (PLC$_{\beta 2}$), the inositol trisphosphate (IP$_3$) receptor (IP$_3$R) and the transient receptor potential channel m5 (TRPm5) are key elements in sweet taste transduction. Thus, at this time, evidence exists for initiation of the human sweet taste response by activation of the single receptor T1R2/T1R3, the G protein gustducin, the affecter enzyme (PLC$_{\beta 2}$), the 2nd messenger receptor IP$_3$R and the ion channel TRPm5 in sweet-sensing TBCs. Recognition of the relatively high concentrations of non-caloric sweeteners commonly employed in foods and beverages and the fact that such lipophilic molecules are generally absorbed into cells, led to speculation by the author that some sweeteners may initiate their activity at intracellular elements of the transduction cascade.[30] Support for this idea was provided by Naim and associates (Hebrew University) who reported that some non-caloric sweeteners have the capability to directly activate G proteins.[49] And further support was provided in recent work by the Naim group in studies showing that some sweeteners (i.e., saccharin and D-tryptophan) are rapidly taken up into TBCs.[50] However, in view of the finding that every sweetener tested, activated T1R2/T1R3-receptor-expressing HEK-293 cells, while otherwise identical control cells, lacking the T1R2/T1R3 receptor, are unaffected, it remains to be demonstrated that any sweeteners do actually act at downstream elements in the sweet-sensing TBC activation cascade. Nonetheless, since Naim and coworkers have demonstrated that small molecules are readily taken up into TBCs, it is logical to expect that some may.

In summary, transduction of sweet taste is generally accepted as proceeding via activation of the heterodimeric sweetener receptor T1R2/T1R3 with subsequent activation of the G protein gustducin. In this generally accepted transduction pathway, the gustducin G$_\beta$G$_\gamma$ subunit is thought to activate PLC$_{\beta 2}$, thus enabling it to act on membrane phosphatidylinositol bisphosphate to produce IP$_3$, which acts at its receptor IP$_3$R on intracellular Ca^{2+} storage sites thus promoting Ca^{2+} release into the cytoplasm. And finally, Ca^{2+} is thought to gate the TRPm5 ion channel enabling the inward flow of Na$^+$, depolarizing the

sweet-sensing TBC and initiating the signaling to the CNS. Evidence from the Zuker/Ryba laboratories argues that this gustducin $G_\beta G_\gamma$ pathway is the only sweet taste transduction pathway since mice in which the TRPm5 gene was partially deleted were observed to lack all behavioral and nerve responses to sweeteners.[47] Earlier work, however, reported in 2000 by Varkevisser and Kinnamon,[51] and later by Margolskee,[52] argues for involvement of gustducin G_α in sweet taste transduction. And very recent work from the Margolskee laboratory in which TRPm5 gene expression in the mouse was fully blocked, continues to argue for a sweet taste transduction pathway not mediated by TRPm5.[53] In this work, the TRPm5 knockout mice continue to exhibit weak responses to sweeteners. And, in the electrophysiological component of this work, glossopharyngeal nerve responses were observed while the chorda tympani nerve responses were not, thus suggesting that transduction pathway may vary between TBCs innervated by the two different nerve systems. Thus, at this time, while there remains a general consensus that the primary pathway for sweet taste transduction is the gustducin $G_\beta G_\gamma/PLC_{\beta 2}/IP_3R/TRPm5$ pathway, it appears that at least one additional pathway must exist for activation of sweet-sensing TBCs.

What is All the Excitement About?

In brief, the excitement is about the fantastic progress that has been made by biologists in understanding sweet taste and how sweet-sensitive taste bud cells initiate their communication to the CNS. It is now generally accepted that the sweet tastes of all sweeteners are mediated, or at least predominantly mediated, by a single receptor in a single subset of taste bud cells. And we know that this sweetener receptor has multiple sweetener binding sites, all of which cause the receptor to undergo activation and signaling. This receptor can now be expressed in heterologous cell systems and thus is accessible in unlimited quantities for studies that, until now, were unimaginable. Included among such studies are the following:

1)High-throughput-screening of synthetic and natural product libraries for the discovery of novel synthetic and natural non-caloric sweeteners.

2) High-throughput-screening of synthetic and natural product libraries for the discovery of novel synthetic and natural sweetness enhancers and sweetness inhibitors.

3) Mechanistic studies targeted at understanding the reasons for differences Concentration/Response functions of carbohydrate and high-potency sweeteners.

4) Mechanistic studies targeted at understanding the reasons for differences in Temporal Profiles of carbohydrate and high-potency sweeteners.

5) Mechanistic studies targeted at understanding the reasons for differences Adaptation behaviors of carbohydrate and high-potency sweeteners.

In this symposium "Sweetness and Sweeteners", the attempt has been made to comprehensively cover the scientific advances in the field since they were covered in 1989 in a special symposium of the Agricultural & Food Chemistry Division of the American Chemical Society.[54] In this special symposium, the papers presented have been organized into the following 7 sessions:
1) Structural Studies of the Sweetener Receptor.
2) Modeling of the Sweetener Receptor.
3) Sweet Taste Transduction.
4) Quantifying the Responses of Sweet-Sensitive Taste Bud Cells.
5) Modulation of Sweet-Sensitive Taste Bud Cell Signaling.
6) Advances in the Discovery and Commercial Development of Synthetic Non-Caloric Sweeteners.
7) Advances in the Discovery and Commercial Development of Natural Non-Caloric Sweeteners.

References

1. Mintz, S. W. *Sweetness and Power: The Place of Sugar in Modern History*; Penquin Books, New York, NY, 1985.
2. Steiner, J. E., *Olfaction and Taste XI, Proceedings of the 11th International Symposium on Olfaction and Taste and of the 27th Japanese Symposium on Taste and Smell*; Kurihara, K.; Suzuki, N.; Ogawa, H. J., Eds., Springer-Verlag: Tokyo, 1994, pp 284-287.
3. Muspratt, J. T.; Hofmann, A. W. *Ann. Chem.* **1846**, *57*, 201-224.
4. DuBois, G. E. *Sweeteners and Sugar Alternatives in Food Technology, Part II: Non-Nutritive / Low-Calorie Sweeteners: Principles and Practice*; Mitchell, H., Ed., Blackwell Publishing Ltd.: Oxford, UK, 2006, pp 103-129.
5. Fischer, E. *Ber. Dtsch. Chem. Ges.* **1891**, *24*, 2683-2687.
6. Haworth, W. N.; Hirst, E. L. *J. Chem. Soc.* **1926**, 1858-1868.
7. Audrieth, L. F.; Sveda, M. *J. Org. Chem.* **1944**, *9*, 89-101.
8. Phillips, K.C. *Developments in Sweeteners-3*; Grenby, T. H., Ed., Elsevier Applied Science, 1987, pp 1-43.
9. Mazur, R. H.; Schlatter, J. M.; Goldkamp, A. H. *J. Am. Chem. Soc.* **1969**, *91*, 2684-2691.
10. Clauss, K.; Jensen, H. *Angewandte Chemie, International Edition in English*, **1973**, *12*, 869-876.
11. Hough, L.; Phadnis, S. P. *Nature*, **1976**, *263*, 800.
12. Nofre, C.; Tinti, J.-M. *Food Chemistry*, **2000**, *69(3)*, 245-257.

13. Cohn, G. *Die Organischen Geschmacksstoffe*; Franz Siemenroth: Berlin, 1914.
14. Oertly, E.; Myers, R. G. *J. Am. Chem. Soc.*, **1919**, *41*, 855-867.
15. Shallenberger, R.S.; Acree, T. E. *Nature* **1967**, *216*, 480-482.
16. Kier, L. B. *J. Pharm. Sci.* **1972**, *61*, 1394-1396.
17. Rohse, H.; Belitz, H.-D. *Sweeteners: Discovery, Molecular Design and Chemoreception*; Walters, D. E.; Orthoefer, F. T.; DuBois, G. E., Eds., ACS Symposium Series 450, Chapter 13, American Chemical Society: Washington, D.C., 1991, pp 176-192.
18. Tinti, J.-M.; C. Nofre, C. *Sweeteners: Discovery, Molecular Design and Chemoreception*; Walters, D. E.; Orthoefer, F. T.; DuBois, G. E., Eds., ACS Symposium Series 450, Chapter 13, American Chemical Society: Washington, D.C., 1991, pp 206-213.
19. Van der Heijden, A.; Van der Wel, H.; Peer, H. G. *Chemical. Senses*, **1985**, *10*, 57-72.
20. Van der Heijden, A.; Van der Wel, H.; Peer, H. G. *Chemical Senses*, **1985**, *10*, 73-88.
21. Culberson, J. C.; Walters D. E. *Sweeteners: Discovery, Molecular Design and Chemoreception*; Walters, D. E.; Orthoefer, F. T.; DuBois, G. E., Eds., ACS Symposium Series 450, Chapter 13, American Chemical Society: Washington, D.C., 1991.
22. Douglas, A.J.; Goodman, M. *Sweeteners: Discovery, Molecular Design and Chemoreception*; Walters, D. E.; Orthoefer, F. T.; DuBois, G. E., Eds., ACS Symposium Series 450, Chapter 13, American Chemical Society: Washington, D.C., 1991, pp 128-142.
23. Temussi, P.A.; Lelj, F.; Tancredi, T. *Sweeteners: Discovery, Molecular Design and Chemoreception*; Walters, D. E.; Orthoefer, F. T.; DuBois, G. E., Eds., ACS Symposium Series 450, Chapter 13, American Chemical Society: Washington, D.C., 1991, pp 143-161.
24. Bassoli, A.; Drew, M. G. B.; Merlini, L.; Morini, G. *J. Med. Chem.* **2002**, *45*, 4402-4409.
25. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M; Li, X. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14258-14263.
26. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L. A.; Benard, L. M. J.; Osman, R.; Max, M.; Margolskee, R. F. *Journal of Biological Chemistry* **2005**, *280*, 34296-34305.
27. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Bernard, M. J.; Margolskee, R. F.; Max, M. *Journal of Biological Chemistry* **2004**, *279*, 45068-45075.
28. D'Angelo, L. L.; Iacobucci, G. A. Presented at the 210th National Meeting of the American Chemical Society, Chicago, IL, August 20-24, 1995. Book of Abstracts, Pt 1, COMP-083, American Chemical Society, Washington, D.C.
29. McLaughlin, S.K.; Mckinnon, P.J.; Margolskee, R.F. *Nature*, **1992**, *357*, 563-569.

30. Faurion, A. *Progress in Sensory Physiology*; Volume 8, Springer-Verlag: Berlin, 1987, pp 129-201.
31. DuBois, G. E. *Firmenich Jubilee Symposium Olfaction and Taste: A Century for the Senses 1895-1995*; G. Salvadori Ed., Allured Publishing Corp.:Carol Stream, IL, 1997, pp 32-95.
32. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J. P.; Zuker, C. S. *Cell* **2001**, *106*, 381-390.
33. Pin, J.-P.; Galvez, T.; Prezeau, L. *Pharmacology & Therapeutics*, **2003**, *98*, 325-354.
34. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696.
35. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N, J. P; Zuker, C. S. *Cell* **2003**, *115*, 255-266.
36. Bachmanov, A. A.; Li, X.; Reed, D. R.; Ohmen, J. D.; Li, S.; Chen, Z.; Tordoff, M. G.; de Jong, P. J.; Wu, C.; West, D. B.; Chatterjee, A.; Ross, D. A; Beauchamp, G. K. *Chemical Senses* **2001**, *26*, 925-933.
37. Max, M.; Gopi Shanker, Y.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S; Margolskee, R. F. *Nature Genetics* **2001**, *28*, 58-63.
38. Montmayeur, J.-P.; Liberles, S. D.; Matsunami, H; Buck, L. B. *Nature Neuroscience* **2001**, *4(5)*, 492-498.
39. Sainz, E.; Korley, J. N.; Battey, J. F.; Sullivan, S. L. *J. Neurochem.* **2001**, *77*, 896-903.
40. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. *Nature* **2000**, *407*, 971-977.
41. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14258-14263.
42. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Bernard, M. J.; Margolskee, R. F.; Max, M. *Journal of Biological Chemistry* **2004**, *279*, 45068-45075.
43. Hellekant, G.; Ninomiya, Y. *Physiology & Behavior* **1991**, *49*, 927-934.
44. Hellekant, G.; Ninomiya, Y. *Kirin International Symposium on Bitter Taste*; Beauchamp, G. K., Ed., Elsevier Science Ltd.:Tarrytown, NY, 1994, pp 1185-1188. Hellekant, G.; Ninomiya, Y. *Physiology & Behavior* **1994**, *56(6)*, 1185-1188.
45. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. P. *Cell* **2003**, *112*, 293-301.
46. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N, J. P.; Zuker, C. S. *Cell* **2003**, *115*, 255-266.
47. Pérez, C. A.; Huang, L.; Rong, M.; Kozak, J. A.; Preuss, A. K.; Zhang, H.; Max, M.; Margolskee, R. F. *Nature Neuroscience* **2002**, *5 (11)*, 1-8.
48. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. P. *Cell* **2003**, *112*, 293-301.

49. Naim, M.; Seifert, R.; Nürnberg, B.; Grünbaum, L; Schultz, G. *Biochem. J.* **1994**, *297*, 451-454.
50. Zubare-Samuelov, M.; Shaul, M. E.; Peri, I.; Aliluiko, A.; Tirosh, O.; Naim, M. *Am. J. Physiol. Cell Physiol.* **2005**, *289*, C483-C492.
51. Varkevisser, B.; Kinnamon, S. C. *Journal of Neurophysiology* **2000**, *83(5)*, 2526-2532.
52. Margolskee, R. F. *Journal of Biological Chemistry* **2002**, *277*, 1-4.
53. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili; Z., Perez C. A.; Shigemura, N.; Yoshida, R.; Mosinger Jr., B.; Glendinning, J. I.; Ninomiya, Y; Margolskee, R. F. *Chemical Senses* **2006**, *31*, 253-264.
54. *Sweeteners: Discovery, Molecular Design and Chemoreception*; Walters, D. E.; Orthoefer, F. T.; DuBois, G. E., Eds. ACS Symposium Series 450, American Chemical Society, Washington, DC, 1991.

Chapter 2

Genetic Architecture of Sweet Taste

Alexander A. Bachmanov

Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104

Genetic studies help to gain insight into mechanisms of sweet taste, a phenotype with a complex genetic architecture. The genetic approach was instrumental for discovering sweet taste receptors, detecting their functionally important polymorphic sites, and characterizing their ligand specificity. The genetic *in vivo* approach to analyze receptor-ligand interactions complements the *in vitro* approach and overcomes some methodological limitations of *in vitro* studies. Variation of the sweet taste receptor genes contributes to differences in sweet taste perception within and between species. In addition to the sweet taste receptors, a number of other genes influence sweet taste responses. These yet unknown genes are likely to be involved in peripheral or central mechanisms of sweet taste processing or in its interaction with homeostatic systems of ingestive behavior and reward. There is evidence that responses to different sweeteners are affected by different sets of genes. Some data suggest that individual differences in sweet taste perception are associated with obesity and predisposition to alcoholism. Recent advances in development of genomic resources make genetics a powerful approach for understanding mechanisms of sweet taste.

18   © 2008 American Chemical Society

Introduction

The sense of taste has probably evolved to allow animals to choose and consume appropriate food. The most common natural taste stimuli that humans describe as sweet are sugars. Sugars are important nutrients for animals from many different species ranging from insects to mammals. In animals from many species, sugars are recognized by the taste system and evoke appetitive consummatory responses. In addition to sugars, a wide range of other chemicals (referred to here as sweeteners), also evoke the sensation of sweetness in humans and are palatable to many other animals. Numerous studies have shown that the mechanisms of taste perception of sweeteners are similar in humans and non-human mammals. This justifies using laboratory animals, such as mice and rats, as model organisms to study mechanisms of sweet (sucrose-like) taste relevant to humans.

In mammals, sweetness perception is initiated when sweeteners interact with taste receptor proteins from the T1R family expressed in taste receptor cells in taste buds of the oral cavity. Thus, sweeteners function as ligands of the G protein coupled T1R receptors. The sweetener-responsive taste receptor cells transmit afferent taste information to the brain via branches of three cranial nerves, VII (the facial nerve with two gustatory branches, the chorda tympani and greater petrosal nerves), IX (the glossopharyngeal nerve) and X (the vagus nerve) (*1*). These nerves terminate in the nucleus of the solitary tract in the medulla oblongata, a part of the brain stem. From the nucleus of the solitary tract, gustatory signals are transmitted (directly or indirectly) to many other brain structures, including parabrachial nucleus, thalamus, hypothalamus, amygdala and cortex (*2*). Central taste processing results in perception of several different aspects of sweeteners' taste: quality, intensity, and hedonics (pleasantness or unpleasantness).

The wide representation of taste information in the brain is probably necessary to integrate it with interoceptive (hunger, satiety) and exteroceptive (vision, olfaction, somatosensation) signals, and to generate behavioral responses to taste stimuli. Sweet taste stimuli were shown to evoke preabsorptive cephalic phase responses, such as insulin release (*3-5*), activate endogenous opioidergic, dopaminergic and serotonergic systems (*6-13*) and produce analgesic effects in children and young animals (*14-18*). Taste responses to sweeteners are modulated by post-ingestive feedback and hormones (*19-25*). Although appetitive responses to sweet taste stimuli are inborn in many animals (*26, 27*), they are also often modulated by environment and depend on genetic factors (*28, 29*). The interactive mechanisms of sweet taste suggest that it is a part of a complex ingestive behavior and is likely to be determined by multiple genes.

When we want to understand the genetic basis of the sweet taste, we consider it as a phenotype, or a trait. The definition of phenotype is the

observable characteristics of an organism determined by both genetic makeup and environmental influences. The goal of the genetic analysis is to separate the genetic and environmental effects on phenotype. In humans, taste phenotypes are usually assessed using verbal information, e.g., by rating sensation intensity on a scale with verbal descriptors or by reporting a perceptual difference between samples. These techniques allow one to evaluate sensitivity, intensity, quality and hedonic value of the taste sensation. Assessment of taste perception in non-human animals relies on a number of different techniques to record behavior elicited by taste stimuli (30). These techniques include two-bottle preference tests, brief-access lick recording tests, and approaches that require animal conditioning to examine generalization and discrimination between taste stimuli, and to measure recognition thresholds. In addition to behavioral techniques, animal taste phenotypes can be examined using electrophysiological recordings of activity in the afferent gustatory nerves or in the brain. Studies of gustatory nerve activity help to elucidate whether genetic effects on taste perception have peripheral or central origin.

Many taste phenotypes are measured using a continuous quantitative scale (e.g., volume of solution consumed, preference score or lick rate) and thus are considered quantitative traits. Genes with allelic variants that underlie variation of quantitative traits reside in chromosomal regions named quantitative trait loci (QTL). Defining these chromosomal regions through genetic linkage analysis is called QTL mapping. It helps to identify DNA sequences of genes in the QTL regions and to find genes that are responsible for phenotypical variation. Because this approach to identify genes is based on a chromosomal position of a phenotypical locus, it is called positional cloning. Quantitative traits that depend on multiple genetic and environmental factors are considered complex traits. The combined effect of multiple QTL that determine complex traits is often described as genetic architecture, and process of its elucidation is called genetic dissection. Because there is strong evidence that sweet taste responsiveness is a complex trait, the goal of this chapter is to discuss its genetic architecture.

Individual and genetic variation in sweet taste

Humans differ in perception of sweet taste (e.g., (31-38), but genetic determination of this variation has not been unequivocally established (reviewed in (28, 29, 39-43)). One of the best known examples of this variation is a sweet liking phenotype: some people ('sweet-likers') display higher hedonic ratings of sucrose solutions as their concentrations increase, while other people ('sweet-dislikers') decrease the ratings for the higher sucrose concentrations (33, 38). Mechanisms underlying this phenomenon are not known yet. They may involve peripheral or central taste processing and can be genetically determined,

acquired or depend on interaction between genetic and environmental factors. Studies of model organisms help to understand the role of these factors in sweet taste variation.

Compared with humans, laboratory animals offer an important advantage in studying genetic variation in taste responses because for several species, inbred strains are available. Because animals within an inbred strain are genetically homogeneous, the within-strain variation is due to non-genetic (environmental) factors, but differences between strains represent genetic variation. Strain differences in consummatory responses to sweeteners have been reported for rats (*44-46*) and hamsters (*47*), but most research on genetics of taste was conducted in mice.

Prominent genetic differences in taste responses to sweeteners among inbred strains of mice were shown using different experimental techniques and a variety of sweeteners (e.g., sucrose, glucose, dulcin, saccharin, acesulfame, glycine, D-phenylalanine and L-glutamine). Mice from different strains differ in taste responses to sweeteners assessed using long-term preference tests (*48-60*), single-bottle tests (*61*), brief-access tests based on lick recording (*62*), taste detection thresholds (*63*), conditioned taste aversion generalization (*64*), and responses of gustatory nerves (*65-67*). These studies have shown that responses to many of these sweeteners (e.g., sucrose, glucose, dulcin, saccharin and acesulfame) closely correlate among mouse strains, suggesting a common genetic basis for sweet taste. However, responses to some sweet-tasting amino acids display somewhat different patterns of strain differences (*68*).

The most detailed analysis of physiological mechanisms underlying genetic differences in sweet taste responses was conducted using mice from the C57BL/6 (B6) and 129 strains. Compared with 129 mice, B6 mice have higher preferences for a large number of sweeteners, including sugars (sucrose and maltose), sweet-tasting amino acids (glycine, D-phenylalanine, D-tryptophan, L-proline and L-glutamine), and several non-caloric sweeteners (saccharin, acesulfame, dulcin, sucralose and SC-45647) (*53, 57-59, 69, 70*). This phenotypic difference is specific to sweet-taste processing, and is not due to a generalized difference in taste responsiveness or differences in caloric appetite (*70-73*). For many sweeteners, the strain differences in consumption are associated with variation in chorda tympani responses to sweeteners, which are higher in B6 mice compared with 129 mice (*65, 74-76*). Subsequent studies described below showed that this peripheral gustatory mechanism involves allelic variation of the sweet taste receptor gene, *Tas1r3*.

Although differences in peripheral sweet taste mechanisms appear to be the major determinant of the strain differences in sweetener preferences, non-sweet sensory (e.g., bitterness, viscosity, osmolality, or coolness resulting from endothermic reactions with saliva) and postingestive (e.g., caloric value, intestinal osmotic effects) factors may also affect consumption of some

sweeteners (69). A recent detailed analyses of non-sensory factors that can contribute to differences between B6 and 129 mice in consumption of sugars has shown that mice from these two strains have similar postingestive responsiveness to sucrose (77). Although 129 mice drink less sucrose in preference tests, in operant licking tests designed to measure sugar appetite they are as much or more motivated to obtain sucrose compared with B6 mice (78). Prior experience with sucrose increased sucrose preference in 129 mice more than in B6 mice (79). This illustrates a complex interaction of genetics, experience and experimental design, and interplay between sweet taste and sugar appetite in determining sugar preferences (80).

Differences between B6 and 129 mice in preference for a sweet-tasting amino acid glycine (69) appear to depend on mechanisms distinct from those affecting responses to many other sweeteners. Both B6 and 129 mice generalized conditioned taste aversion between glycine and several other sweeteners, demonstrating that they perceive the sucrose-like taste of glycine. Thus, the lack of a strong glycine preference by 129 mice cannot be explained by their inability to perceive its sweetness (81). Despite differences in glycine intakes and preferences, chorda tympani responses to glycine are similar in mice from both strains (65). Neither behavioral nor neural responses to glycine are influenced by the $Tas1r3$ genotype (63, 82), suggesting that variation in taste responses to glycine depends on other genes.

Some genetic analyses of sweetener consumption by mice yielded evidence that it is influenced by a single locus, named Sac (saccharin preference) (49, 53, 57, 83), whereas other experiments indicated that more than one gene is involved (54, 57, 58, 72, 84). The apparent discrepancy in whether the single-gene or the multi-gene model better describes genetic variation in sweetener preferences is likely due to use of different progenitor strains and types of mapping panels, different sweetener solutions tested, and different quantitative analyses used in these studies.

The Sac locus and discovery of the T1R genes

Discovery of three mammalian T1R receptors resulted from two converging lines of studies. The first line was related to identification of the Sac locus. In 1974, using long-term two-bottle tests, Fuller showed that differences in saccharin preferences between the C57BL/6 and DBA/2 inbred strains largely depend on a single locus, Sac, with a dominant Sac^b allele present in the B6 strain and associated with higher saccharin preference, and a recessive Sac^d allele present in the DBA/2 strain and associated with lower saccharin preference (49). Subsequent studies confirmed this finding in the BXD recombinant inbred strains, and in crosses between the C57BL/6 and DBA/2 and between the

C57BL/6 and 129 strains (*53, 57, 74, 83-85*). In addition to sweetener preferences, the *Sac* genotype influences the afferent responses of gustatory nerves to sweeteners (*74, 75*), which indicated that the *Sac* gene is involved in peripheral taste transduction and may encode a sweet taste receptor. The *Sac* locus has been mapped to the subtelomeric region of mouse chromosome 4 (*57, 74, 84, 85*).

The second line of studies stemmed from analyses of a taste-bud-enriched cDNA library (*86*), which resulted in a discovery of two genes[1], *Tas1r1* and *Tas1r2*, encoding putative G protein coupled taste receptors (GPCR), T1R1 and T1R2 (*89*).

Localization of the *Tas1r1* gene in the distal part of mouse chromosome 4, near the *Sac* locus, suggested identity of *Tas1r1* and *Sac*. However, a high-resolution genetic mapping study has rejected this possibility by showing distinct locations of *Tas1r1* and *Sac* (*75*). A positional cloning study at the Monell Chemical Senses Center has shown that the *Sac* locus corresponds to a novel gene, *Tas1r3*, which is the third member of the *Tas1r* family (*60, 90, 91*) (Figure 1). These studies have restricted the genomic position of the *Sac* locus to a critical interval not exceeding 194 kb and identified genes within this region, one of which, *Tas1r3*, was the most likely candidate for the *Sac* locus based on the effects of the *Sac* genotype on peripheral sweet taste responsiveness (*74, 75*) and involvement of the G protein-coupled mechanism in sweet taste transduction (*92*). *Tas1r3* sequence variants were associated with sweetener preference phenotypes in genealogically diverse mouse strains (*60, 90*) (Figure 2). Substitution of *Tas1r3* alleles in congenic mice resulted in phenotypical changes attributed to the *Sac* locus (*90*). These data provided evidence for identity of *Sac* and *Tas1r3* and for the role of the T1R3 receptor in sweet taste.

Several other studies provided additional evidence for identity of *Sac* and *Tas1r3*:

1) A phenotype rescue transgenic experiment, in which a genomic clone containing the *Tas1r3* gene from the C57BL/6 mouse strain with a dominant *Sac* allele determining higher sweetener preference was incorporated in the genome of mice carrying a recessive *Sac* allele (from the 129X1/Sv strain) determining

---

[1] According to the gene nomenclature rules (*87, 88*), names of these genes are "taste receptor, type 1, member 1, 2 or 3." Corresponding gene symbols abbreviate these names to *Tas1r1, Tas1r2* or *Tas1r3* (in mouse or rat) or *TAS1R1, TAS1R2* or *TAS1R3* (in human); corresponding protein symbols are T1R1, T1R2 and T1R3 (uppercase letters and not italicized). For brevity, when we refer to both human (*TAS1R*) and non-human (*Tas1r*) genes, we describe them as T1R genes.
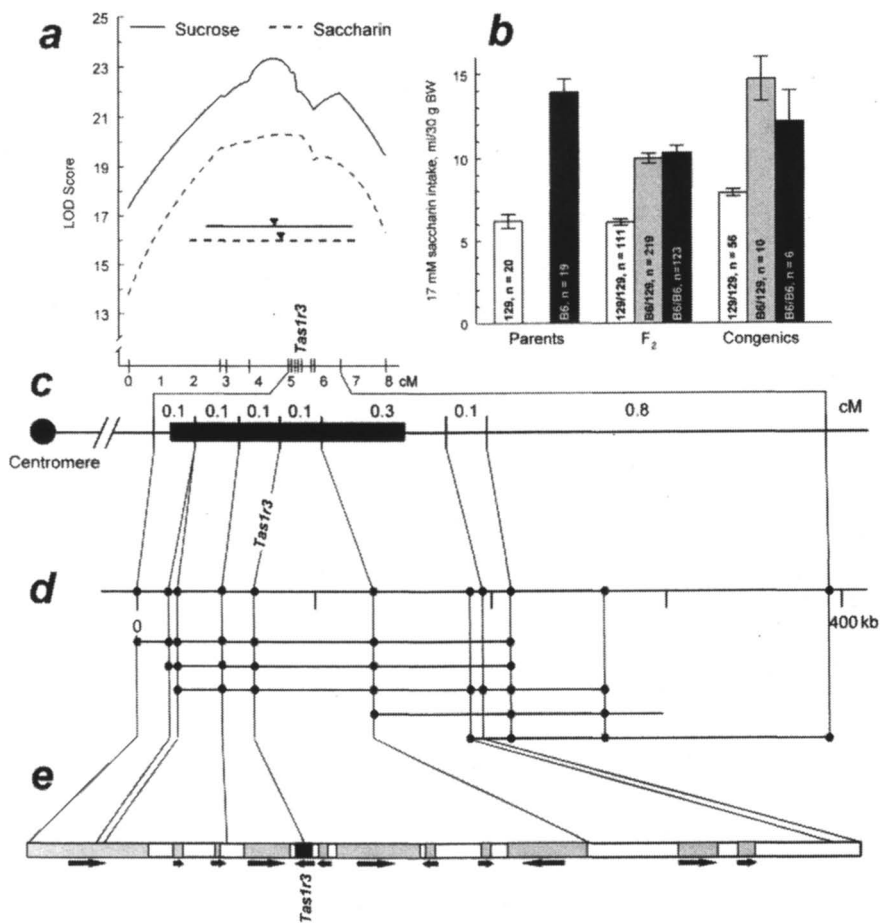
Figure 1. Positional identification of the Sac (saccharin preference) locus. ⟶

a. Linkage map of mouse distal chromosome 4 based on data from the B6 × 129 F2 intercross (n = 629). The X axis shows distances between markers in recombination units (cM). The Y axis shows the logarithm of the odds ratio (LOD) scores for sucrose and saccharin consumption. The LOD score peaks (indicated by black triangles) and confidence intervals (solid horizontal line for sucrose, 4.5 cM, and dotted horizontal line for saccharin, 5.3 cM) define the genomic region of the Sac locus.

b. Average daily 17 mM saccharin consumption by mice from parental 129 and B6 inbred strains (left), B6 × 129 F2 hybrids (center), and congenic 129.B6-Sac mice (right) in 96-hr two-bottle tests with water (Means ± SE). Tas1r3 genotypes of the F2 and congenic mice and mouse numbers are indicated on the bars. Differences between parental strains and among the F2 and congenic genotypes were significant (p < 0.0001, ANOVA). F2 and congenic B6 homozygotes and heterozygotes for Tas1r3 did not differ from each other, and had higher saccharin intakes compared with 129 homozygotes (p < 0.0001, post hoc tests).

c. Linkage map of the Sac-containing region defined based on the size of the donor fragment in the 129.B6-Sac congenic strain (black box). Distances between markers were estimated based on the B6 × 129 F2 intercross (see panel a).

d. A contig of bacterial artificial chromosome (BAC) clones and physical map of the Sac region. BAC clones are represented by horizontal lines. Dots indicate marker content of the BAC clones.

e. Genes within the Sac-containing interval. Filled areas indicate predicted genes. Arrows indicate the predicted direction of transcription.

Reproduced with permission from reference (93). Copyright 2002.
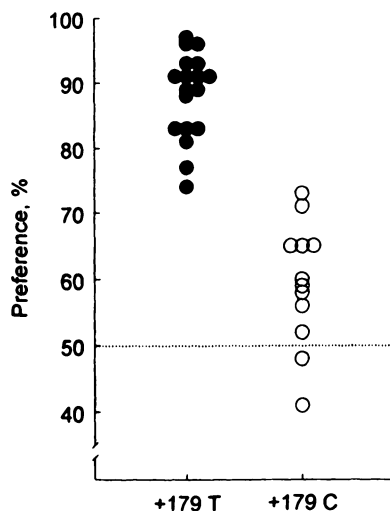
*Figure 2. Preference for 1.6 mM saccharin by mice from inbred strains with
different Tas1r3 genotypes at the T/C variant site at nucleotide position +179
(relative to the first nucleotide in the ATG start codon of the Tas1r3 gene). This
polymorphism results in amino acid substitution of isoleucine to threonine at
position 60 (I60T), in the extracellular N-terminus of the predicted T1R3
protein. Closed circles denote means for C57BL/6, C57L/J, CAST/Ei, CE/J,
FVB/NJ, I/LnJ, IS/Cam, KK/HlJ, NOD/LtJ, NZB/BlNJ, P/J, RBF/DnJ, SEA/GnJ,
SJL/J, SM/J, SPRET/Ei, ST/bJ and SWR/J strains with +179 T genotype. Mice
from these strains strongly preferred saccharin (average preference score
88±2%, Mean ± SE; n=18). Open circles show means for 129P3/J, A/J, AKR/J,
BALB/cByJ, BUB/BnJ, C3H/HeJ, CBA/J, DBA/2J, LP/J, PL/J, RF/J and RIIIS/J
strains with +179 C genotype. Mice from these strains were indifferent to or
only weakly preferred saccharin (average preference score 59±3%, n=12;
p=0.00000000012, two-tailed t-test). Despite the strong phenotypical effect of
the Tas1r3 genotype, there is also substantial variation in saccharin preference
within each genotype group. As a result, Tas1r3 genotype explains only 78% of
genetic variation in saccharin preferences among the inbred strains; the
remaining 22% of genetic variance is attributed to the effect of other genes.
Adapted with permission from reference (60). Copyright 2004 by the
Society for Neuroscience*

lower sweetener preference. The transgenic mice had higher taste preferences for sucrose and saccharin (but not for non-sweet taste solutions) compared with the 129X1/Sv mice (*94*).

2) Genetically engineered mice lacking the *Tas1r3* gene had diminished or abolished taste responses to sweeteners (*95, 96*).

3) Cells with heterologously expressed T1R2 + T1R3 proteins responded to sucrose and saccharin more strongly when C57BL/6 *Tas1r3* allele was used compared with the cell responses when 129X1/Sv *Tas1r3* allele was used (*97*). Interestingly, allelic variation of *Tas1r3* did not affect responses of the T1R1 + T1R3 to amino acids *in vitro* (*97*), consistent with lack of *Tas1r3* effects on responses to umami taste stimuli *in vivo* (*82*).

4) An *in vitro* study (*98*) has shown that binding of several sweeteners to the extracellular N-terminal domain of the T1R3 protein was reduced when isoleucine at position 60 (a predicted sweetener-sensitive *Sac/Tas1r3* allele (*60*)) was substituted to threonine (a predicted hyposensitive *Sac/Tas1r3* allele).

The T1R gene family

The three mouse *Tas1r* genes are located in the distal chromosome 4 in the order: *Tas1r2* (70.0 cM or 139 Mb, NCBI Build 36) - *Tas1r1* (81.5 cM or 151 Mb) - *Tas1r3* (83.0 cM or 155 Mb). Their human orthologs reside in a region of conserved synteny in the short arm of human chromosome 1 (1p36) in the same order: *TAS1R2* (1p36.13) - *TAS1R1* (1p36.23) - *TAS1R3* (1p36.33).

The mouse *Tas1r* genes contain 6 coding exons that are translated into 842 - 858-amino acid proteins. There is evidence for alternative splicing of the T1R genes (*99, 100*; X. Li and D. Reed, unpublished data). The T1R proteins have a predicted secondary structure that includes seven transmembrane helices forming a heptahelical domain, and a large extracellular N-terminus composed of a Venus Flytrap module and a cysteine-rich domain connected to the heptahelical domain. According to a frequently used GPCR classification system that includes receptors of different vertebrate and invertebrate species and groups them into six classes (clans), A, B, C, D, E, and F (*101-103*), T1Rs belong to the class C (metabotropic glutamate/pheromone receptors). More recently, the GRAFTS (glutamate - rhodopsin - adhesion - frizzled/taste2 - secretin) classification system was developed based on phylogenetic analyses of transmembrane parts of human GPCRs (*104*). According to this classification, T1Rs also belong to the glutamate family.

There is strong evidence that T1R2 and T1R3 proteins function as sweet taste receptors:

1) The main sites of expression of these genes are taste receptor cells of the taste buds. In mice and rats, *Tas1r2* and *Tas1r3* are co-expressed in the same

taste cells, but some taste cells express only *Tas1r3* (*94, 99, 105*). Interestingly, co-expression of the T1R2 and T1R3 genes was also found in fish taste cells (*106*). Co-expression of T1R2 and T1R3 in the same taste cells suggested that they may function as heterodimers, which is believed to commonly occur with GPCRs (*107*).

2) Cells heterologously expressing both T1R2 and T1R3 respond to sweeteners (*94, 97, 108*), but T1R3 may also function as a low-affinity sugar receptor alone, probably as a homodimer (*96*). (Heterologously expressed T1R1 + T1R3 functions as a broadly tuned L-amino acid receptor in mice and as a more narrowly tuned umami receptor in humans (*97, 108*).)

3) Genetically engineered mice with targeted mutations of the *Tas1r2* or *Tas1r3* genes have diminished taste responses to sweeteners (*95, 96*).

Evolution of the T1R genes and sweet taste

Numbers of the T1R genes in different vertebrate species range from complete absence in the frog to five in the pufferfish and medaka fish (Table I). With the exception of the frog, all vertebrates have single T1R1 and T1R3 genes, but the number of the T2R genes varies: chicken and Felidae species (domestic cat, tiger and cheetah (*109*)) lack functional *Tas1r2*, but several fish species have 2 - 3 *Tas1r2* genes. The vertebrate T1R receptors are not found in invertebrates (*110*) and are not related to a Drosophila taste receptor for a sugar trehalose encoded by the *Gr5a* gene (*111-116*).

These data allow us to establish changes in the T1R repertoire during evolution. Although many vertebrate and invertebrate animals detect taste of sugars and avidly consume them, receptors for sugars evolved independently in these two lineages. Most vertebrates have three clades of *Tas1r* genes, suggesting that all three of them evolved before separation of tetrapods and teleosts (*117*). However, another group concluded from a similar analysis that fish and mammalian *Tas1r1* and *Tas1r3* are orthologs, but fish and mammalian *Tas1r2* evolved independently (*106*). It appears that after separation of tetrapods and teleosts, duplication of *Tas1r2* occurred in the evolution of different fish species. Several Felidae species (*109*) and the chicken (*117, 119*) lost *Tas1r2*, and the tongueless western clawed frog lost all three *Tas1r* genes (*117*). Thus, pseudogenization of *Tas1r2* occurred multiple times in evolution.

Loss of the *Tas1r2* gene in cats and chickens must result in the absence of the T1R2+T1R3 sweet taste receptor, which corresponds to the lack of taste responses to sweeteners in these species (*121-125*). Interestingly, some birds recognize sugar taste (*126, 127*), suggesting that they may have a functional T1R2. *Tas1r2* pseudogenization and lack of sweet taste responsiveness in cats are probably results of these animals being obligate carnivores that do not seek

Table I. Numbers of functional and putatively functional T1R genes in vertebrate species

Species	T1R1 genes	T1R2 genes	T1R3 genes
Human (*Homo sapiens*)	1	1	1
Mouse (*Mus musculus*)	1	1	1
Rat (*Rattus norvegicus*)	1	1	1
Dog (*Canis familiaris*)	1	1	1
Cat (*Felis catus*)[a]	1	0	1
Opossum (*Monodelphis domestica*)	1	1	1
Chicken (*Gallus gallus*)	1	0	1
Frog (*Xenopus tropicals*)	0	0	0
Fugu fish (*Takifugu rubripes*)	1	2	1
Pufferfish (*Tetraodon nigroviridis*)	1	3	1
Zebrafish (*Danio rerio*)	1	2	1
Medaka fish (*Oryzias latipes*)	1	3	1

Data from (*89, 90, 106, 108-110, 117-120*).

[a]Cat *Tas1r2* and *Tas1r3* data from (*109*); cat *Tas1r1* is based on a cat genome sequence (GenBank accession # AANG01000989) with a corresponding predicted protein 90% identical to dog T1R1.

sugars in their food, and thus do not have a selective advantage of having a functional sweet taste receptor that recognizes sugars. Dogs, which are carnivores from the Canidae family, have a functional *Tas1r2* structure (*109, 117*) and are attracted to sugars (*128, 129*). Thus, loss of *Tas1r2* in cats and chickens may be a consequence of their feeding behavior that does not require a sweet taste receptor for proper food choice. However, a reverse causative relationship cannot be excluded, when a loss-of-function mutation in the *Tas1r2* gene resulted in loss of sweet taste sensation, which in turn altered feeding behavior of these animals. The impact of absence of all *Tas1r* genes in the tongueless western clawed frog on their taste responsiveness is not known.

Sequence divergence among the paralogous *Tas1r* genes appears to be governed by positive Darwinian selection (*117*). Most of the inferred positively selected sites are located in the N-terminal extracellular domains of the T1R proteins (*117*), which participate in ligand binding (*98, 130, 131*). Thus, these evolutionary changes are likely to affect ligand binding properties of T1Rs. This is consistent with currently available data on species differences in ligand specificity of the T1R2+T1R3 receptor (see Table II).

Similar conclusions were made based on analysis of within-species *TAS1R* sequence variation among humans. Evolutionary genetic analysis indicated that *TAS1R* variants have come to their current frequencies under positive natural

selection during population growth, which suggests that the coding sequence variants affect receptor function (*132*).

Although ligands for the T1R receptors have been experimentally confirmed only for humans and rodents, it is likely that their orthologs in other species have similar ligand specificities. However, variation in the T1R genes among species may reflect species differences in feeding behavior, for example presence of sweet proteins in some tropical fruits.

Allelic variation of the T1R genes and its role in individual variation in sweet taste responses

Humans

In humans of African, Asian, European, and Native American origin, all three *TAS1R* genes have multiple polymorphisms, which include those resulting in amino acid changes of the T1R proteins. The majority of amino acid sequence variation occurs in the N-terminus extracellular domain, where taste ligands are likely to bind to the taste receptors. *TAS1R2* was particularly diverse compared with other human genes: its rate of polymorphisms was in the top 5-10% of all human genes surveyed. The high rate of *TAS1R2* variation was predicted to result in variation in sweet taste perception (*132*), but such association has not yet been experimentally confirmed.

Rats

Several rat strains with different saccharin preferences did not differ in protein sequence of T1R3. Some non-protein-coding *Tas1r3* variants found among these strains did not result in marked differences in *Tas1r3* expression and thus are unlikely to affect T1R3 function. Therefore, rat strain differences in saccharin preferences depend on genes other than *Tas1r3* (*133*), even though rat T1R3 is a part of taste receptor responding to saccharin (*94, 108, 134*).

Mice

In initial studies that identified the mouse *Tas1r3* gene, several polymorphisms associated with sweetener preferences were detected (*94, 99, 105, 135, 136*). However, these studies lacked proper quantitative analyses of gene-phenotype associations. Reed et al (*60*) conducted a comprehensive

quantitative analysis of the *Tas1r3* sequence variants associated with saccharin preference using 30 genealogically diverse inbred mouse strains. Of the 89 polymorphisms detected within the ~6.7 kb genomic region including the *Tas1r3* gene, eight were significantly associated with saccharin preferences. Lack of differences in the *Tas1r3* gene expression in the taste tissues of mice with different *Tas1r3* alleles suggested that receptor function is likely to be affected by polymorphisms that change the amino acid sequence of the T1R3 protein. A coding polymorphism with the strongest association with saccharin preferences (Figure 2) resulted in the amino acid substitution of isoleucine to threonine at position 60 (I60T), in the extracellular N-terminus of the predicted T1R3 protein. Modeling of the T1R3 protein using the structure of the related mGluR1 receptor as a prototype has suggested that the I60T substitution introduces an extra N-terminal glycosilation site, which could affect dimerization of the receptor (*105*). However, this was not confirmed in a co-immunoprecipitation experiment (*97*). It was also suggested that this type of polymorphism could affect ligand binding (*60*). This prediction was subsequently confirmed in an *in vitro* study showing that a corresponding site-directed mutation changes binding affinity of the T1R3 protein to several sweeteners (*98*).

Ligands of sweet receptors

Two main approaches have been used to examine interactions of sweeteners with the T1R receptors. 1) *In vitro* heterologous expression experiments analyzed responses to taste stimuli in cultured cells transfected with T1Rs. 2) *In vivo* experiments examined effects of *Tas1r* genotype on sweet taste responses in mice. Two types of gene variation were studied *in vivo*: targeted mutations disrupting a gene (*95, 96*), and natural allelic variation (*53, 57, 60, 74, 75, 82, 85, 94*).

The *in vivo* and *in vitro* approaches to characterize taste receptor-ligand interactions have their own advantages and disadvantages (discussed in (*42, 137, 138*)) and thus should be considered as complementary. Limitations of the *in vitro* approach are related to the artificial character of the heterologous expression systems, which require substantial modification of conditions that exist *in vivo*. This, for example, includes using G proteins and other components of intracellular transduction that are not present in taste receptor cells *in vivo*, an absence of regulatory influences existing *in vivo*, and application of taste stimuli to the whole surface of transfected cells, while only apical ends of taste cells are exposed to tastants *in vivo* (*42, 137-140*).

Despite these limitations, *in vitro* studies were instrumental in characterizing ligand specificity of the T1R receptors and were generally consistent with *in vivo* results. A heterologously expressed combination of T1R2 and T1R3 responds to

a large number of sweeteners (Table II). The *in vitro* system reproduces *in vivo* species differences in sweet taste sensitivity. Several sweeteners (e.g., aspartame, cyclamate, neohesperidin dihydrochalcone, neotame and sweet proteins) are perceived as sweet by humans but not rodents (e.g., (*65, 69, 141*)). Correspondingly, human but not rodent T1R2 + T1R3 responds to these sweeteners *in vivo*. The ligand specificity of human T1R2 was also examined *in vivo* using *Tas1r2* knockout mice that express a human *TAS1R2* transgene under control of mouse *Tas1r2* promoter. While wild-type mice are indifferent to aspartame, glycyrrhizic acid, thaumatin and monellin, the transgenic mice display appetitive responses to these stimuli (*96*).

Consistent with the *in vitro* results, *Tas1r2* and *Tas1r3* knockout mice are deficient in taste responses to sweeteners (*95, 96*). Effects of *Sac/Tas1r3* alleles of existing inbred strains on responses to sweeteners were shown in several studies (*53, 57, 60, 74, 75, 85, 94*), with the most detailed analysis reported in (*82*). Patterns of effects of the natural and genetically engineered *Tas1r3* alleles were not completely identical: the natural allelic variation affected taste responses to sweeteners (including D-amino acids) but not to L-amino acids, non-chiral glycine or umami taste stimuli. This pattern was more similar to changes found in *Tas1r2* knockout mice. The likely reason for this is that the null allele of *Tas1r3* prevents formation of heteromeric receptors with both T1R1 and T1R2, thus affecting responses to all ligands of the T1R1 + T1R3 and T1R2 + T1R3 receptors. Natural allelic variation of *Tas1r3* affects binding affinity of the T1R3 protein for sweeteners (*98*), but it does not affect responses of heterologously expressed T1R1 + T1R3 to L-amino acids (*97*), which corresponds to effects of *Tas1r3* allelic variation *in vivo*. The lack of effect of the natural allelic variation of *Tas1r3* on taste responses to glycine that activates the T1R2 + T1R3 receptor *in vitro* and has a sucrose-like taste to mice (*81*) can be explained by several possible mechanisms: (i) glycine binding to the T1R3 receptor at a site that is not affected by the polymorphic variants; (ii) glycine binding to the T1R2 receptor; (iii) existence of another taste receptor binding glycine.

Although data on ligand specificity of the T1R receptors are generally consistent between the *in vitro* and *in vivo* studies, some results are conflicting. For example, sweet L-amino acids (e.g., L-proline and L-threonine) do not activate the sweet receptor combination of T1R2 and T1R3 (*97*). Perhaps, most difficult for interpretation are negative results of *in vitro* experiments, when lack of response to a taste stimulus may either indicate true lack of receptor-ligand interaction, or it may be an artifact of the *in vitro* system. For example, rat T1R2 + T1R3 responded to galactose, glucose, lactose and maltose in (*108*) but not in (*94*) (Table II).

Although the *in vivo* approach to characterize receptor-ligand interactions is more laborious, it overcomes some methodological limitations of the *in vitro*

Table II. *In vitro* and *in vivo* analyses of ligand specificity
of the T1R receptors.

Ligand	*In vitro* effects (T1R2+T1R3)			*In vivo* effects[a] (mouse)		
	Human	*Rat*	*Mouse*	*Tas1r2-knockout*	*Tas1r3-knockout*	*Natural Tas1r3 alleles[b]*
	(108, 134, 142-146)	*(94, 108, 134)*	*(97, 142, 143)*	*(96)*	*(95, 96)*	*(53, 57, 60, 74, 75, 82, 85, 94)*
Sugars						
Fructose	+	+			+	
Galactose	+	+/-[c]				
Glucose	+	+/-[c]		+	+/-[d]	
Lactose	+	+/-[c]				
Maltose	+	+/-[c]		+	+	
Palatinose	-					
Sucrose	+	+	+	+	+	+
Universal sweeteners						
Acesulfame K	+	+		+	+	+
Dulcin	+	+				
Guanidinoacetic acid sweeteners		+				
Saccharin	+	+	+	+	+	+
SC45647					+	+
Sorbitol					+	
Steviozide	+	+				
Sucralose	+	+			+	
Primate-specific sweeteners						
Aspartame	+	-	-			
Cyclamate	+	-	-			
Neohesperidin dihydrochalcone	+	-				
Neotame	+	-				
Sweet proteins						
Brazzein	+		-			
Monellin	+	-	-			
Neoculin	+					
Thaumatin	+	-				

Continued on next page.

Table II. *Continued.*

Ligand	In vitro effects (T1R2+T1R3)			In vivo effects[a] (mouse)		
	Human	Rat	Mouse	Tas1r2-knockout	Tas1r3-knockout	Natural Tas1r3 alleles[b]
Amino acids						
D-alanine			+	+	+	
D-asparginine			+	+	+	
D-glutamine			+			
D-histidine			+			
D-phenylalanine			+	+	+	+
D-tryptophan	+	+	+	+	+	+
Glycine	+	+	+			-
L-alanine			-	-	+	-
L-arginine				-	+*	
L-asparginine			-	-	+*	
L-aspartate				-	+*	
L-glutamate	-		-	-	+/-[e]	-
L-glutamine						-
L-phenylalanine			-			
L-proline			-			-
L-serine			-	-	+	
L-threonine			-			
L-tryptophan	-	-				
Umami compounds						
IMP				-	+	-
L-AP4				-	+*	

Responses only to sweet-tasting stimuli, umami-tasting stimuli and amino acids are shown.

+ *In vitro*: a response to a taste stimulus. *In vivo*: effect of genotype on a response to a taste stimulus.

- *In vitro*: lack of a response to a taste stimulus. *In vivo*: lack of effect of genotype on a response to a taste stimulus.

*Responses to taste stimuli mixed with IMP.

[a]Behavioral (long-term preference tests and brief-access lick tests) and neural (chorda tympani and/or glossopharyngeal nerve) responses.

Continued on next page.

[b]Effects of natural *Tas1r3* alleles on sweet taste responsiveness were examined in inbred, recombinant inbred, hybrid (*53, 57, 60, 74, 75, 82, 85*), congenic (*75*) and transgenic (*94*) mice.

[c]Rat T1R2 + T1R3 responded to galactose, glucose, lactose and maltose in (*108*) but not in (*94*). The difference in response patterns was suggested to be attributed to different G proteins co-transfected with T1Rs: $G\alpha15$ and $G\alpha16$-Gz in (*94*) or $G\alpha15/i1$ in (*108*).

[d]In *Tas1r3* knockout mice, changes in behavioral and chorda tympani responses to glucose were significant in (*96*) but not significant in (*95*).

[e]In *Tas1r3* knockout mice, chorda tympani responses to MSG were reduced (*95, 96*), but glossopharyngeal nerve responses to MSG were not affected, even in presence of IMP (*95*).

In *Tas1r2* and *Tas1r3* knockout mice, concentrated solutions of sugars elicited reduced but not completely eliminated taste responses (*95, 96*). These residual responses were completely eliminated in *Tas1r2/Tas1r3* double knockout mice (*96*). This suggests that T1R2 and T1R3 may function on their own as low-affinity sugar receptors, probably as homodimers. Consistent with this, heterologously expressed T1R3 alone responded to 0.5 M sucrose, but not to lower sucrose concentrations (<0.3 M) or to artificial sweeteners (*96*).

studies. Thus, the *in vivo* and *in vitro* approaches complement each other in providing conclusive characteristics of taste receptors. Both the *in vivo* and *in vitro* data suggest that perception of most sweet taste stimuli occurs via activation of the T1R2 + T1R3 receptor.

Sweet taste genes other than T1R

Multigenic inheritance of sweetener preferences was shown in a number of studies (*54, 57, 58, 72, 84*). Accordingly, several lines of evidence indicated that allelic variation of the mouse *Tas1r3* locus does not account for all the genetically determined differences in sweetener preferences. Analysis of multiple inbred mouse strains has shown that the *Tas1r3* genotype explains only 78% of genetic variation in saccharin preference (*60*) (Figure 2). In the B6 × 129 F2 cross, the *Tas1r3* genotype explained 64 - 96% of genetic variation in preference scores for different sweeteners, 10 - 35% of genetic variation in sweetener intakes, and 37 - 92% of genetic variation in chorda tympani responses to sweeteners (*74, 82*). Responses to sweeteners in brief-access tests differ among mouse strains but do not seem to be associated with *Tas1r3* alleles (*62*). Thus, a substantial part of the genetic variation in taste responses to sweeteners among mouse strains is attributed to loci other than *Tas1r3*. Taste responses to glycine provide a remarkable example: although there are substantial differences among mouse strains in responses to glycine (*57, 69*), this variation is not attributed to

the *Tas1r3* genotypes (*63, 82*). Consistent with the mouse work suggesting effects of genes other than *Tas1r3*, variation in saccharin preferences in rats is not associated with sequence variants of the rat *Tas1r3* gene (*133*), and therefore it must be attributed to the effects of other genes (see also (*147*)).

One of the genetic loci affecting sweet taste responses is *dpa* (D-phenylalanine aversion), which affects ability of mice to generalize conditioned taste aversion between D-phenylalanine and sucrose, inferring that *dpa* affects ability to detect the sweetness of D-phenylalanine. The *dpa* locus also affects responses of sucrose-sensitive fibers of the chorda tympani nerve to D-phenylalanine. B6 mice carry a dominant allele of *dpa* that determines an ability to recognize the sweetness of D-phenylalanine, whereas BALB/c mice carry a recessive *dpa* allele conferring inability to detect D-phenylalanine sweetness. The *dpa* locus was mapped to proximal chromosome 4, a region distinct from the subtelomeric chromosome 4 harboring the *Tas1r* genes (*148-151*). It was suggested that the *dpa* locus can also affect responses to sweeteners in two-bottle tests (*58*). Consistent with this, a locus on proximal chromosome 4, in the *dpa* region, was found to be suggestively linked to consumption of, and chorda tympani responses to, sucrose (*74*). An epistatic interaction between effects on sucrose intake of this locus and the *Tas1r3* locus suggests that these two loci may encode interacting components of sweet taste transduction (*74*).

To study the non-*Tas1r* genes involved in sweet taste, we began selective breeding of mouse lines divergent in sweetener consumption. To eliminate the *Tas1r3* effects, we crossed B6 inbred mice with 129.B6-*Tas1r3* congenic mice. As a result, all mice in this cross had only B6 *Tas1r3* allele. Despite genetic identity at the *Tas1r3* locus, mice from the F2 generation varied widely in consumption of 20 mM saccharin and 30 mM glycine, but there was no correlation between these two traits. We therefore began selective breeding of mouse lines with high and low saccharin intakes, and with high and low glycine preferences. The substantial divergence of the selected lines (Figure 3) demonstrates that much of genetic variation in mouse sweet taste responses depends on genes other than *Tas1r3* (*152*). These other genes may be involved in peripheral or central sweet taste mechanisms.

Sweet taste, T1R genes and other complex phenotypes

Sweet taste perception involves hedonic processes and is an important factor affecting food choice and consumption. Therefore, genetic variation in sweet taste may affect complex phenotypes that depend on oral consumption of nutrients or drugs, or involve pleasure-seeking behavior. Some (but not all) data suggest that human sweet taste responsiveness is associated with obesity (*39, 41, 153, 154*) and alcohol intake (*155-159*).
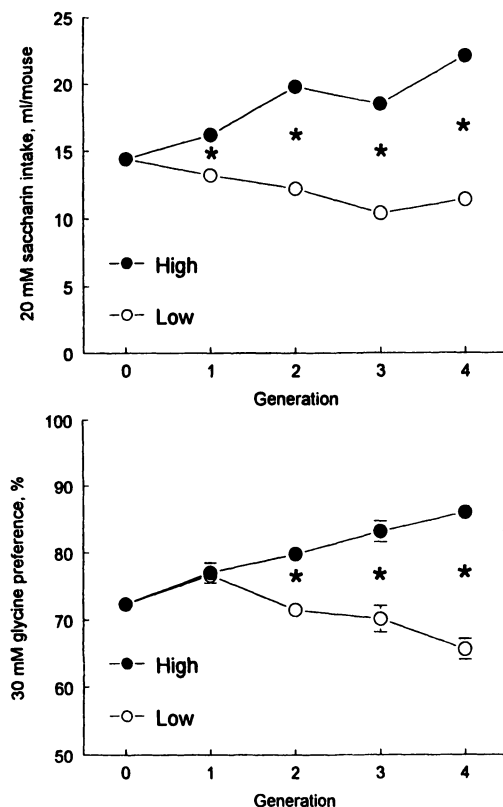
*Figure 3. Selective breeding of mice with divergent sweetener consumption independent of the Tas1r3 gene. Data for B6 × 129.B6-Tas1r3 F2 (S0) mice and mice from four generations of selective breeding (S1 - S4); Means ± SE; *P < 0.05.*

Top. Intake of 20 mM saccharin. "High" – line selected for high saccharin consumption; "Low" – line selected for low saccharin consumption.

Bottom. Preference for 30 mM glycine. "High" – line selected for high glycine preference; "Low" – line selected for low glycine preference.

Studies of rodents elucidated some genetic factors and physiological mechanisms for the association between sweet taste and alcohol. Perception of the sweet taste component of ethanol by rodents was shown in behavioral and neurophysiological experiments (reviewed in (*160*)). Conditioned taste aversion generalizes between ethanol and sucrose (*161-164*). Electrophysiological recordings indicate that lingual application of ethanol activates sweetener-responsive neural fibers in the gustatory nerves (*165, 166*) and sweetener-responsive units in the nucleus of the tractus solitarius (*167, 168*); this activity is blocked by application of gurmarin, a peripheral antagonist of sweet taste (*168*). Central mechanisms that determine hedonic responses to ethanol and sweeteners also overlap and involve opioidergic, serotonergic and dopaminergic brain neurotransmitter systems (*169-173*). In addition, there may be common signals related to the caloric value of ethanol and sugars (*174-179*).

Positive correlations between preferences for ethanol and sweeteners in rats and mice were found among various strains and in segregating crosses (*45, 46, 52, 59, 70, 72, 84, 180-187*). This genetically determined association can be due to any of the mechanisms described above, including peripheral or central taste processing, or postingestive reward.

Genetic analysis of a cross between mice from a high ethanol- and sweetener-preferring B6 strain and a low ethanol- and sweetener-preferring 129 strain suggested that the strain differences in sweetener and ethanol consumption depend on relatively small and partially overlapping sets of genes (*72*). One of these genetic loci, *Ap3q* (alcohol preference 3 QTL), maps to a region of chromosome 4 overlapping with the *Tas1r3* gene. This suggests that the *Tas1r3* gene is identical to the *Ap3q* locus and that its pleiotropic effect on ethanol consumption is mediated by genetic differences in perception of the sweet taste component of ethanol flavor: higher hedonic attractiveness of ethanol sweetness results in higher ethanol intake by B6 mice (*188*). In addition to the *Tas1r3* gene, there are other genetic loci with pleiotropic effects on ethanol and sweetener intake (*189, 190*).

Hedonic responses to sweet taste are considered as a biological marker of predisposition to alcoholism (*46, 156, 157*), but genes responsible for this association are still unknown. Sweet taste phenotypes and alleles of the T1R genes have a potential to be used as biomarkers for diagnosing predisposition to alcoholism and other diseases.

Concluding remarks

The data presented in this review demonstrate that sweet taste has a complex genetic architecture. Variation of the sweet taste receptor genes contributes to differences in sweet taste perception within and between species. In addition to

the sweet taste receptors, a number of other genes influence sweet taste responses. These yet unknown genes are likely to be involved in different stages of sweet taste processing pathway, including taste transduction and transmission in the periphery and in the brain, and interaction of taste processing with homeostatic systems involved in the regulation of ingestive behavior and reward. There is evidence that responses to different sweeteners are affected by different sets of genes. Some data suggest that individual differences in sweet taste perception are associated with obesity and predisposition to alcoholism.

The genetic approach has proven to be instrumental for discovering sweet taste receptors, detecting their functionally important polymorphic sites, and characterizing their ligand specificity. The *in vivo* approach to analyze receptor-ligand interactions overcomes some methodological limitations of *in vitro* studies. Thus, the *in vivo* and *in vitro* approaches complement each other in providing conclusive characteristics of taste receptors.

In recent years, genetics has experienced dramatic progress, with genome sequencing completed for several species, including the mouse and the human. These advances in genomic resources tremendously facilitate genetic studies and make them an even more powerful approach for understanding mechanisms of sweet taste.

Acknowledgements

References

1. Witt, M.; Reutter, K.; Miller, I. J., In *Handbook of Olfaction and Gustation*, 2nd ed.; Doty, R. L., Ed. Marcel Dekker: New York, 2003, pp 651-677.

2. Rolls, E. T.; Scott, T. R., In *Handbook of Olfaction and Gustation*, 2nd ed.; Doty, R. L., Ed. Marcel Dekker: New York, 2003, pp 679-705.

3. Teff, K. L.; Devine, J.; Engelman, K. *Physiol. Behav.* **1995**, *57*, 1089-1095.

4. Grill, H. J.; Berridge, K. C.; Ganster, D. J. *Am. J. Physiol.* **1984**, *246*, R88-R95.

5. Malaisse, W. J.; Vanonderbergen, A.; Louchami, K.; Jijakli, H.; Malaisse-Lagae, F. *Cell Signal* **1998**, *10*, 727-733.

6. Yamamoto, T.; Sako, N.; Maeda, S. *Physiol. Behav.* **2000**, *69*, 345-350.

7. Marks-Kaufman, R.; Hamm, M. W.; Barbato, G. F. *J Am Coll Nutr* **1989**, *8*, 9-14.

40

8. Melchior, J. C.; Rigaud, D.; Colas-Linhart, N.; Petiet, A.; Girard, A.; Apfelbaum, M. *Physiol. Behav.* **1991**, *50*, 941-944.
9. O'Doherty, J. P.; Deichmann, R.; Critchley, H. D.; Dolan, R. J. *Neuron* **2002**, *33*, 815-826.
10. Cooper, S. J.; Barber, D. J. *Pharmacol Biochem Behav* **1994**, *47*, 541-546.
11. Muscat, R.; Kyprianou, T.; Osman, M.; Phillips, G.; Willner, P. *Pharmacol Biochem Behav* **1991**, *40*, 209-213.
12. Schneider, L. H. *Ann N Y Acad Sci* **1989**, *575*, 307-319.
13. Yirmiya, R.; Lieblich, I.; Liebeskind, J. C. *Brain. Res.* **1988**, *438*, 339-342.
14. Blass, E. M.; Watt, L. B. *Pain* **1999**, *83*, 611-623.
15. Blass, E. M.; Shah, A. *Chem. Senses* **1995**, *20*, 29-35.
16. Calcagnetti, D. J.; Holtzman, S. G. *Brain Res Bull* **1992**, *29*, 859-864.
17. Carbajal, R.; Chauvet, X.; Couderc, S.; Olivier-Martin, M. *BMJ* **1999**, *319*, 1393-1397.
18. Ors, R.; Ozek, E.; Baysoy, G.; Cebeci, D.; Bilgen, H.; Turkuner, M.; Basaran, M. *Eur J Pediatr* **1999**, *158*, 63-66.
19. Laeng, B.; Berridge, K. C.; Butter, C. M. *Appetite* **1993**, *21*, 247-254.
20. Hajnal, A.; Takenouchi, K.; Norgren, R. *J. Neurosci.* **1999**, *19*, 7182-7190.
21. Atchley, D. P.; Weaver, K. L.; Eckel, L. A. *Physiol. Behav.* **2005**, *86*, 265-271.
22. Curtis, K. S.; Stratford, J. M.; Contreras, R. J. *Physiol. Behav.* **2005**, *86*, 281-286.
23. Simon, S. A.; Liu, L.; Erickson, R. P. *Am J Physiol Regul Integr Comp Physiol* **2003**, *284*, R1494-1505.
24. Shigemura, N.; Ohta, R.; Kusakabe, Y.; Miura, H.; Hino, A.; Koyano, K.; Nakashima, K.; Ninomiya, Y. *Endocrinol.* **2004**, *145*, 839-847.
25. Mook, D. G. *J. Comp. Physiol. Psychol.* **1963**, *56*, 645-659.
26. Steiner, J. E.; Glaser, D.; Hawilo, M. E.; Berridge, K. C. *Neurosci Biobehav Rev* **2001**, *25*, 53-74.
27. Berridge, K. C. *Neurosci Biobehav Rev* **2000**, *24*, 173-198.
28. Reed, D. R.; McDaniel, A. H. *BMC Oral Health* **2006**, *6 Suppl 1*, S17.
29. Reed, D. R.; Tanaka, T.; McDaniel, A. H. *Physiol. Behav.* **2006**, *88*, 215-226.
30. Spector, A. C., In *Handbook of Olfaction and Gustation*, 2nd ed.; Doty, R. L., Ed. Marcel Dekker: New York, 2003, pp 861-879.
31. Gent, J. F.; Bartoshuk, L. M. *Chem. Senses* **1983**, *7*, 265-272.
32. Looy, H.; Weingarten, H. P. *Physiol. Behav.* **1992**, *52*, 75-82.
33. Looy, H.; Callaghan, S.; Weingarten, H. P. *Physiol. Behav.* **1992**, *52*, 219-225.
34. Beauchamp, G. K.; Moran, M. *Appetite* **1982**, *3*, 139-152.
35. Desor, J. A.; Greene, L. S.; Maller, O. *Science* **1975**, *190*, 686-687.
36. Greene, L. S.; Desor, J. A.; Maller, O. *J. Comp. Physiol. Psychol.* **1975**, *89*, 279-284.

37. Falciglia, G. A.; Norton, P. A. *J. Am. Diet. Ass.* **1994**, *94*, 154-158.
38. Looy, H.; Weingarten, H. P. *Chem. Senses* **1991**, *16*, 123-130.
39. McDaniel, A. H.; Reed, D. R., In *Genomics and Proteomics in Nutrition*; Berdanier, C. D.; Moustaid-Moussa, N., Eds. Marcel Dekker, Inc: New York, 2003, pp 51-70.
40. Reed, D. R.; Li, X.; Bachmanov, A. A.; Mascioli, K.; Beauchamp, G. K., In *Progress in Obesity Research*; Medeiros-Neto, G.; Halpern, A.; Bouchard, C., Eds. John Libbey Eurotext Ltd.: London, 2003; Vol. 9, pp 304-306.
41. Reed, D. R.; Bachmanov, A. A.; Beauchamp, G. K.; Tordoff, M. G.; Price, R. A. *Behav. Genet.* **1997**, *27*, 373-387.
42. Drayna, D. *Annu Rev Genomics Hum Genet* **2005**, *6*, 217-235.
43. Kim, U. K.; Breslin, P. A.; Reed, D.; Drayna, D. *J Dent Res* **2004**, *83*, 448-453.
44. Dess, N. K.; Minor, T. R. *Animal Learn. Behav.* **1996**, *24*, 105-115.
45. Overstreet, D. H.; Rezvani, A. H.; Parsian, A. *Alcohol Alcohol.* **1999**, *34*, 378-385.
46. Kampov-Polevoy, A. B.; Garbutt, J. C.; Janowsky, D. S. *Alcohol Alcohol.* **1999**, *34*, 386-395.
47. Frank, M. E.; Wada, Y.; Makino, J.; Mizutani, M.; Umezawa, H.; Katsuie, Y.; Hettinger, T. P.; Blizard, D. A. *Behav. Genet.* **2004**, *34*, 465-476.
48. Lewis, S. R.; Ahmed, S.; Dym, C.; Khaimova, E.; Kest, B.; Bodnar, R. J. *Physiol. Behav.* **2005**, *85*, 546-556.
49. Fuller, J. L. *J. Hered.* **1974**, *65*, 33-36.
50. Nachman, M. *J Comp Physiol Psychol* **1959**, *52*, 451-457.
51. Hoshishima, K.; Yokoyama, S.; Seto, K. *Am. J. Physiol.* **1962**, *202*, 1200-1204.
52. Rodgers, D. A.; McClearn, G. E. *Q J Stud Alcohol* **1964**, *25*, 26-35.
53. Lush, I. E. *Genet. Res.* **1989**, *53*, 95-99.
54. Ramirez, I.; Fuller, J. L. *Physiol. Behav.* **1976**, *16*, 163-168.
55. Pelz, W. E.; Whitney, G.; and Smith, J. C. *Physiol. Behav.* **1973**, *10*, 263-265.
56. Stockton, M. D.; Whitney, G. *J. Comp. Physiol. Psychol.* **1974**, *86*, 62-68.
57. Lush, I. E.; Hornigold, N.; King, P.; Stoye, J. P. *Genet. Res.* **1995**, *66*, 167-174.
58. Capeless, C. G.; Whitney, G. *Chem. Senses* **1995**, *20*, 291-298.
59. Belknap, J. K.; Crabbe, J. C.; Young, E. R. *Psychopharmacol.* **1993**, *112*, 503-510.
60. Reed, D. R.; Li, S.; Li, X.; Huang, L.; Tordoff, M. G.; Starling-Roney, R.; Taniguchi, K.; West, D. B.; Ohmen, J. D.; Beauchamp, G. K.; Bachmanov, A. A. *J. Neurosci.* **2004**, *24*, 938-946.
61. Kotlus, B. S.; Blizard, D. A. *Physiol. Behav.* **1998**, *64*, 37-47.
62. Dotson, C. D.; Spector, A. C. *Chem. Senses* **2004**, *29*, 489-498.

63. Eylam, S.; Spector, A. C. *Chem. Senses* **2004**, *29*, 639-649.
64. Ninomiya, Y.; Higashi, T.; Katsukawa, H.; Mizukoshi, T.; Funakoshi, M. *Brain Res.* **1984**, *322*, 83-92.
65. Inoue, M.; McCaughey, S. A.; Bachmanov, A. A.; Beauchamp, G. K. *Chem. Senses* **2001**, *26*, 915-923.
66. Frank, M. E.; Blizard, D. A. *Physiol. Behav.* **1999**, *67*, 287-297.
67. Ninomiya, Y.; Mizukoshi, T.; Higashi, T.; Katsukawa, H.; Funakoshi, M. *Brain Res.* **1984**, *302*, 305-314.
68. Bachmanov, A. A.; Li, S.; Li, X.; Lu, K.; Tordoff, M. G.; West, D. B.; Ohmen, J. D.; Reed, D. R.; Beauchamp, G. K. *Chem. Senses* **2002**, *27*, A95-A96.
69. Bachmanov, A. A.; Tordoff, M. G.; Beauchamp, G. K. *Chem. Senses* **2001**, *26*, 905-913.
70. Bachmanov, A. A.; Tordoff, M. G.; Beauchamp, G. K. *Alcohol. Clin. Exp. Res.* **1996**, *20*, 201-206.
71. Bachmanov, A. A.; Reed, D. R.; Tordoff, M. G.; Price, R. A.; Beauchamp, G. K. *Physiol. Behav.* **2001**, *72*, 603-613.
72. Bachmanov, A. A.; Reed, D. R.; Tordoff, M. G.; Price, R. A.; Beauchamp, G. K. *Behav. Genet.* **1996**, *26*, 563-573.
73. Bachmanov, A. A.; Tordoff, M. G.; Beauchamp, G. K. *J. Nutr.* **2000**, *130*, 935S-941S.
74. Bachmanov, A. A.; Reed, D. R.; Ninomiya, Y.; Inoue, M.; Tordoff, M. G.; Price, R. A.; Beauchamp, G. K. *Mamm. Genome* **1997**, *8*, 545-548.
75. Li, X.; Inoue, M.; Reed, D. R.; Huque, T.; Puchalski, R. B.; Tordoff, M. G.; Ninomiya, Y.; Beauchamp, G. K.; Bachmanov, A. A. *Mamm. Genome* **2001**, *12*, 13-16.
76. Bachmanov, A. A.; Reed, D. R.; Ninomiya, Y.; Inoue, M.; Tordoff, M. G.; Price, R. A.; Beauchamp, G. K. *Chem. Senses* **1997**, *22*, 642.
77. Sclafani, A.; Glendinning, J. I. *Am J Physiol Regul Integr Comp Physiol* **2005**, *289*, R712-720.
78. Sclafani, A. *Physiol. Behav.* **2006**, *87*, 734-744.
79. Sclafani, A. *Physiol. Behav.* **2006**, *87*, 745-756.
80. Sclafani, A. *Physiol. Behav.* **2006**, *89*, 525-530.
81. Manita, S.; Bachmanov, A. A.; Li, X.; Beauchamp, G. K.; Inoue, M. *Chem. Senses* **2006**, *31*, 785-793.
82. Inoue, M.; Reed, D. R.; Li, X.; Tordoff, M. G.; Beauchamp, G. K.; Bachmanov, A. A. *J. Neurosci.* **2004**, *24*, 2296-2303.
83. Belknap, J. K.; Crabbe, J. C.; Plomin, R.; McClearn, G. E.; Sampson, K. E.; O'Toole, L. A.; Gora-Maslak, G. *Behav. Genet.* **1992**, *22*, 81-100.
84. Phillips, T. J.; Crabbe, J. C.; Metten, P.; Belknap, J. K. *Alcohol. Clin. Exp. Res.* **1994**, *18*, 931-941.
85. Blizard, D. A.; Kotlus, B.; Frank, M. E. *Chem. Senses* **1999**, *24*, 373-385.
86. Hoon, M. A.; Ryba, N. J. P. *Journal of Dental Research* **1997**, *76*, 831-838.

87. The Human Genome Organisation (HUGO) Gene Nomenclature Committee, http://www.gene.ucl.ac.uk/nomenclature/.

88. Rules for Nomenclature of Genes, Genetic Markers, Alleles, and Mutations in Mouse and Rat, http://www.informatics.jax.org/mgihome/nomen/gene.shtml.

89. Hoon, M. A.; Adler, E.; Lindemeier, J.; Battey, J. F.; Ryba, N. J.; Zuker, C. S. *Cell* **1999**, *96*, 541-551.

90. Bachmanov, A. A.; Li, X.; Reed, D. R.; Ohmen, J. D.; Li, S.; Chen, Z.; Tordoff, M. G.; de Jong, P. J.; Wu, C.; West, D. B.; Chatterjee, A.; Ross, D. A.; Beauchamp, G. K. *Chem. Senses* **2001**, *26*, 925-933.

91. Li, X.; Bachmanov, A. A.; Li, S.; Chen, Z.; Tordoff, M. G.; Beauchamp, G. K.; de Jong, P. J.; Wu, C.; Chen, L.; West, D. B.; Ross, D. A.; Ohmen, J. D.; Reed, D. R. *Mamm. Genome* **2002**, *13*, 5-19.

92. Wong, G. T.; Gannon, K. S.; Margolskee, R. F. *Nature* **1996**, *381*, 796-800.

93. Bachmanov, A. A.; Reed, D. R.; Li, X.; Beauchamp, G. K. *Pure and Applied Chemistry* **2002**, *74*, 1135-1140.

94. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S. *Cell* **2001**, *106*, 381-390.

95. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili, Z.; Varadarajan, V.; Zou, S.; Jiang, P.; Ninomiya, Y.; Margolskee, R. F. *Science* **2003**, *301*, 850-853.

96. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N. J.; Zuker, C. S. *Cell* **2003**, *115*, 255-266.

97. Nelson, G.; Chandrashekar, J.; Hoon, M. A.; Feng, L.; Zhao, G.; Ryba, N. J.; Zuker, C. S. *Nature* **2002**, *416*, 199-202.

98. Nie, Y.; Vigues, S.; Hobbs, J. R.; Conn, G. L.; Munger, S. D. *Curr Biol* **2005**, *15*, 1948-1952.

99. Montmayeur, J. P.; Liberles, S. D.; Matsunami, H.; Buck, L. B. *Nat. Neurosci.* **2001**, *4*, 492-498.

100. Kiuchi, S.; Yamada, T.; Kiyokawa, N.; Saito, T.; Fujimoto, J.; Yasue, H. *Cytogenet Genome Res* **2006**, *115*, 51-61.

101. Kolakowski, L. F., Jr. *Receptors Channels* **1994**, *2*, 1-7.

102. Attwood, T. K.; Findlay, J. B. *Protein Eng* **1994**, *7*, 195-203.

103. GPCRDB: Information system for G protein-coupled receptors (GPCRs), http://www.gpcr.org/7tm/.

104. Fredriksson, R.; Lagerstrom, M. C.; Lundin, L. G.; Schioth, H. B. *Mol Pharmacol* **2003**, *63*, 1256-1272.

105. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. *Nat. Genet.* **2001**, *28*, 58-63.

106. Ishimaru, Y.; Okada, S.; Naito, H.; Nagai, T.; Yasuoka, A.; Matsumoto, I.; Abe, K. *Mech Dev* **2005**, *122*, 1310-1321.

107. Pin, J. P.; Galvez, T.; Prezeau, L. *Pharmacol Ther* **2003**, *98*, 325-354.

108. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696.

109. Li, X.; Li, W.; Wang, H.; Cao, J.; Maehashi, K.; Huang, L.; Bachmanov, A. A.; Reed, D. R.; Legrand-Defretin, V.; Beauchamp, G. K.; Brand, J. G. *PLoS Genet* **2005**, *1*, 27-35.

110. Bjarnadottir, T. K.; Fredriksson, R.; Schioth, H. B. *Gene* **2005**, *362*, 70-84.

111. Chyb, S.; Dahanukar, A.; Wickens, A.; Carlson, J. R. *Proc. Natl. Acad. Sci. USA* **2003**, *100 Suppl 2*, 14526-14530.

112. Dahanukar, A.; Foster, K.; van der Goes van Naters, W. M.; Carlson, J. R. *Nat. Neurosci.* **2001**, *4*, 1182-1186.

113. Hallem, E. A.; Dahanukar, A.; Carlson, J. R. *Annu Rev Entomol* **2006**, *51*, 113-135.

114. Isono, K.; Morita, H.; Kohatsu, S.; Ueno, K.; Matsubayashi, H.; Yamamoto, M. T. *Chem. Senses* **2005**, *30 Suppl 1*, i275-i276.

115. Inomata, N.; Goto, H.; Itoh, M.; Isono, K. *Genetics* **2004**, *167*, 1749-1758.

116. Ueno, K.; Ohta, M.; Morita, H.; Mikuni, Y.; Nakajima, S.; Yamamoto, K.; Isono, K. *Curr Biol* **2001**, *11*, 1451-1455.

117. Shi, P.; Zhang, J. *Mol Biol Evol* **2006**, *23*, 292-300.

118. Liao, J.; Schultz, P. G. *Mamm Genome* **2003**, *14*, 291-301.

119. Lagerstrom, M. C.; Hellstrom, A. R.; Gloriam, D. E.; Larsson, T. P.; Schioth, H. B.; Fredriksson, R. *PLoS Comput Biol* **2006**, *2*, e54.

120. Bjarnadottir, T. K.; Gloriam, D. E.; Hellstrand, S. H.; Kristiansson, H.; Fredriksson, R.; Schioth, H. B. *Genomics* **2006**, *88*, 263-273.

121. Beauchamp, G. K.; Maller, O.; Rogers, J. G. *J. Comp. Physiol. Psychol.* **1977**, *91*, 1118-1127.

122. Bartoshuk, L. M.; Jacobs, H. L.; Nichols, T. L.; Hoff, L. A.; Ryckman, J. J. *J Comp Physiol Psychol* **1975**, *89*, 971-975.

123. Halpern, B. P. *Am J Physiol* **1962**, *203*, 541-544.

124. Ganchrow, J. R.; Steiner, J. E.; Bartana, A. *Dev Psychobiol* **1990**, *23*, 103-117.

125. Kare, M. R., In *Physiological and Behavioral Aspects of Taste*; Kare, M. R.; Halpern, B. P., Eds. The University of Chicago Press: Chicago, 1961, pp 6-15.

126. Matson, K. D.; Millam, J. R.; Klasing, K. C. *Zoo Biol.* **2001**, *20*, 1-13.

127. Matson, K. D.; Millam, J. R.; Klasing, K. C. *Appl Anim Behav Sci* **2000**, *69*, 313-326.

128. Grace, J.; Russek, M. *Physiology & Behavior* **1969**, *4*, 553-558.

129. Ferrell, F. *Neurosci Biobehav Rev* **1984**, *8*, 199-203.

130. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14258-14263.

131. Jiang, P.; Cui, M.; Ji, Q.; Snyder, L.; Liu, Z.; Benard, L.; Margolskee, R. F.; Osman, R.; Max, M. *Chem. Senses* **2005**, *30 Suppl 1*, i17-i18.

132. Kim, U. K.; Wooding, S.; Riaz, N.; Jorde, L. B.; Drayna, D. *Chem. Senses* **2006**, *31*, 599-611.

133. Lu, K.; McDaniel, A. H.; Tordoff, M. G.; Li, X.; Beauchamp, G. K.; Bachmanov, A. A.; VanderWeele, D. A.; Chapman, C. D.; Dess, N. K.; Huang, L.; Wang, H.; Reed, D. R. *Chem. Senses* **2005**, *30*, 231-240.

134. Winnig, M.; Bufe, B.; Meyerhof, W. *BMC Neurosci* **2005**, *6*, 22.

135. Kitagawa, M.; Kusakabe, Y.; Miura, H.; Ninomiya, Y.; Hino, A. *Biochem. Biophys. Res. Commun.* **2001**, *283*, 236-242.

136. Sainz, E.; Korley, J. N.; Battey, J. F.; Sullivan, S. L. *J. Neurochem.* **2001**, *77*, 896-903.

137. Bachmanov, A. A. *Chem. Senses* **2005**, *30 Suppl 1*, i82-i83.

138. Meyerhof, W. *Rev Physiol Biochem Pharmacol* **2005**, *154*, 37-72.

139. Behrens, M.; Bartelt, J.; Reichling, C.; Winnig, M.; Kuhn, C.; Meyerhof, W. *J Biol Chem* **2006**, *281*, 20650-20659.

140. Ueda, T.; Ugawa, S.; Yamamura, H.; Imaizumi, Y.; Shimada, S. *J. Neurosci.* **2003**, *23*, 7376-7380.

141. Danilova, V.; Hellekant, G.; Tinti, J. M.; Nofre, C. *J Neurophysiol* **1998**, *80*, 2102-2112.

142. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Margolskee, R. F.; Max, M. *J Biol Chem* **2004**, *279*, 45068-45075.

143. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L. A.; Benard, L. M.; Osman, R.; Max, M.; Margolskee, R. F. *J Biol Chem* **2005**, *280*, 34296-34305.

144. Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Osman, R.; Margolskee, R. F.; Max, M. *J Biol Chem* **2005**, *280*, 15238-15246.

145. Galindo-Cuspinera, V.; Winnig, M.; Bufe, B.; Meyerhof, W.; Breslin, P. A. *Nature* **2006**, *441*, 354-357.

146. Nakajima, K.; Asakura, T.; Oike, H.; Morita, Y.; Shimizu-Ibuka, A.; Misaka, T.; Sorimachi, H.; Arai, S.; Abe, K. *Neuroreport* **2006**, *17*, 1241-1244.

147. Foroud, T.; Bice, P.; Castelluccio, P.; Bo, R.; Ritchotte, A.; Stewart, R.; Lumeng, L.; Li, T. K.; Carr, L. *Behav. Genet.* **2002**, *32*, 57-67.

148. Ninomiya, Y.; Sako, N.; Katsukawa, H.; Funakoshi, M., In *Genetics of Perception and Communication*; Wysocki, C. J.; Kare, M. R., Eds. Marcel Dekker: New York, 1991; Vol. 3, pp 267-278.

149. Ninomiya, Y.; Higashi, T.; Mizukoshi, T.; Funakoshi, M. *Ann. NY. Acad. Sci.* **1987**, *510*, 527-529.

150. Ninomiya, Y.; Nomura, T.; Katsukawa, H. *Brain Res.* **1992**, *596*, 349-352.

151. Shigemura, N.; Yasumatsu, K.; Yoshida, R.; Sako, N.; Katsukawa, H.; Nakashima, K.; Imoto, T.; Ninomiya, Y. *Chem. Senses* **2005**, *30 Suppl 1*, i84-i85.

152. Bachmanov, A. A.; Bosak, N. P.; Beauchamp, G. K. *Chem. Senses* **2005**, *30*, A171.

153. Drewnowski, A. J.; Brunzell, J. D.; Sande, K.; Iverius, P. H.; Greenwood, M. R. *Physiol. Behav.* **1985**, *35*, 617-622.
154. Salbe, A. D.; DelParigi, A.; Pratley, R. E.; Drewnowski, A.; Tataranni, P. A. *Am J Clin Nutr* **2004**, *79*, 372-378.
155. Yamamoto, M. E.; Block, G. D.; Ishii, E. *Alcohol. Clin. Exp. Res.* **1991**, *15*, 359.
156. Kampov-Polevoy, A. B.; Garbutt, J. C.; Khalitov, E. *Alcohol. Clin. Exp. Res.* **2003**, *27*, 1743-1749.
157. Kampov-Polevoy, A. B.; Eick, C.; Boland, G.; Khalitov, E.; Crews, F. T. *Alcohol. Clin. Exp. Res.* **2004**, *28*, 1291-1298.
158. Kampov-Polevoy, A. B.; Garbutt, J. C.; Davis, C. E.; Janowsky, D. S. *Alcohol. Clin. Exp. Res.* **1998**, *22*, 610-614.
159. Kampov-Polevoy, A. B.; Garbutt, J. C.; Janowsky, D. *Am J Psychiatry* **1997**, *154*, 269-270.
160. Bachmanov, A. A.; Kiefer, S. W.; Molina, J. C.; Tordoff, M. G.; Duffy, V. B.; Bartoshuk; Lm; Mennella, J. A. *Alcohol. Clin. Exp. Res.* **2003**, *27*, 220-231.
161. Kiefer, S. W.; Lawrence, G. J. *Chem. Senses* **1988**, *13*, 633-641.
162. Kiefer, S. W.; Mahadevan, R. S. *Chem. Senses* **1993**, *18*, 509-522.
163. Lawrence, G. J.; Kiefer, S. W. *Chem. Senses* **1987**, *12*, 591-599.
164. Blizard, D. A.; McClearn, G. E. *Alcohol. Clin. Exp. Res.* **2000**, *24*, 253-258.
165. Hellekant, G.; Danilova, V.; Roberts, T.; Ninomiya, Y. *Alcohol* **1997**, *14*, 473-484.
166. Sako, N.; Yamamoto, T. *Am. J. Physiol.* **1999**, *276*, R388-396.
167. Di Lorenzo, P. M.; Kiefer, S. W.; Rice, A. G.; Garcia, J. *Alcohol* **1986**, *3*, 55-61.
168. Lemon, C. H.; Brasser, S. M.; Smith, D. V. *J Neurophysiol* **2004**, *92*, 536-544.
169. Levine, A. S.; Kotz, C. M.; Gosnell, B. A. *Am J Clin Nutr* **2003**, *78*, 834S-842S.
170. Gosnell, B. A.; Majchrzak, M. J. *Pharm. Biochem. Behav.* **1989**, *33*, 805-810.
171. George, S. R.; Roldan, L.; Lui, A.; Naranjo, C. A. *Alcohol. Clin. Exp. Res.* **1991**, *15*, 668-672.
172. Hubell, C. L.; Marglin, S. H.; Spitalnic, S. J.; Abelson, M. L.; Wild, K. D.; Reid, L. D. *Alcohol* **1991**, *8*, 355-367.
173. Pucilowski, O.; Rezvani, A. H.; Janowsky, D. S. *Psychopharmacol.* **1992**, *107*, 447-452.
174. Gentry, R. T.; Dole, V. P. *Life Sci.* **1987**, *40*, 2191-2194.
175. McMillen, B. A.; Williams, H. L. *Alcohol* **1998**, *15*, 193-198.
176. Rodgers, D. A.; McClearn, G. E.; Bennett, E. L.; Hebert, M. *J. Comp. Physiol. Psychol.* **1963**, *56*, 666-672.

177. Rodgers, D. A. *Psychosom. Med.* **1966**, *28*, 498-513.
178. Freed, E. X. *Quart. J. Stud. Alc.* **1972**, *33*, 504-507.
179. Richter, C. P. *Quart. J. Studies Alcohol* **1941**, *1*, 650-662.
180. Overstreet, D. H.; Kampov-Polevoy, A. B.; Rezvani, A. H.; Murelle, L.; Halikas, J. A.; Janowsky, D. S. *Alcohol. Clin. Exp. Res.* **1993**, *17*, 366-369.
181. Stewart, R. B.; Russell, R. N.; Lumeng, L.; Li, T.-K.; Murphy, J. M. *Alcohol. Clin. Exp. Res.* **1994**, *18*, 375-381.
182. McClearn, G. E.; Rodgers, D. A. *Quart. J. Studies Alcohol* **1959**, *20*, 691-695.
183. Rodgers, D. A.; McClearn, G. E. *Q J Stud Alcohol* **1962**, *23*, 26-33.
184. Belknap, J. K.; Crabbe, J. C.; Young, E. R. *Psychopharmacology (Berl)* **1993**, *112*, 503-510.
185. Stewart, R. B.; Russell, R. N.; Lumeng, L.; Li, T. K.; Murphy, J. M. *Alcohol. Clin. Exp. Res.* **1994**, *18*, 375-381.
186. Stewart, R. B.; Bice, P.; Foroud, T.; Lumeng, L.; Li, T. K.; Carr, L. G. *Alcohol. Clin. Exp. Res.* **2003**, *27*, 49A.
187. Dess, N. K.; Badia-Elder, N. E.; Thiele, T. E.; Kiefer, S. W.; Blizard, D. A. *Alcohol* **1998**, *16*, 275-278.
188. Bachmanov, A. A.; Reed, D. R.; Li, X.; Li, S.; Beauchamp, G. K.; Tordoff, M. G. *Genome Res* **2002**, *12*, 1257-1268.
189. Terenina-Rigaldie, E.; Moisan, M. P.; Colas, A.; Beauge, F.; Shah, K. V.; Jones, B. C.; Mormede, P. *Pharmacogenetics* **2003**, *13*, 543-554.
190. Terenina-Rigaldie, E.; Jones, B. C.; Mormede, P. *Genes Brain Behav* **2003**, *2*, 125-131.

Chapter 3

Making Sense of the Sweet Taste Receptor

Peihua Jiang[1], Emeline Maillet[1], Meng Cui[2], Roman Osman[2],
Marianna Max[1], and Robert F. Margolskee[1]

[1]Department of Neuroscience, Mount Sinai School of Medicine,
Mail Stop 1065, 1425 Madison Avenue, New York, NY 10029
[2]Department of Physiology and Biophysics, Mount Sinai School
of Medicine, Mail Stop 1677, 1425 Madison Avenue, New York, NY 10029

Sugars, sweeteners and certain proteins all taste sweet. These
chemically diverse compounds activate the same sweet
receptor, a heterodimer of T1R2 plus T1R3. Humans and
mice each detect the sweetness of sugars, but some compounds
that are intensely sweet to humans are not sweet at all to mice.
Heterologous expression of the mouse or human sweet taste
receptors reproduces, respectively, the mouse or human
response to sweeteners. Expression of mismatched
combinations of human plus mouse T1R subunits, or of
human/mouse chimeras of T1R subunits enabled us to identify
portions of the sweet receptor required for responses to sweet
ligands. Responsiveness to aspartame depends on the
extracellular domain of human T1R2; brazzein depends on the
cysteine-rich domain of human T1R3; the agonist cyclamate
and the antagonist lactisole depend on the transmembrane
domain of human T1R3.

The sense of taste is essential for humans and other organisms to detect the nutritive quality of a potential food source while avoiding environmental toxins (1-3). Taste perception can be categorized into five distinct qualities: sweet, bitter, salty, sour, and umami (amino acid taste) (1-3). The initial event in sweet, bitter, and umami tastes is activation of specific G-protein-coupled receptors (GPCRs) (1,3). Sour and salty tastes, on the other hand, appear to be mediated by direct effects on specialized ion channels (1,4-6).

The sweet taste receptor is a heterodimer of T1R2 and T1R3, two GPCR family C receptors expressed in taste receptor cells (7-13). When expressed *in vitro*, the T1R2 + T1R3 heterodimer responds to a chemically diverse range of sweeteners, including sugars (sucrose, fructose, glucose, maltose), sweet amino acids (D-tryptophan, D-phenylalanine, D-serine), artificial sweeteners (acesulfame-K, aspartame, cyclamate, saccharin, sucralose) and sweet tasting proteins (brazzein, monellin, thaumatin) (8,14,15). With the exception of the sugar trehalose (which apparently activates the T1R3 homodimer (16), all sweeteners activate the T1R2 + T1R3 heterodimer. *In vivo*, genetic ablation in mice of T1R2, T1R3, or both either greatly reduces or eliminates responses to sweet compounds (17,18). Thus, the T1R2 + T1R3 heterodimer is broadly tuned and functions as the principal or sole sweet taste receptor.

Class C GPCRs include T1R1,T1R2,T1R3, metabotropic glutamate receptors (mGluR1-8), the calcium-sensing receptors (CaSR, GPRC6A), γ-aminobutyric acid (GABA) type B receptors, and vomeronasal receptors. Like most other GPCRs, each class C receptor has a heptahelical transmembrane domain (TMD). In contrast to other types of GPCRs, each class C GPCR has a large extracellular domain composed of two parts: a "Venus flytrap module" (VFTM), which is involved in ligand binding, and a cysteine-rich domain (CRD), which contains nine highly conserved cysteines and links the VFTM to the TMD (19). A variable length intracellular C-terminal tail completes the class C receptor. Although the "canonical" ligand-binding site lies within the VFTM, additional "non-canonical" ligand-binding sites have been identified elsewhere in T1R receptors and other class C receptors (15,20-27).

How does the sweet receptor detect and respond to so many chemically diverse compounds? Recent studies have shown that this heterodimeric receptor utilizes multiple ligand binding sites within each subunit. The VFTMs of mouse T1R2 (mT1R2) and mouse T1R3 (mT1R3) both have been shown to bind sugars (28). Furthermore, the human form of T1R2's VFTM is required for responsiveness to aspartame (15). The CRD of human T1R3 (hT1R3) has been implicated in the sweet receptor's interaction with sweet proteins (15). The TMD of hT1R3 is essential for sweet receptor responsiveness to both lactisole (an inverse agonist) and cyclamate (an agonist) (26,27,29-31, E. Maillet et al., in preparation). Thus, there are at least four broadly defined potential binding domains on the heterodimeric sweet receptor, all of which may be capable of mediating receptor activation.

The canonical binding pocket in T1R2's VFTM

For family C GPCRs such as GABA-b, mGluRs and CaSRs the orthosteric ligand binding site resides within the VFTM domain (32-34). Thus, it is to be expected that in the heterodimeric T1R2 + T1R3 sweet receptor one or both VFTMs may provide the binding site for sugars and/or small molecule sweeteners. Because of the low affinity of sugars and other sweeteners binding assays with the sweet receptor have been problematic (although note the recent results of Munger and colleagues (28)), so that most studies of expressed sweet receptors have relied upon activity assays. These activity studies have been aided by species differences in sweet taste that carry over to the expressed T1R2 + T1R3 sweet receptor. For example, aspartame, cyclamate, neohesperidin and the sweet tasting proteins monellin, thaumatin and brazzein all taste sweet to humans, but not to mice; whereas mice and humans both taste the sweetness of sucrose, saccharin and D-tryptophan (35).

The first identification of the interacting site for a sweetener was obtained by examining mismatched pairings of mouse and human T1R2 + T1R3 monomers. Responsiveness to the human-specific sweetener aspartame is obtained by *in vitro* expression of the human + human (hT1R2 + hT1R3) or human+ mouse combination (hT1R2 + mT1R3), but not the mouse + mouse (mT1R2 + mT1R3) receptor (**Figure 1**)(15). This indicates that hT1R2 is required for aspartame to bind to and/or interact with the receptor. Furthermore, the pairing of hT1R3 with h.1-564.mT1R2, a chimeric receptor comprising the entire extracellular domain (VFTM + CRD) of hT1R2 coupled to the TMD and C-terminal tail of mT1R2, yields responsiveness to aspartame and monellin (**Figure 2**), further narrowing down the binding/interaction site of aspartame and monellin to the extracellular domain of hT1R2 (15). Additional human/mouse chimeric receptors have been generated and used to identify which portions of the extracellular domain of hT1R2 are required for human-specific sensitivity to aspartame and monellin (**Figure 2**) (15, P Jiang et al., in preparation, E Maillet et al., in preparation).

Another useful approach to probing candidate binding sites makes use of structural similarity amongst all family C receptors. The solved crystal structure of the VFTM of mGluR1 (20) provides a template for building testable models of the VFTMs of T1Rs (27). From such a model of hT1R2's VFTM (see Meng et al., this volume) it appears that residues R63 and D307 lie within the canonical binding site of hT1R2's VFTM. To test this model D307 was replaced with residues having distinct physicochemical properties (e.g. D307A, D307N, D307K). The D307 mutants had diminished or abolished responses to D-tryptophan, but retained normal responses to sweet proteins (27).
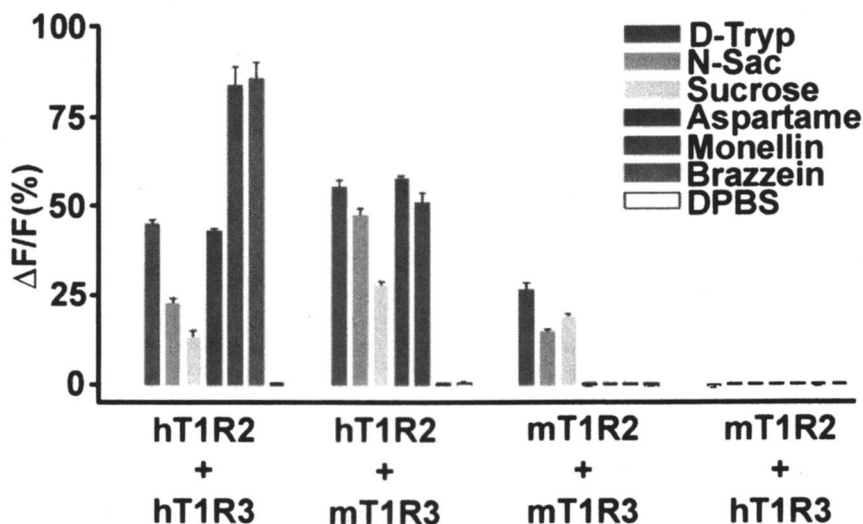
Figure 1. Responsiveness to "human specific" sweeteners requires human T1R2 and/or human T1R3. Human (hT1R2 + hT1R3), mouse (mT1R2 + mT1R3), or human + mouse mismatched (hT1R2 + mT1R3 and mT1R2 + hT1R3) T1R subunits were expressed in HEK293E cells, along with Gα16-i3. Receptor activation in response to sweeteners was measured by Ca^{2+} mobilization. The following sweeteners were tested for each receptor pair: D-tryptophan (10 mM), N-saccharin (1 mM), sucrose (75 mM), aspartame (2.5 mM), monellin (0.1%), and brazzein (0.25%). Buffered saline (DPBS) served as a negative control. F is the baseline level of fluorescence, and ΔF is the change in fluorescence from the baseline level (peak - baseline). Values represent mean ± S.E. of fluorescence changes from three independent experiments.

The cysteine-rich domain determines the receptor's responsiveness to sweet tasting proteins

By mixing and matching sweet receptor monomers from mouse and human, we determined that the human-specific response to brazzein depends on hT1R3 (**Figure 1**)(15). Results with various human/mouse chimeras of T1R3 showed that it was only the extracellular region of hT1R3 that was required for responsiveness to brazzein (**Figure 2**) (15). Note, for example, that T1R3 chimera h.1-567-mT1R3 (with human VFTM, human CRD, mouse TMD and mouse C-terminal tail) is responsive to brazzein, whereas the reverse chimera (mT1R3.h.568-852) is not. Additional chimeras narrowed down the required region of hT1R3 to aa535-545 within the CRD (**Figure 2**). Confirmation of this result came by introducing only residues 535-545 of hT1R3 into mT1R3: this "humanized" mT1R3 in combination with hT1R2 generated a brazzein-responsive receptor(15).

Within this critical region of hT1R3's CRD there are only five amino acid differences between hT1R3 and mT1R3. Loss-of-function experiments in which each one of these critical residues of hT1R3 were mutated to the mouse counterpart, then assayed in the presence of hT1R2 for responses to brazzein showed that A537 of hT1R3 was critical for responses to brazzein but not to other sweeteners (15). Within this part of the CRD of hT1R3 we also determined that F540 contributed to the receptor's responses to brazzein and monellin (15). In a gain-of-function experiment we determined that making two humanizing mutations in mT1R3 (T542A and P545F: the equivalents of A537 and F540) did indeed confer responses to brazzein and monellin on the humanized mT1R3 when paired with hT1R2 (15). Furthermore, mutation to alanine or glutamine of D535, a charged residue within hT1R3's CRD that lies along the same face as A537 and F540 (see model of the CRD in Meng et al. this volume), resulted in complete loss of responsiveness to brazzein without disrupting the receptor's responses to other sweeteners (data not shown). Altogether, these results implicate the CRD in the sweet receptor's responses to sweet proteins such as brazzein.

Cyclamate interacts with the transmembrane domain of human T1R3

Again, using activity assays and mismatched pairings of human and mouse T1Rs we determined that hT1R3 is required for the human-specific response to cyclamate (**Figure 3**). Results with various human/mouse chimeras of T1R3 showed that it was only the TMD of hT1R3 that was required for responsiveness
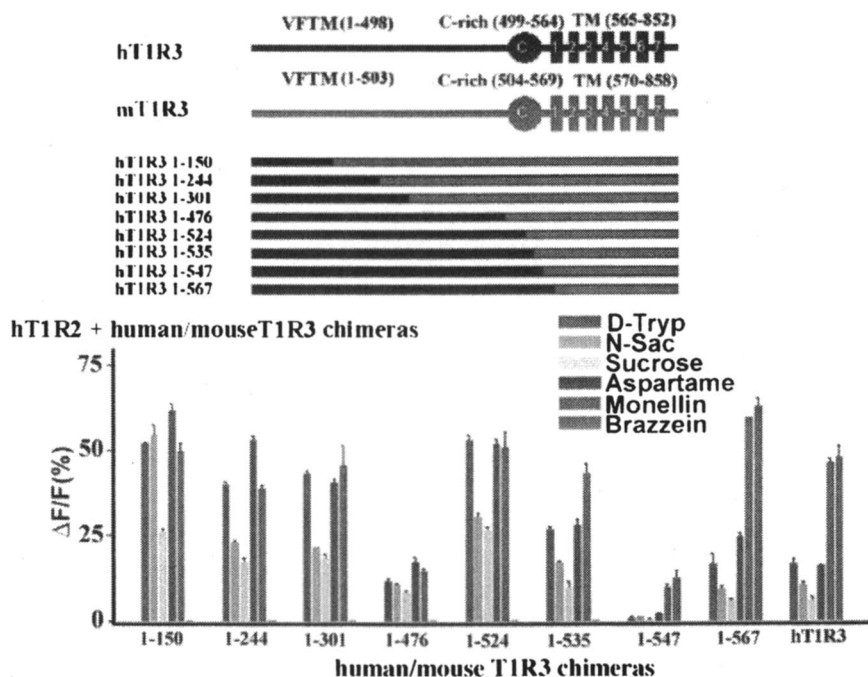
Figure 2. Responsiveness of the sweet taste receptor to brazzein requires the cysteine-rich domain of human T1R3. Upper panel, schematic diagram showing the venus fly trap module (VFTM), cysteine-rich region (C in oval), and transmembrane domain (rectangles 1–7) of human (black) and mouse (dark grey) T1R3. Human/mouse chimeras of T1R3 are indicated by color-coded segments. By our naming convention h.1–150.mT1R3 comprises residues 1–150 from hT1R3 and the remainder from mT1R3. Lower panel, HEK293E cells transiently transfected with the indicated human/mouse T1R3 chimera, hT1R2, and $G\alpha_{16}$-i3 were assayed for sweetener responses.
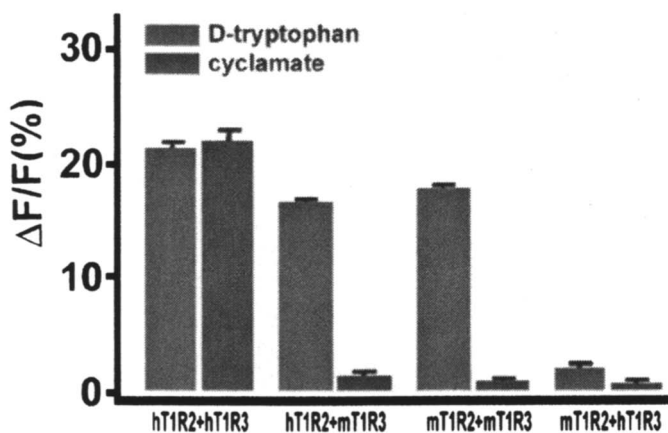
Figure 3. Human T1R3 determines sweet receptor responsiveness to cyclamate. Human (hT1R2 + hT1R3), mouse (mT1R2 + mT1R3), or human + mouse mismatched (hT1R2 + mT1R3 and mT1R2 + hT1R3) T1R subunits were expressed in HEK293E cells, along with Gα$_{16}$-gust44. The responses of the cells to cyclamate (10 mM) and D-tryptophan (10 mM) were assayed by calcium mobilization.
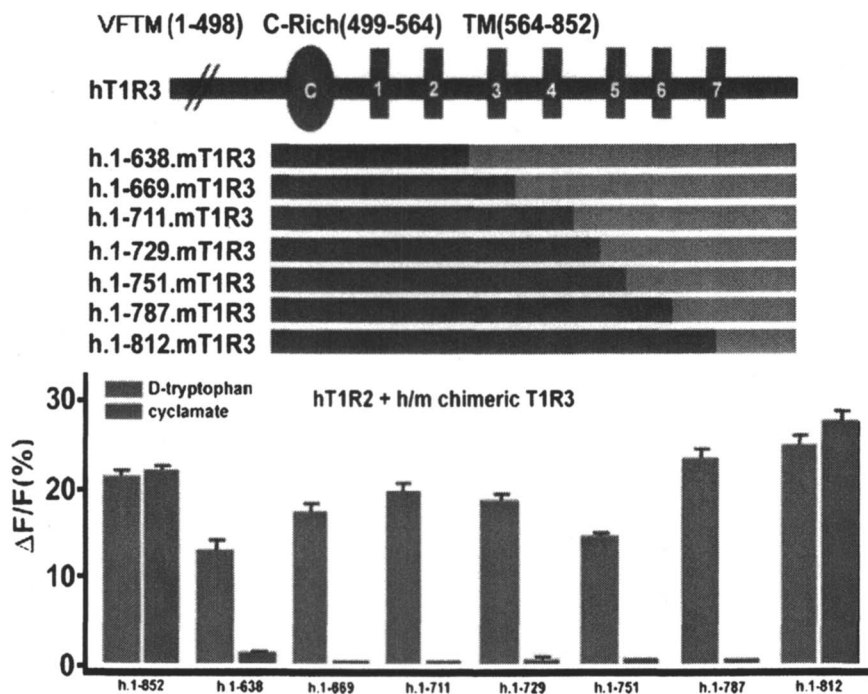
Figure 4. Extracellular loop 3 and/or TM helix 7 of hT1R3 are required for responsiveness to cyclamate. Upper panel, schematic diagram showing chimeras between human (black) and mouse (dark grey) T1R3 within the transmembrane domain (TMD). Lower panel, HEK293E cells transiently transfected with the indicated human/mouse T1R3 chimera, hT1R2, and $G\alpha_{16}$-gust44 were assayed for sweetener responses.

EX3 / TM helix 7

```
                787
Consensus    V---PAVQMGA-L-C-LGIL--F
Human T1R3   .VLR.......L.L.V....AA.
Mouse T1R3   .AYQ.......I.V.A....VT.
```
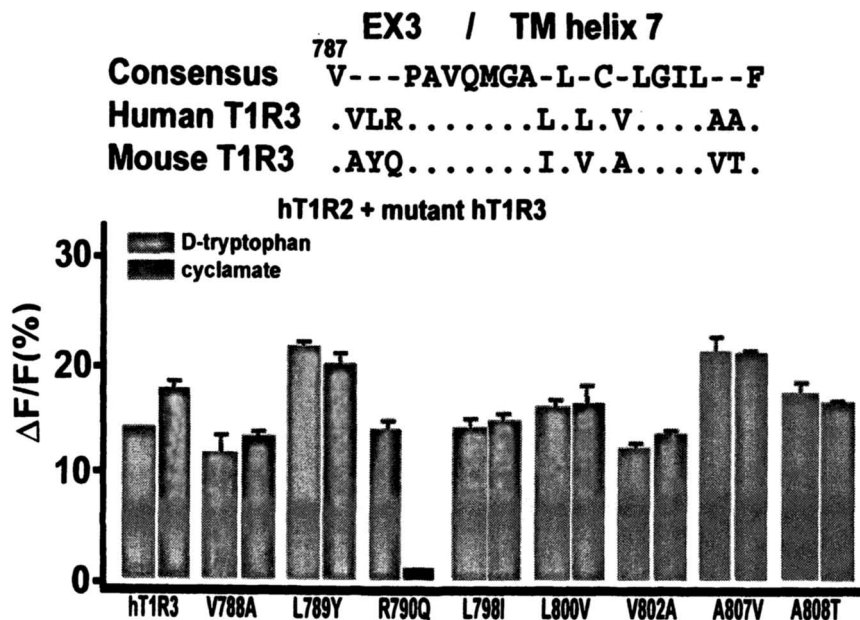
Figure 5. Responsiveness to cyclamate requires human-specific residue in extracellular loop 3. Upper panel, alignment of human and mouse T1R3 sequences showing eight sequence differences in TM helix 7 and adjacent third extracellular loop (EX3). Lower panel, HEK293E cells transiently transfected with the indicated hT1R3 mutants, hT1R2, and $G\alpha_{16}$-gust44 were assayed for responses to cyclamate and D-tryptophan.

to cyclamate. For example, mT1R3-h568-852 when paired with either hT1R2 or mT1R2 responds normally to cyclamate (30). Additional chimeras narrowed down the minimal required region of hT1R3's TMD to aa787-812 (i.e. extracellular loop 3 (EX 3) and TM helix 7) (**Figure 4**). By substituting human specific residues within this region and nearby portions of the TMD with their mouse counterparts we determined that R790 (EX 3) (**Figure 5**) and F730 (TM helix 5) (30) are important determinants for the human specific response to cyclamate.

To identify residues of T1R3 conserved between hT1R3 and mT1R3 that might contribute to the human sweet receptor's interaction with cyclamate we used the following approach. First, we used the solved structure of rhodopsin (36) as a template, after sequence alignment, to model the TMD of hT1R3. Next, we made alanine substitutions of all of the hT1R3 TMD residues predicted to line the inner helical bundle region (based on homology to rhodopsin's retinal binding pocket). The resulting mutant receptors were co-expressed with hT1R2 then tested for responsiveness to D-tryptophan (control) and cyclamate. Of seventeen residues examined in this way we identified the following six positions where alanine substitution yielded receptors with selective defects in responding to cyclamate: Q636, H641, H721, R723, F778 and L782 (**Figure 6**) (30). In sum, our alanine scanning and human-to-mouse mutagenesis results identify a
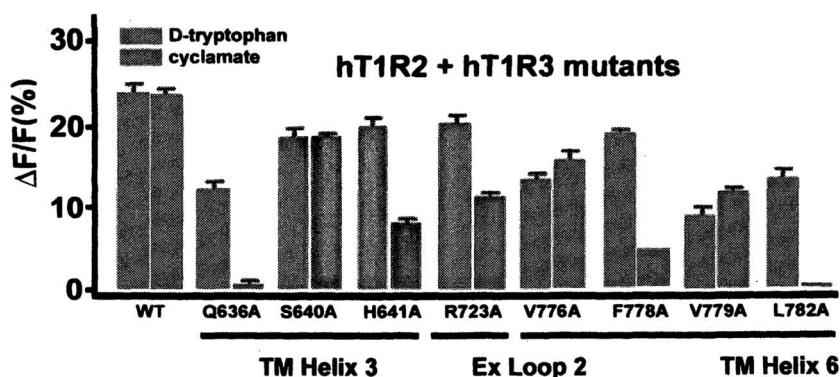
Figure 6. Identification of residues lining potential cyclamate-binding pocket within the TMD of human T1R3. Residues of hT1R3 identified by homology to rhodopsin as potential contributors to a TMD binding pocket for cyclamate were replaced with alanine. HEK293E cells transiently transfected with the indicated hT1R3 mutants, hT1R2, and $G\alpha_{16}$-gust44 were assayed for responses to cyclamate and D-tryptophan.

potential binding pocket for cyclamate bound by TM3, TM5, TM6 and EX 2. Computationally docking cyclamate into a molecular model of hT1R3's TMD also identified this region of hT1R3 as a potential binding site for cylcamate (See Meng et al., this volume).

Lactisole interacts with the transmembrane domain of hT1R3

Lactisole is a remarkable compound that broadly suppresses sweet taste in humans but not in rodents (37, 38). This effect can be reproduced *in vitro*: lactisole suppressed the heterologously-expressed human sweet receptor's responses to all sweeteners tested but had no effect on those of the mouse sweet receptor (**Figure 7**) (29). To identify sites at which lactisole binds to or interacts with the human sweet receptor we once again utilized mismatched pairs of human and mouse T1R monomers and mouse/human chimeras. We found that sweet receptor sensitivity toward lactisole required hT1R3, and within T1R3 it is the human TMD that is needed (**Figure 8**) (29). Analysis of mouse/human chimeric receptors indicated that sensitivity to lactisole depended on aa729-751 of hT1R3 (i.e. TM helix 5) (29). Within TM helix 5 of hT1R3 only 4 residues (F730, A733, A735, and T739) differ between the human and mouse T1R3. Of these four residues, A733 proved critical: substitution here with the corresponding mouse residue (A733V), yielded a generally functional receptor with ~one-fiftieth the sensitivity to lactisole (**Figure 9**) (29). Alanine scanning mutagenesis identified H641,V779 and F778 as also being important for sensitivity to lactisole (**Figure 10**) (29). Interestingly, two alanine mutants showed enhanced sensitivity toward lactisole (S640A, L782A) (**Figure 10**) (29). Thus, residues in TM helices 3, 5 and 6 contribute importantly to the receptor's sensitivity toward lactisole and are likely part of its binding pocket.

Conclusions

We have identified which T1R subunits, and specific sites within each of these subunits, that determine receptor responsiveness to agonists (brazzein, thaumatin, aspartame, neotame, cyclamate) and to the antagonist lactisole. In all likelihood, and consistent with our molecular models, many of these interaction sites are probably directly involved in ligand binding. Results of ours and others demonstrate that there are multiple binding sites on the sweet taste receptors. These findings explain how so many structurally diverse compounds can all taste sweet.
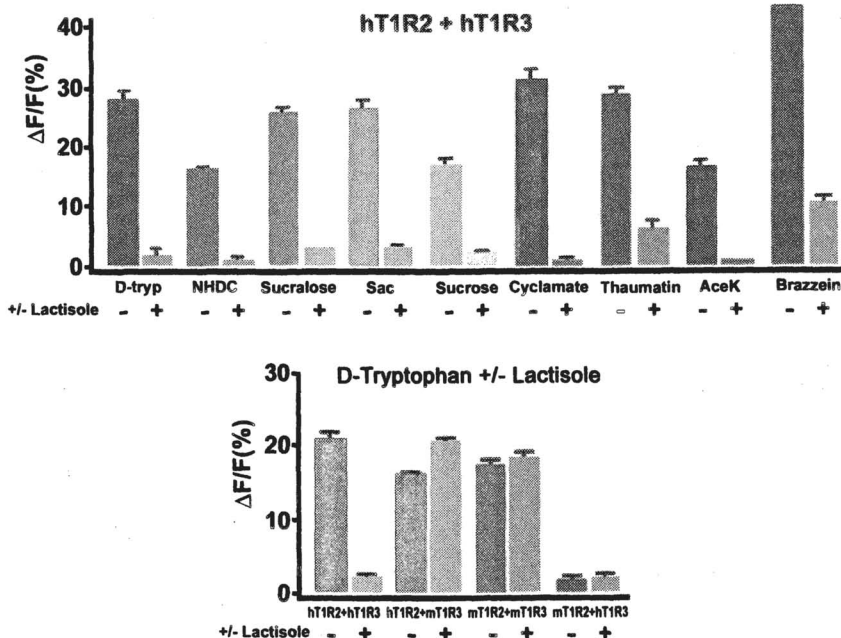
Figure 7. hT1R3 determines the human sweet receptor's sensitivity to lactisole. Upper panel, HEK293E cells transiently transfected with the human sweet receptor (hT1R2+hT1R3) and $G\alpha_{16}$-gust44 were assayed for responses to the following sweeteners without (-) and with lactisole (+, 1.25 mM): acesulfame-K (Acek, 10 mM), brazzein (0.25%), cyclamate (10 mM), D-tryprophan (10 mM), NHDC (0.25 mM), saccharin (1 mM), sucralose (1 mM), sucrose (100 mM), and thaumatin (0.1%). Lower panel, HEK293E cells transiently transfected with human (hT1R2 + hT1R3), mouse (mT1R2 + mT1R3), or human + mouse mismatched (hT1R2 + mT1R3 and mT1R2 + hT1R3) T1R subunits and $G\alpha_{16}$-gust44 were assayed for responses to D-tryptophan (10 mM) without (-) or with lactisole (+, 1.25 mM).
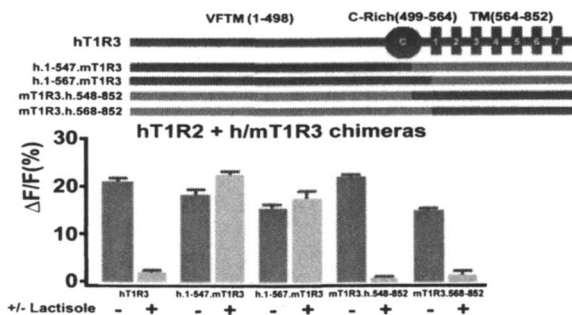
Figure 8. hT1R3's TMD determines sensitivity to lactisole. *Upper panel, schematic diagram showing chimeras between human (black) and mouse (dark grey) T1R3 within the transmembrane domain (TMD). Lower panel, HEK293E cells transiently transfected with the indicated human/mouse T1R3 chimera, hT1R2, and Gα₁₆-gust44 were assayed for responses to D-tryptophan (10 mM) without (-) or with lactisole (+, 1.25 mM).*
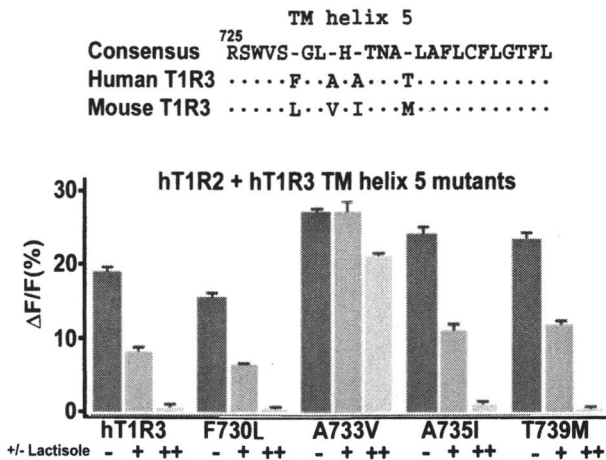
Figure 9. Sensitivity to lactisole depends on several human-specific residues within the TMD of hT1R3. *Upper panel, alignment of human and mouse T1R3 sequences showing four sequence differences in TM helix 5. Lower panel, HEK293E cells transiently transfected with the indicated hT1R3 mutants, hT1R2, and Gα₁₆-gust44 were assayed for responses to D-tryptophan (10 mM) without (-) or with lactisole (0.125 mM (+), 1.25 mM (++)).*

Acknowledgements
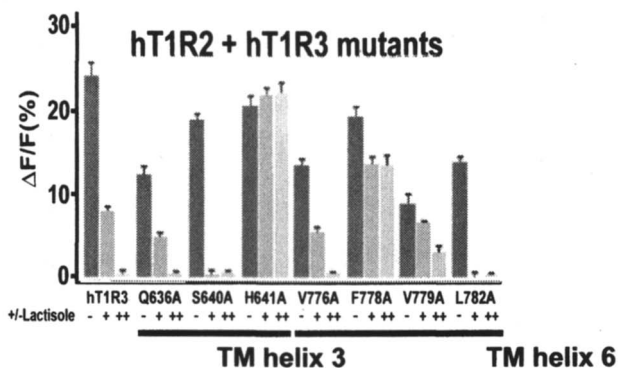
Figure 10. Identification of residues lining potential lactisole-binding pocket within the TMD of human T1R3. Residues of hT1R3 identified by homology to rhodopsin as potential contributors to a TMD binding pocket for lactisole were replaced with alanine. HEK293E cells transiently transfected with the indicated hT1R3 mutants, hT1R2, and Gα₁₆-gust44 were assayed for responses to D-tryptophan (10 mM) without (-) or with lactisole (0.125 mM (+), 1.25 mM (++)).

References

1. Margolskee, R. F. (2002) *J Biol Chem* **277**, *(1)*, 1-4.

2. Lindemann, B. (1996) *Physiol Rev* **76**, *(3)*, 718-766.

3. Lindemann, B. (2001) *Nature* **413**, *(6852)*, 219-225.

4. Ishimaru, Y., Inada, H., Kubota, M., Zhuang, H., Tominaga, M., and Matsunami, H. (2006) *Proc Natl Acad Sci U S A* **103**, *(33)*, 12569-12574

5. Huang, A. L., Chen, X., Hoon, M. A., Chandrashekar, J., Guo, W., Trankner, D., Ryba, N. J., and Zuker, C. S. (2006) *Nature* **442**, *(7105)*, 934-938

6. LopezJimenez, N. D., Cavenagh, M. M., Sainz, E., Cruz-Ithier, M. A., Battey, J. F., and Sullivan, S. L. (2006) *J Neurochem* **98**, *(1)*, 68-77

7. Sainz, E., Korley, J. N., Battey, J. F., and Sullivan, S. L. (2001) *J Neurochem* **77**, *(3)*, 896-903

8. Nelson, G., Hoon, M. A., Chandrashekar, J., Zhang, Y., Ryba, N. J., and Zuker, C. S. (2001) *Cell* **106**, *(3)*, 381-390.

9. Montmayeur, J. P., Liberles, S. D., Matsunami, H., and Buck, L. B. (2001) *Nat Neurosci* **4**, *(5)*, 492-498.

10. Max, M., Shanker, Y. G., Huang, L., Rong, M., Liu, Z., Campagne, F., Weinstein, H., Damak, S., and Margolskee, R. F. (2001) *Nat Genet* **28**, *(1)*, 58-63.

11. Kitagawa, M., Kusakabe, Y., Miura, H., Ninomiya, Y., and Hino, A. (2001) *Biochem Biophys Res Commun* **283**, *(1)*, 236-242.

12. Bachmanov, A. A., Li, X., Reed, D. R., Ohmen, J. D., Li, S., Chen, Z., Tordoff, M. G., de Jong, P. J., Wu, C., West, D. B., Chatterjee, A., Ross, D. A., and Beauchamp, G. K. (2001) *Chem Senses* **26**, *(7)*, 925-933.

13. Hoon, M. A., Adler, E., Lindemeier, J., Battey, J. F., Ryba, N. J., and Zuker, C. S. (1999) *Cell* **96**, *(4)*, 541-551.

14. Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M., and Adler, E. (2002) *Proc Natl Acad Sci U S A* **99**, *(7)*, 4692-4696.

15. Jiang, P., Ji, Q., Liu, Z., Snyder, L. A., Benard, L. M., Margolskee, R. F., and Max, M. (2004) *J Biol Chem* **279**, *(43)*, 45068-45075.

16. Koseki, T., Koganezawa, M., Furuyama, A., Isono, K., and Shimada, I. (2004) *Chem Senses.* **29**, *(8)*, 703-11.

17. Damak, S., Rong, M., Yasumatsu, K., Kokrashvili, Z., Varadarajan, V., Zou, S., Jiang, P., Ninomiya, Y., and Margolskee, R. F. (2003) *Science* **301**, *(5634)*, 850-853

18. Zhao, G. Q., Zhang, Y., Hoon, M. A., Chandrashekar, J., Erlenbach, I., Ryba, N. J., and Zuker, C. S. (2003) *Cell* **115**, *(3)*, 255-266

19. Pin, J. P., Galvez, T., and Prezeau, L. (2003) *Pharmacol Ther* **98**, *(3)*, 325-354

20. Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., and Morikawa, K. (2000) *Nature* **407**, *(6807)*, 971-977.

21. Binet, V., Brajon, C., Le Corre, L., Acher, F., Pin, J. P., and Prezeau, L. (2004) *J Biol Chem* **279**, *(28)*, 29085-29091. Epub 22004 May 29084.

22. Malherbe, P., Knoflach, F., Broger, C., Ohresser, S., Kratzeisen, C., Adam, G., Stadler, H., Kemp, J. A., and Mutel, V. (2001) *Mol Pharmacol* **60**, *(5)*, 944-954.

23. Pagano, A., Ruegg, D., Litschig, S., Stoehr, N., Stierlin, C., Heinrich, M., Floersheim, P., Prezeau, L., Carroll, F., Pin, J. P., Cambria, A., Vranesic, I., Flor, P. J., Gasparini, F., and Kuhn, R. (2000) *J Biol Chem* **275**, *(43)*, 33750-33758.

24. Petrel, C., Kessler, A., Maslah, F., Dauban, P., Dodd, R. H., Rognan, D., and Ruat, M. (2003) *J Biol Chem* **278**, *(49)*, 49487-49494

25. Petrel, C., Kessler, A., Dauban, P., Dodd, R. H., Rognan, D., and Ruat, M. (2004) *J Biol Chem* **279**, *(18)*, 18990-18997

26. Xu, H., Staszewski, L., Tang, H., Adler, E., Zoller, M., and Li, X. (2004) *Proc Natl Acad Sci U S A* **101**, *(39)*, 14258-14263

27. Jiang, P., Cui, M., Ji, Q., Snyder, L., Liu, Z., Benard, L., Margolskee, R. F., Osman, R., and Max, M. (2005) *Chem Senses* **30 Suppl 1**, i17-i18

28. Nie, Y., Vigues, S., Hobbs, J. R., Conn, G. L., and Munger, S. D. (2005) *Curr Biol* **15**, *(21)*, 1948-1952

29. Jiang, P., Cui, M., Zhao, B., Liu, Z., Snyder, L. A., Benard, L. M., Osman, R., Margolskee, R. F., and Max, M. (2005) *J Biol Chem* **280**, *(15)*, 15238-15246

30. Jiang, P., Cui, M., Zhao, B., Snyder, L. A., Benard, L. M., Osman, R., Max, M., and Margolskee, R. F. (2005) *J Biol Chem* **280**, *(40)*, 34296-34305

31. Winnig, M., Bufe, B., and Meyerhof, W. (2005) *BMC neuroscience [electronic resource]* **6**, *(1)*, 22

32. Malitschek, B., Schweizer, C., Keir, M., Heid, J., Froestl, W., Mosbacher, J., Kuhn, R., Henley, J., Joly, C., Pin, J. P., Kaupmann, K., and Bettler, B. (1999) *Mol Pharmacol* **56**, *(2)*, 448-454.

33. Takahashi, K., Tsuchida, K., Tanabe, Y., Masu, M., and Nakanishi, S. (1993) *J Biol Chem* **268**, *(26)*, 19341-19345

34. Brauner-Osborne, H., Jensen, A. A., Sheppard, P. O., O'Hara, P., and Krogsgaard-Larsen, P. (1999) *J Biol Chem* **274**, *(26)*, 18382-18386

35. Bachmanov, A. A., Tordoff, M. G., and Beauchamp, G. K. (2001) *Chem Senses* **26**, *(7)*, 905-913

36. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Trong, I. L., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science* **289**, *(5480)*, 739-745

37. Schiffman, S. S., Booth, B. J., Sattely-Miller, E. A., Graham, B. G., and Gibes, K. M. (1999) *Chem Senses* **24**, *(4)*, 439-447.

38. DuBois, G. E. (2004) *Proc Natl Acad Sci U S A* **101**, *(39)*, 13972-13973

Chapter 4

T1R2, T1R3, and the Detection of Sweet Stimuli

Stephan Vigues[1], Jeanette R. Hobbs[2], Yiling Nie[1], Graeme L. Conn[2], and Steven D. Munger[1],*

[1]Department of Anatomy and Neurobiology, University of Maryland School of Medicine, 20 Penn Street, Baltimore, MD 21201
[2]Manchester Interdisciplinary Biocentre, Faculty of Life Sciences, University of Manchester, Manchester M1 7DN, United Kingdom

G protein-coupled receptors (GPCRs) play a central role in the detection of chemosensory cues, including sweet-tasting stimuli. To fully understand the basis of stimulus sensitivity and selectivity in chemosensory systems, it is essential to characterize the structural basis of receptor-ligand interactions. Efforts to express chemosensory receptors at levels suitable for detailed structure-function studies have met with limited success. We have developed a novel strategy for expressing and purifying functional domains of T1R taste receptors. Using these purified proteins in concert with spectroscopic analyses, we determined that each of the two subunits of the heteromeric T1R2:T1R3 sweet taste receptor binds sugar stimuli, though with different affinities and distinct conformational changes. Furthermore, a T1R3 variant associated with reduced sweet taste sensitivity in mice exhibits reduced affinities for sugars. These results provide fundamental new insights into the function of the sweet taste receptor and an important new strategy for studying the receptor basis of taste.

© 2008 American Chemical Society

65

Introduction

Taste stimuli elicit five basic perceptual qualities in humans: sweet, bitter, sour, salty, and umami *(1-3)*. The ability to detect and discriminate taste stimuli is essential for health and survival, as taste stimuli convey important information about the nutritional value and quality of food. Mammals use a small number of G protein-coupled receptors (GPCRs) to detect sweet-, umami- and bitter-tasting stimuli *(4)*. All classes of sweet-tasting stimuli, including natural sugars (e.g., sucrose, glucose), synthetic sweeteners (e.g., sucralose, saccharin, aspartame, cyclamate), sweet-tasting amino acids (e.g., D-phenylalanine, D-tryptophan) and sweet proteins (e.g., monellin, thaumatin, brazzein) activate a heteromeric GPCR comprised of the T1R2 and T1R3 subunits *(5,6)*. T1R3 also combines with the T1R1 receptor to form an umami taste receptor sensitive to some L-amino acids, including L-glutamate *(5,7)*. T1R2:T1R3 sweet taste receptors are expressed in a unique subset of taste receptor cells within taste buds of the tongue and palate.

T1R receptors are Class C GPCRs, a group that includes metabotropic glutamate receptors (mGluRs) and γ-aminobutyric acid type B receptors (GABA$_B$Rs) *(8)*. This class of GPCR is distinguished by a long extracellular N-terminal domain (NTD) containing a venus-flytrap module (VFTM) motif. The VFTM of mGluRs and GABA$_B$Rs contains the orthosteric ligand binding site for these receptors *(8,9)*. However, it remained unclear if the same were true for the T1Rs. Modeling studies of the T1R2 and T1R3 NTDs, based on the crystal structures of the mGluR1 NTD, suggested that small molecule sweeteners could bind to both subunits *(10)*. This prediction was consistent with *in vitro* activity assays and *in vivo* behavioral experiments indicating that homomeric T1R3 receptors can function as a low efficacy sweet receptor, though the heteromeric T1R2:T1R3 receptor is required for full activity *(11)*. Several elegant studies using a combination of human-rodent T1R2 and T1R3 chimeras, along with stimuli that are sweet to humans but not preferred by rodents, added another layer of complexity *(12-14)*: these studies provide strong evidence that the T1R2:T1R3 receptor contains multiple allosteric binding sites. For example, the T1R2:T1R3 receptor requires the NTD of human T1R2 to respond to the dipeptide sweeteners aspartame and neotame *(14)*, the transmembrane domain of human T1R3 to respond to cyclamate *(12,14)*, and the cysteine-rich linker region of human T1R3 to respond to brazzein *(13)*. A sweet taste receptor with multiple binding sites provides a parsimonious explanation for the broad responsiveness of the receptor to such varied stimuli *(15)*. However, none of these studies could directly address whether small sweet stimuli, including natural sugars that are preferred by both humans and rodents, bind to the NTDs of T1R2 and/or T1R3.

Attempts to answer this question have been hampered by two technical limitations. First, mammalian chemosensory receptors, including the T1R taste

receptors, have been difficult to obtain in quantities suitable for biochemical or structural studies. Since T1R receptors are expressed in few taste receptor cells and at very low levels, isolation of a single chemosensory receptor type from gustatory tissues is not feasible. T1Rs also express poorly in heterologous expression systems, possibly due to the absence of a critical chaperone or co-receptor *(4,16,17)*. Therefore, optimization of *in vitro* expression and purification protocols for obtaining large quantities of functional T1R proteins or protein domains is required. Second, the low potency of most sweeteners, including natural sugars, suggests a low binding affinity that makes conventional binding assays problematic. Therefore, new approaches are needed that can quantify binding to T1Rs and can decouple this binding from subsequent receptor activation events such as intra- and intersubunit interactions and G protein activation. Here we discuss a new strategy for the expression, purification and characterization of T1R ligand binding domains and its application in dissecting the roles of T1R2 and T1R3 in the recognition of sweet stimuli *(18,19)*.

Expression and Purification of the N-terminal Ligand Binding Domains of T1R2 and T1R3

We used two strategies to express and purify the NTDs of the C57BL/6J variants of mouse T1R2 and T1R3 *(18,19)*. In the first, the NTD of T1R3 (lacking a putative signal sequence and the cysteine-rich domain *(20)*; Figure 1) was cloned into the IMPACT™ expression vector pTXB1 (New England Biolabs, Ipswich, MA) and expressed in BL21-CodonPlus(DE3)-RIL *E. coli* (Stratagene, La Jolla, CA) after induction with isopropyl-β-D-thioglycopyranoside (IPTG). Expression from this vector results in the production of a T1R3 NTD fusion protein containing an intein-chitin binding domain (CBD) affinity tag at the C-terminus. After sonication, the fusion protein was purified by chitin affinity chromatography. Cleavage of the T1R3NTD protein from the CBD was induced by reduction with dithiothreitol, and the purified receptor protein eluted in a highly purified form. The second strategy entailed expressing either the T1R2 or T1R3 NTD as a C-terminal fusion to the *E. coli* maltose binding protein (MBP). The fusion construct was built in the pET21a expression vector (New England Biolabs) and expressed in BL21-CodonPlus(DE3)-RIL *E. coli* as above. The MBP-tagged proteins were purified by amylose affinity chromatography followed by anion exchange chromatography, and the MBP fusions left uncleaved to enhance stability of the T1R NTDs. In each case, we obtained highly purified T1R NTD proteins that were stable in solution.
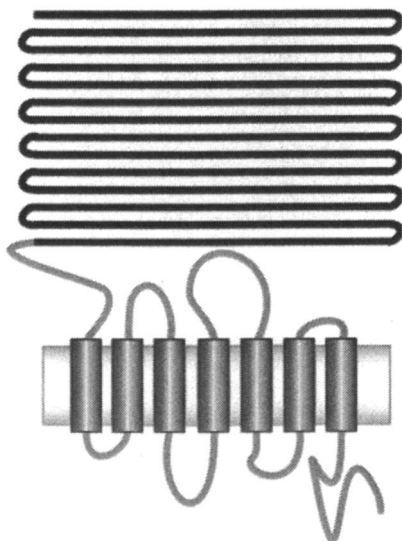
Figure 1. T1Rs have seven transmembrane helices linked to a large extracellular N-terminal domain NTD (black) by a short cysteine-rich linker.

T1R2 and T1R3 N-Terminal Domains Bind Sugars

To quantify ligand binding to T1R NTDs, we used steady-state fluorescence spectroscopy to monitor ligand-dependent changes in steady-state intrinsic tryptophan fluorescence in the receptor proteins *(18,19)*. We measured the interaction of two natural sugars (glucose and sucrose) and one synthetic sweetener (the chlorinated sugar sucralose) with MBP-T1R2NTD, MBP-T1R3NTD and T1R3NTD. For each protein, these ligands induced a dose-dependent quenching of intrinsic tryptophan fluorescence *(18,19)* (Figure 2). In contrast, the umami stimulus L-glutamate and the sulfamate sweetener cyclamate (which binds to the transmembrane domains of human T1R3) had no effect *(18)*. The tryptophan fluorescence of MBP alone, while quenched by maltose, was unaffected by sucrose or sucralose *(18)*. Therefore, we conclude that glucose, sucrose and sucralose bind to the NTDs of both T1R2 and T1R3.

Next, we determined K_d values for glucose, sucrose and sucralose binding to the T1R2 and T1R3 NTDs *(18,19)*. T1R3NTD and MBP-T1R3NTD bound glucose and sucrose with nearly identical K_d values (Figure 2 and Table I), again supporting the specificity of ligand binding to the NTD. All three sugars bound MBP-T1R2NTD, though with affinities somewhat different from those seen for the T1R3 NTD proteins. For example, sucralose exhibited a 20-fold lower K_d
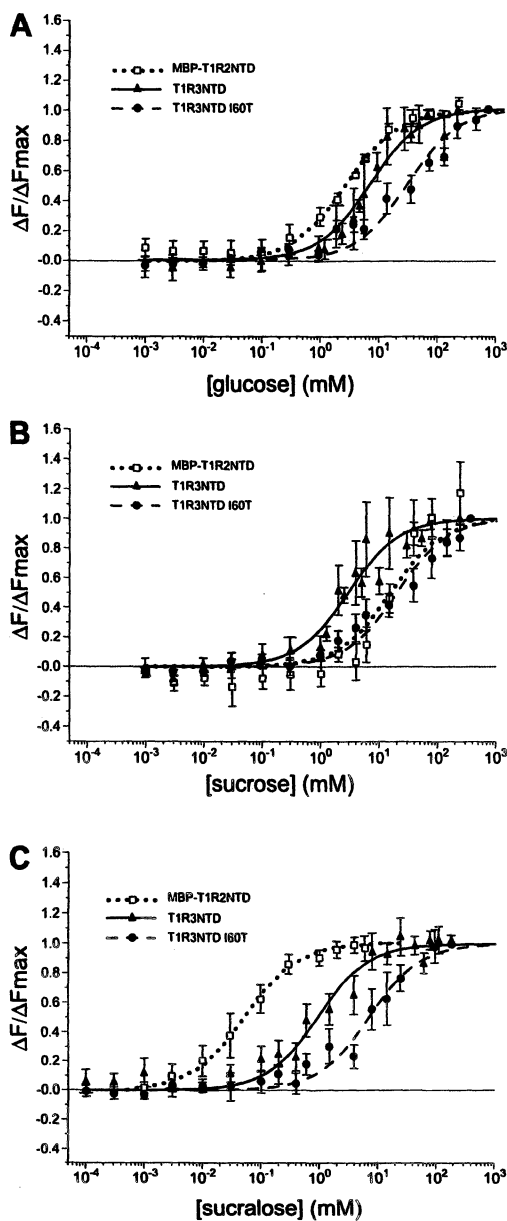
Figure 2. Sugars bind both T1R2 and T1R3 NTDs.
The peak intrinsic tryptophan fluorescence of MBP-T1R2NTD, T1R3NTD
and T1R3NTD$_{I60T}$ was measured as a function of ligand concentration for
(A) glucose (B) sucrose and (C) sucralose.

for MBP-T1R2NTD than for T1R3NTD, while the K_d for sucrose was 5-fold higher for MBP-T1R2NTD than for T1R3NTD or MBP-T1R3NTD.

Table I. K_ds (mM) for T1R2 and T1R3 NTDs

	MBP-T1R2NTD	T1R3NTD	T1R3NTD$_{160T}$	MBP-T1R3NTD
Glucose	2.6 ± 0.2	7.3 ± 0.7	32 ± 5	8.2 ± 1.5
Sucrose	15 ± 5	2.9 ± 0.4	20 ± 3	3.4 ± 0.4
Sucralose	0.0052 ± 0.004	0.091± 0.15	6.9 ± 0.9	ND

ND: not determined

T1R2 and T1R3 NTDs Undergo Distinct Ligand-Dependent Conformational Changes

Ligand binding to the NTDs of class C GPCRs, such as mGluR or GABA$_B$R, stabilizes a conformational change important for receptor activation and subsequent downstream signaling (8). Such changes can be monitored using synchrotron radiation circular dichroism (SRCD) spectroscopy, which is sensitive to changes in the secondary and/or tertiary structure of proteins (21-23). Glucose, sucrose and sucralose induced shifts in the SRCD spectra of MBP-T1R2NTD, MBP-T1R3NTD and T1R3NTD (Figure 3 and data not shown; sucralose was only tested with T1R3NTD) (18,19). Cyclamate induced no change in the spectra of the T1R NTDs, and glucose and sucrose had no effect on the spectrum of MBP alone (data not shown) (18). Interestingly, MBP-T1R2NTD displayed a spectral shift distinct from that seen for either T1R3 NTD protein, indicating that the two subunits undergo different ligand-dependent conformational changes. These results, along with those described above, suggest that each subunit of the native sweet taste receptor makes unique contributions to the conformational coupling of ligand binding to receptor activation.

A Mechanistic Explanation for Reduced Sweet Taste Preference in Inbred Mice

Inbred mouse strains display differences in preference for sugars, saccharin and other sweeteners. This variation has been linked to a single locus on distal chromosome 4 and designated as the saccharin preference (Sac) locus (24).

Research published by a number of groups in 2001 provided convincing evidence that allelic variation of a single gene, *Tas1r3* (which encodes T1R3) is synonymous with *Sac (6,20,25-28)*. Subsequent studies by Reed and colleagues *(29)* examined polymorphisms in *Tas1r3* across thirty inbred mouse strains of defined saccharin preference. Only a single non-synonymous polymorphism, a T→C change at nucleotide position 179 that resulted in an Ile→Thr change at amino acid position 60 (I60T), was significantly associated with reduced sweetener preference across these strains. This amino acid change has been suggested to either affect ligand binding *(29)* or to interfere with the dimerization of T1R2 and T1R3 *(20)*, but the basis of its effects on sweet taste sensitivity remained unclear.

To address this question, we examined the impact of the I60T polymorphism on the ability of sweet ligands to bind T1R3NTD *(18)*. Surprisingly, neither 5 mM glucose, sucrose or sucralose caused a shift in the SRCD spectra of T1R3NTD$_{I60T}$ (not shown) *(18)*, suggesting that either the affinity or the efficacy of ligand binding had been altered in this protein. Titration of these ligands showed that the T1R3NTD$_{I60T}$ protein continues to exhibit a dose-dependent quenching of intrinsic trytophan fluorescence (Figure 2). However, glucose, sucrose and sucralose, each bound T1R3NTD$_{I60T}$ with a lower affinity than that for the C57BL/6J variant of T1R3NTD (Table I). These results provide a mechanistic basis for the reduced sweet taste preference of mice bearing the *Sac* nontaster allele. Higher resolution structural studies will be required to determine whether this residue participates directly in ligand binding or if the mutation indirectly perturbs the binding pocket through a cascade of steric effects.

Conclusions

These studies offer a new approach to understanding the basis of receptor sensitivity and selectivity in sweet taste. The methodologies described here provide several advantages for dissecting the function of T1R taste receptors. First, ligand binding can be examined in the absence of other aspects of receptor activation, such as G protein coupling. Second, the contributions to binding of cooperative interactions between subunits binding can be examined by comparing results with homomeric receptors (as described here) and with heteromeric complexes (e.g., T1R2 + T1R3 NTDs). For example, glutamate can bind each subunit of the homomeric mGluR with the same affinity but induces a negative allosteric interaction as soon as the first subunit is bound *(30)*. The GABA$_B$R forms a heterodimer where only the GABA$_B$R1 subunit binds ligands at physiological concentrations, though the GABA$_B$R2 subunit also exerts cooperative effects *(8)*. Third, binding sites can be mapped for ligands that are
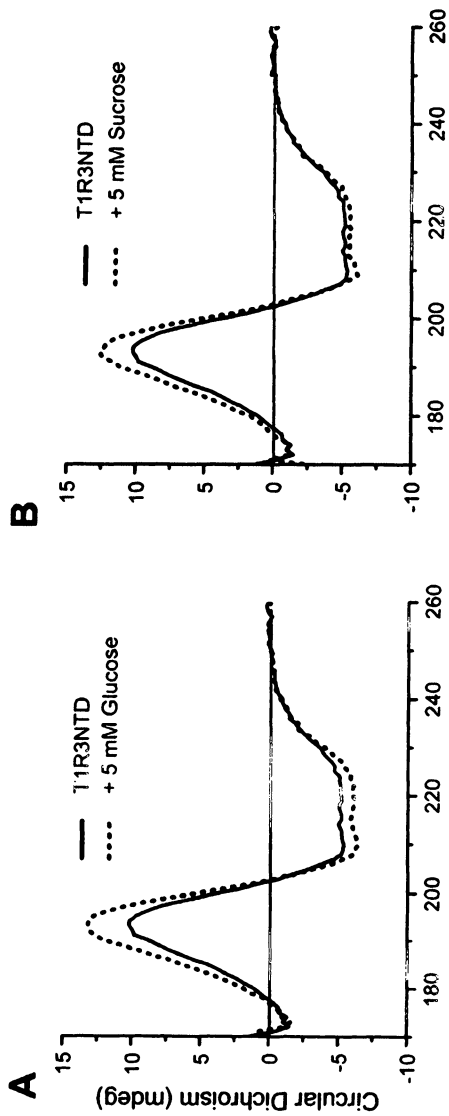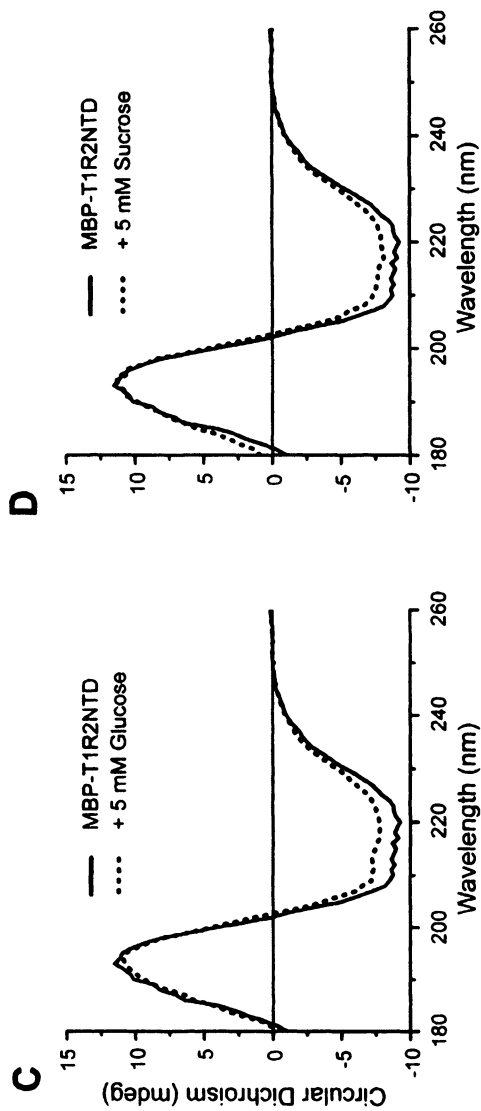
Figure 3. Ligand binding induces distinct conformational changes for T1R2 and T1R3 NTDs. SRCD spectra of T1R3NTD (A, B) and MBP-T1R2NTD (C, D) in the absence (solid) or presence (dashed) of ligand.

broadly preferred across species as the assay does not depend on receptor activation. Fourth, relatively low affinity interactions, even with K_d values in the millimolar range, can be quantified. Fifth, the highly purified state of the proteins eliminates potential non-specific ligand interactions. Sixth, the ability to express and purify functional ligand binding domains in large quantities will be necessary if their structures are to be determined by x-ray crystallography or other means.

Some of this promise has already been realized. Using these approaches we have shown that both T1R2 and T1R3 NTDs bind sugar stimuli at physiologically relevant concentrations, indicating that both subunits play an important role in the detection of sweet stimuli. Furthermore, our studies provide a mechanistic link between sweet taste receptor function and taste behaviors. Future studies should provide interesting insights into the mechanisms of stimulus recognition by T1R taste receptors.

Acknowledgements

References

1. Kinnamon, S. C.; Cummings, T. A. *Annu. Rev. Physiol.* **1992**, *54*, 715-731.
2. Lindemann, B. *Physiol. Rev.* **1996**, *76*, 718-766.
3. Stewart, R. E.; DeSimone, J. A.; Hill, D. L. *Am. J. Physiol.* **1997**, *272*, C1-C26.
4. Mombaerts, P. *Nat. Rev. Neurosci.* **2004**, *5*, 263-278.
5. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc. Natl. Acad. Sci. U S A* **2002**, *99*, 4692-4696.
6. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S. *Cell* **2001**, *106*, 381-390.
7. Nelson, G.; Chandrashekar, J.; Hoon, M. A.; Feng, L.; Zhao, G.; Ryba, N. J.; Zuker, C. S. *Nature* **2002**, *416*, 199-202.
8. Pin, J. P.; Kniazeff, J.; Liu, J.; Binet, V.; Goudet, C.; Rondard, P.; Prezeau, L. *FEBS J* **2005**, *272*, 2947-2955.

9. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. *Nature* **2000**, *407*, 971-977.

10. Morini, G.; Bassoli, A.; Temussi, P. A. *J Med Chem* **2005**, *48*, 5520-5529.

11. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N. J.; Zuker, C. S. *Cell* **2003**, *115*, 255-266.

12. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L. A.; Benard, L. M.; Osman, R.; Max, M.; Margolskee, R. F. *J. Biol. Chem.* **2005**, *280*, 34296-34305.

13. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Margolskee, R. F.; Max, M. *J. Biol. Chem.* **2004**, *279*, 45068-45075.

14. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. *Proc. Natl. Acad. Sci. U S A* **2004**, *101*, 14258-14263.

15. DuBois, G. E. *Proc Natl Acad Sci U S A* **2004**, *101*, 13972-13973.

16. Gimelbrant, A. A.; Haley, S. L.; McClintock, T. S. *J. Biol. Chem.* **2001**, *276*, 7285-7290.

17. Saito, H.; Kubota, M.; Roberts, R. W.; Chi, Q.; Matsunami, H. *Cell* **2004**, *119*, 679-691.

18. Nie, Y.; Vigues, S.; Hobbs, J. R.; Conn, G. L.; Munger, S. D. *Curr. Biol.* **2005**, *15*, 1948-1952.

19. Nie, Y.; Hobbs, J. R.; Vigues, S.; Olson, W. J.; Conn, G. L.; Munger, S. D. *Chem. Senses.* **2006**, *31*, 505-513.

20. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. *Nat. Genet.* **2001**, *28*, 58-63.

21. Manavalan, P., and Johnson, Jr., W. C. *Nature* **1983**, *305*, 831-832.

22. Venyaminov, S.; Vassilenko, K. S. *Anal. Biochem.* **1994**, *222*, 176-184.

23. Wallace, B. A. *Nat. Struct. Biol.* **2000**, *7*, 708-709.

24. Fuller, J. L. *J. Hered.* **1974**, *65*, 33-36.

25. Bachmanov, A. A.; Li, X.; Reed, D. R.; Ohmen, J. D.; Li, S.; Chen, Z.; Tordoff, M. G.; de Jong, P. J.; Wu, C.; West, D. B.; Chatterjee, A.; Ross, D. A.; Beauchamp, G. K. *Chem. Senses* **2001**, *26*, 925-933.

26. Kitagawa, M.; Kusakabe, Y.; Miura, H.; Ninomiya, Y.; Hino, A. *Biochem. Biophys. Res. Commun.* **2001**, *283*, 236-242.

27. Montmayeur, J. P.; Liberles, S. D.; Matsunami, H.; Buck, L. B. *Nat. Neurosci.* **2001**, *4*, 492-498.

28. Sainz, E.; Korley, J. N.; Battey, J. F.; Sullivan, S. L. *J. Neurochem.* **2001**, *77*, 896-903.

29. Reed, D. R.; Li, S.; Li, X.; Huang, L.; Tordoff, M. G.; Starling-Roney, R.; Taniguchi, K.; West, D. B.; Ohmen, J. D.; Beauchamp, G. K.; Bachmanov, A. A. *J. Neurosci.* **2004**, *24*, 938-946.

30. Suzuki, Y.; Moriyoshi, E.; Tsuchiya, D.; Jingami, H. *J. Biol. Chem* **2004**, *279*, 35526-35534.

Chapter 5

In Vitro Models to Study Taste: Can Sweetness Be Modeled in a Dish?

Nancy E. Rawson[*] and M. Hakan Ozdener

Monell Chemical Senses Center, 3500 Market Street,
Philadelphia, PA 19104–3308
[*]Corresponding author: 215–898–0943 (phone), 215–898–2084 (fax), and
rawson@monell.org

Primary and immortalized cultures of many cell types have facilitated efforts to understand the signals involved in proliferation, differentiation and senescence, and yielded tools to rapidly assay new molecules targeting specific receptor pathways. Surprisingly, few studies have reported successful primary culture protocols for taste cells, and those reported have had a limited life span and have not purported to generate new cells *in vitro*. Like other epithelial cells, taste cells are generated throughout life from a basal cell population, although the precise lineage and signaling molecules involved in this process are not well known. We have recently developed a primary taste cell culture method that supports the generation of new cells expressing key molecular and functional features of mature taste cells. These cultures can be maintained for functional assays for over two months. In this chapter we will present insights into the development of this protocol and discuss advantages and disadvantages of the approach. We will also discuss the potential use of these cultures as discovery tools to study the effects of neurotrophic factors on taste cell proliferation and differentiation and as an aid in the development and evaluation of new taste molecules.

76 © 2008 American Chemical Society

Introduction

The discovery of the receptor proteins responsible for detecting sweet, bitter and umami taste stimuli has led to an explosion of research into the peripheral processes of taste detection. Receptor binding can be studied by inserting the genes into tumor cell lines or other non-native systems ('heterologous' cells) which express the proteins in a way that allows stimulus binding to be detected by measuring cellular activity in high throughput assays with fluorescence probes (1, 2, 3). This approach enables screening the taste activity of many compounds in a reasonable amount of time, and a wealth of data regarding basic taste receptor binding activities has been generated. While these methods have been instructive in studies of the initial stimulus binding event, results may fail to reflect actual taste detection processes because several important aspects of the peripheral taste organ are missing. First, the signaling pathways used by these 'heterologous' cells differs from those of the native taste cells. These signaling pathways determine the cellular output and can also play critical roles in modulating receptor binding activity. Second, in their native environment, taste receptor cells exist within a specialized structure, the taste bud, in which close communication between mature taste receptor-containing cells and several other cell types occurs (4, 5, 6). While the details and full implications of this intercellular communication have yet to be elucidated, it is likely to play a key role in shaping the signal received by the taste nerves.

Several alternative approaches to investigate taste receptor cell function exist, such as recording from taste nerves in intact preparations or imaging dissociated single taste cells or semi-intact taste buds in tissue slices. While these methods reflect the behavior of the intact system to a better degree, they are not conducive to screening large numbers of chemicals for taste activity and require the use of a large number of experimental animals. We therefore aimed to develop a method that would retain the intrinsic intracellular signaling mechanisms while being potentially amenable to higher throughput approaches. We have for the first time produced mature taste cells in culture, generating them *in vitro* from taste cell precursors that continue to divide in culture (7). These proliferating cells, obtained from rat taste buds, divided and differentiated into clusters of mature taste cells. The new cells, which were kept alive for up to three months, were similar to mature taste cells in several respects. A variety of methods were used to show that these cells contain marker proteins characteristic of mature functioning taste receptor cells. In addition, functional assays showed characteristic responses to either bitter or sweet taste stimuli in a subset of the cells. No single method can answer all questions, and disadvantages of this approach include a limitation in the types of stimuli that can be used due to the osmotic requirements of these cultured cells, and the loss of the normal taste cell morphological polarity and cell-cell contacts. However, cultured cells are amenable to the use of higher throughput assay methods to

examine responses to single or combined taste stimuli and thus represent one step closer to the *in vivo* system than heterologously expressed receptors, while retaining the advantage of rapid assay capability. Also, control over the growth conditions will allow us to begin to explore mechanisms by which receptor gene expression is controlled and to identify growth factors that act to regulate the processes by which new taste cells are generated.

This chapter will describe the history and current state-of-the art methods for taste cell culture. Primary and immortalized cell cultures provide powerful tools for examining the molecular bases for proliferation and differentiation as well as cellular function, but these systems have limitations as well. We will discuss the potential for these cultures as discovery tools for the study of sweet taste stimuli or modifiers, as well as for understanding control and differentiation of taste receptor cells.

Cellular anatomy and function

Taste receptor cells are highly specialized receptor cells with unique histological, molecular and physiological characteristics designed to detect a wide range of chemical molecules contained in foods. In mammals, taste receptor cells are contained within a specialized cluster termed the taste bud. Taste buds are located in three kinds of papillae (foliate, circumvallate, fungiform) on the tongue and also in the palate. Each bud contains a group of 50-100 taste cells surrounded by basal and epithelial cells which have been classified anatomically into four types: basal, type I, type II, type III. Different taste cell types exhibit neuronal and epithelial properties, and have a limited life span of ~10-12 days (*8, 9, 10*). The taste bud is functionally divided into two parts; the apical region and the basal region. The apical region faces the oral cavity and is where taste chemicals interact with taste receptor cells. Tight junctions function as a barrier between the apical region and lower basal region by limiting diffusion of large molecules into the serosal space. Because of the tight junction, only a few taste bud cells interact directly with stimuli, and the majority of the taste cell body is protected from the harsh environment of the oral cavity. This configuration, which is lost during typical cell isolation and culture procedures, is likely to be important for certain aspects of taste receptor cell signaling, and the loss of such orientation represents one disadvantage of dissociated and cultured cell methods (*11*).

Mammalian taste cells are heterogeneous in terms of morphological structures, immunochemical features and functional characteristics. Anatomical studies depend on particular profiles of protein markers to delineate subsets of taste cells, but most studies find some cells that do not fit these classes. The GTP- binding regulatory protein gustducin (guanine nucleotide-binding protein), phospholipase C-β2 (PLCβ2) and inositol 1,4,5 triphosphate receptor type 3

(IP3R3), along with taste receptors, are expressed in subsets of taste cells, typically considered 'Type II' cells (*12, 13, 14, 15, 16*). Gustducin generally appears in a subset of PLCβ2-expressing cells. However, not all gustducin labeled cells are PLCβ2 immunoreactive (*17*), and it is not clear whether this is a developmental difference or represents a distinct class of mature cells. Serotonin, neuron-specific enolase (NSE), ubiquitin carboxyl terminal hydrolase (PGP 9.5), and neural cell adhesion molecule (NCAM) have been used as markers for morphologically defined Type III taste cells in rats, although not all 'Type III' cells express all of these markers (*18, 19*). Nerve transection studies suggest that NCAM expression in taste cells depends on innervation by the IX nerve - suggesting that taste cells expressing NCAM communicate with nerves (*20*). In most studies, gustducin does not appear to colocalize with PGP 9.5, NSE or NCAM, although this distinction is not always complete (*18*). Species and methods differences may account for some of the lack of consistency in the anatomical data throughout the literature. Taken together, however, a model for the organization of the rodent taste bud suggests at least three populations of differentiated cells – type I cells that express none of the markers of receptor function or neurotransmission and have been identified by the expression of the glial glutamate transporter GLAST1 (*21*); cells expressing receptor and transduction-elements that are primarily Type II cells, and cells expressing neural and synaptic vesicle markers, primarily Type III cells.

The functional classification of taste bud cells also remains somewhat controversial. Some authors propose a model organized as a 'labeled line', in which cells are segregated by function as 'sweet detectors or bitter detectors', and transmit their signals to particular nerves projecting to specific regions of the gustatory cortex (*22*). In contrast, physiological data suggest a less-discrete arrangement, in which cells responsive to multiple taste qualities transmit information to each other and to associated nerves in a fashion that is modulated both spatially and temporally to generate a particular activity pattern in the cortex that is interpreted as a taste quality (*23, 24*). Some of these physiological studies are performed using dissociated cells that may enable activation via pathways not accessible to the stimulus under *in vivo* conditions. However, even under conditions where taste bud morphology is preserved (e.g., optical recording from thick tissue sections), receptor cells do not appear to be 100% selective for a single taste modality (*25, 26*). In general, however, broad classes of functionally distinct cells have been described: one group which responds to one or more sweet, bitter and umami stimuli but does not exhibit voltage-sensitive calcium channels (VSCC), and another that does not respond to these taste stimuli directly, but does exhibit a calcium increase in response to depolarization with high K^+ stimulation, indicating the presence of VSCCs (*28, 29, 30*). Species differences have also been reported, and the classification of taste cells based on anatomical, functional or molecular traits alone is challenging. An ideal approach would be to combine all three types of

characterizations. One study approached this by combining functional and molecular (RT-PCR) methods to study isolated mouse taste cells (*31*). This study found that cells responsive to tastants (a bitter and a sweet stimulus) but not to depolarization expressed mRNA for PLCβ2, but did not express the synaptic marker SNAP-25, while the converse was true for cells responding to depolarization (*31*). Taken together, two functional classes may be proposed – cells expressing receptor and transduction proteins and responding to taste stimuli with changes in intracellular calcium, probably representing the majority if not all 'Type II' cells, and those expressing neuronal markers including synaptic vesicle proteins and responding to depolarization. The recently discovered ion channel involved in sour taste, PKD2L1, appears to localize to a subset of taste cells that do not express PLCβ2, but this paper did not examine whether or not these sour detecting cells expressed neuronal markers. Finally, mapping of salt taste mechanisms to these classification schemes awaits definitive identification of these mechanisms (*32, 33*).

The identification of cell types in culture thus requires both anatomical and functional methods to compare cellular characteristics to their *in vivo* counterparts. The fact that the literature regarding the phenotypic characterization of those counterparts remains incomplete in some respects represents both a challenge and an opportunity. In culture, we have the opportunity to explore the phenotypic capacity of cells derived from mature taste buds and to trace their behavior over time, in a system that is amenable to examining molecular and functional characteristics simultaneously. The lack of a polarized structure represents a limitation of the approach, but the development of 3-D cell culture matrices may enable this limitation to be overcome in the future.

Taste Bud Development and Cell Lineage

Why has it been so difficult to generate taste cells *in vitro*? The historical perspective of taste bud development and regeneration can shed light on this question. Studies of taste bud development suggest that these specialized sensory receptors arise from stem cells residing in and around the taste bud (ecto- or endodermal epithelia) (*34, 35*). These studies have relied in part on approaches that examine regeneration of taste buds following nerve transection, or explant cultures to examine early embryonic development. A few studies have employed lineage tracing techniques. The results of these lines of research have led to two apparently conflicting models of taste bud development.

The neural induction model proposes that peripheral nerve fibers induce taste bud formation (*36, 37, 38*). Studies of lingual explants and tongue cultures show that the structural integrity of the mammalian taste bud is dependent on the presence of gustatory nerves (cranial nerves VII, IX and X), but not motor or

other sensory nerves (*39*). Severe injury or transection of gustatory nerves resulted in degeneration of taste buds (*40, 41*) and taste cells only reappeared after gustatory nerves entered the taste papillae (*42, 43*). After sectioning cranial nerve IX (glossopharyngeal nerve) in mice, the number of cells expressing T1R3, gustducin, Mash1, shh and Nkx2.2 was reduced (*44, 45, 46*). However, it was noted that fungiform taste bud cells were more resistant to denervation than vallate and foliate taste buds, suggesting differences between development of taste buds in these regions of the tongue (*47, 48*). These studies suggest that trophic factors are released by the nerve that are necessary to stimulate taste bud regeneration. Uchida et al. demonstrated that BDNF and TrkB colocalized with NCAM (neural cell adhesion molecule) in some taste bud cells. After denervation, the number of taste bud and nerve fibers decreased, but remaining intact taste buds still contained cells expressing BDNF and TrkB. Interestingly, 4 weeks after denervation, TrkB expressing nerve fibers entered taste papillae, and new taste buds expressing BDNF and TrkB re-appeared (*49*). This result may indicate that BDNF acts as a neurotrophic factor acting both on TrkB expressing taste bud cells and on the growing sensory nerve. However, studies with transgenic mice lacking the TrkB receptor showed that this receptor is not essential to taste bud development (*50*). If nerve-derived trophic factors are an absolute requirement for the differentiation of a mature taste cell, cultures in which nerves or their derived factors are absent will not generate phenotypically complete, mature taste cells. Nevertheless, primary cultures would still represent a tool to help clarify the roles of specific growth factors involved in promoting proliferation of progenitor cells and their specification toward a mature taste cell phenotype.

Other lines of evidence have led to the 'early specification model'. According to this perspective, taste cells arise from local endodermally-derived epithelium of the oropharynx in the absence of innervation (*34, 52, 53*). The majority of evidence for this developmental path has been obtained from experiments studying the development of axolotls. These studies point to an early specification of the oropharyngeal epithelium during gastrulation and a process that is dependent on signals from the axial mesoderm but independent of neuroectoderm or other tissue sources. (*54*). Taste bud progenitor cells originate from the oropharyngeal endoderm as a result of cell-cell signaling (*55*), and taste buds are induced and begin to develop prior to innervation. As the taste bud continues to differentiate, synapses are formed with the afferent fibers (*52*).

These two models may not, in fact, be contradictory. Rather, they may reflect true biological processes occurring under distinctly different experimental conditions - regeneration vs. development, and/or different animal models. Alternatively, innervation may be needed for proper assembly of a complete taste bud, but a population of basal cells may exist that remain capable of cell division yet are pre-specified to differentiate into mature taste cells regardless of their innervation status. The data thus leave open the question of whether the capacity to generate taste cells is programmed into peri- or

intragemmal basal cells such that they can be directed to this fate under certain experimental conditions *in vitro*.

Various models for understanding the lineage of taste cells have been explored. In one model, different taste cell types arise from pre-specified progenitors that generate distinct lineages and maintain a stable phenotype throughout their lifespan (*56, 57*). Alternatively, a single progenitor may give rise to a multipotent immature phenotype that becomes specified as maturation proceeds (*53, 58*). Stone et al. reported (Figure 1) that this lineage restriction could occur via multipotent embryonic progenitor cells that give rise to multipotent basal cells which generate mature phenotypes that are a stable phenotype throughout their lifespan (*53*). Thus, a particular basal cell might generate only a single type of taste cell (Figure 1 A). Conversely, a single embryonic progenitor cell may give rise to multiple basal cells, which are fate-restricted to generate only certain types of mature taste cells (Figure 1 B). The potential and ultimate phenotype of proliferative, immature and mature taste cells can be examined *in vitro* under conditions where cells at all stages can be followed over time and characterized using functional and molecular methods. Cell culture studies may therefore yield new insight into the process by which a sweet sensitive cell arises and to examine the factors involved in controlling expression of the genes involved in detection of specific taste qualities.

Culture systems to study taste cells: From whole tongue to cell culture

Whole tongue cultures and lingual explants have been used primarily to investigate differentiation and development of embryonic taste cells and regeneration after nerve surgeries (*59, 60, 61*). These studies have shown that maintenance of the relationships between different types of taste cells, surrounding cells and the nerves that innervate taste buds during development and regeneration requires complex cell – cell signaling (*62, 63, 64*). While these approaches provide a physiologically natural environment, lingual explants and tongue cultures have disadvantages. Primary among these is the need for a large number of animals as each preparation or experiment generally requires one animal and can be used for a limited amount of time, typically ranging from days to a week (60, 61). In addition, manipulation of the external environment to assess the importance of specific molecules to specific steps in the developmental process can be problematic.

To date, no immortalized taste cell lines have been reported. Asamoto and colleagues described a metastasizing neuroblastoma arising from a taste bud during establishment of a prostate cancer model in transgenic rats (*65*). However, these cells fail to express classical markers of mature taste cells and do not respond to taste stimuli (Ozdener and Rawson, unpublished
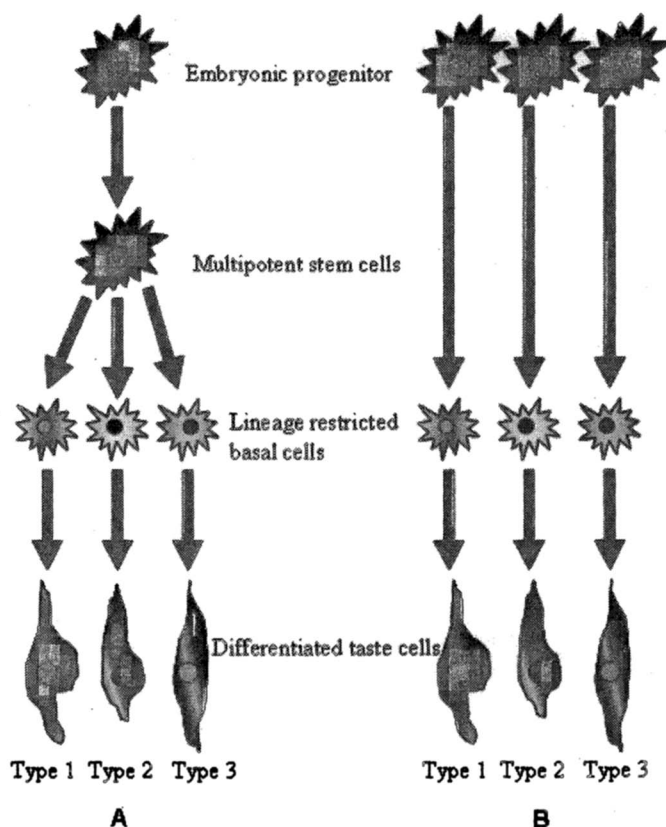
Figure 1. Diagram represents proposed taste cell lineage models. Taste cell development may occur in two ways. (A): Embryonic progenitor cells give rise to multipotent stem cells which generate lineage restricted basal cells which are capable of generating a stable mature phenotype; (B) Each single embryonic progenitor cell give rises to multiple basal cells which give rise to lineage restricted mature taste cells (Adapted from Stone et al 2002, (53)).

observations). Other studies have investigated the responses of several existing cell lines to taste stimuli. The mouse neuroblastoma cell (N-18 clone) was found to be reversibly depolarized by various bitter substances, and NG108-15 (mouse neuroblastoma x rat glioma hybrid) cells were broadly responsive to several potent sweeteners and bitter compounds (*66, 67*). However, no further studies have reported the use of these cells for detailed studies of taste detection, and it is not known whether the mechanisms accounting for these responses resemble those present in taste receptor cells.

A number of researchers have carried out studies in which freshly dissociated taste cells are maintained *in vitro* for functional or molecular studies for up to a week (*68, 69, 70, 71*). Only two reports describe longer term cultures, and in both cases no effort was made to establish whether new cells were generated *in vitro*. In one report, cells derived from mammalian taste buds were maintained at room temperature, which is believed to slow down various cellular processes, and survived for up to 14 days. Notably, when cells were kept at 37°C under the same conditions, they could be maintained for only 3-4 days, as had been previously reported (*68*). A second study maintained a primary culture of taste receptor cells from the labella of blowfly pupa for up to 17 days *in vitro*. These cells were functional for up to a week after isolation, but there was no effort to assess whether any new cells were generated *in vitro*. Finally, attempts to isolate a particular type of cell from mouse taste epithelium, sorted based on their expression of integrin β1 marker generated a semi-purified culture of proliferating cells, some of which expressed NCAM as well as integrin β1. However, this integrin β1 positive mouse cell culture did not generate cells similar to those responsible for the primary detection of taste stimuli and further work with them has not been reported (*72*).

Development of a primary taste cell culture protocol

In view of the potential value of such a system, we decided to revisit the problem of long-term taste cell culture using modern methods of cell culture and an open mind. Our goal was first, to replicate earlier studies maintaining mature cells for at least two weeks *in vitro*; second, to maintain a population of cells that could proliferate *in vitro*, and finally, to establish whether any of these proliferating cells proceeded to differentiate to cells exhibiting phenotypic traits of a mature taste cell. The protocol, developed through an extensive empirical process, allows the maintenance and generation of cells exhibiting both molecular and functional characteristics of mature taste cells. The detailed methods have been published elsewhere (*7*); here, we review the critical steps in this process, summarize the successful protocol and highlight key methodological issues.

Age of the animal, euthanization method and dissection speed can all influence the ultimate outcome of any primary cell culture process. Although cultures could be generated from mature as well as weanling rats, rats ranging from 1-2 months euthanized by CO_2 inhalation followed by cervical dislocation generated the most consistent results. The tongue was immediately dissected proximal to circumvallate papillae and placed into a chilled isolation solution (26mM $NaHCO_3$, 2.5mM NaH_2PO4, 20mM glucose, 65mM NaCl, 20mM KCl, and 1mM EDTA) for 5-10 min on ice. The EDTA in solution serves to reduce the concentration of calcium, a factor critical to disrupting cell to cell adhesion (*73, 74*). Taste buds are well-protected beneath a thick keratinized epithelium and can be challenging to disrupt. For this reason, most isolation protocols include harsh proteolytic enzymes that may degrade receptors and ion channels on the taste cell surface. Additionally, mechanical stress during isolation may also reduce viability of cells (*69, 70, 72*). While it is necessary to disrupt cells sufficiently to seed the cells into culture, over-digestion or mechanical disruption can make cells too fragile and increase cell death during isolation. To accomplish taste bud removal and disruption, we injected approximately 1 ml of the isolation buffer mixed with 1.5mg/ml pronase E (Sigma, St. Louis, MO) and 1mg/ml elastase (Sigma, St. Louis, MO) uniformly with a 25 gauge Norm-Ject syringe under and around the lingual epithelium of circumvallated and foliate papillae of the dissected tongue. The preparation was then removed from ice and incubated for 15-20 min in isolation buffer at room temperature. The enzymes, concentrations, temperature and time are key and designed to be the minimum needed to enable the epithelium to be gently peeled from the underlying muscle layer under a dissecting microscope (Stereomaster, Fischer Scientific, Pittsburgh, PA). The isolated epithelium was then transferred to Iscove's Modified Dulbecco's medium (Gibco BRL, New York, NY) containing 10% fetal bovine serum ((FBS) BTI, Stoughton, MA), 1:5 ratio of MCDB 153 (Sigma), and a triple cocktail of antibiotics (100U/ml / 100µg/ml, Penicillin/Streptomycin, 2.5 µg/ml Gentamycin and 0.25 µg/ml Fungizone) and cut into small pieces with a razor blade. This process can be done with scissors or a scalpel, but the razor blade typically results in less tearing and cell disruption. The pieces were seeded onto tissue culture plates coated with rat tail collagen type 1 to promote adhesion, and incubated at 36°C in a humidified environment containing 5% CO_2. Culture medium was replaced after 24-48 h and then every 5-7 days. A large variety of cellular attachment substrates are now available that are designed to encourage selective adhesion of different cell types. In preliminary studies, attachment, viability and growth were compared among cells seeded on coverslips coated with either mouse fibroblasts (ATCC, Manassas, VA), matrix gel (2 ml/l, ATCC), or poly-D-lysine (BD Biosciences, San Jose CA) or on uncoated polystyrene plates (Corning, Corning, NY). Results among these substrates varied considerably, and collagen was selected for further studies due to its effectiveness and comparatively low cost. A wide array of culture media and supplements are now available that can also be

critical to cell attachment, growth and differentiation. While the possible combinations of these are virtually limitless, we initially selected a set of media and supplements to test based on their use in other primary cell systems or their advertised characteristics. Among the media tested were: Iscove's Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), DMEM + MCDB 153 (all available from Sigma), and each media was tested with various concentrations of fetal bovine serum. Serum is a comprehensive source of growth factors and stabilizing proteins that can help to emulate the normal *in vivo* environment. However, the exact composition of different sources, types and even batches can vary making the use of FBS potentially difficult. This also prevents attributing a successful culture to a particular growth factor or combination of factors, and can complicate the interpretation of experiments examining growth factor effects. Thus, an ideal in cell culture is the development of a 'defined media' with specific concentrations of particular growth factors and supplements that generates the desired result. As yet, our efforts with taste cells have been limited to the use of sera, rather than entirely defined media, but this remains a goal for the future.

Under the conditions described (7), we maintained isolated rat taste cells from circumvallate and foliate papillae in culture without loosing viability or cellular and physiological functions for more than two months. We also maintained a population of cells in the culture that divided and differentiated. To demonstrate this, we incubated the cells with bromo-deoxyuridine (BrdU), which is incorporated into the DNA of actively dividing cells, allowed cells to grow for several additional days, then assessed the expression of taste specific cell markers (gustducin, PLCβ2, T1R3, T2R5, β-actin) by PCR and Western blot (gustducin, PLCβ2) after 60 days in culture. A significant proportion of biomarker-immunoreactive cells were also labeled with BrdU, demonstrating that the method developed enables the generation of new mature taste cells *in vitro* (Figure 2).

Doubling time was approximately 7 days, and cells derived from one initial culture could be studied for up to two months. Initial taste stimuli were selected to avoid artifacts that could arise from large perturbations in osmolarity, refractive index or autoflourescence, and included denatonium, cycloheximide, sucralose, Acesulfame K, and aspartame. Cultured cells responded to one or more of the tested stimuli with increases in intracellular calcium, based on experiments using ratiometric Ca^{+2}-imaging (7 and Ozdener and Rawson unpublished observations). No cells responded to all stimuli, and only one cell responded to depolarization with high potassium, indicative of voltage-sensitive calcium channels. This observation suggests that the cultures are preferentially preserving or generating cells more typical of the 'Type II' phenotype, which does not exhibit voltage-gated calcium currents and expresses taste receptors and transduction elements. While it is not yet known whether these cells possess the full complement of taste receptors and associated ion channels, demonstration of molecular and functional properties reflecting those of mature
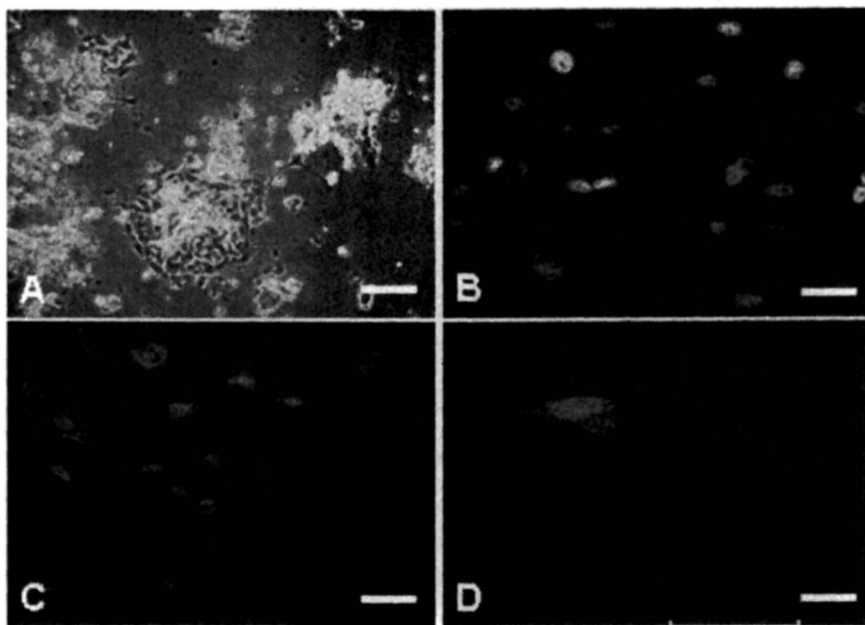
*Figure 2. Morphology and taste cell specific immunoreactivity of cultured taste
cells and foliate papillae obtained from rat tongue. Rat taste cell cultured on
rat tail collagen type 1 coated plates were imaged after 2 days (A) Individual,
bud-type and cell clusters were observed in short term culture. Double
immunofluorescence labeling indicates cultured taste cells immunoreactive with
BrdU (green; mouse anti-BrdU 1:100, Sigma B-2531) and gustducin (red;
rabbit polyclonal, 1:500, Santa Cruz,) (B); and PLCβ2 (red; rabbit polyclonal,
1:500, Santa Cruz,). Goat anti-mouse Alexa 488 (green; 1:500, Molecular
Probe) and goat anti-rabbit Alexa 633 antibodies (red; 1:500, Molecular Probe)
were used as secondary antibody for BrdU and Gustducin and PLCβ2,
respectively. (C). Labeling with BrdU and a taste cell marker indicates
proliferation and differentiation in vitro. A small number of taste cells were
immunoreactive with NCAM antibody (mouse monoclonal, 1:500, Sigma C9672)
suggesting the presence of type III cells. Goat anti-mouse Alexa 488 (1:500,
Molecular Probe) was used as secondary antibody for NCAM staining
(D). Σχαλε βαρσ = 50 μm (A) and 80 μm (B-D)
(See page 1 of color inserts.)*

taste receptor-expressing cells also positions these cells as tools to assess the ability of chemical stimuli to elicit taste responses or modulate responses to other stimuli. With this opportunity, taste cells from a single rat can be studied over many days, thus reducing the number of animals necessary and sufficient cells can be generated for studies of the effects of trophic factors or other defined growth conditions on the generation of cells expressing particular phenotypic characteristics.

Why cell culture: benefits and limitations

The art of primary cell culture aims to provide an environment similar to the physiological conditions that a cell would experience in animals. These cells represent the genotype of their donor and can exhibit many phenotypic characteristics of their *in vivo* counterparts. Thus, they may be useful not only for examining cellular function in a more representative system than heterologously expressed receptors, but also for the study of individual diversity in aspects of taste cell function beyond the receptor, that may be derived from genotypic variations among species or individuals.

While these applications amply justify work with primary cultures, a number of limitations must be mentioned. Foremost among these is that each batch of primary cultured cells can vary due to differences in the initial population of cells used to start the culture. While there is no way to entirely prevent this variability, careful attention to dissection and dissociation procedures, documentation of culture appearance during development and maintaining consistent culture conditions can help the investigator minimize batch to batch variability. The identification of a specific marker or set of markers and/or a control stimulus that can be used to compare cell profiles among batches is helpful in interpreting the results of functional or molecular assays. Unfortunately, there is no single stimulus that can be used to identify a 'mature taste cell'. However, expression of a set of markers or a stimulus battery can be used to compare the effects of growth factor treatments to determine relative differences among groups.

Contamination is a common problem when dealing with primary cell cultures, particularly with cells derived from areas exposed to the external environment such as the tongue. Pre-rinsing with sterile buffer and the use of antibiotics in the initial culture can help prevent this problem, but there is no substitute for strict adherence to maintaining a sterile culture environment and the use of sterile techniques. Bacteria and mold destroy the culture, while more subtle contaminants such as mycoplasma can change physiological and immunochemical characteristics of cultured cells and take up nutrients preventing the cells from growing (75, 76).

On a more subtle level, primary cells are also subject to dedifferentiation and exhibit a general plasticity according to the culture conditions. Chromosomal instability, characterized by losses or gains of chromosomes during cell replication in continuous cell lines may be another problem (77). Thus, cells in primary culture may show differences in behavior in comparison with the *in vivo* situation due to the difference in physiological conditions (78). Constant awareness of these issues and monitoring of key phenotypic traits is needed to insure that results are reliable and reproducible.

While variations among cultures can present a challenge for the researcher attempting to determine the effects of any given stimulus on cellular responses, it can also provide an advantage for studying the cell and molecular basis for individual differences in disease susceptibility, response to medication or other therapies, or sensitivity to different taste stimuli.

Future directions in taste cell research

One potential application of taste cell culture systems is to study how taste cell receptors interact with and trigger signaling pathways. Sweet detection is thought to be mediated by dimers of the G-protein coupled receptors Tas1R2 and Tas1R3 (79, 80; see also Chapter 4, this volume). Physiological, molecular and biochemical data suggest that sweet molecules may activate multiple signaling pathways – one involving inhibition of adenylyl cyclase and another involving PLCβ2 and generation of IP_3/DAG (81, 82). Molecular and immunocytochemical data support the expression of the PLCβ2 dependent pathway in cultured taste cells, but additional studies are needed to examine what other signaling pathways may be present in these cells and how they may contribute to responses to taste stimuli. The ability to directly manipulate specific signaling pathways through transfection or pharmacological treatments will facilitate these studies. These cells are also amenable to the use of higher throughput assay methods enabling the evaluation of new molecules aimed at activating, inhibiting or modifying taste cell output within a phenotypic context more similar to their *in vivo* counterparts than a heterologous expression system.

Another promising avenue for further research lies in the clinical arena. Although a rare condition, taste loss is devastating to the quality of life and can even threaten survival of the patient. Radiation therapy for head and neck tumors results in taste loss when the radiation field includes anterior tongue (83). In these patients, the loss becomes complete after repeated treatments and recovery can be delayed for months after the therapy ends (84). During treatment, oropharyngeal irritation and nausea combine to make eating extremely aversive. Taste loss further exacerbates this problem, leading to malnutrition that can threaten survival independently of the primary cancer (85,

86). The primary cell culture model described may provide a valuable aid in the understanding of how radiation injures taste cells and a platform with which to test molecules that may reduce injury and promote regeneration.

Great progress has been made toward understanding the development of taste receptor cells and the mechanisms by which they respond to chemical stimuli, yet many open questions remain. These questions will require the use of many experimental approaches and model systems before our understanding is complete. The protocol we have developed for primary taste cell culture represents one approach we hope will contribute to this effort.

Acknowledgements

References

1. Conte, C; Guarin, E; Marcuz, A; Andres-Barquin, PJ. *Biochimie.* **2006**, *88*, 801-806.
2. Behrens, M; Brockhoff ,A; Kuhn, C; Bufe, B; Winnig, M; Meyerhof, W. *Biochem Biophys Res Commun.* **2004**, *319*, 479-485
3. Versele, M; Lemaire, K; Thevelein, J.M. *SEMBO Rep.* **2001**, *2*, 574-579.
4. Roper, S.D. *Cell Mol Life Sci.* **2006**, *63*, 1494-1500.
5. Finger, T.E; Danilova, V; Barrows, J; Bartel, D.L; Vigers, A.J; Stone, L; Hellekant, G; Kinnamon, S.C. *Science.* **2005**, *310*,1495-1499
6. Kaya, N; Shen, T; Lu, S.G; Zhao, F.L; Herness, S. *Am. J Physiol Regul Integr Comp Physiol.* **2004**, *286*, R649-R658
7. Ozdener, H; Yee, K.K; Cao, J; Brand, J.G; Teeter, J.H; Rawson NE. *Chem Senses.* **2006**, *31*, 279-290
8. Farbman, A.I. *J Ultrastruct Res.* **1965**, *2*, 328-350
9. Kinnamon, J.C; Taylor, B.J; Delay, R.J; Roper, S.D. *J Comp Neurol.* **1985**, *235*, 48-60
10. Yee, C.L; Yang, R; Bottger, B; Finger, T.E; Kinnamon, J.C. *J Comp Neurol.* **2001**, *440*, 97-108
11. Spielman, A.I; Mody, I; Brand, J.G; Whitney, G; MacDonald, J.F; Salter, M.W. *Brain Res.* **1989**, *503*, 326-329
12. Rossler, P; Kroner, C; Freitag, J; Noe, J; Breer, H. *Eur J Cell Biol.* **1998**, *77*, 253-261

13. McLaughlin, S.K; McKinnon, P.J; Margolskee, R.F. *Nature.* **1992**, *357*, 563-569
14. Clapp, T.R; Yang, R; Stoick, C.L; Kinnamon, S.C; Kinnamon, J.C. *J Comp Neurol.* **2004**, *468*, 311-321.
15. Huang, Y.J; Lu, K.S. *Arch Histol Cytol.* **1996**, *59*, 433-441
16. Clapp, T.R; Stone, L.M; Margolskee, R.F; Kinnamon, S.C. *BMC Neurosci.* **2001**, *2*, 6
17. Asano-Miyoshi, M; Abe, K; Emori, Y. *Neurosci Lett.* **2000**, *283*, 61-64.
18. Yee, C.L; Yang, R; Bottger, B; Finger, T.E; Kinnamon, J.C. *J Comp Neurol.* **2001**, *440*, 97-108.
19. Miura, H; Kato, H; Kusakabe, Y; Ninomiya, Y; Hino, A. *Chem Senses.* **2005**, *30*, 367-375.
20. Smith, D.V; Klevitsky, R; Akeson, R.A; Shipley, M.T., *J. Comp Neurol* **1994**, *347*, 187-196
21. Lawton, D.M; Furness, D.N; Lindemann, B; Hackney, C.M; *Eur J Neurosci.* **2000**, *2*, 3163-3171.
22. Smith, D.V; John, S.J; Boughter, J.D. *Physiol Behav.* **2000**, *69*, 77-85.
23. Lindemann, B; *Nature.* **2001**, *413*, 219-225.
24. Bealer, S.L; Smith, D.V. *Physiol Behav.* **1975**, *14*, 795-799
25. Bigiani, A; Roper, S.D. *J Gen Physiol.* **1993**, *102*, 143-170
26. Caicedo, A; Pereira, E; Margolskee, R.F; Roper, S.D. *J Neurosci.* **2003**, *23*, 9947-9952.
27. Chen, M.C; Wu, S.V; Reeve, J.R. Jr; Rozengurt, E. *Am J Physiol Cell Physiol.* **2006**, *291*, C726-C739.
28. Clapp, T.R; Medler, K.F; Damak, S; Margolskee, R.F; Kinnamon, S.C. *BMC Biol.* **2006**, *30*, 4-7.
29. Medler, K.F; Margolskee, R.F; Kinnamon, S.C. *J Neurosci.* **2003**, *23*, 2608-2617
30. Bigiani, A; Kim, D.J; Roper, S.D. *J Neurophysiol.* **1996**, *75*, 1944-1956
31. DeFazio, R.A; Dvoryanchikov, G; Maruyama, Y; Kim, J.W; Pereira, E; Roper, S.D; Chaudhari, N. *J Neurosci.* **2006**, *26*, 3971-3980.
32. LopezJimenez, N.D; Cavenagh, M.M; Sainz, E; Cruz-Ithier, M.A; Battey, J.F; Sullivan, S.L. *J Neurochem.* **2006**, *98*, 68-77.
33. Ishimaru, Y; Inada, H; Kubota, M; Zhuang, H; Tominaga, M; Matsunami, H; *Proc Natl Acad Sci U S A.* **2006**, *103*, 12569-12574.
34. Barlow, L.A; Northcutt, R.G. *Development.* **1997**, *124*, 949-957
35. Stone, L.M; Finger, T.E; Tam, P.P; Tan, S.S. *Proc Natl Acad Sci U S A.* **1995**, *92*, 1916-1920
36. Yee, C; Bartel, D.L; Finger, T.E. *J Comp Neurol.* **2005**, *490*, 371-390.
37. Sollars, S.I. *J Neurobiol.* **2005**, *64*, 310-320.
38. Cheal, M; Oakley, B. *J. Comp Neurol.* **1977**, *72*, 609-626.
39. Zalewski, A.A. *Exp Neurol.* **1969**, *25*, 429-437
40. Guth, L. *Anat Rec.* **1957**, *28*, 715-731

41. Farbman, A.I. *J Embryol Exp Morphol.* **1969**, *22*, 55-68
42. Morris-Wiman, J; Brinkley, L; Sego, R. *Brain Res Dev Brain Res.* **1999**, *16*, 141-150.
43. Guth, L. *Anat Rec.* **1958**, *130*, 25-37.
44. Miura, H; Kato, H; Kusakabe, Y; Tagami, M; Miura-Ohnuma, J; Ninomiya, Y; Hino, A. *Chem Senses.* **2004**, *29*, 823-831.
45. Kusakabe, Y; Miura, H; Hashimoto, R; Sugiyama, C; Ninomiya, Y; Hino, A. *Chem Senses.* **2002**, *27*, 445-451.
46. Miura, H; Kusakabe, Y; Kato, H; Miura-Ohnuma, J; Tagami, M; Ninomiya, Y; Hino, A. *Gene Expr Patterns.* **2003**, *4*, 427-430.
47. Whitehead, M.C; Frank, M.E; Hettinger, T.P; Hou, L.T; Nah, H.D. *Brain Res.* **1987**, *405*, 192-195
48. St John, S.J; Garcea, M; Spector, A.C. Chem Senses. **2003**, *28*, 33-43.
49. Uchida, N; Kanazawa, M; Suzuki, Y; Takeda, M. *Arch Histol Cytol.* **2003**, *66*, 17-25
50. Fritzsch, B; Sarai, P.A; Barbacid, M; Silos-Santiago, I. *Int J Dev Neurosci.* **1997**, *5*, 563-576.
51. Northcutt, R.G. *Brain Behav Evol.* **2004**, *64*, 198-206
52. Barlow, L.A; Chien, C.B; Northcuttm R.G. *Development.* **1996**, *122*, 1103-1111
53. Stone, L.M; Tan, S.S; Tam, P.P; Finger, T.E. *J Neurosci.* **2002**, *22*, 4522-4529
54. Barlow, L.A. *Development.* **2001**, *128*, 4573-4583.
55. Barlow, L.A; Northcutt, R.G. *Dev Biol.* **1995**, *169*, 273-285.
56. Pumplin, D.W; Yu, C; Smith, D.V. *J Comp Neurol.* **1997**, *378*, 389-410
57. Fujimoto, S; Murray, R.G. *Anat Rec.* **1970**, *168*, 383-413.
58. Delay, R.J; Kinnamon, J.C; Roper, S.D. *J Comp Neurol.* **1986**, *253*, 242-252
59. Mbiene, J.P; Maccallum, D.K; Mistretta, C.M. *J Comp Neurol.* **1997**, *377*, 324-340
60. Farbman, A.I. *Ann N Y Acad Sci.* **1974**, *228*, 350-354.
61. Farbman, A.I. *J Cell Biol.* **1972**, *52*, 489-493,
62. Hirota, M; Ito, T; Okudela, K; Kawabe, R; Hayashi, H; Yazawa, T; Fujita, K; Kitamura, H; Farbman, A.I; Mbiene, J.P. *J Comp Neurol.* **1991**, *304*, 172-186
63. Kim, J.Y; Mochizuki, T; Akita, K; Jung, H.S. *Exp Cell Res.* **2003**, *290*, 217-226
64. Ganchrow, J.R. *Physiol Behav.* **2000**, *69*, 29-40
65. Asamoto, M; Hokaiwado, N; Cho, Y.M; Ikeda, Y; Takahashi, S; Shirai, T. *Toxicol Pathol.* **2001**, *29*, 363-368.
66. Kumazawa, T; Kashiwayanagi, M; Kurihara, K. *Brain Res.* **1985**, *333*, 27-33
67. Khare, S; Gokulan, K; Linthicumm D.S. *J Neurosci Res.* **2001**, *63*, 64-71.

68. Ruiz, C.J; Stone, L.M; McPheeters, M; Ogura, T; Bottger, B; Lasher, R.S; Finger, T.E; Kinnamon, S.C. *Chem Senses.* **2001**, *26*, 861-873.

69. Spielman, A.I; Mody, I; Brand, J.G; Whitney, G; MacDonald, J.F; Salter, M.W. *Brain Res.* **1989**, *503*, 326-329

70. Kishi, M; Emori, Y; Tsukamoto, Y; Abe, K. *Neuroscience.* **2001**, *106*, 217-225

71. Herness, M.S. *Neurosci Lett.* **1989**, *106*, 60-64

72. Ookura, T; Kawamoto, K; Tsuzaki, H; Mikami, Y; Ito, Y; Oh, S.L; Hino, A. *In Vitro Cell Dev Biol Anim.* **2002**, *38*, 365-372

73. Wang, S.R; Renaud, G; Infante, J; Catala, D; Infante, R. *In Vitro Cell Dev Biol.* **1985**, *2*, 1526-1530.

74. Kishi, M; Emori, Y; Tsukamoto, Y; Abe, K. *Biosci Biotechnol Biochem.* **2002**, *66*, 484-487

75. Lincoln, C.K; Gabridge, M.G. *Methods Cell Biol.* **1998**, *57*, 49-65.

76. Schiff, L.J. *In Vitro Cell Dev Biol Anim.* **2005**, *41*, 65-70.

77. Hanson, C; Caisander, G. *APMIS.* **2005**, *113*, 751-755.

78. Cervenkova, K; Belejova, M; Vesely, J; Chmela, Z; Rypka, M; Ulrichova, J; Modriansky, M; Maurel, P. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* **2001**, *145*, 57-60

79. Li, X; Staszewski, L; Xu, H; Durick, K; Zoller, M; Adler, E. *Proc Natl Acad Sci U S A.* **2002**, *99*, 4692-4696.

80. Zhao, G.Q; Zhang, Y; Hoon, M.A; Chandrashekar, J; Erlenbach, I; Ryba, N.J; Zuker, C.S. *Cell.* **2003**, *15*, 255-266.

81. Bernhardt, S.J; Naim, M; Zehavi, U; Lindemann, B. *J Physiol.* **1996**, *490*, 325-336.

82. Nakashima, K; Ninomiya, Y. *Cell Physiol Biochem.* **1999**, *9*, 90-98.

83. Yamashita, H; Nakagawa, K; Nakamura, N; Abe, K; Asakage, T; Ohmotom M; Okadam S; Matsumoto, I; Hosoi, Y; Sasano, N; Yamakawa, S; Ohtomo, K. *Int J Radiat Oncol Biol Phys.* **2006**, *66*, 1311-1314.

84. Sandow, P.L; Hejrat-Yazdi, M; Heftm M.W. *J Dent Res.* **2006**, *85*, 608-611

85. Yamashita, H; Nakagawa, K; Tago, M; Nakamura, N; Shiraishi, K; Eda, M; Nakata, H; Nagamatsu, N; Yokoyama, R; Onimura, M; Ohtomo, K. *Head Neck.* **2006**, *28*, 508-516

86. Vissink, A; Burlage, F.R; Spijkervet, F.K; Jansma, J; Coppes, R.P. *Crit Rev Oral Biol Med.* **2003**, *4*, 213-225.

Chapter 6

Structure–Activity Relationship and AH-B after 40 Years

Terry E. Acree[1] and Michael Lindley[2]

[1]Department of Food Science and Technology, Cornell University,
630 West North Street, Geneva, NY 14456
[2]Lindley Consulting, 17, Highway, Crowthorne,
Berkshire, United Kingdom

For over a century the relationship between chemical structure and sweet taste has interested scientists, not only to explain food perception but also to direct the search for low calorie sweeteners. Notwithstanding, most low calorie sweeteners of commercial value were discovered by serendipity, but the AH-B theory published in 1967 did stimulate research that led directly to the discovery of the sweetness antagonist lactisole and the development of the 'multi-point attachment theory' of Tinti and Nofre used to design 'neotame'. This paper will outline the history of AH-B, its role in the discovery of lactisole and its relevance to the present view of taste perception.

96   © 2008 American Chemical Society

Introduction

In the early 1960's sweetness was known to be a property of a number of ligands that some animals evolved to detect and whose detection was described by humans as 'sweet'. Bitterness, however, was known to be stimulated by scores of different ligands distributed throughout the natural environment indicating many different receptor proteins, but not as many as were ultimately discovered for olfaction and certainly not as few as were responsible for ionic tastes: salty and sour. Over the next 20 years several non-carbohydrate sweet ligands were discovered during the search for new low calorie sweeteners of improved technical characteristics and/or to replace those banned by governments. In the absence of tools to study the initial chemistry of sweetness, it was believed that comparing patterns of taste behaviour with patterns of chemical structure could produce some insight (*1*). Known as Structure Activity Relationships (SAR), these techniques are often used in drug discovery, particularly when computer simulations replace mechanical models. Earlier attempts to use infra-red spectrometry to identify intra-molecular hydrogen bonds in crystalline sugars was abandoned when it was recognized that in solution compounds like glucose and fructose are in equilibrium with several tautomers and that each tautomer is likely in equilibrium with several conformers. The tautomeric composition could be determined directly by forming sugar derivatives in solutions quickly frozen with liquid nitrogen (*2*) but the conformational composition remained a mystery. An attempt to use the kinetics and thermodynamics of sugar tautomerization in boric acid solution, measuring changes in both pH and optical rotation, produced theories, but no way of validating them. Reviews of this period include those of Walters (*3*) and Shallenberger (*4*).

Early SAR of Sugar Taste

Robert Sands Shallenberger first described his thoughts about sweetness in California in April 1963. He had read everything remotely related to the subject and was convinced that sweetness was a direct reflection and an indication of selective bonding between sweeteners and specific proteins that functioned as chemoreceptors. He imagined that this was a reversible bonding reaction and it initiated transduction when the concentration of the bound form reached a threshold; highly potent sweeteners just had higher bonding coefficients. To him, it was simply a matter of relating the structure of sweet tasting chemicals to their activity, as defined by the conscious perception of sweetness. Understanding sweetness might be revealed by SAR analysis of sweet tasting chemicals. When Terry Acree joined his laboratory at the New York State Agricultural Experiment Station as a Cornell University graduate student in July of 1963 the only tools available for structural studies were spectrometers, chromatographs, crude molecular models and rulers. That Fall, Shallenberger

began to machine molecular models from steel in the shop in his basement. The models had bonds that rotated and bent and with the addition of foam plastic balls to approximate electron clouds, conformational energy barriers were simulated by the force needed to bend the steel bonds as the models were deformed through all the possible conformations of a single structure without, of course, breaking any covalent bonds.

The 1960's was marked by the work of Jacob, Monod and Changeux with their ideas that protein - ligand binding results in conformational changes in protein structure: the allosteric effect. In the case of enzymes this explained non-competitive "activation" and "inhibition" among other inductive phenomena and it provided a model for extra-cellular activation of intercellular receptor protein reactions, i.e., transduction. These binding reactions were presumed not to require the formation of covalent bonds but could be accomplished through the formation and disruption of hydrogen bonds, hydrophobic associations and other London dispersion forces (5).

Called the MWC model it proposed that a ligand, L, would bind to a receptor protein RP on the outside of a receptor cell and that remote to the binding site (as it turns out on the inside of the receptor cell) the protein changes structure enough to initiate transduction. This was an allosteric effect. The challenge was then to envision the minimum set of structural features necessary to elicit sweetness, i.e., bind to a receptor. Many sweet compounds were compared to similar non-sweet compounds and two sets were used to formulate the AH-B theory (6); the chlorinated alkanes and the cyclic diols. The sweetest chlorinated alkane was chloroform (Figure 1b). This molecule had an acidic or electropositive proton capable of forming a hydrogen bond with a negative site on the receptor protein and any of the chlorines could also form a simultaneous hydrogen bond with a suitable proton on the protein if it was approximately 3Å from the electronegative center. Finally, when a cyclic diol had the "gauche" configuration (Figure 1a), it was sweet and when it was anti-clinal or eclipsed the compound was not sweet.

Most importantly, the distance between an OH proton on any one of the diols and a full p-orbital on an adjacent oxygen could be separated by 3 Å (6). However, which of the many diols located on all carbohydrates formed the AH-B in sweet sugars and just why most sugars were not sweet; even though they bristled with diols (Figure 1c) remained unclear. Although AH-B functional groups could be located on many sweet compounds (Figure 2), it was obvious that AH-B might be a necessary condition for sweetness, but it certainly was not sufficient.

Variations on the Shallenberger/Acree AH-B Model

To explain why there were hundreds of compounds with AH-B structures that did not taste sweet, a comparison of the sweet with the non-sweet amino acids yielded part of the answer (7). Glycine and D- and L-alanine are all sweet.

a.

anti-clinal 180°
(tasteless)

gauch 60°
(sweet)

eclipsed 180°
(tasteless)

AH

B:

b.

AH

B:

c.
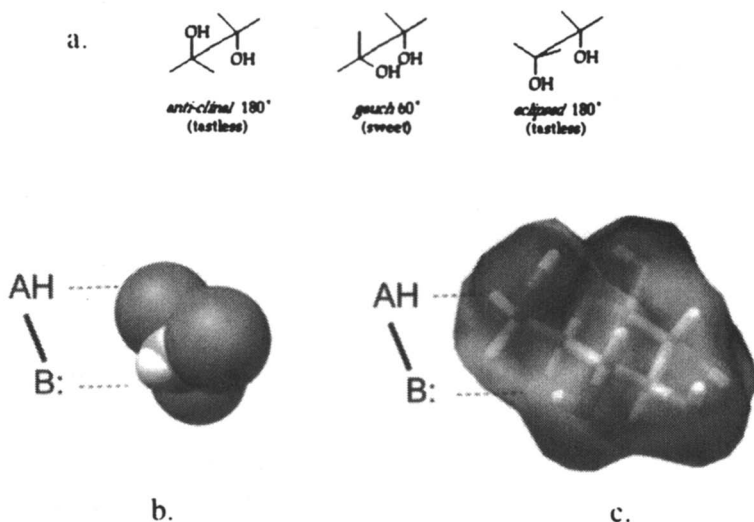
Figure 1. a) Diol configurations and their sweet taste. b) The putative AH-B interaction with chloroform c) One of several AH-B interactions possible on a monosaccharide. (See color insert in this chapter.)

However, extending the length of the alanine side chain by one carbon, as in leucine, causes taste to become chiral; **D**-leucine is sweet and **L**-leucine is tasteless (Figure 3).

Limits to sweetness imposed by the structure of the side chain implies that more than a two point AH-B attachment is involved in the binding of amino acid and carbohydrate sweeteners to their receptor sites. The fact that α-**D**-mannose is sweet while β-**D**-mannose is bitter (a very subtle change in structure) while the **D**-monosaccharides taste more or less the same as the **L**-monosaccharides (even though such a modification involves a change in the chirality at every carbon atom) indicates that the precise requirement for sweetness in carbohydrate structure includes more than just the AH-B functional group. Kier (8) proposed a three-point attachment called AH-B-X, where X is a hydrophobic binding site, thereby forming a tripartite glycophore. In this AH-B-X model, hydrogen bonds form at A and B while X acts as a lipophilic region. Although the AH-B-X model explains why **L**-leucine is not sweet, binding at X is not required, as glycine is sweet. Thus, although the AH-B-X model is a refinement of the original AH-B, this too could not explain all the SAR observed for sweet compounds and over the next 20 years attempts to refine a model that summarizes all the features necessary and sufficient to describe a sweetener culminated in the Tinti and Nofre model shown in Figure 4 (9).

In the AH-B model, nitro (NO_2) and carboxylic (COOH) groups were both thought to act as the B moiety (the hydrogen bond acceptor), implying that
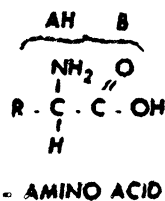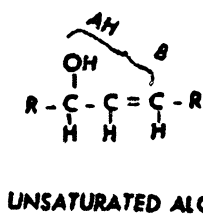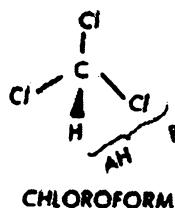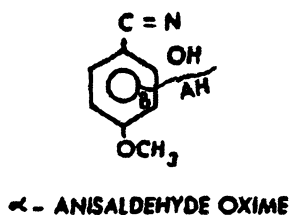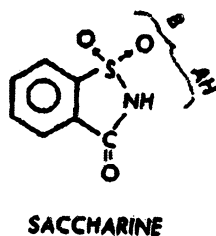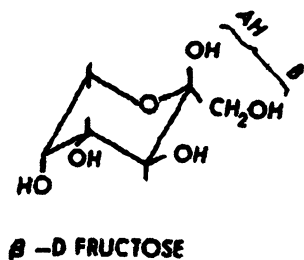
Figure 2. Putative AH-B functional groups on identified on different sweeteners (1).
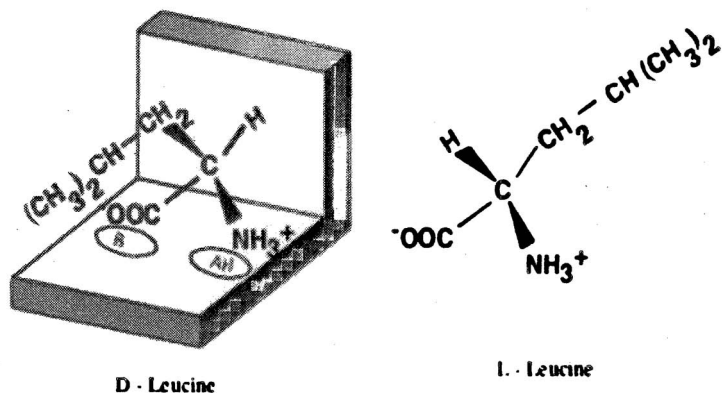
D · Leucine   **L. · Leucine**

Figure 3. The chiral structures D- and L-leucine as they sit on a chiral AH-B receptor site.
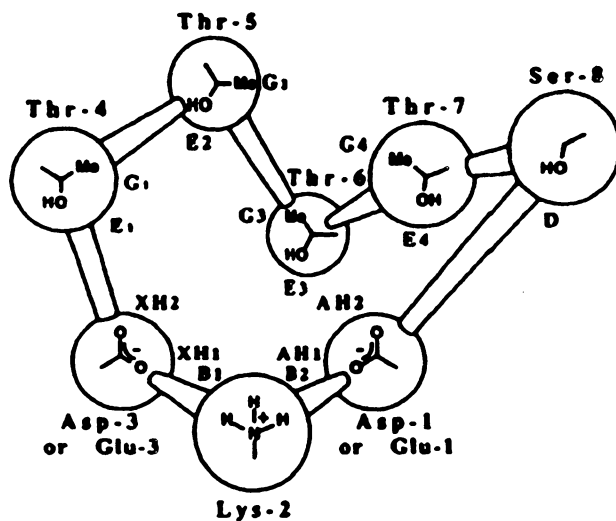
Figure 4. The Nofre and Tinti model of the sweet receptor.

aspartame (carboxylic group) and P4000 (nitro group) would bind at a common site on the receptor. Instead, substitution of nitro groups for carboxylic groups and *vice versa* alters the taste activity of some compounds leading to identification of separate binding sites for CO_2^- and NO_2 (or CN) groups, called B and D, respectively (*10*). Later, the central importance of the D binding site became clear with the synthesis of a new sweetener that was measured to be approximately 14,000 times as sweet as sucrose under given conditions. Its high sweetness was rationalized on the basis that it was able to bind to all four sites; A, B, D and Kier's hydrophobic site, now termed G. Subsequent analysis revealed an additional four potential binding sites and this analysis led to the synthesis of sucrononic acid, a compound rated ·as 200,000 times as sweet as sucrose. Tinti and Nofre then described these findings as a 'multi-point attachment' theory. This model holds that a total of eight sites (AH, B, G, D, Y, XH, E1 and E2) may be found in sweet compounds that interact at the receptor, although attachment to all eight is not required for sweetness. Notwithstanding this significant evolution of Shallenberger and Acree's bipartite AH-B to the multi-point attachment arrangement of Tinti and Nofre, it is a frustrating reality that complete prediction of novel sweet structures remains an elusive goal.

This inability to correlate structure and sweetness uniformly over all compounds, even with the substantially more sophisticated multi-point attachment theory, may be rationalized if we consider the possibility that ligands can act as both an agonist and an antagonist; i.e., a sweetener and a sweetness inhibitor, at one and the same time. This possibility became stunningly clear with the discovery of lactisole. Simultaneous activation and inhibition, especially competitive inhibition, would require an SAR analysis that included a quantitative understanding of both of these two activities. Exactly how to do this is not obvious, if it is at all possible with our present knowledge of ligand binding. Certainly, studying the phenomenon of sweetness inhibition and its structural features has potential.

Sweetness Inhibitors

Compounds that inhibit sweet taste have been known for many years. Extracts of the leaves of *Gymnema sylvestra*, a woody shrub found in parts of the Indian sub-Continent, have been studied extensively by sensory psychologists and, although its inhibitory effects have been demonstrated against sweeteners from many different structural classes of compound, sweetness inhibition is not immediately reversible. In addition, pre-treatment of the tongue with a *Gymnema* tea is required and the inhibition effect lasts for an hour or more, leading to speculation that sweetness inhibition induced by *Gymnema* is due to physical disruption of the taste cell membrane rather than a specific receptor interaction. In contrast, the sweet taste inhibitor known as lactisole appears to exert its inhibitory effects through interaction at the

T1R2/T1R3 dimeric sweet receptor. In addition, the inhibitory effects of lactisole are immediate and immediately reversible, and this originally lead to speculation that lactisole might be a competitive inhibitor acting at a single receptor for sweetness (*11*). Not surprisingly, it has proved to be a helpful tool in developing understanding of the physiology of sweet taste perception.

The Discovery of Lactisole

Lactisole is the common name of 2-(±)-4-methoxyphenoxypropanoic acid. It inhibits the sweetness of all sweet compounds and was discovered in the mid-1980s by Tate & Lyle PLC during a research program that, paradoxically, was searching for compounds capable of potentiating sweetness. The rationale for this search for sweetness potentiators was that since sucrose elicits a sweet taste quality preferred by consumers, potentiating its intrinsic sweetness might permit preparation of lower calorie products that delivered the high quality sweet taste associated with sucrose.

At about the same time as the discovery of lactisole, similar research was also underway within the Central Research facility of General Foods Corporation and this research also identified compounds capable of inhibiting sweetness (*12, 13, 14*). In both laboratories, the innovative step was that compounds of interest were being evaluated not for their intrinsic sensory characteristics, but for their sensory impact on sweeteners with which they were blended.

In the search for sweetness potentiators by Tate & Lyle, neohesperidin dihydrochalcone (NHDC) was a compound of particular interest. NHDC is a potent sweetener and a glycoside of ß-neohesperidose and its aglycone. Since the NHDC glycoside is potently sweet and the ß-neohesperidose sugar is also sweet, although only weakly sweet, it was reasoned that perhaps the aglycone was acting as a potentiator of the sweetness of the sugar. Consequently, a series of dihydrochalcone aglycones, including the aglycones of NHDC and naringin dihydrochalcone, were evaluated for their sweetness enhancing potential. In both cases, low concentrations of aglycone were identified as delivering no or minimal perceptible sweetness on their own, but were found to enhance the sweetness of sucrose solutions by around 30-40%, e.g., making 5% sucrose taste as if it were at c.7% concentration.

Although an interesting observation, it was reasoned that neither dihydrochalcone structure was capable of commercialization as the magnitudes of their potentiating effects were small and they were also unlikely to be able to be produced in quantity from natural sources. Therefore, these compounds became leads in a search for more simplified structures, but structures that would exhibit similar taste modifying properties. The aglycone of NHDC can be considered as a dimer of 2,4,6-trihydroxybenzoic acid and 3-hydroxy-4-

methoxypropanoic acid. When blended with sweeteners, 2,4,6-hydroxybenzoic acid was found to have no impact on their perceived intensity of sweet taste. In contrast, the propanoic acid derivative was found to inhibit rather than potentiate sweetness (Figure 5). Following a standard structure-activity synthetic program, the racemic mixture of 2-(4-methoxyphenoxy)propanoic acid was identified for commercial development (Figure 6).

During this program it was noted that on tasting an NHDC solution, the first sip is sweet, but on second and subsequent sips perceived sweetness levels fall dramatically. It therefore seems possible that NHDC is inhibiting its sweetness and, if true, opens up the intriguing possibility that some structure-activity relationships described in the literature ultimately fall down because moieties of some structures act as self-inhibitors.

Structure-Activity Relationships of Sweetness Inhibitors

The relationships between molecular structure and sweetness inhibition effects have not been studied to the extent of that which has been reported for sweeteners. It is interesting to note, however, that those sweetness inhibitor compounds identified by General Foods and Tate & Lyle all lack a putative AH-B couple, but in many other respects exhibit structure-function relationships remarkably similar to those for sweetness (*11*). The AH,B,X tripartite arrangement of many sweet compounds (*8*) and Tinti and Nofre's multi-point attachment theory (*9*) both demonstrate the relevance of lipophilicity as an influence on sweetness potency. Deutsch and Hansch (*15*) had earlier also demonstrated the role of lipophilicity and the importance of a balance between lipophilicity and the AH-B couple. They demonstrated that within an homologous series such as the ρ-alkoxy derivatives of 2-amino-4-nitrobenzene, as the lipophilicity of the ρ-alkoxy substituent is increased, so relative sweetness increases to a maximum after which further increases in lipophilicity result in reductions in sweetness. In the case of these sweetness antagonists, within the ρ-substituted phenoxypropanoic acids, as lipophilicity increases, so the dose required for a given degree of inhibition is reduced to a minimum (i.e., biological response increases) after which the concentration of inhibitor required to achieve the same degree of inhibition must be increased (*11*). Similarly, replacing the methoxy substituent of lactisole with a halogen atom such as chlorine or fluorine enhances the potency of inhibition significantly (Figure 7). Analogous observations have been made with some sweet compounds. For example, modification of **D**-tryptophan by the introduction of a chlorine atom to form 6-chloro-**D**-tryptophan substantially increases sweetness potency.

Finally, perhaps the most relevant observation of conformity between sweetness inhibitors and sweeteners is that lactisole is an optically active compound whose enantiomers elicit different sensory effects; the S-(-) enantiomer being an inhibitor of sweetness whereas the R-(+) enantiomer is

Aglycone of neohesperidin dihydrochalcone

2,4,6-trihydroxybenzoic acid
Does not modify sweet taste

3-(3'-hydroxy-4'-methoxyphenyl)-propanoic acid
A potent sweet taste inhibitor
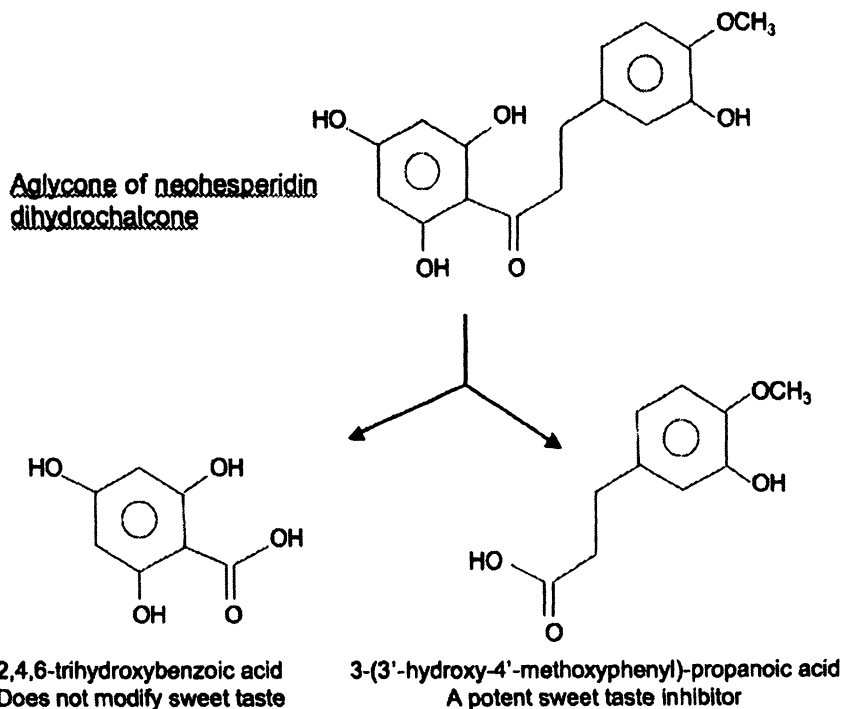
Figure 5. Identification of a sweetness inhibitor derived from NHDC

3-(3'-hydroxy-4'-methoxyphenyl)-
propanoic acid

2-(4-methoxyphenoxy) propanoic acid
(lactisole)

(* chiral centre)
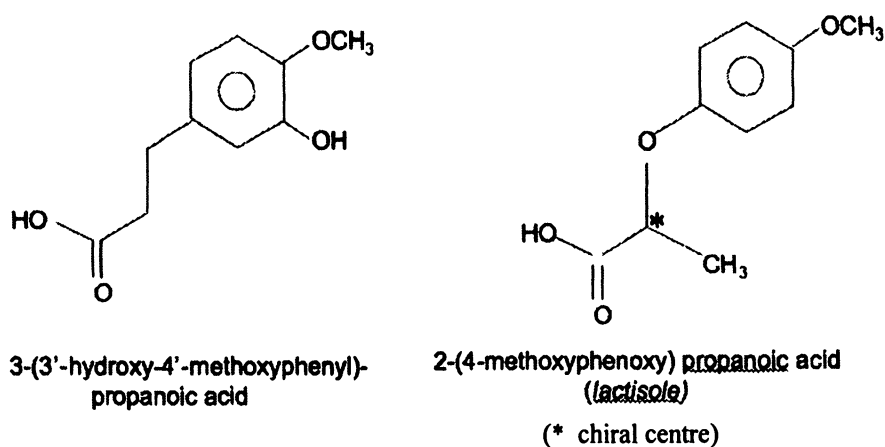
*Figure 6. The structure of lactisole (c.10x as effective at inhibiting sweetness
than 3-(3'-hydroxy-4'-methoxyphenyl)propanoic acid).*
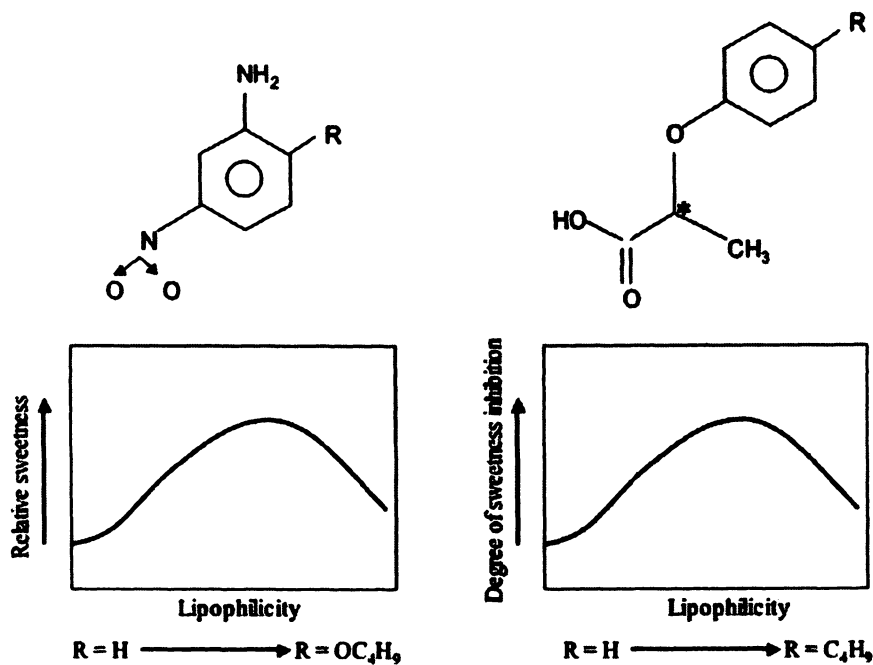
Figure 7. Structure-activity conformity between sweeteners and sweetness inhibitors

inactive. The obvious parallel with compounds that elicit sweetness is the amino acid series where all **D**-enantiomers taste sweet and most **L**-enantiomers are tasteless or elicit bitterness (7).

Lactisole – Taste Receptor Interactions

Although the development of lactisole was undertaken purely for commercial reasons, it has proved subsequently to be a very useful tool in the identification of receptors and the identification of specific sweetener-binding site interactions. Lactisole has been shown to bind to the human T1R3 binding pocket of the transmembrane helices of the dimeric sweet receptor structure (16), but although it binds in the T1R3 binding pocket, lactisole inhibits the sweetness of all sweeteners, including those such as aspartame and neotame that have been shown to bind at the Venus flytrap module of the T1R2 monomer (16, 17). Possibly, when lactisole binds at the T1R3 binding pocket, it may induce an allosteric change in conformation throughout the dimeric T1R2/T1R3 sweet

receptor structure that then ensures all sweet compounds fail to bind to their respective sites on the T1R2/T1R3 dimer at which they would otherwise interact. Cyclamate also binds at the extracellular loops of T1R3 and is both sweetener and sweet potentiator. Interestingly, lactisole inhibits sweetness and also inhibits the taste of monosodium glutamate (umami) by interacting on the T1R3 moiety of the T1R1/T1R3 dimeric umami receptor. On the other hand, cyclamate enhances the sweetness of other sweeteners and the umami taste of monosodium glutamate (*16*).

Conclusions

The other chapters of this book reveal the enormous advance in our knowledge of the psychology, neurobiology and chemistry of sweeteners achieved in the last 40 years. The original "AH-B model" of Shallenberger and Acree was advanced by Tinti and Nofre into the "multi-component attachment theory" (Figure 8a) and, in recent years has been adapted further to include specific amino acids in a pseudoreceptor protein model interacting with sweet ligands (17, 18) (Figure 8b).

Tinti & Nofre   pseudoreceptor
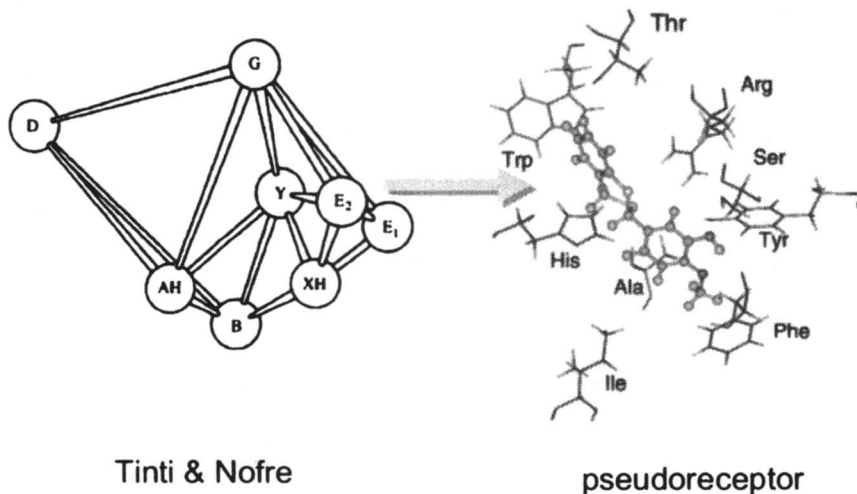
Figure 8. The Tinti and Nofre "multipoint attachment model" of the sweet receptor. Compared with the pseudo-receptor model of Bassoli et al (17).

However, not knowing the balance between agonist and antigonist properties of sweet ligands, the conformational structure of the ligand at the receptor and just how to extrapolate data from crystalline structures to solutions still clouds our interpretation of psychophysical data used to do SAR of sweet tasting

compounds. Perhaps modelling the complete structure of the receptor protein especially as it docks with a sweet ligand will give us a more complete picture. It is ironic that the techniques we sought to develop commercially profitable sweeteners became in the end the tools we used to explain what we have already found serendipitously.

References

1. Moncrieff, R.W. *The Chemical Senses*, Leonard Hill, Ltd., London, **1951**
2. Acree, T.E.; Shallenberger, R.S.; Lee, C.Y.; Einset, J.W.; **1969**, *Thermodynamics and kinetics of D-galactose tautomerism during mutarotation*, in *Carbohyd. Res.* p. 355-360.
3. Walters, D. E.; Orthoefer, F. T.; DuBois, F.T.; **1991**, *Sweetners: Discovery, Molecular Design and Chemoreception*, ASC Symposium Series. 450, American Chemical Society, Washington DC.
4. Shallenberger, R. S.; **1993**, *Taste Chemistry*, Blackie Academic and Professional, Wester Cleddens Road, Bishopbriggs, Glasgow.
5. Jacob, Monot, Changeaux, MWJ *J Mol Biol 12*, 88-118 **(1965)**
6. Shallenberger, R.S.; Acree, T.E. *Nature*, **1967**, *216*, 480.
7. Shallenberger, R.S.; Acree, T.E.; Lee, C.-Y. *Nature*, **1969**, *221*, 555
8. Kier, L.B. *J. Pharm. Sci.*, **1972**, *61*, 1394
9. Tinti, J.-M.; Nofre, C. *Why does a sweetener taste sweet? A new model*. In: *Sweeteners: Discovery, Molecular Design and Chemoreception. ACS Symposium Series*; Walters, D.E.; Orthoefer, F.T.; DuBois, G.E., Eds.; American Chemical Society, Washington, DC., **1991**; p 206.
10. Tinti, J.-M.; Durozard, D.; Nofre, C. *Naturwissenschaften*, **1980**, *67*, 193
11. Lindley, M.G. *Phenoxyalkanoic acid sweetness inhibitors*. In: *Sweeteners: Discovery, Molecular Design and Chemoreception. ACS Symposium Series*; Walters, D.E.; Orthoefer, F.T.; DuBois, G.E., Eds.; American Chemical Society, Washington, DC., **1991**; p 251.
12. Barnett, R.E. *US Patent 4,544,565* **1985**
13. Barnett, R.E.; Yarger, R.G. *US Patent 4,642,240* **1987**
14. Lindley, M.G.; Rathbone, E.B. *US Patent 5,045,336* **1991**
15. Deutsch, E.W.; Hansch, C. *Nature*, **1966**, *211*, 216
16. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. *Proc. Natl. Acad. Sci. USA*, **2004**, *101*, 14258.
17. Angela Bassoli, Lucio Merlini, Gabriella Morini, Angelo Vedani, *J. Chem. Soc.*, Perkin Trans. Q., **1998**, *2*, 1449-1454.
18. Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L.A.; Benard, L.M.; Osman, R.; Margolskee, R.F.; Max, M. *J. Biol. Chem.* **2005**, *280*, 15238.

Chapter 7

Crystal Structures of the Sweet Protein MNEI: Insights into Sweet Protein–Receptor Interactions

Jeanette R. Hobbs[1], Steven D. Munger[2], and Graeme L. Conn[1,*]

[1]Manchester Interdisciplinary Biocentre, Faculty of Life Sciences, University of Manchester, Manchester M1 7DN, United Kingdom
[2]Department of Anatomy and Neurobiology, University of Maryland School of Medicine, 20 Penn Street, Baltimore, MD 21201

Monellin is one of a small number of highly potent, sweet-tasting proteins. X-ray crystal structures of a wild-type single chain monellin (MNEI) and a 10-fold less sweet mutant (G16A) were determined in order to understand the cause of this reduction in sweetness and gain insight into sweet protein-receptor interactions. Comparison of the two structures reveals little change to the global protein fold. However, alterations of amino acid side chain position and exposure adjacent to the site of mutation result in a reorganization of key functional groups on the surface of MNEI. This finding supports the idea that an extensive surface of monellin is involved in binding the T1R2:T1R3 receptor.

© 2008 American Chemical Society

The protein monellin is a highly potent sweet stimulus: on a molar basis, it is many thousands of times sweeter than sucrose *(1)*. It is sweet to humans and some Old World primates, but is not preferred by other mammals. Natural monellin from the African 'Serendipity Berry' (*Dioscoreophyllum cumminsii*) is composed of two chains, A and B, of 44 and 50 amino acids respectively. Single chain monellin proteins were created that enhance its thermal and chemical stability; the two natural chains (B-A) are either directly connected (SCM) *(2)* or joined by a dipeptide linker (MNEI) *(3)*. Despite extensive characterization of the sweet protein *(4-12)*, little is known about the interaction of monellin with the T1R2:T1R3 sweet taste receptor. The concept that sweet proteins might share a common structural motif *(13)*, a so-called 'sweet finger', that in some way mimics the binding of small molecular weight ligands has been largely discarded as no such motif has been identified *(14)*. To date, modeling studies using a T1R2:T1R3 model based on the mGluR1 receptor ectodomain *(15)*, have provided the best route to understanding sweet protein-receptor interactions. Such modeling studies suggest that the major binding site for several sweet proteins resides within the T1R amino terminal domains (NTDs) *(16, 17)*, as has been demonstrated experimentally for some small molecule ligands *(18)*. However, the interaction surface may be more extensive than for small molecule sweeteners and such a recognition mechanism, with the high affinity it suggests, can readily provide an explanation for the high potency and persistent aftertaste of sweet proteins. Experimentally, much less has been determined to date. Activation of the T1R2:T1R3 receptor by the sweet protein brazzein is dependent upon the cysteine-rich linker between the transmembrane domain and the Venus Flytrap Module (VFTM) of T1R3 *(19)*, in addition to any interactions with the VFTM itself. Very recently, mutants of MNEI with altered charges on the protein surface provided some initial experimental validation of the 'wedge model' *(16)*. These results suggest that surface and charge complementarity are important components of the MNEI-receptor interaction. We sought to learn more about how MNEI binds the sweet receptor by examining the structure of mutant MNEI proteins with known reductions in sweet taste.

Wild-type and G16A Mutant MNEI Crystal Structures

The crystal structures of wild-type MNEI and G16A mutant were determined by molecular replacement. The wild-type structure has been refined to the highest resolution (1.15 Å) of any monellin structure available to date *(20)*. Electron density maps for both proteins showed well-defined connected density but with some indications of disorder for G16A revealed by broken density around the more dynamic regions of the protein (i.e. loop regions). The mutation at position 16 was clearly visible in a $F_o - F_c$ difference density map.

Monellin has a secondary structure consisting of five β-strands that form an antiparallel β-sheet (β1 to β5), and a 17-residue α-helix (α1) cradled in the concave face of strands β2-β5 (Figure 1A). In single chain monellins, such as MNEI, the β-strands β2 and β3 are joined by the engineered loop L_{23} (residues 47 to 56). The polypeptide chain ends with a short sequence containing four proline residues; three of these, Pro94-96 form a 3_1-polyproline II helix. Both structures are fully refined and of high quality; Ramachandran plots *(21)* indicate all 96 residues are in the 'Favorable' or 'Allowed' regions for both proteins.
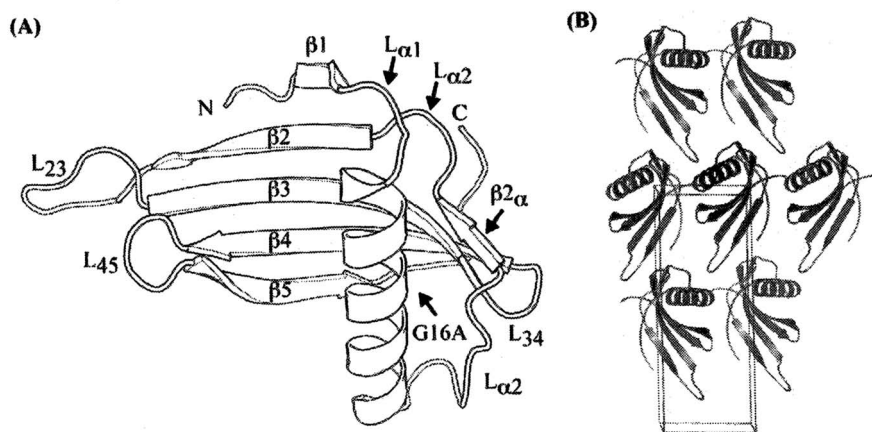
Figure 1. Crystal structure of wild-type MNEI.

A Monomeric Crystal Form of Monellin (MNEI)

Previous natural and single chain monellin structures *(4-6)* have invariably contained the protein packed in such a way as to suggest a homodimeric complex may be present (in the case of natural monellin comprising two copies of both Chain A and Chain B). This observation led to the suggestion that this might be the functional form of the protein. However, this was contradicted by native gel analysis *(22)* and other solution studies *(7, 23)* that argued monellin exists as a monomer. While the G16A MNEI crystal shows the typical monellin dimer, generated by rotation of the protein about a crystallographic two-fold axis, the wild-type crystal is remarkably different. Although in a space group (P2₁) previously observed for monellin *(5)*, this crystal has a markedly different crystal packing arrangement: a single monellin molecule is contained in the asymmetric unit and no dimer interface is observed (Figure 1B). Light scattering

measurements on protein samples used for crystallization yielded molecular weights of ~11.8 kDa, corresponding to the MNEI monomer, for both wild-type and G16A MNEI proteins. Thus the two proteins, one of which crystallizes as a monomer and the other as a dimer, both exist as monomeric proteins in solution.

The MNEI Protein Fold is Unaffected by the G16A Mutation

The wild-type and G16A MNEI crystal structures are globally very similar to each other and to other monellin structures (Figure 2), indicating that the G16A mutation has little effect on the protein fold.
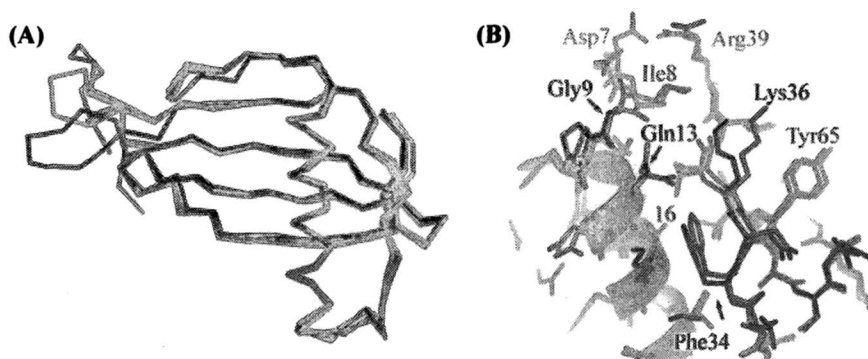
Figure 2. The G16A mutation does not affect the global fold of MNEI but does cause sidechain alterations that extend across the protein surface.

This was confirmed using pairwise superpositions of the Cα atoms of residues 1-46 and 57-96 (residues located on L_{23} were excluded, as these are intrinsically flexible). This confirmed that the wild-type and G16A polypeptide backbones are very similar to each other (r.m.s.d. 0.65 Å), and to natural monellin (3MON) *(5)*, orthorhombic natural monellin (4MON) *(6)*, and SCM (1MOL) *(5)*, with r.m.s.d. values < 0.86 Å for each possible alignment. Comparison of both our crystal structures to the solution NMR structure of wild-type MNEI (1FA3) *(7)* was similarly favorable, with an r.m.s.d. for alignment of approximately 1.4 Å for both. In contrast, the solution structure of G16A-MNEI (1M9G) *(24)* gave considerably larger r.m.s.d. values for alignment to both wild-type (r.m.s.d. 4.57 Å) and G16A MNEI (r.m.s.d. 4.46 Å) crystal structures. Our G16A MNEI crystal structure indicates that this wild-type backbone fold is an energetically favorable one for the mutant protein. However, we note that the

large differences observed in the G16A MNEI solution structure, due to an apparently greater degree of flexibility (24), may reflect an important property of the mutant protein that contributes to a reduced binding affinity for T1R2:T1R3 and therefore sweetness.

Changes Due to the G16A Mutation Extend Across the Surface of MNEI

The G16A mutation is located in the inside of the α-helix, opposite the short β2a strand. The addition of the methyl group directly perturbs the surrounding residues, in particular, Val37 directly opposite the site of mutation. The terminal methyl groups on Val37 are rotated away from Ala16 and displaced by approximately 0.6 Å to avoid a steric clash. Several residues important for monellin sweet taste (Table I) located in the vicinity of Ala16 and Val37 are also found in different conformations, apparently affected by the mutation. One helical turn above the mutation site, Gln13 adopts an alternate rotamer. In the wild-type MNEI structure, the amine group of Gln13 points towards the core of the protein, hydrogen bonding to the backbone of Val37, while the carbonyl group is exposed on the surface of the helix. In the mutant structure, the head of the Gln13 sidechain is rotated, such that both the amine and carbonyl groups are exposed on the surface, roughly parallel with the helical axis. Two other important residues, Phe34 and Lys36, are located near Val37. Again, each is found in a different rotamer in the G16A mutant structure and has a significantly altered side chain position. Beyond the N-terminus of the α-helix, a change is also observed in the position of Asp7. In the wild-type structure, Asp7 forms a salt bridge with Arg39 on the surface of the protein; this interaction is maintained in the mutant but Asp7 is rotated to a roughly orthogonal orientation (Arg39 also adopts an alternate rotamer). As a result, the functional groups of Asp7 are significantly altered in orientation and now point downwards in the direction of the helix.

Table I. The Effect of the G16A Mutation on Other Residues Important for MNEI Sweetness

Residue	Fold reduction in sweetness[a]	Alignment r.m.s.d. (Å)[b]	Surface Exposure Change (%)
Asp 7	>200	2.10 (1.01)	9.4
Gln13	8	1.96 (0.10)	42.1
Phe34	7-10	1.74 (0.48)	4.9

[a]Values from various studies (8, 9, 12). Some mutations were to non-natural amino acids. [b]Values for sidechain and backbone atoms (in parenthesis).

Implications for MNEI Sweet Taste and Receptor Binding

The G16A mutation causes negligible change to the protein fold and, as a buried position, is unlikely to directly contribute to the reduction in sweetness observed for the mutant protein (25). We sought to quantitate the observed alterations in amino acid sidechain conformations surrounding G16A, including several that are important for sweet taste. The wild-type and G16A MNEI structures were aligned with all sidechain atoms included in the calculation (but excluding residues 47-57 as before). The calculation indicated that the positions of several important residues were altered in the mutant. As shown in Table I, each of the residues around the site of mutation, Asp7, Gln13, and Phe34, has a sidechain r.m.s.d. value at least two times greater than that for the mainchain atoms. In addition to sidechain position/ conformation, a major influence on the interaction of MNEI with the T1R2:T1R3 receptor will arise from the accessibility of these important residues on the protein surface. We therefore measured the solvent exposed surface area and protein volume of each structure. Two residues, Gln13 and Lys36, are significantly more exposed on the G16A-MNEI surface due to the changes in their conformation. Overall, the changes are small as expected given the similarity of the protein backbones: G16A MNEI has a slightly reduced total surface area (~2.4 %) but, appropriately for addition of a bulkier side chain in the protein core, an increased protein volume (~1.0 %). Thus, major changes in conformation and surface exposure due to the G16A mutation are only present at the level of individual residues, several of which are important for monellin sweet taste.

Differences in crystallization solution conditions and crystal packing could potentially cause similar alterations in surface amino acid conformations. However, here the protein samples were prepared in the same buffer and crystallized at the same pH. Furthermore, other crystal forms of monellin indicate that Asp7 is invariably found in the same conformation that we observe in our wild-type MNEI structure, regardless of crystallization conditions or crystal packing. Phe34 is found in several similar positions but its position in the G16A structure is at one extreme of the positions observed. Gln13 is found in the same conformation as for our wild-type MNEI structure in all other structures except one, the structure of SCM (5). Interestingly, here the position of Val37 also aligns perfectly in SCM and our G16A mutant structure. While in the case of SCM it is not clear what is the cause of the movement in Val37 (there is no steric clash in the protein core as for G16A), the resulting effect on the key residue Gln13 is the same. Together, these observations indicate that the differences observed between wild-type MNEI and G16A-MNEI in key residues for MNEI sweetness across the surface of the protein can be attributed to the G16A mutation.

Conclusions

Sweet proteins have great potential as sweeteners and could be particularly beneficial to individuals such as diabetics who must control sugar intake. For this potential to be realized, a full understanding of the interaction between sweet proteins such as monellin and the T1R2:T1R3 sweet receptor is needed.

We determined high resolution crystal structures of the sweet protein MNEI and a 10-fold less sweet mutant. The G16A mutation does not directly cause the loss of sweetness: there is no major change in protein structure nor a dramatic alteration to any one critical determinant for binding T1R2:T1R3. Instead, more subtle alterations in sidechain conformation and accessibility extend across the surface of MNEI affecting several key residues for sweetness. Modeling studies suggest that sweet proteins bind the receptor NTD(s) through an extended surface. This idea is supported by our observation that an extended surface of MNEI is affected by the G16A mutation. It is not yet possible to distinguish the contribution of key residues within this extended surface to binding, the induction of changes in receptor conformation and/ or receptor activation. This will require further detailed analysis of T1R-monellin interactions such has recently been begun for low molecular weight sweeteners *(18)*.

Acknowledgements

References

1. Morris, J. A.; Cagan, R. H., *Biochim. Biophys. Acta* 1972, *261*, 114-122.
2. Kim, S. H.; Kang, C. H.; Kim, R.; Cho, J. M.; Lee, Y. B.; Lee, T. K. *Protein Eng.* 1989, *2*, 571-575.
3. Tancredi, T.; Iijima, H.; Saviano, G.; Amodeo, P.; Temussi, P. A. *FEBS Lett.* 1992, *310*, 27-30.
4. Ogata, C.; Hatada, M.; Tomlinson, G.; Shin, W. C.; Kim, S. H. *Nature* 1987, *328*, 739-742.
5. Somoza, J. R.; Jiang, F.; Tong, L.; Kang, C. H.; Cho, J. M.; Kim, S. H. *J. Mol. Biol.* 1993, *234*, 390-404.
6. Bujacz, G.; Miller, M.; Harrison, R.; Thanki, N.; Gilliland, G. L.; Ogata, C. M.; Kim, S. H.; Wlodawer, A. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 1997, *53*, 713-719.

7. Spadaccini, R.; Crescenzi, O.; Tancredi, T.; De Casamassimi, N.; Saviano, G.; Scognamiglio, R.; Di Donato, A.; Temussi, P. A. *J. Mol. Biol.* **2001**, *305*, 505-514.

8. Ariyoshi, Y.; Kohmura, M. *J. Synth. Org. Chem. Jpn.* **1994**, *52*, 359-369.

9. Kohmura, M.; Nio, N.; Ariyoshi, Y. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1937-1942.

10. Kohmura, M.; Nio, N.; Ariyoshi, Y. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 472-476.

11. Niccolai, N.; Spadaccini, R.; Scarselli, M.; Bernini, A.; Crescenzi, O.; Spiga, O.; Ciutti, A.; Di Maro, D.; Bracci, L.; Dalvit, C.; Temussi, P. A. *Protein Sci.* **2001**, *10*, 1498-1507.

12. Somoza, J. R.; Cho, J. M.; Kim, S. H. *Chem. Senses* **1995**, *20*, 61-68.

13. Kim, S. H.; Kang, C. H.; Cho, J. M. *ACS Symp. Ser.* **1991**, *450*, 28-40.

14. Tancredi, T.; Pastore, A.; Salvadori, S.; Esposito, V.; Temussi, P. A. *Eur. J. Biochem.* **2004**, *271*, 2231-2240.

15. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. *Nature* **2000**, *407*, 971-977.

16. Temussi, P. A. *FEBS Lett.* **2002**, *526*, 1-4.

17. Morini, G.; Bassoli, A.; Temussi, P. A. *J. Med. Chem.* **2005**, *48*, 5520-5529.

18. Nie, Y.; Vigues, S.; Hobbs, J. R.; Conn, G. L.; Munger, S. D. *Curr. Biol.* **2005**, *15*, 1948-1952.

19. Jiang, P. H.; Ji, Q. Z.; Liu, Z.; Snyder, L. A.; Benard, L. M. J.; Margolskee, R. F.; Max, M. *J. Biol. Chem.* **2004**, *279*, 45068-45075.

20. Hobbs, J. R.; Munger, S. D.; Conn, G. L. *Acta Crystallogr., Sect. F (In press).*

21. Ramachandran, G. N., Sasisekharan, V. *Adv. Protein Chem.* **1968**, *23*, 283-437.

22. Morris, J. A.; Martenso, R.; Deibler, G.; Cagan, R. H. *J. Biol. Chem.* **1973**, *248*, 534-539.

23. Lee, S. Y.; Lee, J. H.; Chang, H. J.; Cho, J. M.; Jung, J. W.; Lee, W. T. *Biochemistry* **1999**, *38*, 2340-2346.

24. Spadaccini, R.; Trabucco, F.; Saviano, G.; Picone, D.; Crescenzi, O.; Tancredi, T.; Temussi, P. A. *J. Mol. Biol.* **2003**, *328*, 683-692.

25. Iijima, H.; Morimoto, K. *Ann. N.Y. Acad. Sci.* **1995**, *750*, 62-65.

Chapter 8

Molecular Models of Sweet Taste Receptors Provide Insights into Function

Meng Cui[1], Peihua Jiang[2], Emeline Maillet[2], Marianna Max[2], Robert F. Margolskee[2], and Roman Osman[1,*]

Departments of [1]Molecular Physiology and Biophysics and [2]Neuroscience, Mount Sinai School of Medicine, New York, NY 10029

The heterodimer of T1R2 and T1R3 is a broadly acting sweet taste receptor (STR) responsive to natural sugars, artificial sweeteners, D-amino acids, and sweet-tasting proteins. T1Rs are characterized by a large extracellular Venus flytrap module (VFTM), which is linked by a cysteine rich domain (CRD) to the 7-TM-domain (TMD). Although crystal structures are not available for the sweet taste receptor, useful testable homology models can be developed based on appropriate templates. The VFTM, CRD and TMD of T1R2 and T1R3 have been modeled based on the crystal structures of metabotropic glutamate receptor type 1, tumor necrosis factor receptor, and bovine rhodopsin, respectively. We have used homology models of the STR, molecular docking of sweet ligands to the receptors, and directed mutagenesis of the receptors to identify potential ligand binding sites of the STR.

Using genomic and PCR-based approaches multiple groups identified a third member of the T1R gene family, T1R3, as likely being the sweet receptor gene *sac* (1-5). The heterodimer of T1R2 and T1R3 has been shown to act as a broadly acting sweet taste receptor (STR) responsive to natural sugars, artificial sweeteners, D-amino acids, and sweet-tasting proteins (1, 4, 6, 7). The T1Rs, three related family C G protein-coupled receptors (GPCRs) expressed in taste cells of the tongue and palate, are more distantly related to metabotropic glutamate receptors (mGluRs), calcium sensing receptors, and vomeronasal receptors. T1Rs are characterized by a large extracellular amino terminal domain (ATD) (also referred to as the "Venus Flytrap Module" (VFTM)), which is linked by a cysteine rich domain (CRD) to 7-TM-domain (TMD) (8). By analogy with mGluR1's VFTM, for which ligand-bound crystal structures are available (9), it has been proposed that the VFTMs of T1R2 and/or T1R3 may bind some sweeteners (3, 9, 10). Previous experimental studies using sweet receptor chimeras and mutants show that there are at least four potential binding sites in the heterodimer receptor (11-16). Receptor activity induced by the artificial sweeteners aspartame and neotame implicate residues in the VFTM of human T1R2 (14, 15) while natural sugars bind to the VFTMs of both T1R2 and T1R3 (16). In contrast, the sweetener cyclamate and the sweet taste inhibitor lactisole act on the TMD of human T1R3 as demonstrated by specific mutations in that domain (12, 13, 15). Furthermore, receptor activity toward the sweet protein brazzein depends on the CRD of human T1R3 (11).

To better understand the function of STRs, a detailed structure or structural model would be quite useful. Although crystal structures are not available for the STR, useful homology models can be developed based on appropriate templates. The VFTM, CRD and TMD of T1R2 and T1R3 can be modeled based on the crystal structures of mGluR1 VFTM (9), tumor necrosis factor receptor (17), and bovine rhodopsin (18), respectively. We have used homology models of the STR subdomains, molecular docking to the receptor of the sweeteners, aspartame, neotame, cyclamate and brazzein, and sweet inhibitor, lactisole, and directed mutagenesis of the receptors to identify potential ligand binding sites of the STR. Here we present homology models of STRs, and their predictions regarding multiple binding sites in the receptor for sweeteners, sweet proteins, and sweet inhibitors.

The Venus Flytrap Module of the Sweet Taste Receptor

An homology model of the VFTMs of human T1R2 (hT1R2) + human T1R3 (hT1R3) (closed-open/A form) has been constructed by the MODELLER program (19) using mGluR1-VFTM crystal structure (PDB entry: 1EWK) as the template (9). A multiple sequence alignment of the VFTMs of hT1R2, hT1R3, mouse T1R2 (mT1R2), mouse T1R3 (mT1R3), and mGluR1 was generated by

ClustalW program (20), followed by minor manual adjustments in the non-homologous regions. The model of the VFTMs of hT1R2+hT1R3 with 6 Å water shell was refined by the CHARMM program (21). The refined model (as shown in **Figure 1**) was evaluated by the Verify-3D program, which shows a 3D profile score of the model with acceptable values.
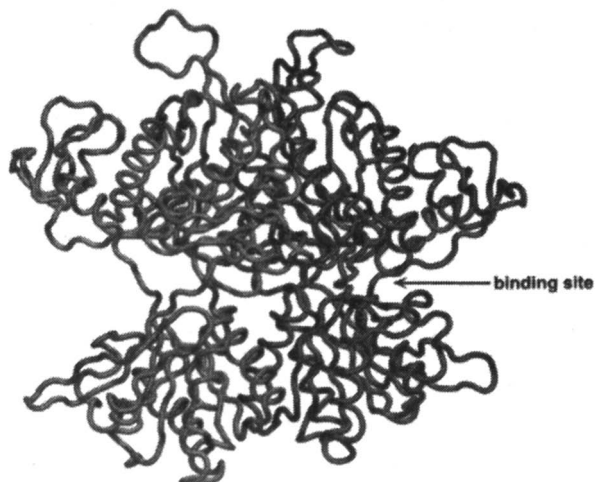
Figure 1. Tube structure representation of the homology model for the Venus Flytrap Module (VFTM) of the sweet taste receptor. hT1R2 and hT1R3 are colored black and grey, respectively.

The binding sites of the VFTM of the STR

Aspartame and neotame are low-calorie peptide sweeteners, ~200 and ~8000 times sweeter, respectively, than sucrose (on a weight basis). Both Xu et al. and Jiang et al. demonstrated that the binding site of the STR for aspartame and neotame is located in the VFTM of hT1R2 (14, 15). To identify potential binding site(s) of the STR for aspartame and neotame, we docked these two sweeteners into the cleft of closed form of the hT1R2-VFTM model using the automatic docking method provided by AUTODOCK program (22). The final dockings of aspartame and neotame were selected based on the docked binding energies and cluster analysis. The docked complexes were then refined by the CHARMM program with 6 Å water shell around the protein. We found that the main interactions of aspartame with the hT1R2-VFTM can be classified as salt bridges (Asp278 and Asp307), hydrogen-bonds (Ser303, Arg383 and Val384) and hydrophobic interactions (Tyr215, Tyr103 and Pro277). Two water molecules were also identified as bridges through hydrogen-bonds between

aspartame and the binding pocket (Asp142 and Leu279). Neotame is predicted to bind to the receptor in a similar configuration to that of aspartame, while the additional N-alkyl group is predicted to interact with a group of five hydrophobic residues of the receptor. The binding pocket of the hT1R2-VFTM with neotame bound is shown in **Figure 2a**. The overlap of the binding conformations of aspartame and neotame is shown in **Figure 2b**. Based on Nofre-Tinti's Multipoint attachment theory (MPA) model (22, 23), we identified the AH, B and D interaction groups for aspartame and neotame, and the G interaction group for neotame. The AH group has been assigned to the amino terminal NH_3^+ of aspartame and NRH_2^+ of neotame, which interact with the negatively charged carboxyl side chains of residues Asp278 and Asp307 through salt bridges, and backbone oxygen atom of Ser303 through a hydrogen-bond interaction. The mutants Asp307Ala and Asp307Asn of hT1R2 were shown to reduce the sweet taste receptor's response to aspartame but not to brazzein (14). The B group has been assigned to the side chain Asp carboxyl groups (COO-) of aspartame and neotame, which interact with the NH backbone atoms of Arg383 and Val384 through hydrogen bonds. The D group has been assigned to the phenyl side chain group of aspartame and neotame, which interact with Tyr215 through π-π interactions, and with Pro277 through hydrophobic interactions. The G group has been assigned to the alkyl group of neotame, which interacts through hydrophobic interactions with the cluster of hydrophobic residues of hT1R2-VFTM. The distances between each of these elements are close to that in Nofre-Tinti MPA model (23). Aspartame covers three high-potency sites (AH, B and D), to make aspartame ~200 times sweeter then sucrose. Neotame covers all four high-potency sites (AH, B, D and G), the additional contact with the G site makes neotame ~40 times sweeter than aspartame.

Brazzein is a small, heat-stable, intensely sweet protein consisting of 54 amino acid residues. It is isolated from the fruit of *Pentadiplandra brazzeana* Baillon (24). The structure of brazzein determined by nuclear magnetic resonance (NMR) spectroscopy shows one short α-helix, and three strands of antiparallel β-sheets held together by four disulfide bonds (25). Site-directed mutagenesis suggested that the N- and C-termini and the β-turn around Arg43 are involved in the sweetness of brazzein (26-28). Our experimental studies suggest that several residues of brazzein, clustered along one face of that sweet protein (29), bind to multiple domains of the sweet receptor. Mutations in the CRD of hT1R3 identified several residues of importance to the receptor's interaction with brazzein. Our recent studies suggest that the VFTM of hT1R2 also contributes to the receptor's responsiveness to brazzein (Maillet et al., in preparation). To further refine our identification of the sweet receptor's binding site of for brazzein, we used the docking feature of Brownian Dynamics (BD) to simulate the brazzein-receptor interaction. This approach has been used in the past to predict protein-protein interactions (30, 31). In earlier work we used BD to successfully simulate the recognition between scorpion toxins and potassium
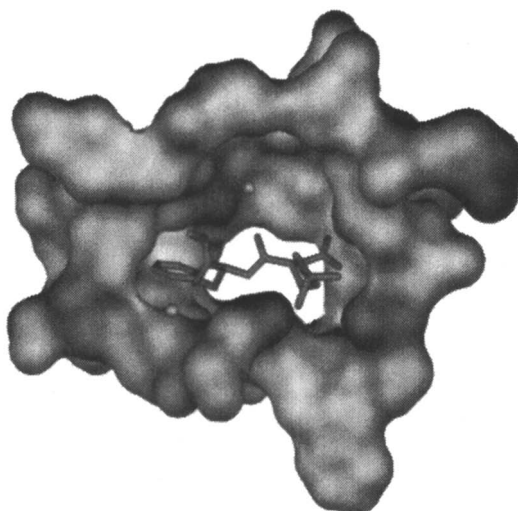
Figure 2a. Neotame complexed with hT1R2's VFTM and associated two water molecules. The binding pocket is represented by molecular surface, and two water molecules shown as spheres.
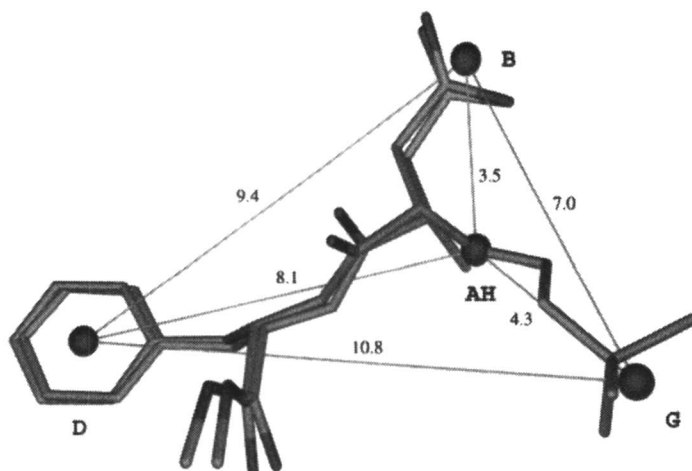
Figure 2b. Mapping the key pharmacophores of Aspartame and Neotame within the context of the Nofre-Tinti MPA model for sweeteners.

122

channels (32-35). During the BD simulations, positions where the distance between the center of masses (COMs) of brazzein and the receptor were smaller than 65 Å were recorded for later analysis. The interaction energies between the monomers in each trajectory with recorded structures were ranked, and the complex with the lowest interaction energy was selected in each trajectory. From the distribution of the COMs of brazzein around the VFTM of STR, we identified a hot spot located on the back of the closed form of hT1R2's VFTM. A typical docked brazzein-receptor complex from this cluster is shown in **Figure 3**.

Figure 3. The docked brazzein-VFTM complex. The VFTM is represented as a molecular surface; brazzein is represented as a coiled structure and it is docked on the back of closed form of VFTM of the hT1R2.

Based on our preliminary studies, we propose that sweet proteins and small molecule sweeteners interact with different binding sites of the STR. Small sweeteners are predicted to bind into the inside binding pocket of the STR ('embedded model'), while sweet proteins bind to the outside of STR ('superficially bound model') (**Figure 4**). Although small sweeteners and sweet proteins bind to different sites, they both can stabilize the closed form of T1R2-VFTM and thereby activate the STR.

The Cysteine-Rich Domain of the Sweet Taste Receptor

Jiang et al. had demonstrated that specific residues in the CRD of hT1R3 were required for eliciting a response to the sweet protein brazzein (11).
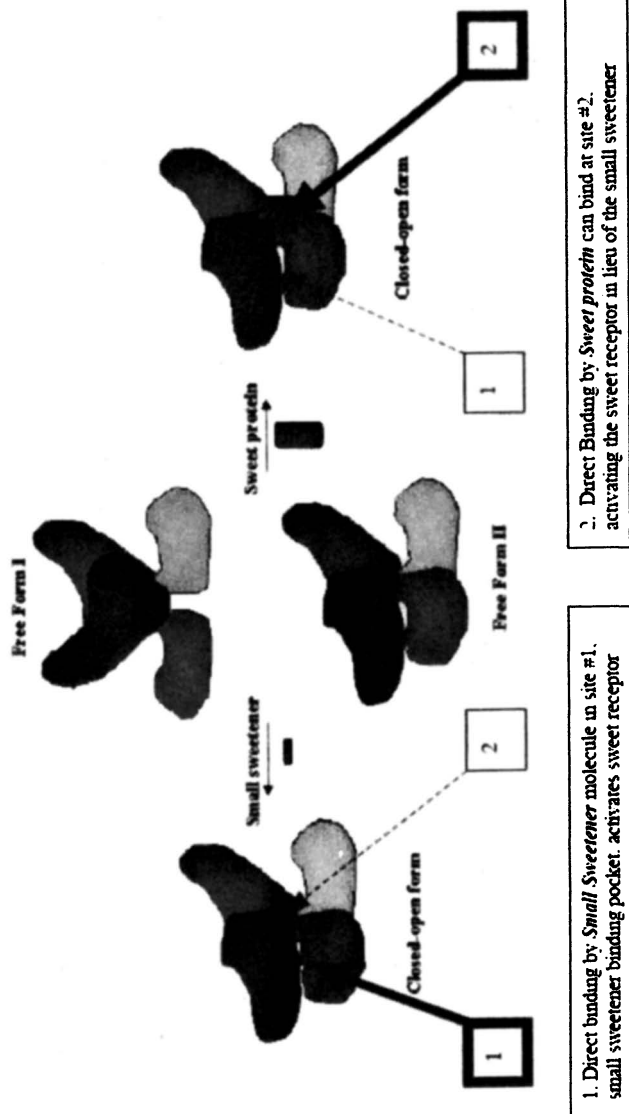
Figure 4. Proposed multiple binding site model for small sweetener vs. sweet protein activation of human T1R2/T1R3 sweet receptor. Small molecule binds to the interior of hT1R2 VFTM cleft whereas sweet protein binding site is on the back of the closed form of hT1R2 VFTM (dark in fruont represents hT1R2, light in back represents hT1R3).

1. Direct binding by *Small Sweetener* molecule in site #1, small sweetener binding pocket, activates sweet receptor

2. Direct Binding by *Sweet protein* can bind at site #2, activating the sweet receptor in lieu of the small sweetener

Free Form I

Free Form II

Small sweetener

Sweet protein

Closed-open form

Closed-open form

However, because the structures of GPCR family C CRDs were unknown there was no mechanistic basis for the interaction of brazzein with the CRD of hT1R3. Liu et al. have proposed that the tumor necrosis factor receptor (TNFR) crystal structure can serve as a template for modeling the CRD of GPCR family C (17). Although the sequence identity between hT1R3's CRD and that of TNFR is only 15%, we hypothesize that they share a similar folding pattern. Based on the TNFR crystal structure (PDB: 1EXT) (36), we have constructed a homology model of hT1R3's CRD (**Figure 5a**). The CRD model was evaluated by Verify-3D program with all positive scores, which indicates that the quality of the model is acceptable (**Figure 5b**). The CRD model shows that six out of the nine conserved cysteines form three disulfide bonds. These disulfide bonds confer considerable rigidity on the CRD, which may be important for the allosteric coupling of the conformational changes that occur upon ligand binding to the VFTM being transmitted to the TMD. Jiang et al. identified two unique residues in hT1R3's CRD (Ala537 and Phe540), which distinguish hT1R3 from mT1R3 and are important for the human specific sweet response to brazzein (11). Ala537 of hT1R3's CRD may interact directly with brazzein or be involved in transmitting the conformational change to the TMD. Mutations to residues with larger side chains, such as, A537T, A537Q and A537V, abolish the sweet receptor's response to brazzein. In contrast, mutants with a smaller side chain, such as A537G, remain responsive to brazzein (11). A homology model of the hT1R3-CRD shows that Ala537 and Phe540 are located close to each other on the surface of the CRD, potentially forming part of the binding site for brazzein. We recently have shown that D535A and D535Q mutations of hT1R3 selectively abolish the mutant receptor's response to brazzein only, sparing responses to other sweeteners (Jiang et al., this publication). Notably, Asp535 lies on the same face of our model of the CRD as do Ala537 and Phe540, suggesting that Asp535 may interact directly with one of brazzein's positively charged residues.

The Transmembrane Domain of the Sweet Taste Receptor

Chimera and mutagenesis studies of hT1R2+hT1R3 have shown that the TMD of hT1R3 is required for the activation of the STR by cyclamate, or for inhibition of the STR by lactisole (12, 13, 15). We have constructed a homology model of hT1R3's TMD based on the bovine rhodopsin crystal structure (PDB: 1F88) (18). A multiple sequence alignment between T1Rs (hT1R2, hT1R3, mT1R2, mT1R3), mGluR1 and bovine rhodopsin was generated by the ClustalW program (20), followed by some manual adjustments in the non-homologous regions. The TM sequence identities (similarities) between hT1R3 and bovine rhodopsin for TM1, TM2, TM3, TM4, TM5, TM6 and TM7 are: 22.6% (61.3%), 13.3% (40%), 22.6% (48.4%), 25.0% (33.3%), 11.1% (40.7), 11.8% (61.8%), 16% (60%), respectively. That key conserved residues in each TM helix of family A GPCRs are also often present in the corresponding TMs of
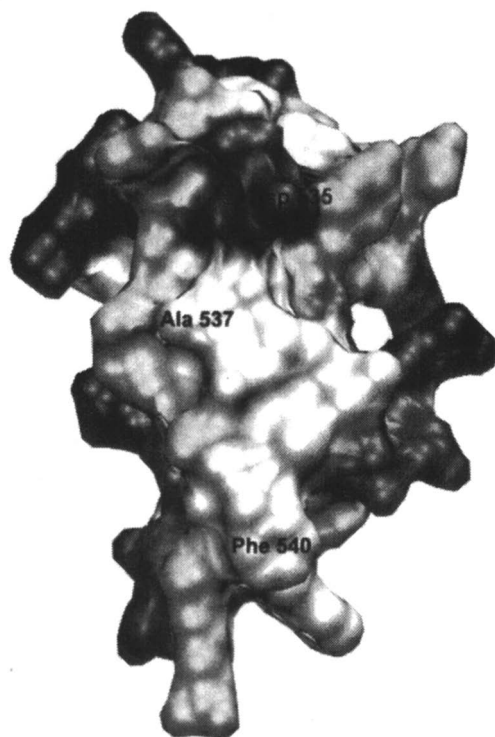
*Figure 5a. Structural model of the Cysteine Rich Domain (CRD) of hT1R3
(The molecular surface with labels on Asp535, Ala537 and Phe540).*

family C receptors increased our confidence in the alignment. The hT1R3 TM homology model was constructed by residue replacement using InsightII (Accelrys, San Diego, CA). The non-conserved Pro kink in TM6 was built by manually shifting it to its proper location in the sequence. Extracellular loop 3 between TM6 and TM7 was generated by *ab initio* loop prediction (37). The other intra- and extra- cellular loops were generated by the MODELLER program (19) based on the bovine rhodopsin template. The model refinements were carried out with CHARMM (21) using the CHARMM27 all atom force field.

The TM binding site of hT1R3 for cyclamate

Our recent docking and mutagenesis studies show that the artificial sweetener cyclamate activates the STR by interacting with the TM region of hT1R3. Experimental studies of chimeras of the T1R2+T1R3 heterodimer show

Verify 3D
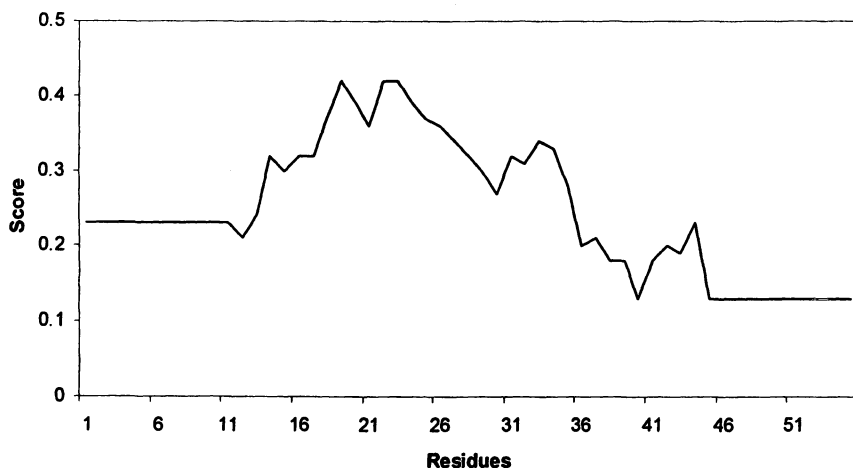
Figure 5b. Homology model of CRD of hT1R3 evaluated by Verify-3D (Higher positive Verify-3D scores indicate higher sequence-structure compativility of the model) .

that hT1R3 is required for STR sensitivity to cyclamate, and the TM domain of hT1R3 is required for cyclamate-induced STR activation (13). The studies of Xu et al. also showed that cyclamate interacts with hT1R3's TM region (15). Alanine-scan mutagenesis of the TM region of hT1R3 show that mutants F778A (TM6) and R723A (extracellular loop2) have a small effect on responsiveness to D-tryptophan (which acts on the VFTM of hT1R2 (14)), but substantially reduce STR responses to cyclamate. This is consistent with the hypothesis that cyclamate activates the receptor through the TM domain. Using an automatic molecular docking method, we identified a possible binding site for cyclamate in the hT1R3 TM domain. The predicted complex of cyclamate with hT1R3's TMD **(Figure 6)** shows that the initial binding pocket is located in a space between TM3, TM5, TM6 and the extracellular loop 2 of hT1R3 TMD. The initial binding pocket consists of hydrophilic and hydrophobic residues. Our homology model predicts that the negatively charged sulfamate group of cyclamate interacts with Arg723, His721, His641, Ser640, and Gln637 through salt bridges or hydrogen bonds. Our mutagenesis results show that mutants R723A, H721F, H641A, S640V and Q637E affect STR responses to cyclamate more than those to D-tryptophan (13). The agreement between our model's predictions and the mutagenesis studies support the validity of our homology models. Based on Kier's A-H/B/X model (38), we identified the AH, B and X

interaction groups for cyclamate (**Figure 7**). The AH group is the NH group of cyclamate, which interacts with the side chain of Gln637 through hydrogen-bond interaction. The B group is the sulfamate group, which interacts with residues Arg723, His 721, and Ser641 through salt bridges and hydrogen bonds. The X group is the cyclohexyl ring of cyclamate, which interacts with residues Phe778 and Leu782 through hydrophobic interactions. The distances between each element are close to those in Kier's A-H/B/X model.

The TM binding site of hT1R3 for sweet inhibitor lactisole

Lactisole (2-(p-Methoxyphenoxy) propionic acid) and its analogs (phenylalkanoic, phenoxyalkanoic and benzylalkanoic acids) were found to inhibit sweet taste responses in humans (39). Xu et al. showed that the TMD of hT1R3 was required for STR sensitivity to lactisole in vitro (15). Our studies of hT1R3+mT1R3 chimeras show that lactisole blocks sweetener-induced activation of hT1R2+hT1R3 but not of the mixed species receptor, hT1R2+mT1R3, indicating that hT1R3 is the major mediator of lactisole antagonism (15). Additional chimeric studies, in which the TM region of hT1R3 replaces that of the mouse in the mT1R2+mT1R3 receptor, show that the presence of the human TM region of hT1R3 is sufficient to support lactisole activity. In contrast, in the absence of the TM region of hT1R3, lactisole shows no blocking effect. Studies of hT1R3 TMD mutants H641A, F778A, Q637E, S640V, A733V have identified specific residues involved in mediating lactisole's inhibitory effect on the STR (15). Using our homology models we identified a possible binding pocket for lactisole in hT1R3's TMD. The predicted model of the lactisole complex with hT1R3 TMD is shown in **Figure 8**. The detailed predictions indicate that lactisole shares the same binding pocket with cyclamate. These interaction sites are with residues His641 and Arg723 through salt bridges; Gln637 and Ser640 through hydrogen bond interactions; and Phe778, Leu782, Leu644 via hydrophobic interactions (12).

Conclusions

The identification and characterization of the sweet taste receptor has opened the door to developing an understanding of the mechanism of sweet taste response. The heterodimer of T1R2 and T1R3 can respond to almost all sweet molecules including natural sugars, artificial sweeteners, and sweet-tasting proteins. Results from experiments and molecular modeling show that the sweet taste receptor has multiple binding sites for different sweeteners (11-15). Although there are no crystal structures of any part of the sweet taste receptor, each part can be modeled based on an appropriate homologous template. We
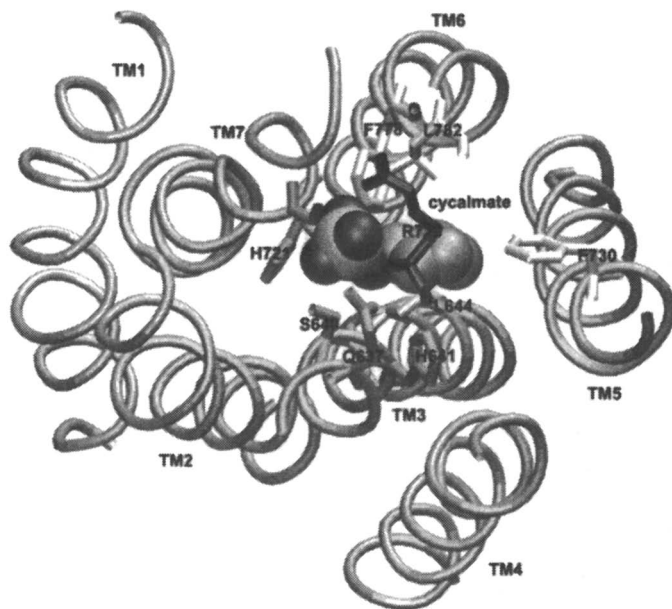
Figure 6. Cyclamate docked in the binding site of hT1R3's TMD (cyclamate is shown in space-filling CPK model, side chains of residues in binding site of TMD are shown as sticks, white represents hydrophobic, grey represents hydrophilic, and black represents positive charged residues) .
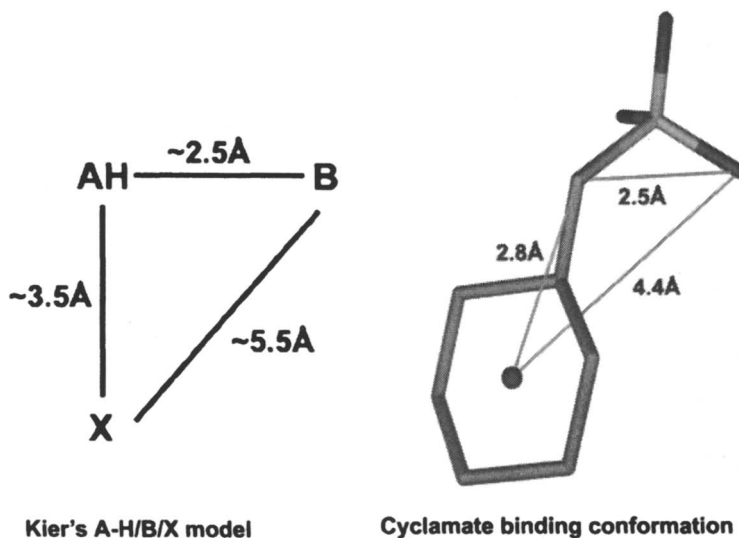
Figure 7. Comparing cyclamate binding conformation with Kier's A-H/B/X model.
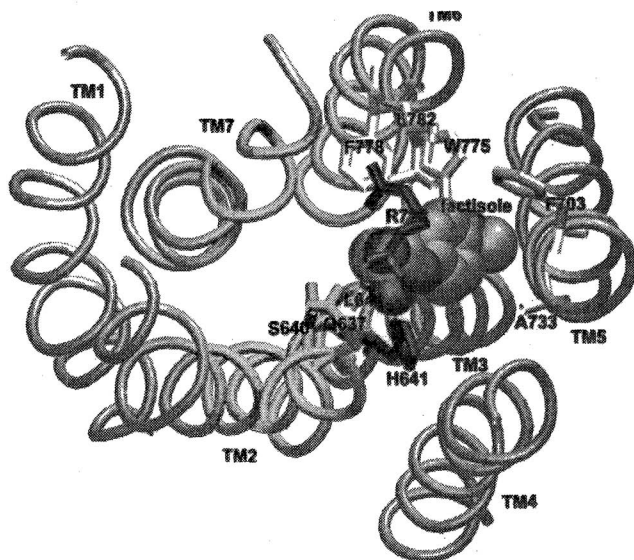
Figure 8. Lactisole docked in the binding site of hT1R3's TMD (Lactisole is shown in space-filling CPK model, side chains of residues in binding site of TMD are shown as sticks, white represents hydrophobic, grey represents hydrophilic, and black represents positive charged residues).

have developed homology models for the VFTM, CRD and TMD of the sweet taste receptor based on, respectively, crystal structures of mGluR1's VFTM, TNFR, and bovine rhodopsin. We have used molecular docking of sweet ligands to these homology models of the receptors, as well as directed mutagenesis of the receptor to identify potential binding sites for sweeteners. We identified that aspartame and neotame bind to hT1R2's VFTM. In contrast, the artificial sweetener cyclamate and sweet taste inhibitor lactisole share a binding pocket in the TMD of hT1R3. Our studies suggest that the CRD of hT1R3 and the VFTM of hT1R2 may form the binding site for sweet-tasting protein brazzein.

Over the past few decades, and based on the structures of existing sweeteners, the following several models have been developed to explain the physico-chemical requirements of a sweet compound: the AH-B model of Shallenberger and Acree (40), the AH, B, X model of Kier (38), and the multipoint attachment theory (MPA) model of Tinti and Nofre (23). However, an intrinsic limitation of these pharmacophore models is that they are not based on direct knowledge of the sweet taste receptor itself. Further complicating the situation is the observation that sweet taste receptors have multiple binding sites for different types of sweeteners. By comparing our predicted aspartame and neotame binding configurations within the VFTM of hT1R2 with the Nofre-Tinti

MPA model, we identified three high affinity sites AH, B and D for aspartame, and four high affinity sites AH, B, D and G for neotame. By comparing our predicted cyclamate binding conformation within the TMD of hT1R3 with Kier's A-H/B/X model, we identified three recognition elements for cyclamate. The agreement of the predicted sweet taste receptor structures with the sweet models indicates that the Nofre-Tinti MPA model describes the closed form of hT1R2's VFTM, and Kier's A-H/B/X model describes the TMD of hT1R3.

Acknowledgements

References

1. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S. *Cell* **2001**, *106*, 381-390.
2. Kitagawa, M.; Kusakabe, Y.; Miura, H.; Ninomiya, Y.; Hino, A. *Biochem Biophys Res Commun* **2001**, *283*, 236-422.
3. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. *Nat Genet* **2001**, *28*, 58-63.
4. Montmayeur, J. P.; Liberles, S. D.; Matsunami, H.; Buck, L. B. *Nat Neurosci* **2001**, *4*, 492-498.
5. Sainz, E.; Korley, J. N.; Battey, J. F.; Sullivan, S. L. *J Neurochem* **2001**, *77*, 896-903.
6. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N. J.; Zuker, C. S. *Cell* **2003**, *115*, 255-266.
7. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc Natl Acad Sci U S A* **2002**, *99*, 4692-4696.
8. Pin, J. P.; Galvez, T.; Prezeau, L. *Pharmacol Ther* **2003**, *98*, 325-354.
9. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. *Nature* **2000**, *407*, 971-977.
10. Montmayeur, J. P.; Matsunami, H. *Curr Opin Neurobiol* **2002**, *12*, 366-371.
11. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Margolskee, R. F.; Max, M. *J Biol Chem* **2004**, *279*, 45068-45075.
12. Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Osman, R.; Margolskee, R. F.; Max, M. *J Biol Chem* **2005**, *280*, 15238-15246.

13. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L. A.; Benard, L. M.; Osman, R.; Max, M.; Margolskee, R. F. *J Biol Chem* **2005**, *280*, 34296-34305.
14. Jiang, P.; Cui, M.; Ji, Q.; Snyder, L.; Liu, Z.; Benard, L.; Margolskee, R. F.; Osman, R.; Max, M. *Chem Senses* **2005**, 30 Suppl 1, i17-i18.
15. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. *Proc Natl Acad Sci U S A* **2004**, *101*, 14258-14263.
16. Nie, Y.; Vigues, S.; Hobbs, J. R.; Conn, G. L.; Munger, S. D. *Curr Biol* **2005**, *15*, 1948-1952.
17. Liu, X.; He, Q.; Studholme, D. J.; Wu, Q.; Liang, S.; Yu, L. *Trends Biochem Sci* **2004**, *29*, 458-461.
18. Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. *Science* **2000**, *289*, 739-745.
19. Marti-Renom, M. A.; Stuart, A. C.; Fiser, A.; Sanchez, R.; Melo, F.; Sali, A. *Annu Rev Biophys Biomol Struct* **2000**, *29*, 291-325.
20. Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res* **1994**, *22*, 4673-4680.
21. Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comp. Chem.* **1983**, *4*, 187-217.
22. Goodsell, D. S.; Morris, G. M.; Olson, A. J. *J Mol Recognit* **1996**, *9*, 1-5.
23. Tinti, J. M.; Nofre, C., eds. *In: Sweeteners: Discovery, Molecular Design, and Chemoreception. ACS Symposium Series 450.* Why Does a Sweetener Taste Sweet? A New Model, ed. DuBois, G. E. **1991**, American Chemistry Society: Washington, D. C. 206-213.
24. Ming, D.; Hellekant, G. *FEBS Lett* **1994**, *355*, 106-108.
25. Caldwell, J. E.; Abildgaard, F.; Dzakula, Z.; Ming, D.; Hellekant, G.; Markley, J. L. *Nat Struct Biol* **1998**, *5*, 427-431.
26. Assadi-Porter, F. M.; Aceti, D. J.; Cheng, H.; Markley, J. L. *Arch Biochem Biophys* **2000**, *376*, 252-258.
27. Assadi-Porter, F. M.; Aceti, D. J.; Markley, J. L. *Arch Biochem Biophys* **2000**, *376*, 259-265.
28. Jin, Z.; Danilova, V.; Assadi-Porter, F. M.; Aceti, D. J.; Markley, J. L.; Hellekant, G. *FEBS Lett* **2003**, *544*, 33-37.
29. Assadi-Porter, F. M.; Abildgaard, F.; Blad, H.; Cornilescu, C. C.; Markley, J. L. *Chem Senses* **2005**, 30 Suppl 1, i90-i91.
30. Pearson, D. C., Jr.; Gross, E. L. *Biophys J* **1998**, *75*, 2698-2711.
31. Ouporov, I. V.; Knull, H. R.; Thomasson, K. A. *Biophys J* **1999**, *76*, 17-27.
32. Cui, M.; Shen, J.; Briggs, J. M.; Fu, W.; Wu, J.; Zhang, Y.; Luo, X.; Chi, Z.; Ji, R.; Jiang, H.; Chen, K. *J Mol Biol* **2002**, *318*, 417-428.
33. Cui, M.; Shen, J.; Briggs, J. M.; Luo, X.; Tan, X.; Jiang, H.; Chen, K.; Ji, R. *Biophys J* **2001**, *80*, 1659-1669.
34. Fu, W.; Cui, M.; Briggs, J. M.; Huang, X.; Xiong, B.; Zhang, Y.; Luo, X.; Shen, J.; Ji, R.; Jiang, H.; Chen, K. *Biophys J* **2002**, *83*, 2370-2385.

35. Huang, X.; Liu, H.; Cui, M.; Fu, W.; Yu, K.; Chen, K.; Luo, X.; Shen, J.; Jiang, H. *Curr Pharm Des* **2004**, *10*, 1057-1067.

36. Naismith, J. H.; Devine, T. Q.; Kohno, T.; Sprang, S. R. *Structure* **1996**, *4*, 1251-1262.

37. Fiser, A.; Sali, A. *Bioinformatics* **2003**, *19*, 2500-2501.

38. Kier, L. B. *J Pharm Sci* **1972**, *61*, 1394-1397.

39. Lindley, M. G., *Method of inhibiting sweetness.* **1986**: U.S.4,567,053.

40. Shallenberger, R. S.; Acree, T. E. *Nature* **1967**, *216*, 480-482.

Chapter 9

Pharmacophore Modeling of Sweet and Bitter Tasting Molecules

Nicolas Froloff

Department of Molecular Modeling, CEREP, 19 Avenue du Québec, 91951 Courtaboeuf Cedex, France

Numerous experimental data on human peripheral taste system suggest the existence of multiple low affinity and low specificity receptor sites, responsible for the detection and the complete discrimination of an unlimited number of organic molecules. The statistical analysis of intensity estimates, made by dozens of human subjects for diverse molecules, allows the calculation of biological intermolecular distances. We present herein a molecular modeling approach of sweet and bitter tasting molecules to identify pharmacophores that could explain their experimental taste distances, and hence be good candidates to be recognized by taste receptor sites. We show the results of the approach on 14 molecules where 7 pharmacophores have been identified which best account for all experimental distances. We also show how these identified pharmacophores could explain experimental cross-adaptation results among four sweeteners. Generalization of the approach as well as possible applications to the design of sweeteners and taste-masking agents will be outlined.

Multiple taste receptor sites

Electrophysiological recordings of taste nerve fibers show that each fiber has a unique sensitivity spectrum to molecular stimuli (*1*): each fiber responds to a large variety of stimuli, and response profiles are never identical from one fiber to another. Statistical analyses show that more than 10 independent factors are responsible for the production of taste responses. These factors are likely to reflect the diversity of peripheral taste receptor mechanisms responsible for the detection of organic molecules. Based on the information content of taste nerve responses, we hypothesize that more than 10 low affinity, low specificity, distinct receptor sites should be found cooperatively signaling the responses of sweet and bitter tasting organic molecules. This hypothesis is largely substantiated by the recent discovery of distinct receptors for sweet taste (T1R2/T1R3) and of about 30 different receptors for bitter taste (T2R family) (*2-6*).

The differential sensitivity of taste nerve fibers to stimuli is also observed in psychophysical experiments (*7*). For example, strong interindividual differences in sensitivity to sucrose have been evidenced: some subjects need ten times more concentrated sucrose to perceive the same intensity as other subjects (as compared to the intensity of a reference NaCl solution). Moreover, these interindividual differences in sensitivity to sucrose do not translate to the same differences for another sweetener such as dulcin: the sensitivity profile of the same panel of human subjects to dulcin is different and cannot be predicted from the sucrose profile alone: the correlation between the sensitivity profiles to sucrose and dulcin as measured in a study on 58 human subjects with 39 molecules (*8*) is significantly low (r = 0.53). This indicates that taste receptor sites involved in the recognition of sucrose are different, at least partially, from those involved in the detection of dulcin.

The non-covariance between taste sensitivity profiles is a general phenomenon as revealed *e.g.* in the studies by Faurion *et al.* (see chapter by Faurion in this book). Statistical analysis (PCA) of the covariance matrix reveals about 10 significant independent factors. Again, this suggests at least 10 different information channels (receptor sites) used by the peripheral taste system for molecular recognition of organic molecules.

Molecular modeling of 14 sweet and bitter tasting molecules

In a first molecular modeling study (*9*), we followed a ligand-based strategy in order to understand the similarities (or dissimilarities) in taste responses for 14 sweet and bitter tasting molecules, as the nature and 3D structure of taste receptors was not known at that time. We hypothesized that each taste receptor

site can bind a large number of compounds. Symmetrically, a given sweet or bitter tasting compound is likely to bind to several distinct taste receptor sites, such that the taste response is the result of these multiple binding events which constitute a unique signature of the compound (*10-12*). This multireceptor component (polypharmacology) of any taste response to organic stimuli needs to be explicitly taken into account in molecular modeling approaches to structure-taste relationships.

We modeled 14 sweet and bitter tasting molecules in order to identify common binding motifs (pharmacophores) which would account for their taste response similarities (or differences), as measured in human psychophysical experiments (*8*). We hypothesized that two molecules produce similar taste responses in humans because they share similar pharmacophores, and hence bind similarly to common GPCRs. The biological covariance matrix among the 14 tastants (Figure 1) indicates the proportion of common receptor sites between two tastants, and thus should indicate the proportion of common pharmacophores. We developed a pharmacophore modeling approach analog to drug design (ligand-based molecular modeling), and we searched for several pharmacophores common to sweet and bitter tastants which would best account for the experimental biological covariance matrix among the 14 tastants.

The 14 organic compounds selected for molecular modeling were the following: 5 synthetic sweeteners (saccharin, SAC, cyclamate, CYC, perillartine, PER, dulcin, DUL and 1-propoxy-2-amino-4-nitrobenzene, PAN), 2 amino acids (glycine, GLY and L-threonine, LTH), 5 acidic substituted benzenes (picric acid, PIC, 3-aminobenzoic acid, ABZ, 3-nitrobenzenesulfonic acid, NSA, 2- and 3-nitrobenzoic acids, ONB and MNB respectively), and 2 xanthines (caffeine, CAF and theophylline, TOF). Of note, these chemicals are quite diverse and belong to quite distinct chemical classes, although they are qualified as "sweet" or "bitter".

A systematic extraction of pharmacophores (*9*) was done for each of the 14 modeled chemical structures. One 3D conformation was modeled per molecule (the experimental crystal structure when available, an energy-minimized conformation otherwise). Ionization states (at pH=7.3) were specified according to known or measured acidity constants. H-bond acceptors, H-bond donors, and both (-OH groups) were all identified. Hydrophobic regions were defined as the portions of molecule surface closest to nonpolar atoms. Then, molecular surfaces were systematically sliced in smaller fragments of about three pharmacophore features. This resulted in a sampling of 240 fragments from the 14 molecules, each fragment containing 0-4 H-bonding atoms and about 30 Å^2 hydrophobic patch.

An algorithm was developed for pairwise comparisons of fragments. This algorithm was optimized to favor the superimposition of H-bond acceptor or donor atoms, as well as of a large proportion of both the hydrophobic patches of the two fragments. Twelve clusters of common fragments extracted from the 14

CAF 0,59

CYC 0,07 0,00

DUL 0,78 0,39 0,22

GLY 0,23 0,09 0,55 0,40

LTH 0,53 0,08 0,45 0,31 0,63

MNB 0,54 0,52 0,15 0,38 0,14 0,36

NSA 0,61 0,60 0,00 0,41 0,20 0,37 0,78

ONB 0,79 0,64 0,20 0,63 0,42 0,34 0,60 0,63

PAN 0,73 0,48 0,38 0,66 0,52 0,62 0,58 0,46 0,68

PER 0,32 0,38 0,38 0,58 0,45 0,32 0,22 0,06 0,38 0,69

PIC 0,27 0,51 0,00 0,22 0,25 0,08 0,65 0,65 0,42 0,29 0,01

SAC 0,59 0,50 0,48 0,50 0,64 0,50 0,49 0,43 0,52 0,27 0,43 0,21

TOF 0,51 0,50 0,46 0,27 0,45 0,44 0,53 0,45 0,47 0,36 0,00 0,32 0,58

    ABZ CAF CYC DUL GLY LTH MNB NSA ONB PAN PER PIC SAC
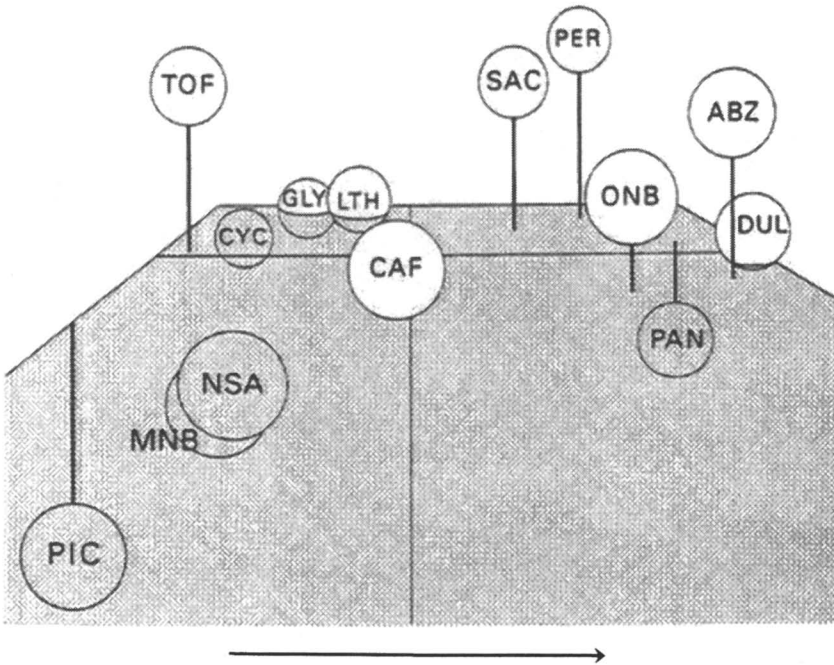
modeled molecules were thus identified (see (*9*) for details of the computations and the clustering). These 12 clusters correspond to 12 pharmacophores possibly recognized by the same number of distinct taste receptor sites. They are made of various spatial arrangements of H-bond donors, acceptors and hydrophobic patches. Each pharmacophore was found on at least 3 different molecules. The spatial arrangements of H-bond acceptors and donors in the 12 identified pharmacophores are depicted in Figure 2.

An algorithm was then developed to select, among those 12 pharmacophores, the ones which would best account for the biological (taste) distances among the isointensity profiles of the 14 molecules. Structural distances among compounds were computed as the proportion of their common pharmacophores, as measured by the city-block distance between bit strings counting the presence ("1") or absence ("0") of a given pharmacophore (Figure 3a). The resulting distance matrix was then compared to the taste distance matrix, where taste distances (d) were calculated from the 91 Pearson correlation coefficient values (r) among the 14 isointensity profiles measured in humans (see Figure 1) as $d^2 = 1 - r^2$. The Spearman rank correlation coefficient was used as an indicator of the fit between the 91 structure distances and the 91 taste distances. The similarity between the two distance matrices was done by looking at all possible combinations of the 12 pharmacophores. By this process, an optimal subset of 7 pharmacophores out of the 12 was identified (Figure 3b), which significantly and best accounted for the 91 pairwise taste distances between all 14 modeled molecules. These 7 pharmacophores are good candidates to be recognized by the same number of distinct taste receptor sites as they are best accounting for their taste responses in humans.

When one looks at the pharmacophore description of the molecules in our final model (Figure 3b), it is interesting to note that there is no clear link between the semantic description of the molecules ("sweet" or "bitter") and the

Figure 1. Biological covariance matrix (top) and corresponding biological space (bottom) for the 14 tastants. The triangular matrix gives the 91 Pearson correlation coefficient values (r) among the 14 isointensity profiles measured in humans (8). The biological space was obtained by principal component analysis (PCA) on the 91 biological distances (d), where $d^2 = 1 - r^2$. It is made of 7 significant dimensions, which account for 77% of the information of the distance matrix. Here the 3 most significant dimensions are shown, which account for 45% of the distance information. A high correlation value (e.g. ONB-ABZ: 0.79) indicates that the 2 compounds probably bind similarly to the taste receptor sites. A low value (e.g. CYC-ABZ: 0.07) indicates that the 2 compounds do not bind to the same receptor sites. It is interesting to note some low correlations between sweet tasting molecules (e.g. CYC-ABZ) and higher correlations between sweet-bitter molecules (e.g. CYC-TOF), which can be visualized in the corresponding biological space, where CYC is closer to TOF than to ABZ.

138
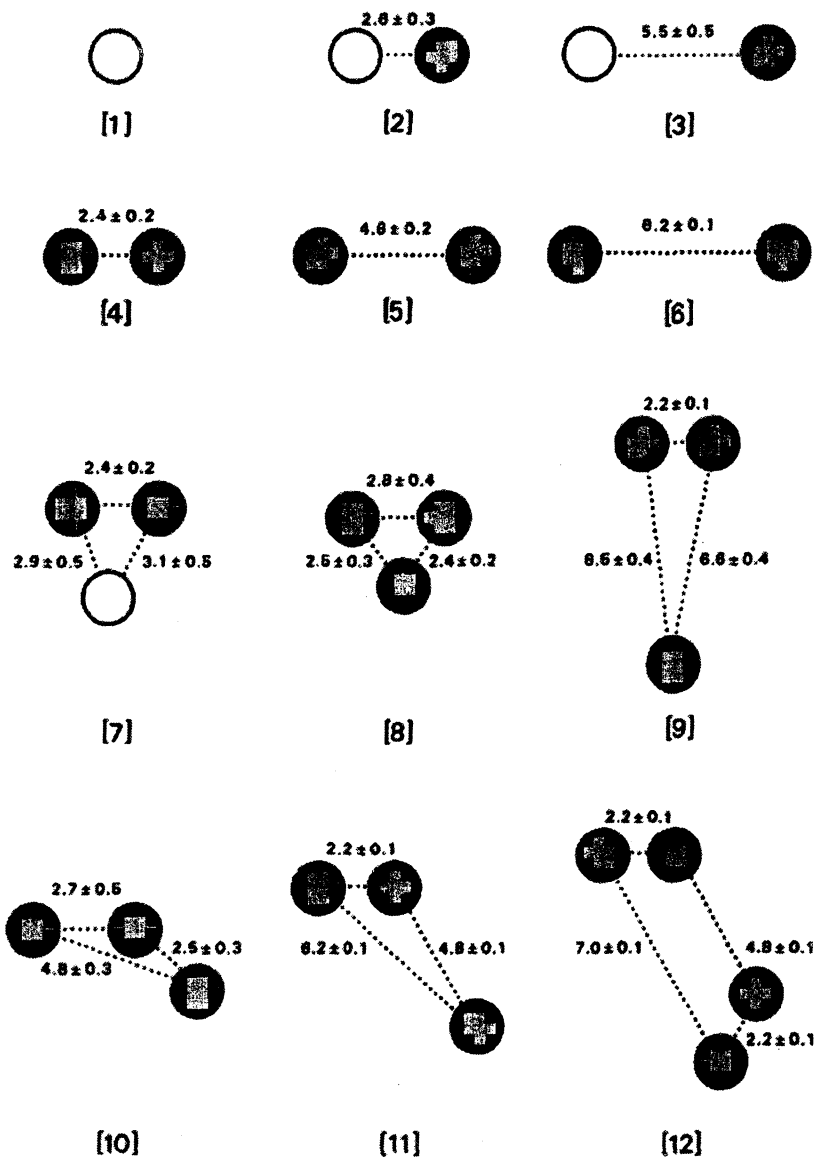
Figure 2. H-bond acceptor (black) and donor (white) average positions for each of the 12 pharmacophores extracted for the 14 modeled sweet and bitter tasting molecules. Each pharmacophore is present on at least 3 molecules. Average distances between H-bond acceptors and donors are indicated in Angströms. (Reproduced from reference 9. Copyright 1996 Oxford University Press.)

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
ABZ	1	0	1	1	0	0	0	0	0	0	0	0
CAF	0	0	0	0	1	0	0	0	0	0	0	0
CYC	0	1	0	1	0	0	1	1	0	0	0	0
DUL	1	1	1	0	0	1	0	0	0	0	0	0
GLY	1	1	0	1	0	0	1	0	0	0	0	0
LTH	1	1	0	1	1	0	1	0	0	1	0	0
MNB	0	0	0	1	1	1	0	0	1	0	1	1
NSA	0	0	0	1	1	1	0	1	1	0	1	1
ONB	0	0	0	1	1	0	0	1	0	1	0	0
PAN	1	1	1	1	0	1	0	0	1	0	0	0
PER	1	1	0	0	0	0	0	0	0	0	0	0
PIC	0	0	0	0	1	1	0	1	1	1	1	1
SAC	0	0	0	1	1	0	0	1	0	1	0	0
TOF	0	1	1	1	1	0	0	0	0	1	1	0

	[1]	[2]	[4]	[5]	[7]	[11]	[12]
ABZ	1	0	1	0	0	0	0
CAF	0	0	0	1	0	0	0
CYC	0	1	1	0	1	0	0
DUL	1	1	0	0	0	0	0
GLY	1	1	1	0	1	0	0
LTH	1	1	1	1	1	0	0
MNB	0	0	1	1	0	1	1
NSA	0	0	1	1	0	1	1
ONB	0	0	1	1	0	0	0
PAN	1	1	1	0	0	0	0
PER	1	1	0	0	0	0	0
PIC	0	0	0	1	0	1	1
SAC	0	0	1	1	0	0	0
TOF	0	1	1	1	0	1	0

Figure 3. (a) Description of the 14 modeled molecules as bit strings indicating the presence ("1") or absence ("0") of a given pharmacophore type on their 3D structure (pharmacophores are numbered from [1] to [12] as in Figure 2); (b) Best selection of 7 pharmacophores accounting for the taste similarities or dissimilarities between the 14 modeled molecules.

way they are likely to be recognized by the taste receptors. For example, molecules generally described as "sweet" by a panel of human subjects do not share a common pharmacophore in our model (these molecules are ABZ, CYC, DUL, GLY, LTH, ONB, PAN, PER and SAC). Moreover, they are all described by at least two pharmacophores. This indicates that one pharmacophore is probably not sufficient to account for sweet taste. As for "bitter" molecules (CAF, MNB, NSA, PIC, TOF), they all share the pharmacophore number 5, but this pharmacophore is also present on the sweet molecules LTH, ONB and SAC. This may also explain the known bitter after taste of SAC. Finally, sweet molecules can share up to 3 pharmacophores with bitter molecules (e.g. LTH and TOF). Our modeling approach is thus able to account for taste distances that can be shorter between sweet and bitter molecules than between two sweet molecules. The taste sensation is more likely to be the result of a particular binding signature (profile) of a compound with the taste receptors, rather than the result of a tight binding to a single sweet or a bitter taste receptor.

In conclusion for this modeling study, this set of 7 pharmacophores is sufficient to explain a significant portion of the information contained in the experimental taste distances between the 14 molecules, as visualized by the good correspondence between the taste and structure dendrograms (Figure 4). However, this set of pharmacophores is far from being exhaustive, and this work needs to be continued by including more molecules and more experimental data, and by refining the molecular modeling approach using more sophisticated pharmacophore description of the compounds. Also, this modeling approach will prove useful when it will be shown generalizable to new compounds not included in the training of the model. It will also probably greatly benefit from direct structure-based modeling approaches of taste receptors (16-18).

Validation by cross adaptation experiments

In a second molecular modeling study (13) we have performed cross adaptation experiments in humans with 2 carbohydrates (SUCrose and FRUctose), and 2 synthetic sweeteners (acesulfame-K, MOD, and DULcin). Only DUL is common to our first modeling study. Seven subjects were asked to match concentrations eliciting the same intensity as a 30g/l sucrose solution. Cross adaptation levels were calculated as the ratio of isointense concentrations for a given stimulus before and under adaptation.

The results of cross adaptation experiments are summarized in Table 1. A first finding is that cross adaptation between SUC and FRU is low and apparently reciprocal. For example, one needs only a 10% more concentrated solution of FRU (on average) to obtain the isointensity after adaptation with a solution of SUC, as compared without prior adaptation (Table 1). By contrast, cross adaptation between SUC and MOD is clearly non-reciprocal: SUC adapts
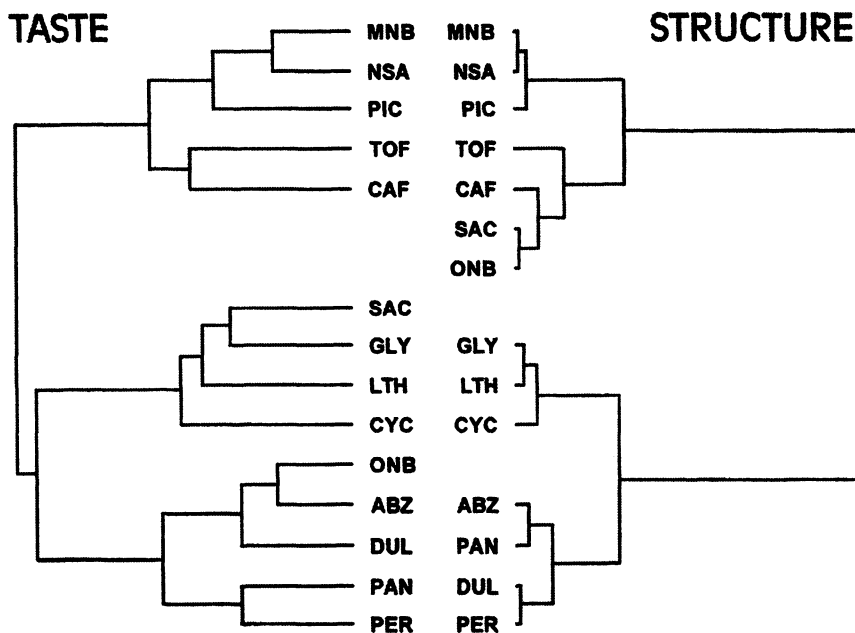
*Figure 4. comparison of the experimental and the molecular modeling
dendrograms. The dendrograms were obtained by hierarchical
clustering of the corresponding taste and structural distance matrices.
(Reproduced from reference 9. Copyright 1996 Oxford University Press.)*

MOD significantly (24%), but MOD fails to adapt SUC (2%). Interestingly, no cross adaptation but significant and reciprocal cross enhancement was measured between DUL and MOD, and between SUC and DUL. For example, one needs on average a 25% less concentrated solution of DUL to perceive the isointensity after adaptation with a MOD solution, than without prior adaptation.

We used molecular modeling of the 4 molecules to understand whether the pharmacophores disclosed in our first modeling study would account for the observed cross adaptation results. We used the same modeling approach, except that we modeled several 3D structures for SUC and FRU, as these chemical structures are quite flexible. SUC is flexible around the glycosidic linkage between the α-glucopyranosyl and the β-fructofuranosyl rings, and is likely to adopt several stable or metastable conformations in aqueous solution as evidenced by NMR experiments (14). For FRU, we modeled the 3 major tautomers in water (15), namely β-D-fructopyranose, β-D-fructofuranose and α-D-fructofuranose.

The results of molecular modeling are displayed in Figure 5. FRU and SUC show almost all of the 12 pharmacophore types identified in the first modeling

Table 1. cross adaptation results.

	SUC/SUC	MOD/MOD	DUL/DUL	SUC/MOD	MOD/SUC	SUC/DUL	DUL/SUC	SUC/FRU	FRU/SUC	MOD/DUL	DUL/MOD
C_1 (mM)	87±4	0.63±0.10	0.52±0.19	0.66±0.13	86±7	0.54±0.15	87±7	158±29	91±11	0.66±0.22	0.62±0.11
C_2 (mM)	107±13	1.12±0.23	0.91±0.06	0.82±0.13	88±15	0.45±0.11	75±6	174±30	98±7	0.50±0.12	0.52±0.08
$100x(C_2-C_1)/C_1$	24%	77%	76%	24%	2%	-16%	-13%	10%	8%	-25%	-17%
p	0.006	0.0002	0.002	0.005	0.79	0.08	0.04	0.23	0.17	0.01	0.03

First tastant is adapting, second tastant is adapted; C_1: concentration of second tastant eliciting isointense perception with respect to 88mM (30g/l) sucrose without adaptation (averaged over 7 subjects +/- s.d.); C_2: concentration of second tastant eliciting isointense perception with respect to 88mM sucrose under adaptation; p: Student's t test double-sided significance level on paired individual C_1 and C_2 values.
SOURCE: Reproduced with permission from reference 13. Copyright 1998 Oxford University Press.
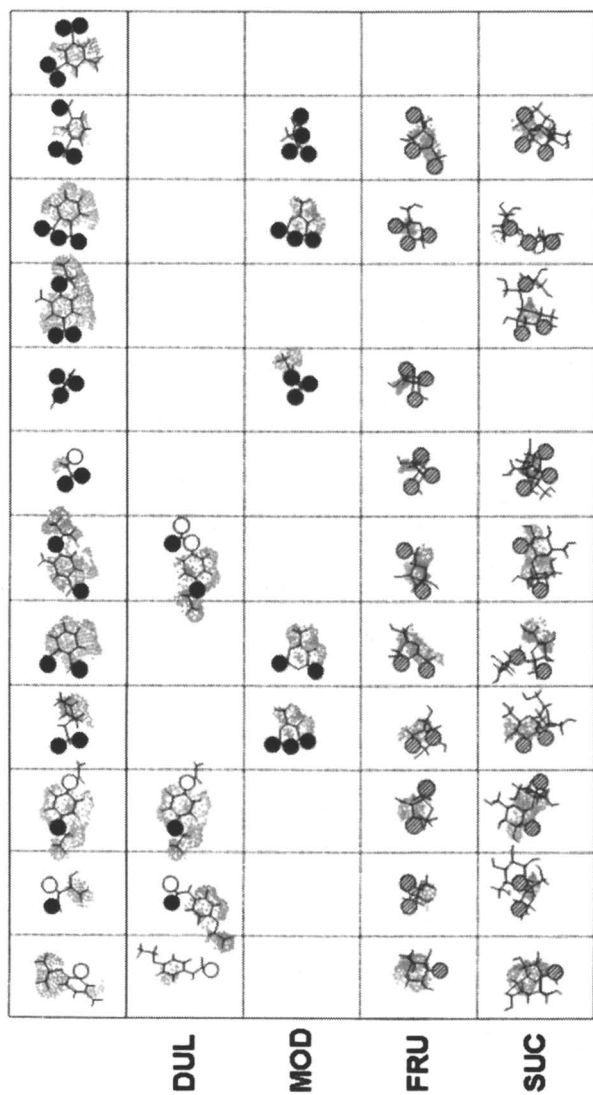
Figure 5. molecular fragments extracted from the 4 modeled molecules and corresponding to the 12 types of Figure 2. The top row displays representative fragments extracted from the 14 molecules modeled in the first study. (Reproduced from reference 9. Copyright 1996 Oxford University Press.)

study (9), due to their intrinsic conformational flexibility as well as the numerous donor/acceptor hydroxyl groups that they display. Consequently, they have a majority of pharmacophores in common and at the same time they show mutual cross adaptation. By contrast, MOD has 4 pharmacophores in common with the 10 pharmacophores of SUC, which may explain why SUC cross adapts MOD but not vice versa. Finally, DUL and MOD do not have pharmacophores in common and do not cross adapt. These findings suggest, at least qualitatively, that experimental cross adaptation levels seem to correlate well with the number of pharmacophores that molecules have in common.

However, one must find an additional hypothesis to explain the cross enhancement results which are measured in the cases where DUL is either the adapting or the adapted stimulus. Is it due to the recruitment of additional receptors, or of distinct transduction pathways? Indeed, cross enhancement could occur if two tastants elicit distinct transduction pathways which yet coexist in the same taste cells (synergy). The case of DUL shows the need for further molecular modeling, as well as for further understanding of the peripheral molecular mechanisms which occur inside taste receptor cells at any step that leads from stimulus binding to neurotransmitter exocytosis.

In conclusion, the findings of this second study are compatible overall with the hypothesis that cross adaptation occurs when two molecules are recognized by common taste receptor sites. It shows that the pharmacophores previously identified by molecular modeling might also prove useful to predict cross adaptation between organic tastants, and that at least part of cross adaptation data can be related to peripheral receptor events. However there is an urge for more biochemical and functional information on taste receptors and transduction pathways in order to get a full picture of the molecular events which are leading to the build-up of the peripheral taste response.

Perspectives

The peripheral system is likely to use several transmembrane receptors of the GPCR family to detect and discriminate organic molecules that are solubilized in the saliva (2-6). In a sense, the taste response can be seen as a "pharmacological profile" over an array of several receptors. Each organic tastant has a specific signature on that array, i.e. a particular combination of affinities and onsets of activation and desensitization for each of the taste receptors. We have shown that a molecular modeling approach which explicitly takes into account the likely multireceptor nature of taste is able to identify pharmacophores which can account for the similarities or dissimilarities of taste responses. This ligand-based modeling approach will probably benefit greatly from the direct structure-based modeling approaches of taste GPCR-ligand binding (16-18), as well as from the understanding of functional aspects of taste

cell responses such as the various second-messenger signaling pathways inside the taste receptor cells.

These modeling studies show that the design of tastants may be difficult, because of the intrinsic multireceptor nature of taste recognition. For example, if one wishes to design a tastant with a taste similar to that of sucrose, yet with a distinct chemical scaffold (so as to minimize its caloric content), one would probably have to build the same pattern of pharmacophores on that new chemical structure to ensure that it displays the same response profile over the taste receptors. More systematic description of the pharmacophores with fingerprint descriptors that are well validated against *in vitro* pharmacological data (*19-21*) may help better identify the pharmacophore signatures that are responsible for the variety of observed peripheral taste responses to organic tastants.

References

1. Faurion, A.; Vayssettes-Courchay, C. *Brain Res.* **1990**, *512*, 317-332.
2. Chandrashekar, J.; Hoon, M.A.; Ryba, N.J.; Zuker, C.S. *Nature* **2006**, *444*, 288-294.
3. Sugita, M. *Cell Mol. Life Sci.* **2006**, *63*, 2000-2015.
4. Reed, D.R.; Tanaka, T.; McDaniel, A.H. *Physiol. Behav.* **2006**, *88*, 215-226.
5. Meyerhof, W. *Rev. Physiol. Biochem. Pharmacol.* **2005**, *154*, 37-72.
6. Montmayeur, J.P.; Matsunami, H. *Curr. Opin. Neurobiol.* **2002**, *12*, 366-371.
7. Faurion, A.; Saito, S.; Mac Leod, P. *Chem. Senses* **1980**, *5*, 107-121.
8. Faurion, A. *Sweet Taste Chemoreception;* Elsevier: Amsterdam, 1993; p 291.
9. Froloff, N.; Faurion, A.; Mac Leod, P. *Chem. Senses* **1996**, *21*, 425-445.
10. Faurion, A.; Saito, S.; Mac Leod, P. *Chem. Senses* **1980**, *5*, 107-121.
11. Faurion, A.; Mac Leod, P. *Nutritive Sweeteners;* Applied Science: London, 1982; p 247.
12. Faurion, A. *Progress in Sensory Physiology 8;* Springer-Verlag: Berlin; p 129.
13. Froloff, N.; Lloret, E.; Martinez, J.-M.; Faurion, A. *Chem. Senses* **1998**, *23*, 197-206.
14. Hervé du Penhoat, C.; Imberty, A.; Roques, N.; Michon, V.; Mentech, J.; Descotes, G.; Pérez, S. *J. Am. Chem. Soc.* **1991**, *113*, 3720-3727.
15. Cockman, M.; Kubler, D.G.; Oswald, A.S.; Wilson, L. *J. Carbohydrate Chem.* **1987**, *6*, 181-201.
16. Cui, M.; Jiang, P.; Maillet, E.; Max, M.; Margolskee, R.F.; Osman, R. *Curr. Pharm. Des.* **2006**, *12*, 4591-4600.

17. Temussi, P. *J. Mol. Recognit.* **2006**, *19*, 188-199.
18. Walters, D.E.; Hellekant, G. *J. Agric. Food Chem.* **2006**, *54*, 10129-10133.
19. Horvath, D.; Jeandenans, C. *J. Chem. Inf. Comp. Sci.* **2003**, *43*, 680-690.
20. Horvath, D.; Jeandenans, C. *J. Chem. Inf. Comp. Sci.* **2003**, *43*, 691-698.
21. Froloff, N. *Trends Biotech.* **2005**, *23*, 488-490.

Chapter 10

Multiple Receptors or Multiple Sites? Modeling the Human T1R2–T1R3 Sweet Taste Receptor

Gabriella Morini[1,2], Angela Bassoli[1], and Piero A. Temussi[3,*]

[1]DISMA, Università di Milano, Via Celoria 2, I-20133 Milano, Italy
[2]Università di Scienze Gastronomiche, Piazza Vittorio Emanuele 9,
I-12060 Pollenzo (CN), Italy
[3]Dipartimento di Chimica, Università di Napoli Federico II, Via Cinthia 45,
I-80126 Napoli, Italy
*Corresponding author: temussi@unina.it

T1R2-T1R3, the sweet taste receptor, is activated by a great variety of sweet compounds. In order to understand its ability to bind molecules belonging to diverse chemical classes and a wide range of dimensions, we undertook a systematic homology modelling, using the metabotropic glutamate mGluR1 receptor as template. All possible heterodimers that can be formed by human T1R2 and T1R3 subunits, modelled on the different conformations of mGluR1 protomers yield four different ligand binding sites for low-molecular weight sweeteners. These sites, when probed by docking a set of molecules representative of all classes of sweet compounds, yield free energies of binding consistent with relative sweetness. They are not accessible to sweet proteins which, however, can bind to a secondary site. Our models are consistent with most experimental observation on sweet taste, including sweetness synergy, and can help to design new sweeteners.

147

Before the discovery of the sweet receptor, the only possible approach to understand why sweet molecules are sweet was based on indirect mapping of the active site of the receptor, exploiting the comparison of the structures and the activities of the several sweet compounds, possibly conformationally rigid. The shape and the electronic properties of small molecular weight ligands were used to infer the nature and topological arrangement of key molecular moieties, in the attempt to design new ideal sweet compounds. The state of the art is well illustrated by the book that collects the papers of the previous ACS Symposium on sweeteners, held during the 199th national ACS meeting (Boston, April 1990): over a period of many decades indirect mapping studies had led to the development of different models of the receptor active site (*1-8*).

Although these models have been very useful in the design of new sweet compounds, many questions were left unanswered. One such question, arising when comparing different classes of sweet compounds, was whether there is a single receptor or several receptors. In particular, the size of sweet proteins, is so different from that of most sweeteners, that, for a long time, the prevailing belief was that they were likely to interact with a receptor different from that accepting small molecular weight sweeteners.

The sweet receptor was identified in 2001 as a result of a major collective effort (*9-15*). The mere knowledge of the nature of the receptor increased our understanding of the molecular bases of sweet taste. First of all, it was shown that small and large molecular weight sweet molecules interact with the same T1R2-T1R3 receptor (*16*). Then, exhaustive modelling studies of the sweet taste receptor, docking calculations with small ligands and sweet proteins (*17,18*) and, later on, in vitro tests (*19-25*) showed that the receptor has multiple active sites. An important implication of the mode of binding of sweet proteins, called 'wedge model', and of the results of some in vitro tests, is that the T1R3 protomer, although common to the umami receptor, plays a crucial role also in the sweet receptor.

Here we shall describe the possible mode of action of small molecular weight sweeteners, the unique mechanism of interaction of sweet proteins with the T1R2-T1R3 receptor and the first explanation of the synergy shown by combinations of some sweeteners.

Glucophores of sweet proteins

An important preliminary consideration to understand the structure-activity relationship of sweet proteins is to understand whether their glucophores are localized on a small part of their surface or spread on a large area. Among the few known sweet proteins (*26*) the best characterized, particularly from a

structural point of view, are thaumatin (*27*), monellin (*28*), and brazzein (*29*). Previous indirect models of active site based on the shape of small sweeteners (*1-8*) can be consistent also with the interaction of sweet proteins only if, on their surface, there are 'sweet fingers' with glucophores chemically similar to those of small sweeteners, that can probe the active site.

The most direct attempt to find these sweet fingers was made by Tancredi *et al.* (*30*). These authors examined the surface of brazzein (*31*), monellin (*32-35*) and thaumatin (*36, 37*), the three proteins of known structure, looking for the presence of common structural motifs. The only common elements found for the three proteins are small β-sheet hairpins, which could indeed correspond to sweet fingers since they host residues with side chains chemically consistent with the glucophores of small sweeteners. Following this lead, Tancredi *et al.* (*30*) designed three cyclic peptides corresponding to the hairpins, stabilized by S-S bonds. However, none of the cyclic peptides designed to mimic these potential "sweet fingers", although assuming conformations consistent with those of the same sequences in the parent proteins, was able to elicit sweet taste (*30*). This result speaks strongly against the sweet fingers hypothesis and in favor of a large area of interaction. Another evidence is the fact that mutants affecting sweetness of monellin (*38*) and brazzein (*39*) are distributed over a large area. Other experimental evidences favoring a large area of interaction were accumulated for MNEI, a single chain monellin. Surveying its surface with a paramagnetic probe and combining the attenuations induced by the probe with an accurate analysis of water-protein NOEs (*40*), it was possible to show that a completely blind physico-chemical technique can circle out, as feasible interacting residues, not only those located on the possible sweet finger of loop L_{34} but also several residues consistent with previous mutagenesis studies, e.g. D7, G9, I6, R72, and R88 (*40*). Another surprising experimental evidence came from the study of a construct of MNEI obtained by the point mutation of an internal residue, i.e. a residue that cannot directly interact with the receptor but that can eventually exert its influence in an 'allosteric' way (*41*). Substituting Gly16, a residue buried in the hydrophobic core, with Ala16 the sweetness decreases by one order of magnitude (*42*). A structural comparison of MNEI and of its Gly16Ala mutant revealed that the basic architecture of the protein was little affected. It was possible to observe only a small change of the tertiary structure, but no secondary structure element was much affected by the mutation. In particular, the putative sweet finger Y65-D68, corresponding to the L_{34} loop of cystatins, is slightly displaced with respect to other secondary structure elements but its conformation is essentially unperturbed. Figure 1 shows a comparison of the average solution structures of MNEI and of its G16A mutant. The displacement of the loop can not affect the interaction of a sweet finger with its receptor but can be critical if the surface of interaction is more extended (*41*).
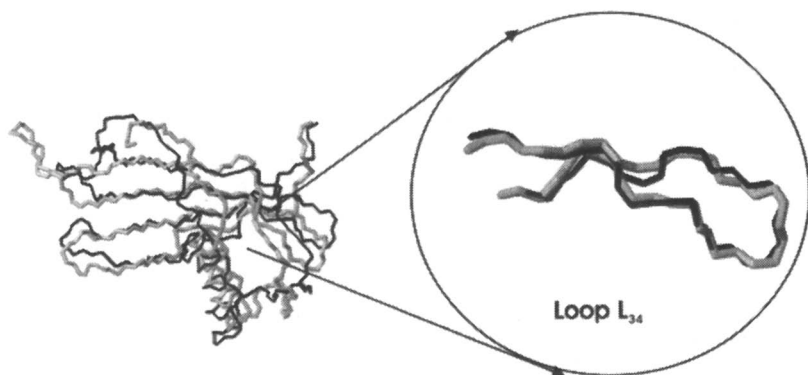
Figure 1. Comparison of the average solution structures of MNEI and of its G16A mutant. Both models are shown as neon representation of backbone atoms, MNEI in black, G16A in grey. The left-hand side panel shows a superposition of backbone atoms of all regular secondary structure elements, and the right-hand side panel shows a superposition of backbone atoms of loop L$_{34}$. Models were generated by MOLMOL (43).

Homology models of the receptor

As mentioned in the introduction, independent laboratories (*9-15*) suggested that T1R3 was a likely candidate for the sweet receptor. T1R3 was characterized as a class C metabotropic GPCR, which presents a large N-terminal extracellular domain (Venus Flytrap domain -VFTD), containing the active site for ligands, and a heptahelical transmembrane domain (7TMD) responsible for G-protein activation. Since T1R3 has 20% identity to homodimeric mGluR1, the first homology model of a sweet receptor was built as a homodimer of two T1R3 chains (*12*), using the X-ray diffraction structure of mGluR1 as template (*44*). A similar homodimeric model, also from mouse T1R3, was used to show that the active site could host three paradigmatic sweeteners (*45*). After Li *et al.* proved that the actual receptor is the T1R2-T1R3 heterodimer (*16*) it was possible to build more realistic homology models. A preliminary step in such models is the choice of the proper template, since Kunishima *et al.* (*44*) have determined the structure of three forms of the extracellular N-terminal domain of mGluR1. The active form of the complex with glutamate has a conformation called open-closed, but there are also two ligand-free forms: an open-open free form I (inactive) and an open-closed free form II (active) that, although does not contain glutamate, is conformationally identical to the complexed active receptor. The combination of the T1R2 and

T1R3 sequences with the open-open and open-closed conformations leads in principle to four heterodimers. The first heterodimeric model based on the mouse T1R2-T1R3 sequences and on the complexed form as template was one of the four possible models (*17*). Recently Morini *et al.* (*18*) generated all possible models using the human sequences.

The four T1R2-T1R3 heterodimers are two models for the open-open form, and two models for the closed-open form. Roo_AB stands for Resting open-open, with T1R2 sequence modelled on chain A and T1R3 modelled on chain B of the template; Roo_BA stands for Resting open-open, with T1R3 modelled on chain A and T1R2 modelled on chain B of the template. Aoc_AB stands for Active open-closed with T1R2 modelled on chain A and T1R3 modelled on chain B of the template; Aoc_BA stands for Active open-closed with T1R3 modelled on chain A and T1R2 modelled on chain B of the template.

Binding of sweet proteins

It seems fair to assume that the sweet receptor has the same general features of mGluR1. Therefore the T1R2-T1R3 receptor should also exist as a mixture of three forms: a complexed one containing a low molecular weight sweetener, and two ligand-free forms, free form I, the 'inactive' conformation and free form II, whose structure is nearly identical to that of the 'active', complexed form. As shown in Figure 2A, the equilibrium between form I and form II is normally shifted towards the active form by the binding of a small molecular weight sweetener that transforms free form I into the complexed form. Another way to shift the equilibrium between form I and form II in favor of form II is by binding of a sweet protein on an external large cavity of the surface of the receptor (Figure 2B). This cavity, constituted mainly by the T1R3 moiety, is a secondary binding site, distinct from the two active sites that can host small sweeteners. This mechanism of interaction of sweet proteins, termed "wedge model", was originally proposed on the basis of docking calculations of brazzein, monellin and thaumatin to a homology model of the receptor built using the mouse sequences of T1R2 and T1R3 for the closed and open conformations of mGluR1 respectively (*17*). Molecular models of all three sweet proteins fit the external cavity with wedge-shaped sides of their surface.

In order to prove that this cavity is the only possible binding site for sweet proteins, Morini *et al.* (*18*) performed docking calculations on all receptor models derived from the human sequences of T1R2 and T1R3: the active ones Aoc_AB and Aoc_BA, and, as a negative check, the inactive ones Roo_AB and Roo_BA. The results for all three proteins were consistent with those described previously for the mouse receptor. Figure 3 shows the interaction of brazzein, MNEI and thaumatin with Aoc_AB, the most likely of the two possible activated forms of the receptor. Figure 3A shows the human Aoc_AB form
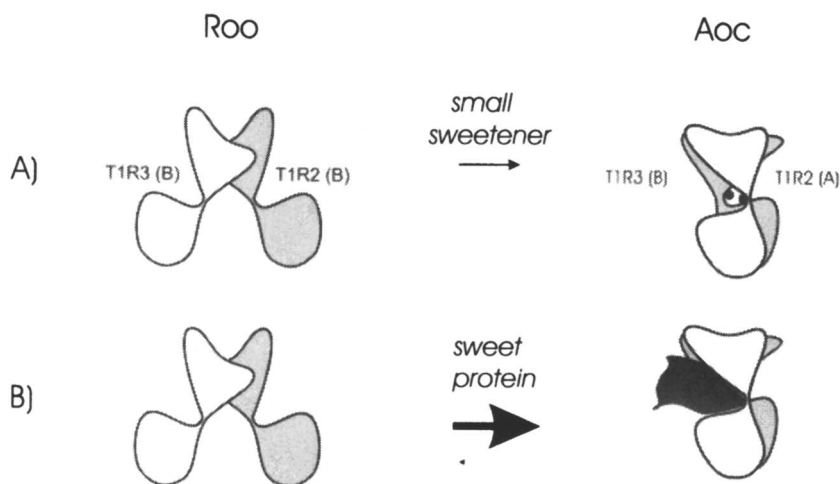
Figure 2. Modes of binding of small sweeteners and sweet proteins.
A) Binding of small molecular weight ligands transforms inactive free form I
(Roo, left) into the complexed form (Aoc, right), identical to free form II.
Small ligands in the two cavities of Aoc are shown as black balls.
B) Free form II, stabilized by protein complexation (active form, right),
activates long lasting signal transduction. The 'wedge' protein is shown in
black.

together with fifteen of the molecules of brazzein calculated by the docking procedure. All fifteen molecules are found in the same spot of the surface, mainly belonging to the T1R3(B) chain. They are oriented in a similar, albeit not identical, way. Efficient binding is assured both by shape and charge complementarity since the cavity is predominantly negative and the interacting surfaces of the proteins are mainly positive. Figures 3B and 3C show the corresponding complexes of human Aoc_AB with ten molecules of MNEI and with ten molecules of thaumatin as calculated by the docking procedure respectively.

The molecules of the three sweet proteins with Roo_AB and/or Roo_BA models bind to a larger area of the receptor, without any apparent regularity. This study suggested that T1R3 plays a major role in the activation of the sweet taste receptor, although at first the fact that the sweet (T1R2-T1R3) and the umami (T1R1-T1R3) taste receptors share the common subunit T1R3, apparently hinted at a passive role (16).

However, the semiquantitative fitting of many small molecular weight sweeteners (*vide infra*) confirmed that both T1R2 and T1R3 can accept several sweet molecules in their binding sites (18). Subsequently, this view received experimental support by the finding that T1R2 and T1R3 can both contribute to the detection of sweet stimuli (23).
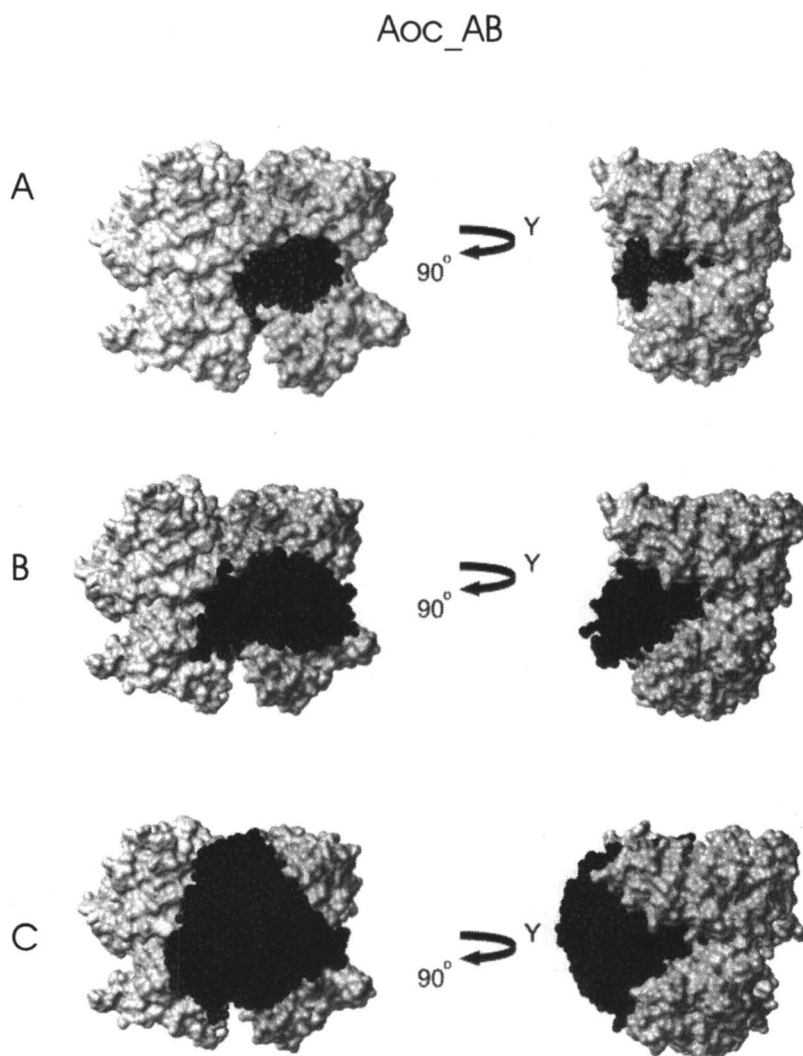
Aoc_AB

Figure 3. Interaction of brazzein, MNEI and thaumatin with Aoc_AB, the activated form of the receptor. The protomers are shown as contact surfaces: T1R2 is colored in light grey, T1R3 is colored in grey. The sweet proteins are shown in black heavy atom representation. A) Two views of Aoc_AB together with fifteen of the molecules of brazzein. B) Two views of Aoc_AB together with ten of the molecules of MNEI calculated by the docking procedure. C) Two views of Aoc_AB together with ten of the molecules of thaumatin calculated by the docking procedure. Models were generated with MOLMOL (43).

Active sites for 'small' molecules

The sweet taste receptor can be activated by a variety of molecules including hydrophobic amino acids and synthetic dipeptides, notably aspartame, that we will call 'small' to distinguish them from sweet proteins. Amino acids and peptides, like glutamate, have an amino group adjacent to a carboxyl group, the moiety typical of all α-amino acids. Therefore, it is fair to hypothesize that residues lining the wall of the part of the cavity that binds the amino acidic moieties should be highly conserved in going from mGluR1 to T1R2-T1R3. On the contrary, the residues of the other part of the cavity, i.e. those binding the side chain of glutamate in mGluR1, are expected to turn from polar to hydrophobic in T1R2-T1R3 since in the sweet taste receptor they have to accommodate molecular fragments of different chemical constitution, but on the average more hydrophobic than glutamate. Indeed in the alignments corresponding to the four models described by Morini *et al.* (*18*) residues binding the amino acidic moiety of glutamate are well conserved in all protomers, while residues binding the glutamate side chain in mGluR1 are changed to apolar or uncharged residues.

Since sweeteners have widely different chemical constitution and size, one cannot be sure *a priori* whether in the sweet taste receptor both binding sites in the VFTDs of the active forms (Aoc_AB, and Aoc_BA) are available for sweet ligands. Therefore, Morini *et al.* examined the quantitative binding of a large number of sweeteners in each cavity of the two models (*18*). Molecules chosen to probe the binding were representative of different families including sugars, peptides and synthetic sweeteners. The fit in the active sites was evaluated by means of PrGen (*46*), a program able to generate surrogate receptors that allows a semi-quantitative prediction of binding affinity for ligands and its comparison with the experimental biological activity, i.e. sweetness. In this case it was used just as a tool to evaluate the reliability of active binding sites identified in the derived homology models. The program was fed with a large number of sweeteners (training set) to derive the final models and subsequently tested with a blind set. Further confirmation of the predictive power of the models stems from the good prediction of the sweetness of some R,R-monatin derivatives and aspartame-based sweeteners recently synthesised (*47, 48*) (data not shown).

In mGluR1 structural comparison between the active and resting dimers suggests that glutamate induces domain closing, increasing the population of the conformer designated as "Active open-closed", indicating that the closed form is needed for the activation (*44, 49*). It was soon clear that active sites located in closed protomers, i.e. T1R2(A) and T1R3(A), are so small that they can only host the smallest sweeteners, due to the difference in size of glutamate with respect to most sweet compounds. The active sites of open protomers, i.e. T1R2(B) and T1R3(B) can bind several larger sweeteners using both interfaces of subdomains LB1 and LB2 (*18*), whereas in mGluR1 only LB1 is used in the

open protomer (*44, 49*). In any case, also for the sweet taste receptor it is reasonable to hypothesize that ligand binding induces the closure of the subunit necessary to obtain the active open-closed conformation.

Figure 4 shows the plot of predicted vs experimental free energies of binding for the T1R3(B) site. Open squares refer to compounds used in the training set, whereas black squares represent compounds of the test set. The compounds are those reported in the corresponding table of Morini *et al.* (*18*). The molar relative sweetening powers are comprised between the figures of 200,000 (corresponding to a ΔG of -13,8 kcal mol-*1*) of sucrononic acid and 0.26 (corresponding to a ΔG of -5.9 kcal mol-*1*) of D-glucose, referred to sucrose as 1.

The docking of small sweeteners to the different possible combinations of T1R2 and T1R3 suggests that both T1R2 and T1R3 can host a very large number of small molecular weight sweeteners with a good correlation between experimental and calculated binding affinity. Although the docking with small sweet ligands does not allow a clear-cut distinction between T1R2(A)_T1R3(B) and T1R2(B)_T1R3(A) as the actual form of the sweet taste receptor, once again it suggests that the role of T1R3 in the mechanism of sweet taste reception is not a mere passive one, as already shown by Temussi in the external binding site for proteins (*17*).

A multiple site receptor model

Our modeling identified three binding sites: the wedge site for sweet proteins and two binding sites in each of the VFTD of T1R2 and T1R3. Moreover, a fourth binding site in the 7TMD of T1R3 for sweet compounds and sweet taste modulators has been localised through in vitro test with chimeric receptors (*20-22*). These findings furnish a possible interpretation of a puzzling phenomenon that had so far eluded explanation, i.e. the synergy between pairs of sweet molecules.

In mGluR1 the closure of one VFT through binding of one glutamate molecule is sufficient for activation, but full response is obtained when both binding sites are occupied by a molecule of glutamate (*50*). Likewise, in the sweet taste receptor the binding of a sweet tastant in at least one of the binding sites is required for activation, while the binding of another ligand in another binding site increases the response (synergy). In fact, the synergic effect is not exerted by every couple of sweet compounds, since not every compound can equally fit all the cavities. In Figure 5 we hypothesize a molecular explanation of some of the sweetener combinations exhibiting synergy reported in the literature (*51*).

Stevioside is reported to be synergic with aspartame, cyclamate, acesulfame–K and thaumatin, which in our model occupy all the four available
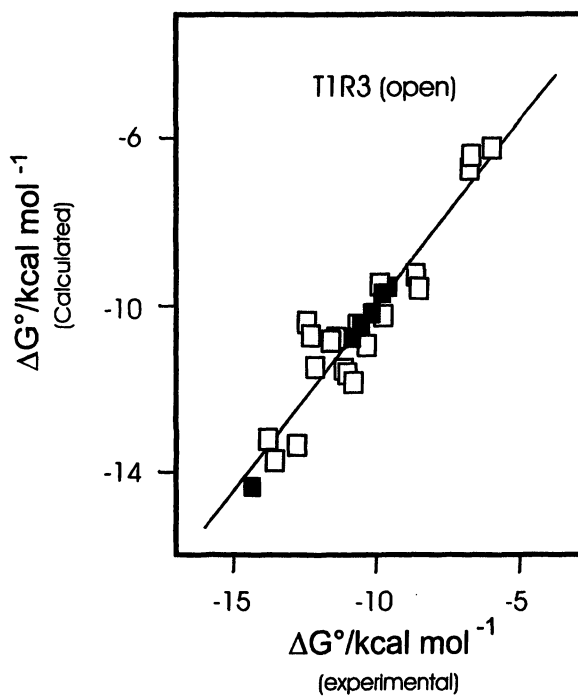
Figure 4. Correlation between calculated and experimental binding affinities of sweet compounds inserted in the T1R3(open) active site. Open squares represent compounds used in the training set, whereas black squares represent compounds of the test set.
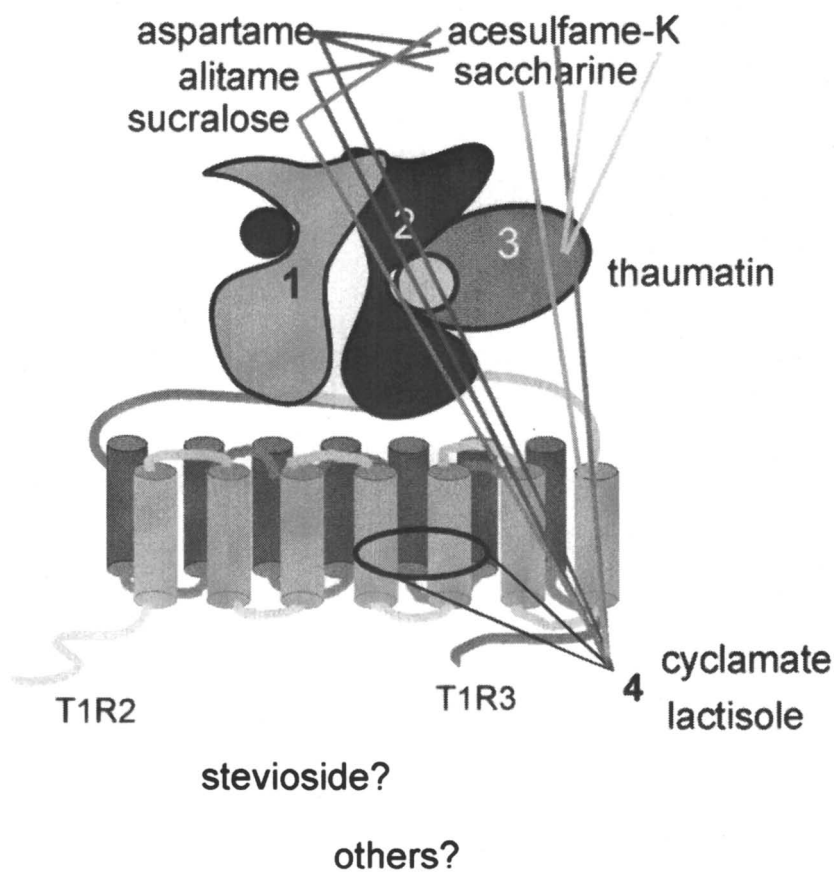
Figure 5. Schematic molecular basis of the phenomenon of synergy. Each of the four active sites (1 and 2 in the VFTDs, 3 wedge site for proteins and 4 in the 7TMD site) can specifically accommodate some ligands. The lines link synergistic compounds.

binding sites. Therefore we suggest that another site for it (and likely for other sweet compounds) has to be present in the sweet taste receptor.

Conclusion

The possibility to build reliable homology models of the sweet taste receptor improves dramatically our understanding of the taste of sweet molecules. Although some points still need a more detailed explanation, the main aspects of the interaction of sweet molecules with their receptor, including the mechanism of action of sweet proteins, have been elucidated. One of the main goals of structure-acitivity studies of sweet molecules has always been the design of new, safer sweeteners. Detailed homology models of the two active sites of the active form of the human sweet receptor can indeed suggest entirely new scaffolds, but it is not yet clear whether the resolution of the models is sufficient for accurate design. Moreover, the described models of sweet taste receptor, although necessarily static models that reflect only in part the dynamic situation of the receptor in vivo, are very useful as a complementary tool to the experimental approaches, suggesting possible point mutations and giving the possibility to visualize ligands-sweet taste receptor interactions.

On the other hand, proteins, although so far little used as sweeteners, seem very promising. The detailed knowledge of their mode of action opens the way to modifications of existing proteins or to entirely *de novo* design of sweet proteins: the structural work on existing proteins may stimulate the search for new ones or even the design of mutations on non sweet proteins that can change them into new very sweet proteins.

Acknowledgments

References

1. Shallenberger, R.S.; Acree, T. *Nature* **1967**, *216*, 480-482.
2. Kier, L.B. *J. Pharm. Sci.* **1972**, *61*, 1394-1397.
3. Temussi, P.A.; Lelj, F.; Tancredi, T. *J. Med. Chem.* **1978**, *21*, 1154-1158.

4. Temussi, P.A.; Lelj, F.; Tancredi, T.; Castiglione-Morelli, M.A.; Pastore, A. *Int. J. Quantum Chem.* **1984**, *26*, 889-906.
5. Temussi, P.A.; Lelj, F.; Tancredi, T. *Sweeteners: Discovery, Molecular Design and Chemoreception;* Walters DE, Orthofer FT, DuBois GE (eds) ACS Symposium Series 450; ACS: Washington DC; 1991, pp.143-161.
6. Iwamura, H. *J. Med. Chem.* **1981**, *24*, 572-578.
7. Goodman, M.; Coddington, J.; Mierke, D.F. *J. Amer. Chem. Soc.* **1987**, *109*, 4712-4714.
8. Tinti, J.M.; Nofre, C. *Sweeteners: Discovery, Molecular Design and Chemoreception;* Walters DE, Orthofer FT, DuBois GE (eds) ACS Symposium Series 450; ACS: Washington DC; 1991, pp. 88-99.
9. Bachmanov, A.A.; Li, X.; Reed, D.R.; Ohmen, J.D.; Li, S.; Chen, Z.; Tordoff, M.G.; de Jong, P.J.; Wu, C.; West, D.B.; Chatterjee, A.; Ross, D.A.; Beauchamp, G.K. *Chem Senses.* **2001**, *26*, 925-933.
10. Kitagawa, M.; Kusakabe, Y.; Miura, H.; Ninomiya, Y. Hino *Biochem. Biophys. Res. Commun.* **2001**, *283*, 236-242.
11. Li, X.; Inoue, M.; Reed, D.R.; Hunque, T.; Puchalski, R.B.; Tordoff, M.G.; Ninomiiya, Y.; Beauchamp, G.K.; Bachmanov, A.A. *Mamm. Genome.* **2001**, *12*, 13-16.
12. Max, M.; Shanker, Y.G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R.F. *Nat. Genet.* **2001**, *28*, 58-63.
13. Montmayeur, J.P.; Liberles, S.D.; Matsunami, H.; Buck, L.B. A. *Nat. Neurosci.* **2001**, *4*, 492-498.
14. Nelson, G.; Hoon, M.A.; Chandrashekar, J.; Zhang, Y.; Ryba, N.J.; Zuker, C.S. *Cell* **2001**, *106*, 381-390.
15. Sainz, E.; Korley, J.N.; Battey, J.F.; Sullivan, S.L. *J. Neurochem.* **2001**, *77*, 896-903.
16. Li X.; Staszewski L.; Xu H.; Durick K.; Zoller M.; Adler E. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696.
17. Temussi, P.A. *FEBS Lett.* **2002**, *526*, 1-3.
18. Morini, G.; Bassoli, A.; Temussi, P.A. *J. Med. Chem.* **2005**, *48*, 5520-5529.
19. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14258-14263.
20. Jang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L.A.; Benard, L.M.J.; Osman, R.; Margolskee, R.F.; Max, M. *J. Biol. Chem.* **2005**, *280*, 15238-15246.
21. Winning, M.; Bufe, B.; Meyerhof, W. *BMC Neurosci.*, **2005**, *6*, 1-7.
22. Jang, P.; Cui, M.; Zhao, B.; Snyder, L.A.; Benard, L.M.J.; Osman, R.; Max, M.; Margolskee, R.F. *J. Biol. Chem.* **2005**, *280*, 34296-34306.
23. Nie, Y.; Vigues, S.; Hobbs, J.R.;, Conn, G.L.; Munger, S.D. *Curr. Biol.* **2005**, *15*, 48-52.
24. Nie, Y.; Vigues, S.; Hobbs, J.R.; Olson, W.J.; Conn, G.L.; Munger, S.D. *Chem.. Senses.* **2006**, *31*, 505-513.

25. Cuspinera, V.G.; Winning, M.; Bufe, B.; Meyerhof, W.; Breslin, P.A.S. *Nature* **2006**, *441*, 354-357.

26. Morris, J. A. *Lloydia.* **1976**, *39*, 25-38.

27. van der Wel, H., and Loeve, K. *Eur J Biochem.* **1972**, *31*, 221-225.

28. Inglett, G. E.; May, J. F. *J. Food Sci.* **1969**, *34*, 408-411.

29. Ming, D.; Hellekant, G. *FEBS Lett.* **1994**, *355*, 106-108.

30. Tancredi, T.; Pastore, A.; Salvadori, S.; Esposito, V.; Temussi, P. A. *Eur. J. Biochem.* **2004**, *271*, 2231-2240.

31. Caldwell, J E.; Abildgaard, F.; Dzakula, Z.; Ming, D.; Hellekant, G.; Markley, J. L. *Nat. Struc.t Biol.* **1998**, *5*, 427-431.

32. Somoza, J. R.; Jiang, F.; Tong, L.; Kang, C. H.; Cho, J. M.; Kim, S. H. *J. Mol. Biol.* **1993**, *234*, 390-404.

33. Hung, L. W.; Kohmura, M.; Ariyoshi, Y.; Kim, S. H. *Acta Crystallogr. D Biol. Crystallogr.* **1998**, *54*, 494-500.

34. Lee, S. Y.; Lee, J. H.; Chang, H. J.; Cho, J. M.; Jung, J. W.; Lee, W. *Biochemistry* **1999**, *38*, 2340-2346.

35. Spadaccini, R.; Crescenzi, O.; Tancredi, T.; De Casamassimi, N.; Saviano, G.; Scognamiglio, R.; Di Donato, A.; Temussi, P. A. *J. Mol. Biol.* **2001**, *305*, 505-514.

36. de Vos, A. M.; Hatada, M.; van der Wel, H.; Krabbendam, H.; Peerdeman, A. F.; Kim, S. H.. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 1406-1409.

37. Ogata, C. M.; Gordon, P. F.; de Vos, A. M.; Kim, S. H. *J. Mol. Biol.* **1992**, *228*, 893-908.

38. Somoza, J. R.; Cho, J. M.; Kim, S. H. *Chem. Senses* **1995**, *20*, 61-68.

39. Jin, Z.; Danilova, V.; Assadi-Porter, F.M.; Aceti, D. J.; Markley, J. L.; Hellekant, G. *FEBS Lett.* **2003**, *544*, 33-37.

40. Niccolai, N.; Spadaccini, R.; Scarselli, M.; Bernini, A.; Crescenzi, O.; Spiga, O.; Ciutti, A.; Di Maro, D.; Bracci, L.; Dalvit, C.; Molinari, H.; Esposito, G.; Temussi, P. A. *Protein Sci.* **2001**, *10*, 1498-1507.

41. Spadaccini, R.; Trabucco, F.; Saviano, G.; Picone, D.; Crescenzi, O.; Tancredi, T.; Temussi, P. A. *J Mol Biol* **2003**, *328*, 683-692.

42. Iijima, H.; Morimoto, K. *Enzyme Engineering XII, Ann. New York Acad. Sciences* **1995**, *750*, 62-65.

43. Koradi, R.; Billeter, M.; Wüthrich, K. *J. Mol. Graph.* **1996**, *14*, 51-55.

44. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. *Nature* **2000**, *407*, 971-977.

45. Walters, D. E. *Pure Appl. Chem.* **2002**, *74*, 1117-1123.

46. Vedani, A.; Zbinden, P.; Snyder, J. P.; Greenidge, P. A. *J. Amer. Chem. Soc.* **1995**, *117*, 4987-4994.

47. Patent US2005/0118317.

48. De Capua, A.; Goodman, M.; Amino, Y.; Saviano, M.; Benedetti, E. *ChemBioChem* **2006**, *7*, 377-387.

49. Tsuchiya, D.; Kunishima, N.; Kamiya, N.; Jingami, H.; Morikawa, K. *Proc Natl Acad Sci USA* **2002**, *99*, 2660-2665.

50. Kniazeff, J.; Bessis, A.S.; Maurel, D.; Ansanay, H.; Prézeau, L.; Pin, J.P. *Nature Struct. Mol. Biol.* **2004**, *11*, 706-713.

51. Schiffman, S.S.; Booth, B.J.; Carr, B.T.; Losee, M.L.; Sattely-Miller, E.A.; Graham, B.G. *Brain Res. Bull.* **1995**, *38*, 105-120.

Chapter 11

Computational Docking to Sweet Taste Receptor Models

D. Eric Walters

Department of Biochemistry and Molecular Biology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064

Computer modeling tools are extremely useful in understanding the ways that sweeteners interact with their receptors. This chapter describes homolgy-based models of the N-terminal ligand binding domains of the sweet taste receptor, T1R2 + T1R3. These models are used in combination with docking calculations and structure-taste data to identify a likely binding mode for the sweet protein brazzein. The taste-modifying protein miraculin has been modeled, and possible modes of miraculin-taste receptor interaction are also identified.

Introduction

The chemical structures of sweet tasting compounds are incredibly diverse. Sucrose and other sugars are the natural ligands for sweet receptors, but other compounds have been known to trigger sweet taste at least since the discovery of saccharin, reported in 1879. The list is long, and it includes polyols (sorbitol, maltitol, lactitol), heterocyclics (saccharin, acesulfame K); amino acids (glycine, D-tryptophan), dipeptides (aspartame, neotame), sulfamates (cyclamate), halogenated sugars (sucralose), terpenes and terpene glycosides (hernandulcin, glycyrrhizin, stevioside, rebaudiosides), urea derivatives (dulcin, superaspartame, suosan), nitroanilines (P-4000), oximes (perillartine). In addition, a number of proteins also have a sweet taste. These include monellin, thaumatin, and brazzein. Curiously, miraculin, a glycoprotein, induces sweet

taste when it is applied to the tongue and then followed with acidic solutions. It would appear to bind to the sweet receptor and induce the appropriate conformational change when exposed to low pH.

The diversity of sweet tasting structures long ago convinced most workers that there must be multiple receptors responsible for detecting sweetness. It was therefore surprising when a single receptor was shown to be responsible for all sweetness transduction (*1-3*). This receptor is a heterodimer formed by two members of the T1R taste receptor family, T1R2 and T1R3. Subsequently, it has been demonstrated that this receptor has several different binding sites (*4-6*). Sequence homology of the taste receptor proteins with a metabotropic glutamate receptor indicates that the T1R receptors are G protein coupled receptors of class C. As illustrated in Figure 1, these proteins have a large extracellular N-terminal domain that is linked to the 7-helix transmembrane domain by a small cysteine-rich domain. Since the N-terminal ligand binding domain of the brain metabotropic glutamate receptor, mGluR1, has been studied using X-ray crystallography (*7*), it has been possible to construct homology-based models of the N-terminal ligand binding domains of T1R2 and T1R3 (*8-11*).
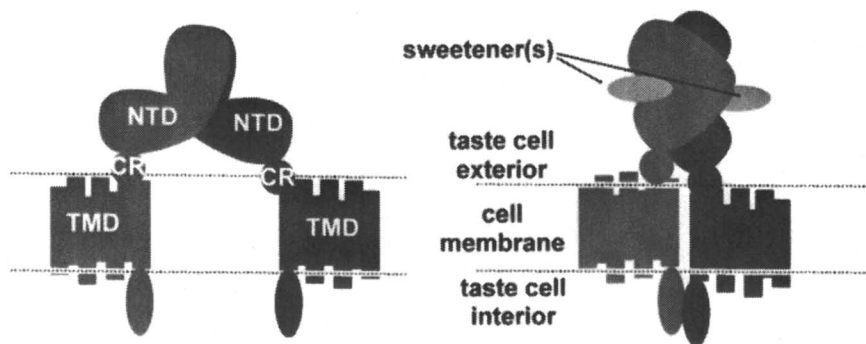
Figure 1. Schematic representation of the T1R2/T1R3 sweet taste receptor, based upon homology to the mGluR1 receptor (7). NTD = N-terminal domain; CR = cysteine-rich domain; TMD = 7 helix transmembrane domain. In the unliganded state (left), both NTDs are open. With ligand(s) bound (right), one of the subunits closes, and a conformational change occurs at the dimer interface, bringing the CR domains closer together.

Here I discuss the construction of homology-based models of the T1R2/T1R3 N-terminal domains. These models have been used in docking calculations with the sweet protein brazzein. I also describe the homology-based modeling of miraculin and docking of a miraculin model with the receptor models.

Receptor Models

The T1R2 and T1R3 proteins have significant sequence homology to a brain metabotropic glutamate receptor, mGluR1, which functions as a homodimer. The N-terminal domain of mGluR1 has been expressed, purified, and crystallized both with and without bound glutamate (*7*). In the ligand-activated form, one monomer exists in a fairly open form, and the other in a closed conformation. In using this structure as a template for homology modeling of T1R2 + T1R3, it is possible to model two activated forms of the receptor. One, which is designated Form 1, has T1R2 closed and T1R3 open. The other, designated Form 2, has T1R2 open and T1R3 closed. Homology modeling of both forms has been described recently.

Brazzein Docking

Brazzein is a potently sweet protein (54 amino acids) produced by the African plant *Pentadiplandra brazzeana* (*12*). It is 2,000 times as sweet as sucrose on a weight basis, and 37,500 times as sweet as sucrose on a molar basis. Its three-dimensional structure has been determined by NMR spectroscopy (*13*). We have used Vakser's GRAMM software (*14, 15*) to carry out docking of brazzein to both forms of our receptor model. Protein-protein docking is a challenging problem, and results are generally only approximate. In the case of docking brazzein to models of the T1R2/T1R3 N-terminal domains, GRAMM consistently places brazzein in the apparent binding site of the open subunit, but it does not consistently orient the brazzein in the same way. We made use of the extensive brazzein structure-taste results (*16, 17*) to assess 20 different docking orientations. We were able to identify one in which brazzein interacts with the T1R2 subunit in an orientation that is consistent with the structure-taste relationships of 21 of 23 brazzein mutants (*11*). This model is now being tested through the design and evaluation of additional brazzein variants.

Miraculin Model

Miraculin is a glycoprotein produced by *Richadella dulcifica*, a plant native to West Africa (*18-21*). It is composed of 191 amino acids and two N-linked polysaccharides. Glycosylation occurs at Asn-42 and Asn-186. Miraculin is a homodimer, covalently linked by an intermolecular disulfide at Cys-138. Each miraculin monomer has 4 intramolecular disulfide linkages as well.

The miraculin protein sequence was used to search the Pfam database (*22*). Miraculin is a member of a family of protease inhibitors that includes the Kunitz zoybean trypsin inhibitor. The Pfam database provided an alignment of miraculin with 337 related protein sequences. It also provided links to 15 related

crystal structures in the Protein Data Bank (*23*). The barley subtilisin inhibitor structure (*24*: PDB code 1AVA) was chosen as a template for homology modeling miraculin because it has 33% sequence identity and 1.9 Å resolution. Homology modeling was carried out using the Homology Model module of Molecular Operating Environment, version 2005.06 (Chemical Computing Group, Montreal). Twenty models of miraculin (monomer) were generated. Miraculin has one more intramolecular disulfide than do the crystal structures in the PDB; several of the miraculin models placed the two additional cysteines in close proximity, so it was possible to generate the fourth disulfide linkage and carry out minimization of the final model using the CHARMM22 force field.

Miraculin Docking

GRAMM was again used to dock the miraculin model (as a monomer) to the two forms of the sweet receptor model. As in the case of brazzein, the apparent binding site of the open subunit was consistently found by GRAMM, but there is not a body of miraculin mutation data to use in evaluating the various docking orientations. Instead, we made the assumption that miraculin must dock in a way that leaves Cys-138 (site of dimerization) and Asn-42 and Asn-186 (sites of glycosylation) oriented away from the binding site. Two orientations of miraculin in the open form of T1R2 and two orientations of miraculin in the open form of T1R3 were identified. It would be necessary to carry out mutations of miraculin or of the receptor (or both) to definitively identify the way in which miraculin binds to the receptor; these models could facilitate design of such experiments.

Conclusion

Computer modeling facilitates an understanding of the ways that sweet proteins may interact with taste receptors. It also assists in the design of new experiments that will further our knowledge of such interactions.

References

1. Nelson, G.; Hoon, M.A.; Chandrashekar, J.; Zhang, Y.; Ryba, N.J.; Zuker, C.S. Mammalian sweet taste receptors. *Cell* **2001**, *106*, 381-390.
2. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. Human receptors for sweet and umami taste. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696.

3. Zhao, G.Q.; Zhang, Y.; Hoon, M.A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N.J.P.; Zuker, C.S. The receptors for mammalian sweet and umami taste. *Cell* **2003**, *115*, 256-266.

4. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L.A.; Benard, L.M.J.; Margolskee, R.F.; Max, M. The cysteine-rich region of T1R3 determines responses to intensely sweet proteins. *J. Biol. Chem.* **2004**, *279*, 45068-45075.

5. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. Different functional roles of T1R subunits in the heteromeric taste receptors. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 14258-14263.

6. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L.A.; Benard, L.M.J.; Osman, R.; Max, M.; Margolskee, R.F. Identification of the cyclamate interaction site within the transmembrane domain of the human sweet taste receptor subunit T1R3. *J. Biol. Chem.* **2005**, *280*, 34296-34305.

7. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **2000**, *407*, 971-977.

8. Max, M.; Shanker, Y.G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R.F. *Tas1r3*, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus *Sac*. *Nature Genet.* **2001**, *28*, 58-63.

9. Temussi, P.A. Why are sweet proteins sweet? Interaction of brazzein, monellin and thaumatin with the T1R2-T1R3 receptor. *FEBS Lett.* **2002**, *526*, 1-4.

10. Walters, D.E. Homology-based model of the extracellular domain of the taste receptor T1R3. *Pure & Appl. Chem.* **2002**, *74*, 1117-1123.

11. Walters, D.E.; Hellekant, G. Interactions of the sweet protein brazzein with the sweet taste receptor. *J. Agric. Food Chem.* **2006**, *54*, in press.

12. Ming, D.; Hellekant, G. Brazzein, a new high-potency thermostable sweet protein from *Pentadiplandra brazzeana* B. *FEBS Lett.* **1994**, *355*, 106-108.

13. Caldwell, J.E.; Abildgaard, F.; Dzakula, Z.; Ming, D.; Hellekant, G.; Markley, J.L. Solution structure of the thermostable sweet-tasting protein brazzein. *Nat. Struct. Biol.* **1998**, *5*, 427-431.

14. Vakser, I.A. Low-resolution docking: Prediction of complexes for underdetermined structures. *Biopolymers*, **1996**, *39*, 455-464.

15. Vakser, I.A.; Matar, O.G.; Lam, C.F. A systematic study of low-resolution recognition in protein-protein complexes. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8477-8482.

16. Assadi-Porter, F.M.; Aceti, D.J.; Markley, J.L. Sweetness determinant sites of brazzein, a small, heat-stable, sweet-tasting protein. *Arch. Biochem. Biophys.* **2000**, *376*, 259-265.

17. Jin, Z.; Danilova, V.; Assadi-Porter, F.M.; Aceti, D.J.; Markley, J.L.; Hellekant, G. Critical regions for the sweetness of brazzein. *FEBS Lett.* **2003**, *544*, 33-37.

18. Kurihara, K.; Beidler, L.M. Taste-modifying protein from miracle fruit. *Science* **1968**, *161*, 1241-1243.

19. Theerasilp, S.; Hitotsuya, H.; Nakajo, S.; Nakaya, K.; Nakamura, Y.; Kurihara, Y. Complete amino acid sequence and structure characterization of the taste-modifying protein, miraculin. *J. Biol. Chem.* **1989**, *264*, 6655-6659.

20. Takahashi, N.; Hitotsuya, H.; Hanzawa, H.; Arata, Y.; Kurihara, Y. Structural study of asparagine-linked oligosaccharide moiety of taste-modifying protein, miraculin. *J. Biol. Chem.* **1990**, *265*, 7793-7798.

21. Igeta, h.; Tamura, Y.; Nakaya, K.; Nakamura, Y.; Kurihara, Y. Determination of disulfide array and subunit structure of taste-modifying protein, miraculin. *Biochim. Biophys. Acta* **1991**, *1079*, 303-307.

22. Finn, R.D.; Mistry, J.; Schuster-Böckler, B.; Griffiths-Jones, S.; Hollich, V.; Lassmann, T.; Moxon, S.; Marshall, M.; Khanna, A.; Durbin, R.; Eddy, S.R.; Sonnhammer, E.L.L.; Bateman, A. Pfam: clans, web tools and services. *Nucl. Acids Res.* **2006**, *34*, D247-D251.

23. Berman, H.M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T.N.; Weissig, H.; Shindyalov, I.N.; Bourne, P.E. The Protein Data Bank. *Nucl. Acids Res.* **2000**, *28*, 235-242.

24. Vallee, F.; Kadziola, A.; Bourne, Y.; Juy, M.; Rodenburg, K.W.; Svensson, B.; Haser, R. Barley alpha-amylase bound to its endogenous protein inhibitor BASI: crystal structure of the complex at 1.9 A resolution. *Structure* **1998**, *6*, 649-659.

Chapter 12

What Can Psychophysical Studies with Sweetness Inhibitors Teach Us about Taste?

Veronica Galindo-Cuspinera and Paul A. S. Breslin

Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104

In this chapter we demonstrate how perceptual studies with inhibitors help guide molecular studies toward an understanding of taste mechanisms. We illustrate this point through the use of sweet taste inhibitors in three different perceptual paradigms: water-taste induction, enhancement of differential sensitivity (decreasing Weber's fraction), and cross-modal inhibition. We show how perceptual studies might complement molecular/functional studies to develop more complete understandings of taste physiology.

The phenomenon of 'Water-Taste': gustatory after-images

The perception of a sensory quality, such as sweetness, is based upon specific patterns of activity in modality-specific sensory cortex, usually initiated by the activation of receptors in the periphery. After-images can be useful for revealing these underlying qualitative codes, such as the red-green/blue-yellow opponency mechanisms unveiled by color after-images. In the gustatory system after-images are labeled 'water-tastes', as water becomes the neutral substrate for the 'after-image', and can appear sweet, sour, bitter or salty depending on the perceived quality of the previously tasted chemical and its chemical structure [1, 2]. In general, the phenomenon of 'water-taste' has received little attention, and there have been no clear explanations of this phenomenon. The term 'Water-taste' refers to the taste elicited by water after a chemical solution is rinsed from the mouth. One theory posits that water-tastes are adaptation phenomena, wherein adaptation to one taste solution causes the water presented

170   © 2008 American Chemical Society

subsequently to act as a taste stimulus [3, 4]. According to this hypothesis, pure water will stimulate a taste when the normal pattern of neural activity is altered in just the subset of neurons utilized in common with the adapting stimulus; hence, these water rinses produce an atypical pattern of activity which may be perceived as taste. However, there is little physiological or molecular data at present that accounts for this theory. An alternative hypothesis is that the removal of the stimulus when rinsing generates a receptor-based, positive, off-response in taste receptor cells, ultimately inducing a gustatory perception [5, 6]. By studying the interaction that compounds, which elicit sweet water-taste, have with the T1R2-T1R3 sweet receptor, we have shown that the phenomenon of sweet 'water-taste' is directly related to sweet taste inhibition and that the perceived sweetness from water is the result of releasing the receptor from an inhibitory state [7].

Na-Saccharin: sweetener or sweetness inhibitor?

Saccharin was discovered in 1879 at Johns Hopkins University by Remsen and Fahlberg and is the first artificial sweetener (figure 1).
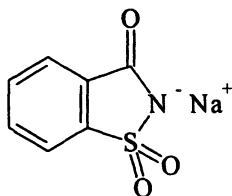
Figure 1. Sodium Saccharin

Throughout the 1970s, saccharin was used as a low-calorie sweetener in the United States, and today it continues to be important for a wide range of low-calorie and sugar-free foods and beverages. Na-saccharin is a peculiar compound because when it is tasted at low concentrations a characteristic sweet taste is perceived, accompanied by a low level of bitterness; however, when the concentration of saccharin increases the sweetness perception diminishes. At high concentrations, Na-Saccharin curiously elicits little sweetness and tastes mostly bitter to many observers (Figure 2a). A potential explanation for the low sweetness perceived at high concentrations is a mixture-suppression effect caused by the high bitterness elicited by strong Na-saccharin. This explanation is unlikely, as there is no correlation between perceived bitterness and sweetness at any concentration (figure 2a -- inset). In addition, high concentrations of Na-saccharin elicit a strong sweet water-taste when they are rinsed from the mouth (Figure 2b). The apparent ability of Na-saccharin to inhibit its own sweet taste at
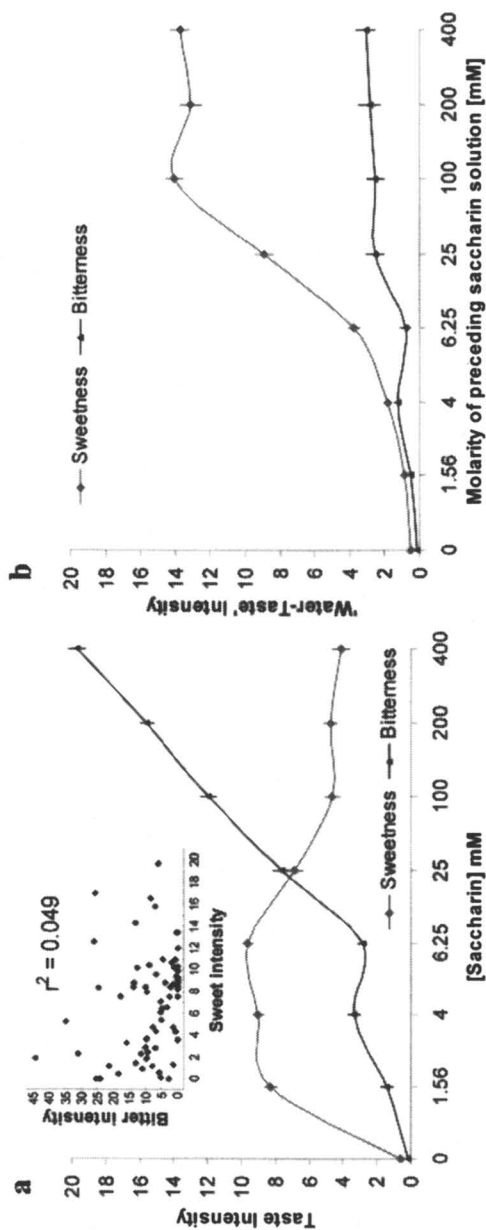
Figure 2. .a) Concentration- intensity curve for Na-Saccharin (second tasting), black line (▲) indicates bitterness, dark gray line(◆) sweeteness. n=14subjects. Inset shows the correlation of bitterness with sweetness for Na-saccharin. b) Water-taste intensity measured after exposure to different concentrations of Na-Saccharin. Adapted by permission from Macmillan Publishers Ltd: Nature [7], copyright 2006. (See color insert in this chapter.)

high concentrations is very revealing, as it requires the sweetener to act as both an agonist as well as an antagonist on the same receptor, albeit at different conentration ranges. This surprising observation led to an investigation of the sweet water-taste phenomenon [7].

By screening compounds for sweet water-taste, we found that the well-known sweet taste blocker "lactisole", despite not eliciting sweet taste on its own, produces a pronounced sweet 'water-taste' when it is rinsed from the mouth. This raised the question of whether there is a causal relationship between sweetness inhibition and sweet water-taste. Given the parallel effects elicited by both strong Na-saccharin and lactisole, we determined if Na-saccharin might also be a general sweetener inhibitor. By mixing high concentrations of Na-saccharin with several chemically-diverse, intensity-matched sweeteners, we demonstrated the general inhibitory effect of Na-saccharin on these sweeteners (Figure 3a and b).   We further demonstated that other compounds which demonstrate a sweet 'water-taste' (Acesulfame-K and $MgSO_4$) are also sweetener inhibitors. Furthermore, both the inhibition effect of concentrated saccharin and the sweet water-taste phenomenon were demonstrated *in vitro* with the sweetener receptor hTAS1R2-hTAS1R3, which was heterologously expressed in immortalized human kidney cells (HEK293/ Ga16gust44). Thus, these perceptual phenomena are explained at the receptor level.

But how do we understand that Na-saccharin is both a sweetener and a sweetness inhibitor? This is possible if the sweetener receptor has more than one binding site for Na-saccharin. The first is an orthostheric site with high affinity for Na-saccharin, and the second is an allosteric site with lower affinity. When the receptor is exposed to low concentrations of Na-saccharin, the molecules bind to the orthostheric site activating the receptor and consequently eliciting sweet taste perception. But at higher concentrations, Na-saccharin will additionly bind to the low affinity allosteric site, which happens to inhibit the receptor and block sweetness. It is the release of the molecules from the inhibitory allosteric site by water rinses that triggers the sweet 'water-taste' perception. In general, we propose that certain stimuli elicit sweet water-tastes because they are hTAS1R2-hTAS1R3 allosteric inhibitors and their removal activates the receptor. This would occur due to the equilibrium forces of a two-state allosteric receptor resulting in a coordinated rebound to the activated state after most receptors had been locked in the inactive state by the inhibitor. Perceptual studies of water-tastes have, therefore, indicated that taste receptors have multiple states and can be activated and inhibited by a single molecule.

Alternatively, adaptation effects might also explain the low level of sweetness perceived with high Na-Saccharin concentrations. Taste adaptation is caused by exposure to a taste stimulus over some interval(s) (either short or long periods) and is manifested as the subsequent decrease in the perceived intensity of the stimulus or decresed activation by the stimulus. In comparison, inhibition also causes a decrease in the perceived intensity of the stimulus, but

174
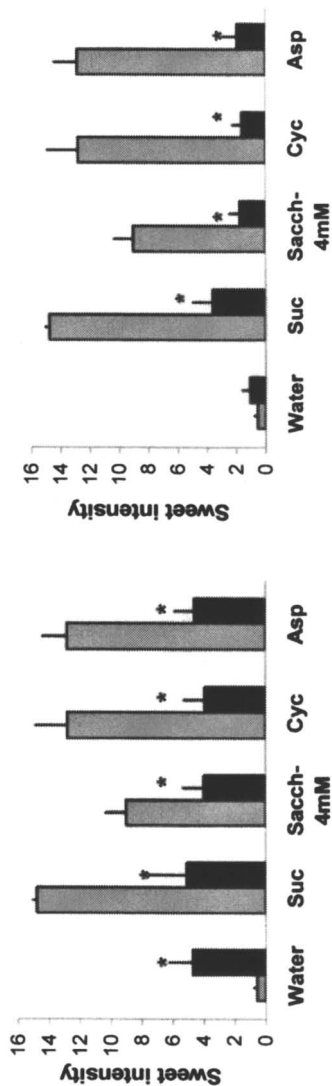
Figure 3. Sweetness blocking effects of a)200 mM Na-Saccharin and b) 1 mM lactisole. Gray bars refer to the mean intensity of the pure sweeteners listed on the x axis (n = 14); black bars reflect the mean sweetness for mixtures of sweetener and blocker. The agonists tested were 300mM sucrose (Suc.), 4mM Na-saccharin (Sacch-4), 17.5mM Na-cyclamate (Cyc.) and 3mM aspartame (Asp.). Error bars indicate s.e.m. Asterisks indicate significance at α=0.05 using repeated measures ANOVA. Adapted by permission from Macmillan Publishers Ltd: Nature [7], copyright 2006.

does so via a different molecular interaction with the taste receptor. To test this hypothesis directly, we designed an experiment where 14 subjects were repeatedly exposed to a mixture of sucrose and high Na-saccharin, after which the saccharin was removed from the solution to assess the level of adaptation to sucrose. Interestingly, after the removal of Na-saccharin from the mixture, sucrose was perceived quite strongly which indicates the lack of adaptation despite multiple sucrose presentations immediately prior (Figure 4). The failure of sucrose to adapt after 10 consecutive exposures indicates that the sucrose response was likely inhibited by the Na-saccharin; but when Na-saccharin was removed, the receptor returned to the active state and was normally activated by sucrose. It should be noted that sucrose presented alone ten times in a similar way without Na-saccharin adapts strongly.
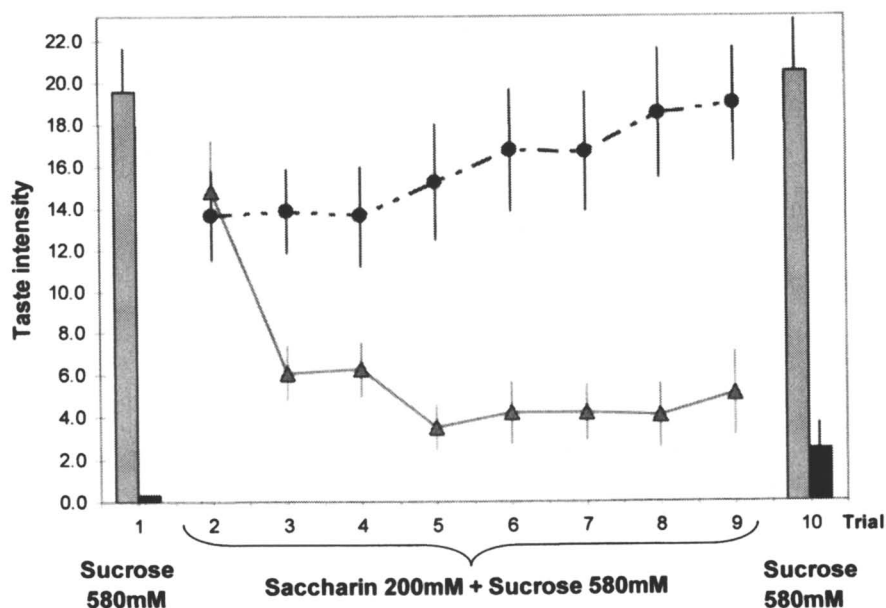
Figure 4. A test of sweetness adaptation versus sweetness inhibition as an explanations for NaSacharin's sweetness suppressing effects. Mean sweetness and bitterness of 580 mM sucrose and a mixture of this sucrose with 200 mM NaSaccharin. Fourteen subjects tasted the solutions shown at 10 sec intervals (90 sec total) and rated sweetness on a general labeled magnitude scale (gLMS) without rinsing in between tastings. Gray bars indicate sucrose sweetness and black bars its bitterness. Triangles indicate the sweetness of the mixture and circles its bitterness. Error bas indicate standard eror of the mean (s.e.m.). Reprinted by permission from Macmillan Publishers Ltd: Nature [7], copyright 2006.

Sensitivity increases with inhibition

Following the logic that there are similarities between inhibition and adaptation, we next wished to determine whether inhibition affected the slope of the concentration-intensity curve of sucrose, hence the sensitivity to sucrose. This comparison is based on the common observation that adaptation to stimuli increases observers' sensitivity to changes in stimulus strength. We measured the psychophysical curve for sucrose alone and in mixture with 50 mM Na-saccharin, as an inhibitor. Fifteen people rated all stimuli on a gLMS scale in triplicate and showed that the presence of the sweetener inhibitor Na-saccharin causes a rightward shift of the psychophysical function of sucrose, demonstrating saccharin's inhibition of sweetness over the whole concentration range (figure 5a). We examined the changes in slope by plotting the data in log-log coordinates (figure 5b). The addition of saccharin, as expected, lowers the perceived sweetness intensity but at the same time increases the slope of the curve from 0.48 to approximately 0.85, which means sensitivity increased. To confirm this sensitivity shift independently, Weber fractions were obtained for 400 mM sucrose alone and in the presence of 50 mM Na-saccharin. We show that standard Weber fractions for sucrose differential thresholds are between 12 to 14% (figure 5c). When testing sucrose mixed with inhibitory consentrations of Na-saccharin the average weber fraction appears lower than for sucrose alone, although the mean difference between conditions was not significant. When analyzing individual responses, we find significant decreases in weber fractions for several people, indicating an increase in differential sensitivity. However, this trend was not consistent in other subjects. We believe that when Na-sacharin is used as an inhibitor the strong bitterness stimulated at high concentrations, interferes with some subject's ability to differentiate solutions based solely on sweetness. These subjects rated the bitterness close to 'very strong' on the gLMS, which could explain their low differential sensitivity, while others rated the bitterness between moderate and strong. Sweet receptor inhibition might make sweet receptor cells more sensitive to changes in concentration because small increases in concentration can stimulate against a low level of background activity. More studies are needed to assess the impact of inhibition on differential sensitivity.

Intramodal interactions of sweet taste inhibitors.

Lactisole. A broad spectrum sweetener inhibitor

Lactisole (Na, p-methoxy-phenoxy-propionate, figure 7A), is a potent broad-acting sweetener inhibitor specific to humans and other primates and has no effect on rodents response to sweet taste. Lactisole, despite not eliciting

a

b
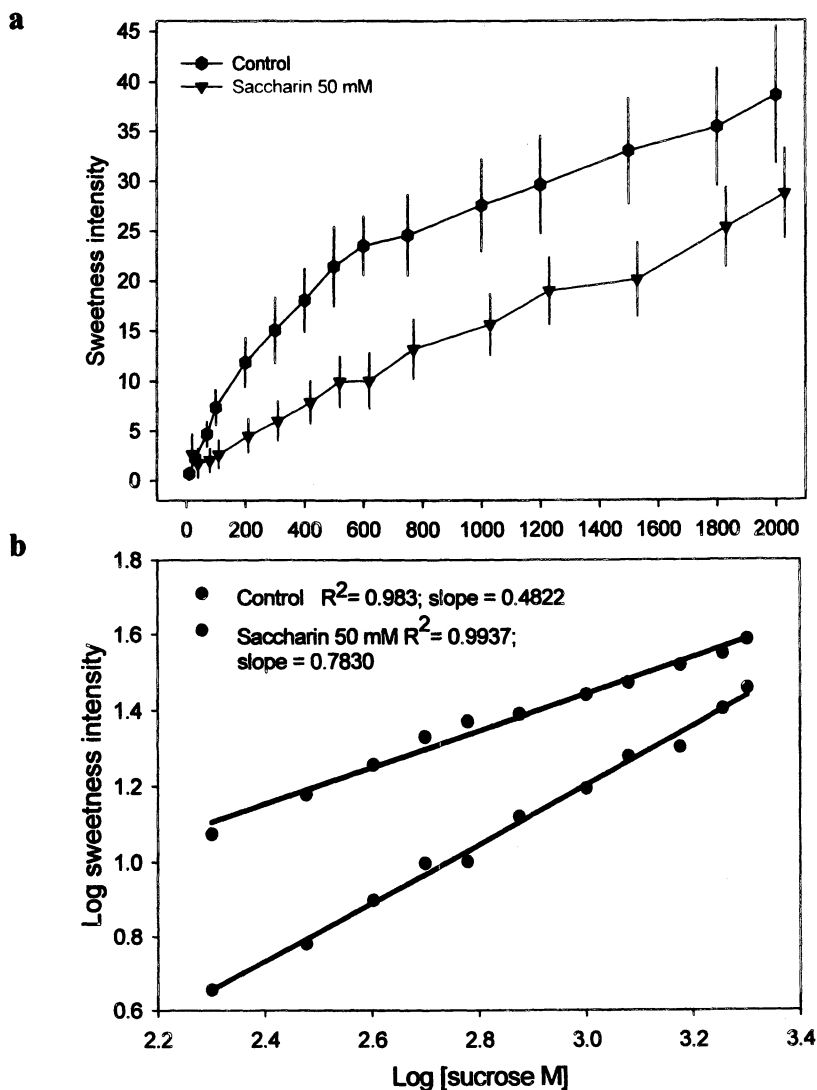
Figure 5. Concentration intensity curves of sucrose (control-top line) and mixtures of sucrose with 50mM Na-Saccharin (bottom line). a) normal space, intensity measured on a gLMS, b) log-log space, linear regression analysis. Error bars indicate SEM, n=15. (See color insert in this chapter.)

a
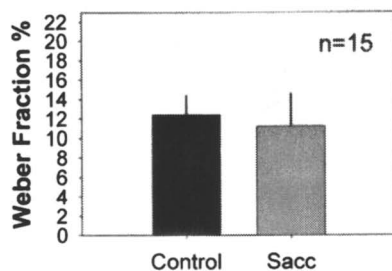
b
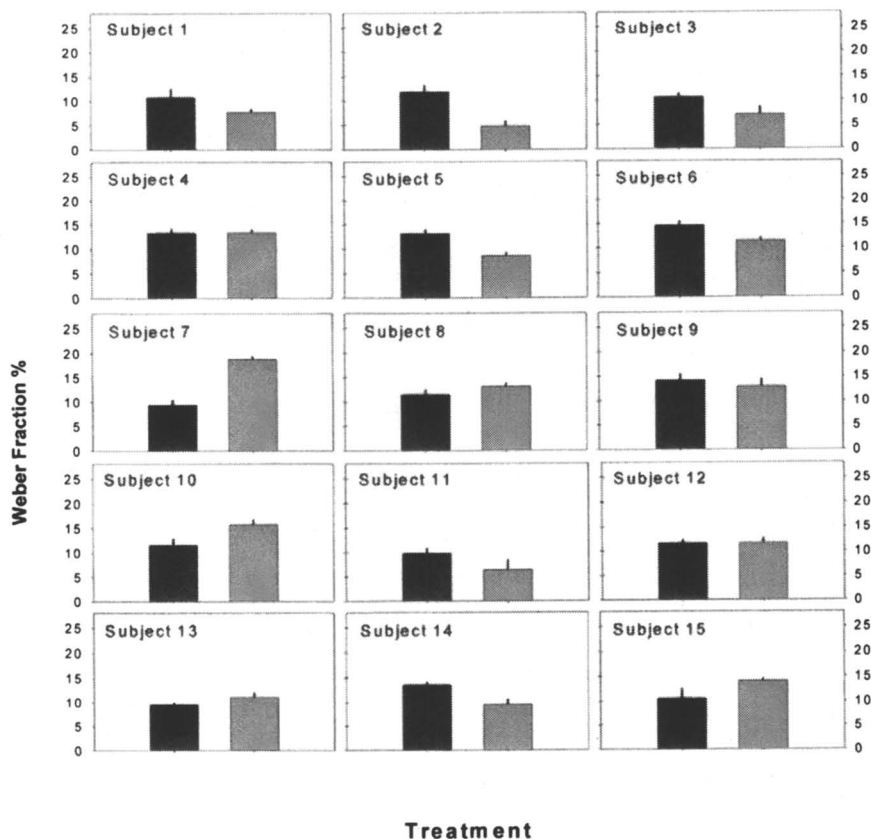
Figure 6. a) Weber's fractions for sucrose (black) and sucrose + Na-saccharin
(gray). Standard concentration =400 mM sucrose. b) individual Weber's
fractions for sucrose and sucrose (black) + Na-saccharin (gray). Error bars
indicate SEM for 3 replicates. (See color insert in this chapter.)

sweet taste per se, suppresses the sweet taste of most sugars, protein sweeteners, and other high potency sweeteners [8]. An early study suggested that lactisole does not inhibit all sweeteners to the same degree [9]. However it is not clear if the lack of inhibition observed in that study is due to the temporal properties of the sweeteners or the temporal dynamics of the inhibition. Recent *in vitro* studies have shown that lactisole binds specifically to the human T1R3 trans-membrane domains causing inhibition of the T1R2-T1R3 receptor's response to sweeteners [10-12]. Jiang et al showed that the TM helices 3, 5, and 6 of hT1R3 are involved in lactisole binding; by using chimeric and mutational studies they also identified Leu-7987.36 in TM helix 7 and Arg-790ex3 in extracellular loop 3 (which connects TM helices 6 and 7) as human-specific residues that affect responsiveness to lactisole [10]. Further research revealed that the exchange of valine 738 in the fifth transmembrane domain of rTas1r3 with an alanine is sufficient to confer lactisole sensitivity to the rat sweet taste receptor [12]. Given the broad effect that lactisole has on sweet taste and the specificity of its binding site, lactisole has become a useful tool to study taste modulation.
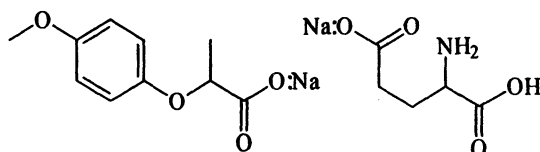
Figure 7. Chemical structures of a) lactisole and b) Monosodium glutamate MSG.

A molecular link between sweet and umami tastes.

The T1R receptors comprise a family of taste-specific class C G-protein coupled receptors, which mediate mammalian sweet and umami tastes. A glutamate or umami receptor (T1R1-T1R3) and a sweetener receptor (T1R2-T1R3) share a common entity, the T1R3 GPCR [13-16]. Given that perception of both taste qualities likely involves the presence of the T1R3 receptor [5, 17], it is logical to think that compounds that bind to this monomer will have an effect in perception of both taste qualities. Using a psychophysical approach we tested this hypothesis. In this experiment we turned again to lactisole, which interacts specifically with the transmembrane domain of T1R3. Multiple concentrations of lactisole were tasted in mixtures with 20 and 100 mM monosodium glutamate (MSG, figure 7) and the umami intensity ratings recorded. Our results showed a concentration dependent decrease in umami taste [18]. Lactisole also inhibited the perception of umami taste from MSG, albeit with less efficacy than it inhibits sweeteners, demonstrating that the shared

monomer (T1R3) allosterically moderates activation of T1R1 and T1R2 in humans. Given that the concentrations needed to inhibit umaminess were aprox. 16 times higher than those needed to inhibit sweet taste, we assessed the effect of lactisole on other taste modalities using the same concentrations. Exemplars of each taste quality were mixed with 16 mM lactisole. Subjects tasted these mixtures but found that lactisole inhibits only sweet and umami tastes (figure 8).

The differences in the level of inhibition between the umami and sweet modalities suggest either potential changes in conformation due to interactions with the other receptor protein (figure 9), or different mechanism of modulation given that other compounds such as cyclamate which also bind to T1R3 does not have a significant effect on umami taste (unpublished observation).

A distinctive characteristic of human umami taste is its powerful synergism derived from mixing 5'ribonucleotides with glutamate (figure 9 C, E & F) [19]. To test the effect of lactisole on the umami synergy of MSG with 5'ribonucleotides, increasing amounts of lactisole were added to constant mixtures of 20 mM MSG plus 3mM of either IMP or GMP or to ribonucleotides alone. We found no significant effect of lactisole on the umami taste of the synergized mixture with lactisole concentrations as high as 32 mM in human perceptual studies [18]. Based on the *in vitro* observation of MSG and 5' ribonucleotide synergy with hT1R1-hT1R3, if we assume synergy occurs, at least in part, within the hT1R1-T1R3 receptor, then our data suggest that 5'ribonucleotides bind to the T1R1 subunit but not the T1R2 subunit and alter the T1R1-T1R3 heterodimer, preventing lactisole from inhibiting umami taste [20]. Thus, we infer from lactisole's differential ability to inhibit both sweet
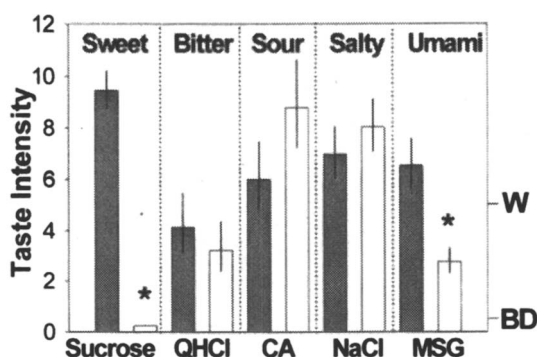
*Figure 8. Effect of 16 mM lactisole (clear bars) on standard quality solutions (gray bars); 200 mM sucrose, 2.5e-2 mM quinine-HCl, 2 mM citric Acid, 100 mM NaCl and 100 mM monosodium glutamate. Data analysis: Repeated Measures ANOVA and Tukey pairwise post-hoc comparisons (n=12). * Significant at α=0.05. W=weak intensity, BD=barely detectable intensity. Reprinted by permission from Oxford University Press: Chemical Senses, [18] copyright 2006.*
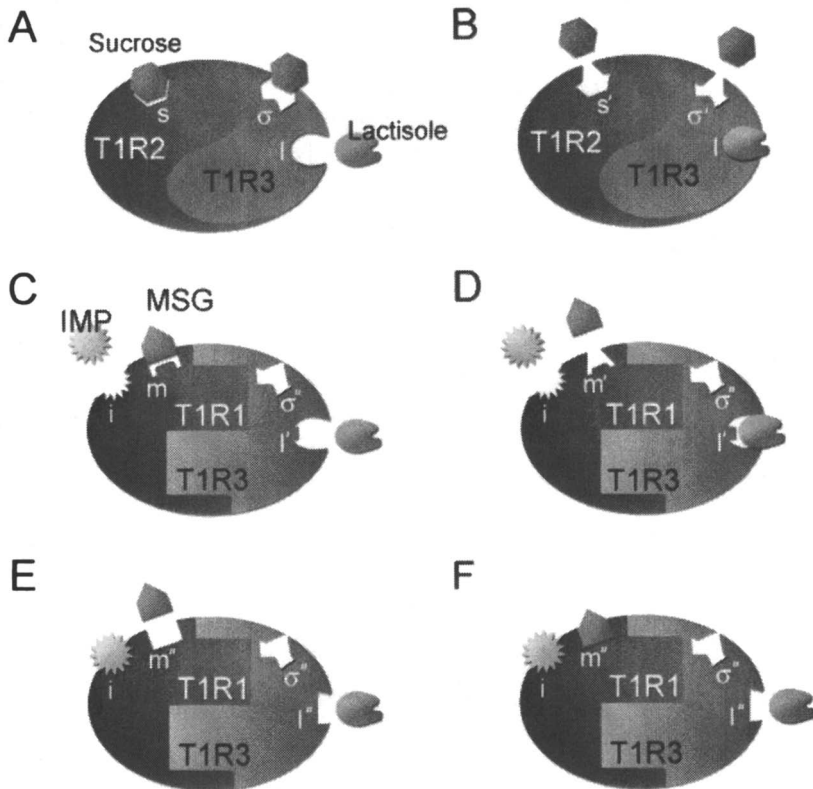
*Figure 9. Human sweet (T1R2(blue)-T1R3(purple)) and umami (T1R1(dark gray)-T1R3(medium gray)) taste heteromer receptor schematics: Inhibition of sucrose's (gray hexagon) sweet taste by the compound lactisole (small gray oval) (**A & B**); inhibition of monosodium glutamate's (MSG) (gray pentagon) umami taste by lactisole (**C & D**); and modulatory effects of 5'ribonucleotides, such as inosine monophosphate (IMP) (light gray star), on MSG binding and IMP's blockade of lactisole's inhibition (**E & F**). Reprinted by permission from Oxford University Press: Chemical Senses, [18] copyright 2006. (See color insert in this chapter.)*

(figure 9-A&B) and umami taste (figure 9-C&D) and from 5'ribonucleotide's ability to block lactisole's inhibition of umami but not sweet taste that the identity of a receptor subunit and/or its activation by ligands can alter the conformation of the partner subunit and hence its ability to be activated or inhibited. The following schematic model is based on previous *in vitro* models and has been modified to accommodate our psychophysical data.

Conclusions

We have used taste inhibition as a tool to demonstrate the utlity of combining perceptual & psychophysical studies for inferring the molecular mechanisms of several perception phenomena. First, we show that a sweet receptor agonist may simultaneously be an antagonist of sweet taste. This is illustrated with Na-saccharin, which both stimulates sweet taste at low concentrations and inhibits sweet taste at high concentrations. A parallel effect was also demonstrated with saccharin *in vitro* on heterologously expressed sweet taste receptors. We argue that the sweet receptor is a multi-state receptor that when released from its inhibited state will rebound to an activated state, acounting for the sweet water-taste that follows rinses. Similarly, *in vitro* responses when rinsing Na-saccharin away with water closely parallels the perceptual phenomenon. Second, we demonstrate that the inhibition of sucrose can increase the sensitivity to changes in concentration, much as adaptation is known to do. These data suggest that suprathreshold sensitivity to stimuli like sucrose is determined by the ability of the peripheral system to adjust responsiveness to ambient or background levels, so that changes in stimulus levels are perceived as large percent changes perceptually. This will result in the ability to detect physical intensity changes, even if these changes are very small relative to background concentration levels. The observation of heightened sensitivity when an inhibitor is mixed with the agonist, indicates that this phenomenon may be explained at the receptor or cellular level, as opposed to higher in the signal processing pathway. Third, we demonstrate that the sweetener inhibitor lactisole, which binds to the T1R3 subunit and inhibits the T1R2-T1R3 sweetener receptor, also inhibits the savory taste of glutamate, albeit with low efficacy. In humans, this taste is believed to be transduced, in part, by the T1R1-T1R3 receptor, which shares the T1R3 subunit with the sweetener receptor. These data support the hypothesis that T1R1-T1R3 receptor plays a role in human savory taste, but appears only to convey part of the glutamate signal. Since the addition of 5' ribonucleotides to agonists does not affect lactisole's ability to inhibit the sweet taste of sugars but does prevent lactisole from inhibiting the savory taste of glutamate, we infer that the binding and activation of each T1R subunit by compounds may be allosterically modulated by the identity and state of its partner subunit. These three examples

of perceptual research with taste inhibitors, therefore, illustrate the utility of this approach for highlighting the underlying molecular mechanisms of chemosensory perception.

References

1. Bartoshuk, L.M. (1968). Water taste in man. Perception and Psychophysics *3*, 69-72.
2. Bartoshuk, L.M. (1977). Water taste in Mammals. In Drinking Behavior, J.A.W.W. Weijnen and J. Mendelson, eds. (New Haven, CT: Plenum Publishing Co.), pp. 317-339.
3. McBurney, D.H., and Bartoshuk, L.M. (1971). Water taste in mammals. Paper presented at the fourth international symposium on olfaction and taste.
4. McBurney, D.H., and Bartoshuk, L.M. (1973). Interactions between stimuli with different taste qualities. Physiology and Behaviour *10*, 1101-1106.
5. DuBois, G.E. (2004). Unraveling the biochemistry of sweet and umami tastes. Proc Natl Acad Sci *101*, 13972-13973.
6. Dubois, G. (1999). Selective sweetness inhibitors and a biological mechanism for sweet water aftertaste. In *Annual Meeting of the Association of Chemoreception Scientists* vol. 21. pp. Abstract: Sarasota.
7. Galindo-Cuspinera, V., Winnig, M., Bufe, B., Meyerhof, W., and Breslin, P.A.S. (2006). A TAS1R receptor-based explanation of sweet water-taste. Nature *441*, 354-357.
8. Dubois, G.E. (1997). New insights on the coding of the sweet taste message in chemical structure. In Firmenich Jubilee Symposium 1895-1995, G. Salvadori, ed. (Carol Stream, IL: Allured Publishing Corp), pp. 32-95.
9. Schiffman, S.S., Booth, B.J., Sattely-Miller, E.A., Graham, B.G., and Gibes, K.M. (1999). Selective inhibition of sweetness by the sodium salt of (+/-)2-(4-methosyphenoxy)propanoic acid. Chemical Senses *24*, 439-447.
10. Jiang, P., Cui, M., Zhao, B., Liu, Z., Snyder, L.A., Benard, L.M.J., Osman, R., Margolskee, R.F., and Max, M. (2005). Lactisole Interacts with the Transmembrane Domains of Human T1R3 to Inhibit Sweet Taste. Journal of Biolgical Chemistry *280*, 15238-15246.
11. Xu, H., Staszewski, L., Tang, H., Adler, E., Zoller, M., and Li, X. (2004). Different functional roles of T1R subunits in the heteromeric taste receptors. Proc. Natl. Acad. Sci. *101*, 14258-14263.
12. Winnig, M., Bufe, B., and Meyerhof, W. (2005). Valine 738 and lysine 735 in the fifth transmembrane domain of rTas1r3 mediate insensitivity towards lactisole of the rat sweet taste receptor. BMC Neuroscience *6*, 1-7.

184

13. Damak, S., Rong, M., Yasumatsu, K., Kokrashvili, A., Varadarajan, V., Zou, S., Jiang, P., Ninomiya, Y., and Margolskee, R.F. (2003). Detection of sweet and umami taste in the absence of taste receptor T1R3. Science *301*, 850-853.

14. Zhao, G.Q., Zhang, Y., Hoon, M.A., Chandrashekar, J., Erlenback, I., Ryba, N.J.P., and Zuker, C.S. (2003). The receptors for mammalian sweet and umami taste. Cell *115*, 255-266.

15. Jiang, P., Ji, Q., Liu, Z., Snyder, L.A., Benard, L.M.J., Margolskee, R.F., and Max, M. (2004). The Cysteine-rich Region of T1R3 Determines Responses to Intensely Sweet Proteins. Journal of Biological Chemistry *279*, 45068-45075.

16. Nelson, G., Chandrashekar, J., Hoon, M.A., Feng, L., Zhao, G., Ryba, N.J.P., and Zucker, C.S. (2002). An amino-acid taste receptor. Nature *416*, 199-202.

17. Li X., Staszewski L., Xu H., Durick K., Zoller M., and E., A. (2002). Human receptors for sweet and umami taste. Proc Natl Acad Sci *99*, 4692-4696.

18. Galindo Cuspinera, V., and Breslin, P.A. (2006). The liaison of sweet and savory. Chemical Senses *31*, 221-225.

19. Yamaguchi, S. (1967). The synergistic taste effect of MSG and disodium 5'-inosinate. Journal of Food Science *32*, 473-478.

20. Jensen, A.A., and Spalding, T.A. (2004). Allosteric modulation of G-protein coupled receptors. European Journal of Pharmaceutical Sciences *21*, 407-420.

Chapter 13

A Method to Measure Taste Qualities, Taste Intensity, and Temporal Profile of Compounds Aimed at Human Consumption by Taste Nerve Recordings in Monkeys

Göran Hellekant and Yiwen Wang

Department of Physiology and Pharmacology, Medical School, University of Minnesota, Duluth, MN 55812

A method is described to measure taste qualities and intensity of compounds aimed at human consumption by recording from taste nerve fibers of monkeys. Here we demonstrate its usefulness by presenting results of a comparison of sweetness of brazzein derivatives and bitterness of denatonium benzoate analogs as assessed by a human taste panel and recorded from monkey single taste fibers. The correlation between the responses in sweet sensitive fibers in monkeys and the estimates of sweetness by a human taste panel was 0.78 for 25 analogs of the sweet protein brazzein, and 0.9 between the responses of bitter sensitive fibers and human bitterness estimates for 6 analogs of the bitter compound denatonium benzoate.

185

Introduction

Evaluation of the taste of compounds aimed for oral consumption is a necessary step in the development and marketing process. Data acquisition from human taste panels can be tedious, especially since toxicological concerns have to be put to rest prior to the taste tests in humans. This is not a concern in taste nerve recordings from animals because the compounds never enter the body of the animal. Instead, the main concern is: how applicable are the animal data to human taste?

With regard to the sweet and bitter taste qualities, all mammals tested so far, recognize these taste qualities, as judged by behavioral tests, but the compounds that elicit these qualities differ among species. Although some earlier investigators clearly were aware of the existence and importance of species differences in taste [1, 2], it was not generally well understood, as judged by the many attempts to relate human taste qualities with the taste fibers from cats, rodents and other non-primate species [3, 4]. This makes the choice of animal model crucial.

We became aware of both qualitative and quantitative species differences in taste when our taste nerve recordings from human and monkey showed a large enhancement of the taste nerve response to acids after miraculin, whereas none was recorded in rat [5]. Miraculin is a taste modifier that adds sweetness to acids in humans and monkeys but not rats. This and later studies [6, 7] lead us to the conclusion that mammals have different types of sweet receptors [8]. Our further studies suggested that the sense of taste is more similar among primates, than between primates and non-primates, and that combinations of behavioral observations and taste nerve recordings from non-human primates could be used to elucidate human taste mechanisms, including how information from the taste buds is mediated to the brain, that is, how taste is coded.

Recordings from taste nerves of several primates over more than 30 years, in particular from three higher primates, have revealed that indeed there are nerve fibers in the taste nerves, whose response spectrum mirrors the human taste qualities. Thus, we and others have found that sweet and bitter taste are linked to activity in two groups of taste fibers, one responding to sweet, the other to bitter tasting compounds, and that nerve impulses in these fibers evoke either a sweet or bitter taste quality [9-16]. This coding of taste has been called labeled lines. These findings refute the idea that all taste fibers have to participate in creating a taste quality, the across-fiber pattern theory, which originally was proposed to explain the lack of connection between human taste qualities and single fiber response spectrum in the cat[3]. It was later expanded, using rodent recordings [4] and recently supported again [17-19]

Since then supportive data for the labeled line theory, obtained with other techniques, have been published. Genetic engineering, combined with behavioral

experiments in mice, have shown that bitter and sweet tastes are sensed by separate sets of receptors, and, what is more important from the point of coding, these receptors are located in different, not overlapping taste bud cells [20-27]. This is an important condition, because if bitter and sweet receptors were co-localized, it is difficult to visualize how a taste fiber, synapsing with a cell with two different receptors, could carry only one taste quality.

In the following we present data from an Old-World primate, the rhesus monkey, *M. mulatta*. It provides data on the intensity of a sweet and a bitter compound, which are well correlated with assessments by a human taste panel.

Methods, Animals, Subjects and Stimuli

The taste nerve responses were recorded under general anesthesia from the front of the tongue of rhesus monkeys, *M. mulatta,* through the chorda tympani proper (CT) and the back of the tongue, through the glossopharyngeal nerve (NG). Some 30 taste stimuli, representing the sweet, bitter, salty, sour and umami taste qualities, were applied to the tongue while the responses in single taste fibers were recorded [28]. The responses in the fibers were subjected to hierarchical cluster analyses. The analyses resulted in three to four clusters, which were linked to the sweet, bitter, salty or sour tastes. The fibers in these clusters are labeled S, Q, N and H fibers.

The responses in single nerve fibers, belonging to the S cluster, were then used as a tool to assess the sweetness of the sweet protein Brazzein [29] and 25 structurally closely related derivatives of brazzein [30]. As a measure we used the impulses over 5 sec of stimulation. Similarly we used the responses in Q fibers to assess the bitterness of denatonium benzoate and 6 of its derivatives.

The neurophysiological data were then compared with data from a human taste panel using the Labeled Magnitude Scale to estimate the sweetness or bitterness of the same compounds [31, 32]. The panel consisted of 14 volunteers, 6 females and 8 males.

Results

Figures 1 and 2 present the result of the cluster analyses of the fibers in the CT and NG nerves as dendrograms. The analyses took into account the responses to all stimuli. The cluster analyses distinguished four major clusters, which, based on the fibers' response to salty, sour, sweet and bitter compounds, were labeled, either the N, H, S or Q cluster. The dendrograms show that the taste fibers can be arranged according to their responses to the compounds humans consider salty, sour, bitter and sweet.

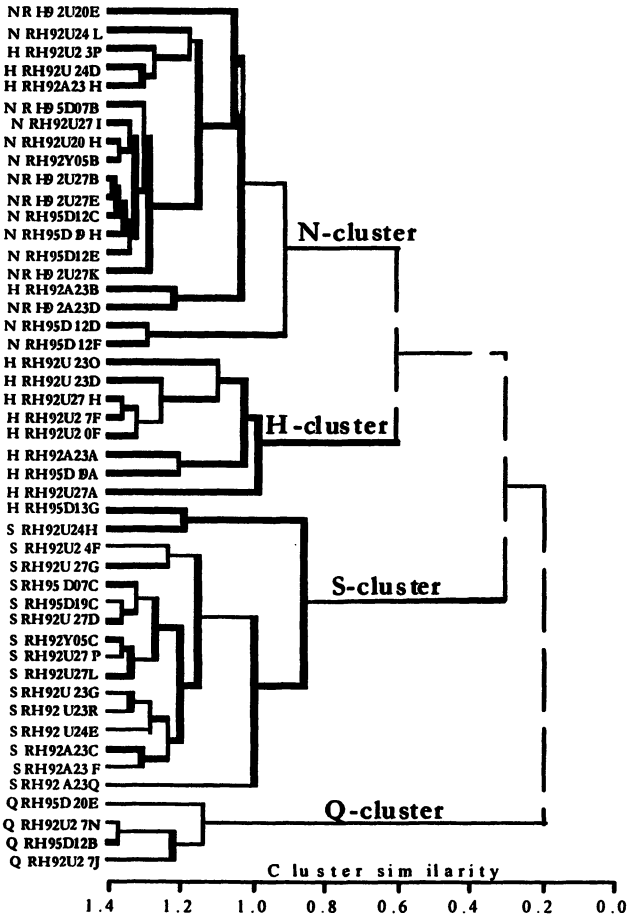Figure 1. Hierarchial cluster analysis of the response profiles for 47 CT nerve fibers. Fiber number and response category on the basis of its response to the 4 basic solutions are listed on the left.
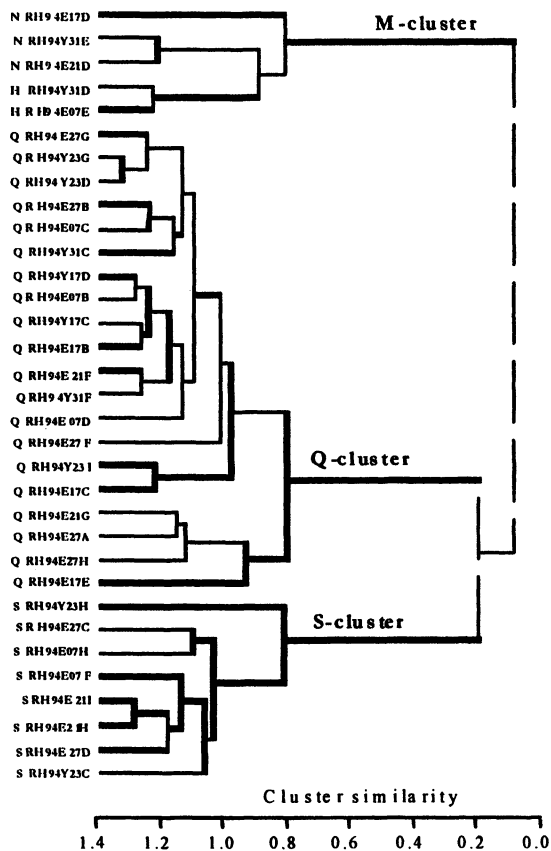
Figure 2. Hierarchial cluster analysis of the response profiles for 33 NG nerve fibers. Fiber number and response category on the basis of its response to the 4 basic solutions are listed on the left.

Figures 3 and 4 show the responses of the individual taste fibers of the CT and NG fibers. The stimuli have been arranged along the X-axis in order of salty, umami, sour, bitter and sweet stimuli. The identity of the fibers is presented along the Y-axis to allow their identification in the dendrogram. The heights of the bars depict the response during the first 5 sec of stimulation of the compounds listed along the X-axis.

Figure 3, from the CT nerve, shows one group of fibers responding only to NaCl. These fibers did not respond to KCl. This indicates that the taste of NaCl and of KCl are very different to the rhesus monkey, a difference, which also is very evident to humans. A second group in Figure 3, part of the N cluster, responded to monosodium glutamate (MSG), but not to KCl. Citric and aspartic acid stimulated the largest range of CT fibers, which also included some S fibers. We noticed the same feature in our first single fiber study in rhesus monkey [33]. This is not surprising, since these acids also have a sweet component, in contrast to HCl, which lacks sweetness, and consequently with one exception, did not stimulate any S fibers. A small number of CT fibers responded to the bitter compounds, quinine, caffeine and denatonium. Instead Q fibers are found in and dominate the NG recordings in Figure 4, where there really is no well defined S cluster. This indicates that bitter is the dominant taste quality on the back of the tongue. The fact that the largest cluster of S fibers were found in the CT nerve supports the notion that sweet is perceived from the tip of the tongue. The final observation, which we think is important from the point of view of taste quality, is that the S fibers responded to virtually every sweet compound applied. This corroborates our conclusion that these fibers are responsible for the sweet taste quality.

The S fibers were then used to measure the intensity of the brazzein compounds. Figure 5 compares the results obtained from humans and monkeys, when sweetness scores and nerve responses are expressed relative to those of brazzein. As can be seen, the results are highly correlated (r=0.78, p<0.001). Figure 5 shows that the same four mutants, Asp29Ala, Asp29Lys, Asp29Asn, and Glu41Lys, were scored significantly sweeter than WT brazzein by both the human panel and the monkey data. Similarly, both methods suggested that the sweetness of 8 other mutants was significantly decreased. Ten brazzein mutants were scored similar to water by both methods. The response to three brazzein mutants and both types of monellins did not differ from that of the WT. It is notable that the results of the nerve recordings stressed the differences in sweetness between the compounds more than the psychophysical method. However, most interesting in the present context is that the results of the two methods were highly correlated.

We used the same approach to estimate bitterness of 8 derivatives of denatonium benzoate by recording from fibers, which cluster analyses had
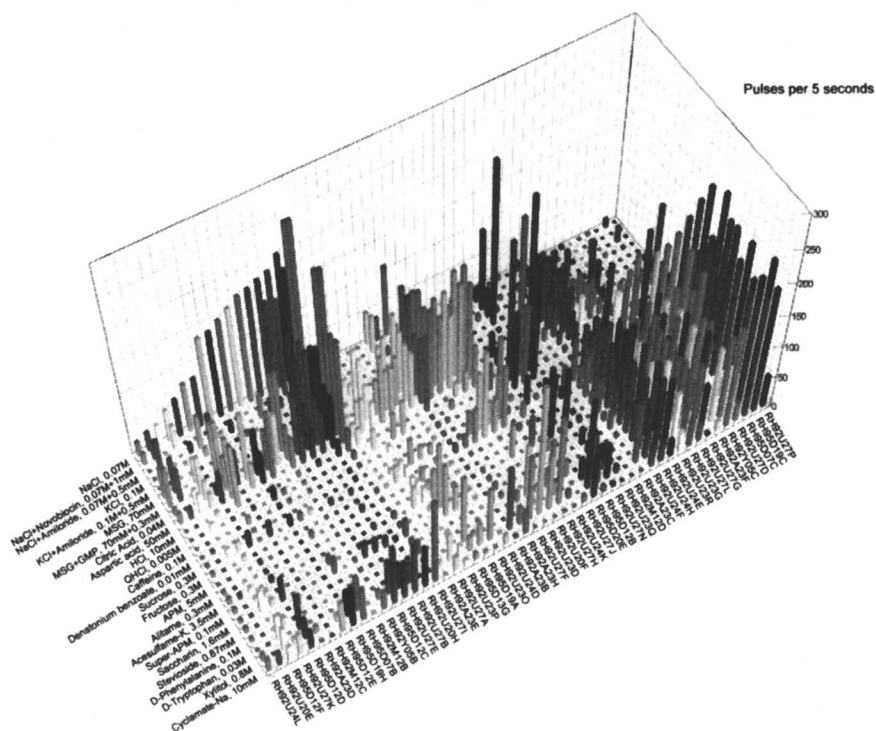
Figure 3. Overview of the response profiles of 51 CT single fibers. The stimuli were arranged along the X- axis in order of salt, sour, bitter and sweet. The fibers were arranged along the Y- axis in groups: NaCl, acid, Quinine hydrochloride and sucrose best fibers. MSG denotes monosodium glutamate; GMP, guanosine 5'-monophosphate. (See color insert in this chapter.)
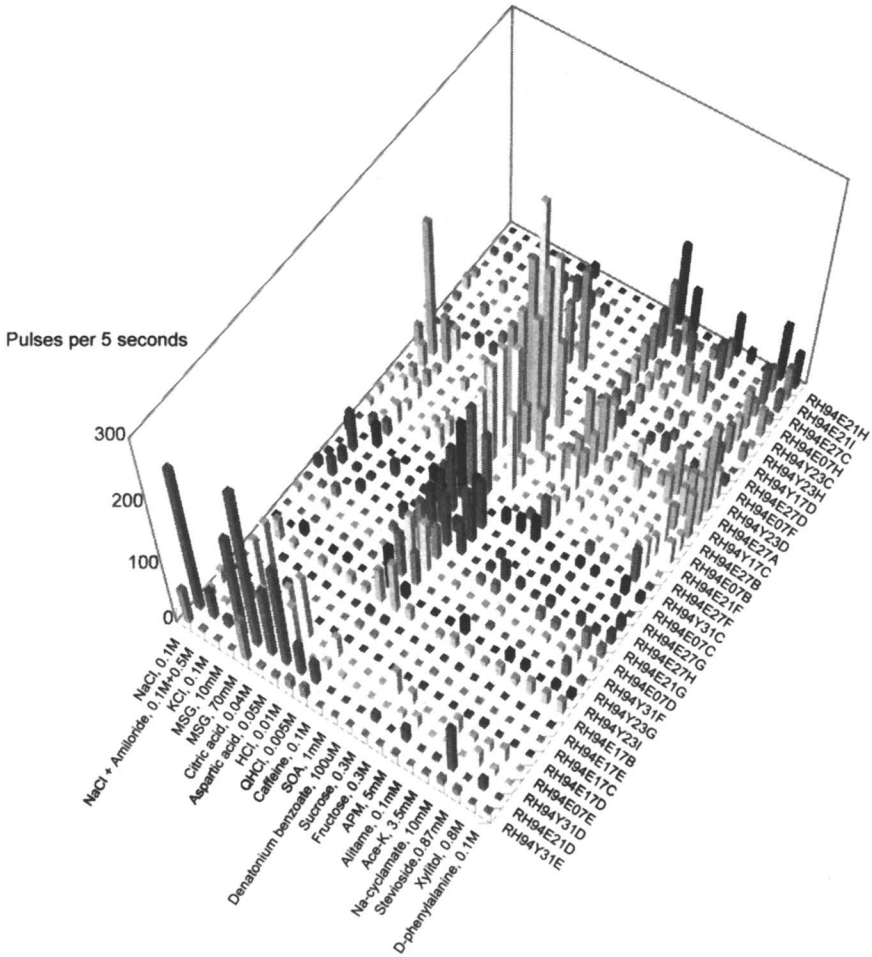
Figure 4. Overview of the response profiles of 33 NG single fibers. The stimuli and fibers were arranged as for the CT. (See color insert in this chapter.)
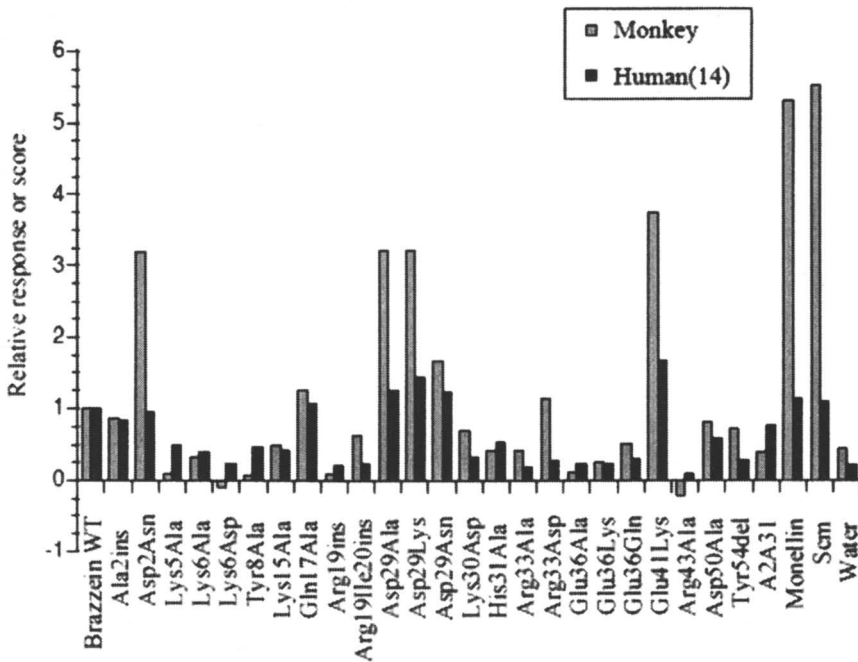
Figure 5. Comparison between human psychophysical and monkey electrophysiological results for brazzein, brazzein mutants, monellin, single chain monellin and water. (See color insert in this chapter.)

identified as bitter responding fibers, Q fibers. In a similar manner as for the sweet compounds above, we obtained data on the taste intensity of these compounds from a human panel using the Label Magnitude Scale. As was done for brazzein, the average response to denatonium benzoate was used as standard and assigned the value 100. The bitterness scores of the psychophysical experiments and number of impulses in electrophysiological experiments were then expressed in percent of the standard. Figure 6 shows the results. It is evident that the human assessment of intensity of bitterness and the responses in the bitter sensitive fibers of the monkey model are highly positively correlated. The correlation coefficient is 0.9 [34].

To summarize, here we demonstrate a close positive correlation between estimates of sweet and bitter by humans and the nerve response in monkey S and Q taste fibers. This suggests that recordings from S and Q fibers can be used to assess the taste to humans of sweet and bitter compounds. We suggest that the method can replace human taste panels. The results serve also as a further verification of the relevance of the labeled line theory in sweet and bitter taste.
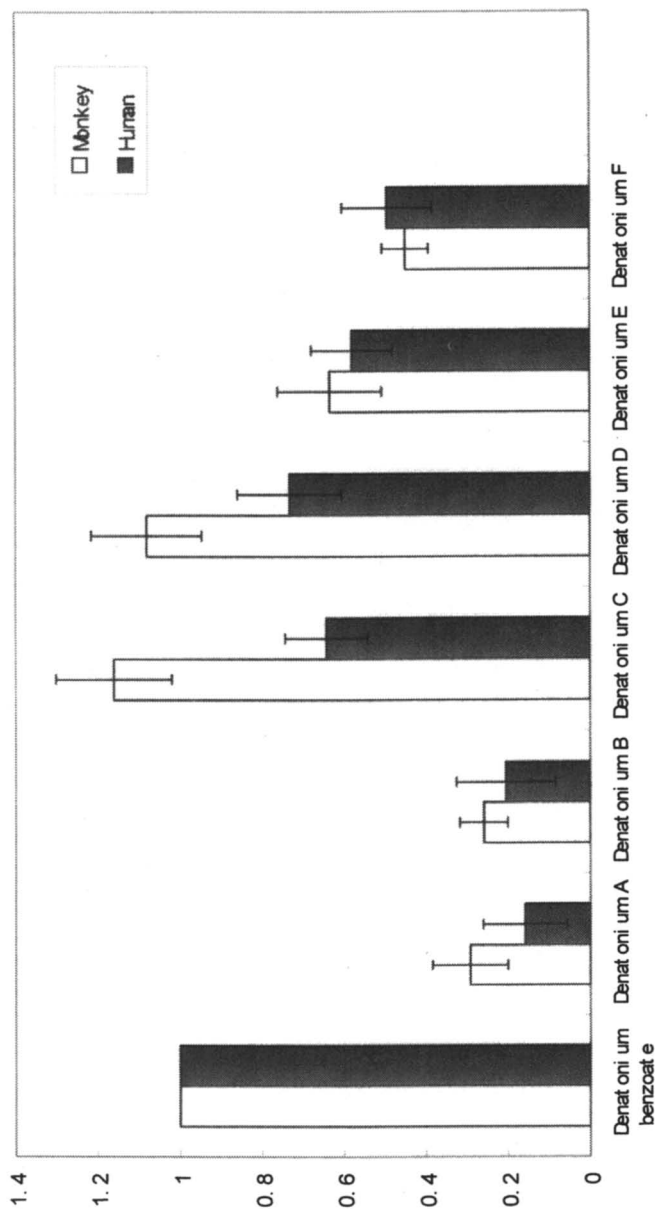
Figure 6. Comparison between human psychophysical and monkey electrophysiological results for denatonium benzoate, denatonium benzoate derivates and water. (See color insert in this chapter.)

Discussion

The use of taste fiber recordings from animals as replacement of human assessments depends on the fulfillment one condition; that the compounds to be assessed elicit a similar taste quality in the animal model as it does to humans. This is not always the case as we and other authors have found in a number of animal species [13, 35-39]. Aspartame and monellin are well known as lacking sweet taste to non-catarrhine species but there are several more examples known. On the other hand, in all Old-world primates we have found that all compounds that are sweet or bitter to humans seem to elicit the same taste quality.

Furthermore our research in non-human primates has shown that their taste nerves contain fibers that are either particularly sensitive to sweet or to bitter stimuli. That is, they essentially don't respond to compounds representing other taste qualities. This conclusion is supported not only by the results of taste stimulation with a large number of sweet or bitter compounds, both natural and artificial, but also by results of two modifiers of sweet taste, miraculin and gymnemic acid [33, 40-44]. Our most convincing results have been obtained in the chimpanzee, where S fibers don't respond to any other tastes, but Q fibers are also very specific in the chimpanzee [13, 40, 41, 43, 45-49]. Since no species is more closely related to humans than the chimpanzee, this indicates that these features exist in humans.

Among macaques we have studied most extensively the *M. mulatta*, but also *M. fascicularis*. We have also in a series of electrophysiological recordings showed that the same groups of taste fibers exist in marmosets [15, 42, 44, 50, 51]. The conclusion of these studies is that sweet and bitter are "hardwired", in the sense that S taste fibers mediate sweetness and Q taste fibers elicit a bitter taste in primates.

Since each cluster elicits a different taste quality, one can deduce from the proportion between responses in the S and Q cluster, if a compound tastes both sweet and bitter. Furthermore, using a series of concentrations of the compound, one can learn if the taste quality of the compound changes with concentration. Saccharin is a prime example of this. To humans it gets more and more bitter with increase of concentration and the Q fiber response increases with increase of saccharin concentration.

The nerve response to a compound differs from that to another compound, not only in what taste fibers it stimulates and number of impulses within the response, but also in its temporal profile, also called temporal pattern or temporal intensity (TI). Thus, for example, aspartame elicits a nerve response with the largest magnitude within the first few sec of stimulation, while the response to brazzein grows slowly over several seconds of stimulation. This feature is reflected in the taste sensations that increase slowly, as is the case for brazzein, and more rapidly for aspartame. The actual nerve recording can then

provide a numerical value of this parameter for comparison with the TI of other compounds. This may be of considerable importance in the search for new sweeteners, because one shortcoming of many new sweeteners is a TI profile that differs substantially from that of sugar.

Although we have not dealt with other taste qualities as extensively as with sweet and bitter, our non-published data and others' data on the specificity of the taste cells in the taste buds, e.g. [27], suggest that other taste qualities of a compound can be assessed using taste nerve recordings from animals. Thus, for example, some of the human taste qualities have been found in hamsters, such as saltiness of a compound, which is reflected and can be monitored by recording from taste fibers sensitive to NaCl, so called N fibers [52-57].

In humans the back of the human tongue has been reported as especially sensitive to bitter and the front to sweet. A comparison between Figures 1 and 3, on one side, and 2 and 4, on the other, shows that a larger number of S fibers were obtained from the tip of the tongue than from the back, while the numbers of Q fibers were larger in the NG nerve from the back. The proportion between S and Q fibers were the same in chimpanzee CT as here found in monkey CT. Since the chimpanzee is phylogenetically closer to humans than any other species, it suggests that the prevalence of sensitivity to sweet on the front and bitter on the back in humans may be the result of a larger number of S fibers in the human CT and Q fibers in the NG nerve.

However, this does not mean that the ability to taste sweet is confined to the front and bitter to the back. Figure 4 and 5 show that sweet also stimulated the back and bitter the front; it is only the proportion between Q and S fibers that differ. ·

Finally, we and other authors have shown that some oral compounds, in addition to giving a taste nerve response, also stimulate non-gustatory fibers. Alcohol presents a striking example, because it also elicits a powerful trigeminal response in primates[58]. The fact that a tastant can stimulate nerve fibers in the trigeminal lingual nerve, normally considered non-gustatory, shows that a taste sensation in a wider sense can be composed of more than the responses from taste nerve fibers. Recordings from the other sensory nerve fibers from the oral region may offer insight into qualities of an oral compound that cannot be acquired in a human taste panel.

Acknowledgements

References

1. Kare, M. R., Comparative aspects of the sense of taste. In Physiological and Behavioral Aspects of Taste, Kare, M. R.; Halpern, B. P., Eds. University of Ghicago Press: Chicago, 1961; pp 6-15.

2. Kitchell, R. L., Comparative anatomical and physiological studies of gustatory mechanisms. Pergamon Press: London., 1963; p 235-255.

3. Pfaffmann, C., Gustatory Afferent Impulses. J. Cell. Comp. Physiol. **1941**, *17*, 243-258.

4. Erickson, R. P.; Doetsch, G. S.; Marshall, D. A., The Gustatory Neural Response Function. Journal of General Physiology **1965** *49, (2)*, 247-263.

5. Diamant, H.; Hellekant, G.; Zotterman, Y., The effect of miraculin on the taste buds of man, monkey and rat. In Olfaction and Taste IV, Schneider, D., Ed. Wissenschaftlige Verlagsgesellschaft MB4 Stuttgart: Stuttgart, 1972; pp 241-244.

6. Brouwer, J. N.; Hellekant, G.; Kasahara, Y.; van der Wel, H.; Zotterman, Y., Electrophysiological study of the gustatory effects of the sweet proteins monellin and thaumatin in monkey, guinea pig and rat. Acta Physol. Scand. **1973**, *89*, 550-557.

7. Hellekant, G.; Hagstrom, E. C.; Kasahara, Y.; Zotterman, Y., On the gustatory effects of miraculin and gymnemic acid in monkey. Chem Sens Flavor **1974**, *1*, 137-145.

8. Hellekant, G., Different types of sweet receptors in mammals. In Olfaction and Taste V, Denton, D.; Cagan, R., Eds. Academic press: New York, San Francisco, London, 1975; Vol. 5, pp 15-21.

9. Glaser, D.; Hellekant, G.; Brouwer, J. N.; van der Wel, H., The Taste Responses in Primates to the Proteins Thaumatin and Monellin and their Phylogenetic Implications. Folia Primatologica **1978**, *29*, 56-63.

10. Hellekant, G.; Roberts, T. W., Whole nerve and single fiber recordings in non-human primates. In Experimental Cell Biology of Taste and Olfaction: Current Techniques and Protocols, Spielman, A. I.; Brand, J. G., Eds. CRC Press: Boca Raton, New York, London, Tokyo, 1995; pp 277-290.

11. Hellekant, G.; Glaser, D.; Brouwer, J.; van der Wel, H., Gustatory responses in three prosimian and two simian primate species (Tupaia glis, Nycticebus coucang, Galago senegalensis, Callithrix jacchus jacchus and Saginus midas niger) to six sweeteners and miraculin and their phylogenetic implications. Chem. Senses **1981**, *6, (3)*, 165-173.

12. Hellekant, G.; Danilova, V.; Ninomiya, Y., Primate sense of taste: behavioral and single chorda tympani and glossopharyngeal nerve fiber recordings in the rhesus monkey, Macaca mulatta. J. Neurophysiol. **1997**, *77, (2)*, 978-993.

13. Hellekant, G.; Ninomiya, Y.; DuBois, G. E.; Danilova, V.; Roberts, T. W., Taste in chimpanzee: I. The summated response to sweeteners and the effect of gymnemic acid. Physiology & Behavior **1996**, *60, (2)*, 469-479.

14. Danilova, V.; Hellekant, G., The taste of ethanol in a primate model. II. Glossopharyngeal nerve response in Macaca mulatta. Alcohol **2000**, *21, (3)*, 259-269.

15. Danilova, V.; Danilov, Y.; Roberts, T.; Tinti, J.-M.; Nofre, C.; Hellekant, G., The sense of taste in a New World monkey, the common marmoset: recordings from the chorda tympani and glossopharyngeal nerves. J Neurophysiol **2002**, *88*, 579-594.

16. Jin, Z.; Danilova, V.; Assadi-Porter, F. M.; Markley, J. L.; Hellekant, G., Monkey electrophysiological and human psychophysical responses to mutants of the sweet protein brazzein: delineating brazzein sweetness. Chem Senses **2003**, *28, (6)*, 491-498.

17. Schiffman, S. S., Taste quality and neural coding: implications from psychophysics and neurophysiology. Physiol Behav **2000**, *69, (1-2)*, 147-159.

18. Smith, D. V.; John, S. J.; Boughter, J. D., Neuronal cell types and taste quality coding. Physiol Behav **2000**, *69, (1-2)*, 77-85.

19. Contreras, R. J.; Lundy, R. F., Gustatory neuron types in the periphery: a functional perspective. Physiol Behav **2000**, *69, (1-2)*, 41-52.

20. Adler, E.; Hoon, M. A.; Mueller, K. L.; Chandrashekar, J.; Ryba, N. J.; Zuker, C. S., A novel family of mammalian taste receptors. Cell **2000**, *100, (6)*, 693-702.

21. Chandrashekar, J.; Mueller, K. L.; Hoon, M. A.; Adler, E.; Feng, L.; Guo, W.; Zuker, C. S.; Ryba, N. J., T2Rs function as bitter taste receptors. Cell **2000**, *100, (6)*, 703-711.

22. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F., Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac. Nat Genet **2001**, *28, (1)*, 58-63.

23. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S., Mammalian sweet taste receptors. Cell **2001**, *106, (3)*, 381-390.

24. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J., Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. Cell **2003**, *112, (3)*, 293-301.

25. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N. J.; Zuker, C. S., The receptors for mammalian sweet and umami taste. Cell **2003**, *115, (3)*, 255-266.

26. Mueller, K. L.; Hoon, M. A.; Erlenbach, I.; Chandrashekar, J.; Zuker, C. S.; Ryba, N. J., The receptors and coding logic for bitter taste. Nature **2005**, *434, (7030)*, 225-229.

27. Chandrashekar, J.; Hoon, M.; Ryba, N.; Zuker, C., The receptors and cells for mammalian taste Nature **2006**, *444*, 16 November 288-294

28. Danilova, V.; Danilov, Y.; Roberts, T.; Elmer, D.; Hellekant, G., Electrophysiological recordings of mammalian taste nerves. In Methods and Frontiers in Neuroscience, Simon, S. A.; Nicolelis, M. A. L., Eds. CRC Press: Boka Raton, London, New York, Washington D.C., 2002; pp 239-264.

29. Ming, D. Brazzein, a new sweet protein from pentadiplandra brazzeana. Ph.D. Thesis, University of Wisconsin at Madison, 1994.

30. Jin, Z.; Danilova, V.; Assadi-Porter, F.; Zhao, Q.; Aceti, D.; Markley, J.; Hellekant, G. In Monkey electrophysiological responses to brazzein mutants-where are the critical regions for brazzein's sweet taste, 2nd International Symposium on Sweetener, Hiroshima, Japan, 2002; Hiroshima, Japan, 2002; pp 143, P-28.

31. Green, B. G.; Dalton, P.; Cowart, B.; Shaffer, G.; Rankin, K.; Higgins, J., Evaluating the 'Labeled Magnitude Scale' for measuring sensations of taste and smell. Chem Senses **1996**, *21, (3)*, 323-334.

32. Green, B. G.; Shaffer, G. S.; Gilmore, M. M., Derivation and evaluation of a semantic scale of oral sensation magnitude with apparent ratio properties. Chem. Senses **1993**, *18, (6)*, 683-702.

33. Brouwer, J. N.; Glaser, D.; Hard Af Segerstad, C.; Hellekant, G.; Ninomiya, Y.; Van der Wel, H., The sweetness-inducing effect of miraculin; behavioural and neurophysiological experiments in the rhesus monkey Macaca mulatta. Journal of Physiology **1983**, *337*, 221-240.

34. Yang, R.; Tabata, S.; Crowley, H. H.; Margolskee, R. F.; Kinnamon, J. C., Ultrastructural localization of gustducin immunoreactivity in microvilli of type II taste cells in the rat. J Comp Neurol **2000**, *425, (1)*, 139-151.

35. Pfaffmann, C., Species differences in taste sensitivity. Science **1953**, *117*, 470.

36. Beidler, L. M.; Fishman, J. Y.; Hardiman, C. W., Species differences in taste responses. American Journal of Physiology **1955**, *181*, 945-949.

37. Carpenter, J. A., Species differences in taste preferences. J. Comp. Physiol. Psychol **1956**, *49*, 139-144.

38. Zotterman, Y., Species differences in the water taste. Acta physiol. scand. **1956**, *37*, 60-70.

39. Glaser, D., Specialization and phyletic trends of sweetness reception in animals. Pure Appl Chem **2002**, *74, (2)*, 1153-1158.

40. Hellekant, G.; Segerstad, C. H.; Roberts, T.; van der Wel, H.; Brouwer, J. N.; Glaser, D.; Haynes, R.; Eichberg, J. W., Effects of gymnemic acid on the chorda tympani proper nerve responses to sweet, sour, salty and bitter taste stimuli in the chimpanzee. Acta Physiology Scand **1985**, *124, (3)*, 399-408.

41. Hellekant, G.; Ninomiya, Y.; Danilova, V., Taste in chimpanzees II: single chorda tympani fibers. Physiol. Behav. **1997**, *61, (6)*, 829-841.
42. Danilova, V.; Hellekant, G.; Jin, Z. In Effect of miraculin on behavioral and single taste fibers responses in common marmoset, Callithrix jacchus jacchus., Chem Senses, Sarasota FL, 1998; Sarasota FL, 1998; p 550.
43. Hellekant, G.; Ninomiya, Y.; Danilova, V., Taste in chimpanzees III: Labeled line coding in sweet taste. Physiol. Behav. 1998, 65, (2), 191-200.
44. Danilova, V.; Hellekant, G., Elucidating coding of taste qualities with the taste modifier miraculin in the common marmoset. Brain Res Bull **2006**, *68, (5)*, 315-321.
45. Hellekant, G.; Ninomiya, Y., On the taste of umami in chimpanzee. Physiology & Behavior **1991**, *49*, 927-934.
46. Ninomiya, Y.; Hellekant G. In Specific taste sensitivity of single chorda tympani fibers in chimpanzee, Proc. Jpn. Symp. Taste and Smell, 1991; 1991; pp 313-316.
47. Hellekant, G.; Ninomiya, Y., Bitter taste in single chorda tympani taste fibers from chimpanzee. Physiology & Behavior **1994**, *56, (6)*, 1185-1188.
48. Hellekant, G.; Y., N., Umami taste in chimpanzee (Pan troglodytes) and rhesus monkey (M. mulatta). In Olfaction and Taste XI, Kurihara, K.; Suzuki, N.; Ogawa, H., Eds. Springer Verlag: Tokyo, Berlin, Heidelberg, New York, London, Paris, HongKong, Barcelona, Budapest, 1994; Vol. XI, pp 365-368.
49. Hellekant, G.; Danilova, V., Species differences toward sweeteners. Food Chem. **1996**, *56*, 323-328.
50. Danilova, V.; Hellekant, G.; Roberts, R.; Tinti, J. M.; Nofre, C., Behavioral and single chorda tympani taste fiber responses in the common marmoset, Callithrix jacchus jacchus. An. NY Acad. Sci. Olfaction and Taste XII **1998**, *855*, 160-164.
51. Danilova, V.; Hellekant, G., Sense of taste in a New World monkey, the common marmoset. II. Link between behavior and nerve activity. J Neurophysiol **2004**, *92, (2)*, 1067-1076.
52. Frank, M., Taste responses of single hamster chorda tympani nerve fibers. In Olfaction and Taste IV, Schneider, D., Ed. Wissenschaftliche: Stuttgart, 1972; Vol. IV, pp 287-293.
53. Frank, M. E., An analysis of hamster afferent taste nerve response functions. Journal of General Psychology **1973**, *61*, 588-618.
54. Nowlis, G. H.; Frank, M. E. In Qualities in hamster taste: behavioral and neural evidence, Olfaction and Taste VI, Paris, 1977; Paris, 1977; pp 241-248.
55. Frank, M. E.; Bieber, S. L.; Smith, D. V., The organization of taste sensibilities in hamster chorda tympani nerve fibers. J. Gen. Physiol. **1988**, *91, (6)*, 861-896.

56. Frank, M. E., Neuron types, receptors, behavior, and taste quality. Physiol Behav **2000**, *69, (1-2)*, 53-62.

57. Frank, M. E.; Formaker, B. K.; Hettinger, T. P., Peripheral gustatory processing of sweet stimuli by golden hamsters. Brain Res Bull **2005**, *66, (1)*, 70-84.

58. Danilova, V.; Hellekant, G., Oral sensation of ethanol in a primate model III: responses in the lingual branch of the trigeminal nerve of Macaca mulatta. Alcohol **2002**, *26, (1)*, 3-16.

Chapter 14

The Bittersweet Search for Bitter–Sweet Interactions: Cell to Cell Communication in the Taste Bud

M. Scott Herness, Fang-li Zhao, and Yu Cao

College of Dentistry, The Ohio State University, 305 West 12th Avenue, Columbus, OH 43210

Bitter and sweet taste qualities are often considered polar opposites of gustatory sensation. Consequently, it seems appropriate that responses to bitter and sweet stimuli demonstrate some degree of segregation at all levels of the neuraxis, from taste buds to the cerebral cortex. Our research suggests that taste receptor cells representing these qualities may interact in the taste bud via cell to cell communication. Though previously thought to communicate only with the afferent nerve fiber that connects them to the central nervous system, taste receptor cells employ both neurotransmitters, such as serotonin and GABA, and neuropeptides, such as cholecystokinin and neuropeptide Y, as mechanisms of cell to cell communication. These signaling agents may elicit either excitatory or inhibitory responses. Expression patterns of these signaling molecules and their receptors appear to be segregated when compared to the expression of bitter (T2R) and sweet (T1R) receptors. These findings suggest that complex hard wiring patterns exist within the taste bud that harbor the potential for bitter T2R expressing cells to inhibit sweet T1R expressing cells. Thus the taste bud may operate as a computational unit, processing and sharpening information contained within the sensory signal prior to initiation of the neural output.

All sensory systems must solve the same problem: stimulus detection requires transduction, that is, the energy inherent in the stimulus must be converted into a form that is decipherable by the nervous system. Moreover, not only must the presence of the stimulus be faithfully detected but also all the full array of the accompanying stimulus dimensions. For example, the retina must detect the presence of light as well as its color, shape, and position in three-dimensional space. Each sensory system has evolved particular strategies that optimize this task for the unique features of its stimulus. These strategies are multifold and utilize not only complex biochemical reactions or specialized biophysical properties of ion channels but also something as pedestrian as anatomy. The spherical structure of the retina encodes the three dimensions of the world; the tonotopic array of the basilar membrane along the turnings of the cochlea is used to encode frequency. Implicit in these observations is that biological purpose is served by anatomical specializations.

Such anatomical specializations may seem less obvious in the gustatory system. However, one strongly conserved and unifying feature of vertebrate gustation is the presence of taste buds. This morphological structure—the taste bud—is highly conserved across virtually all vertebrate species. Whereas other sensory systems have receptor cells more or less evenly distributed across a sheet of epithelium (such as vision, audition, and olfaction), the gustatory system has coalesced its receptor cells into spherical structures. The conservation of this strategy has long lead to suggestions that something inherent in this anatomy is fundamental to its function. However, deciphering this rationale has been problematic and the issue remains unresolved. Recent developments bring to light why this highly conserved anatomy of the taste bud may be essential to gustatory function.

Taste sensations arise from interactions of taste stimuli with individual taste receptor cells that comprise the taste bud. The precise functional roles of these cells are still imprecisely understood. Paring individual receptor cell types to stimuli that give rise to sensations categorized as sweet, sour, salty, bitter has never been straightforward. What has emerged is that receptors for sweet and for bitter are expressed in non-overlapping groups within the bud. The dichotomy of this distribution makes plausible the notion that these cells could be communicating with one another. Indeed, the entire anatomy of the taste bud is optimal anatomy such cell to cell interactions to be occurring. How might cells be communicating with one another in the taste bud? Recent studies have elucidated a number of classic neurotransmitters as well as neuropeptides that are expressed in the taste bud. Cell-to-cell communication provides greater flexibility and more complicated avenues of processing. Both divergent and convergent streams of information processing are possible. Additionally, the possibility of lateral inhibition within the taste bud exists. The identity of taste qualities may be sharpened if, for example, lateral inhibition of sweet and bitter

taste qualities may exist. Cell-to-cell communication thus allows for the taste bud to operate as a complicated processing unit for sensory discrimination.

Taste receptor cells express multiple neurotransmitters and neuropeptides.

Cell-to-cell communication may be a critical feature of how taste buds inform the central nervous system of the identity and intensity of chemical stimuli in the oral cavity. To date a number of potential signaling molecules have been identified that could serve as a substrate for cell-to-cell communication. These signaling molecules include both classic neuron-transmitters and neuropeptides.

The concept that multiple signaling molecules operating simultaneously within the taste bud during gustatory stimulation has received little discussion to date largely because much of these data have been obtained recently. There now exists evidence for at six neurotransmitter systems and potentially four neuropeptide signaling agents in the rodent taste bud. The presence of multiple classic neurotransmitters in the taste bud include cholinergic, adrenergic, purinergic, glutamatergic, gabaergic, and serotonergic systems (*1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14*). Extant evidence for each varies. For adrenergic, purinergic, and serotonergic neurotransmission, there exists evidence for expression of the neurotransmitter and at least one subtype of that it's receptor as well as consequent physiological action when that receptor is activated (*1, 8, 9, 10, 14*). For others, such as acetyl choline, evidence exists for the receptor and patent physiological actions consequent to its activation without any direct evidence of expression of the neurotransmitter in taste receptor cells (*13*). Glutamate remains a special case since it serves as both a neurotransmitter and a umami taste stimulus. Several types of glutamate receptors are likely present in rodent taste buds, including metabotropic receptors and ionotropic receptors and a dimer of the T1R family (T1R1/T1R3) that serves as an amino-acid receptor (*4, 15, 16*). Physiological and pharmacological evidence suggests that rat taste receptor cells respond to stimulation of these receptors in manners appropriate for tastant or transmitter actions (*2, 3, 11*). Purinergic receptors exist on both taste receptor cells and nerve fibers within the taste bud (*1, 14*). These neurotransmitters may produce either excitatory or inhibitory actions. For example, acetyl choline is very stimulatory at increasing elevations of intracellular calcium (*13, 25*). Norepinephrine may act as excitatory through beta receptors whereas it may be inhibitory to those cells expressing alpha adrenergic receptors (*8, 9*). Serotonin appears to inhibit neighboring cells (*5, 6, 7*). Some, such as serotonin and ATP, may be important not only for cell to cell communication and for communication with the afferent nerve fiber (*7, 14*).

In addition to neurotransmitters, taste receptor cells express several neuropeptides. These include vasoactive intestinal peptide (VIP),

cholecystokinin (CCK), neuropeptide Y (NPY) and somatostatin (SST; *25, 18, 19, 20, 21*). In general, less is known of neuropeptides when compared to the neurotransmitters. Of these four candidates, CCK remains the best studied to date. CCK operates as an excitatory peptide that appears to operate in an autocrine manner through CCK-A subtype of the CCK receptors (*19*). Application of exogenous CCK to taste receptor cells results in a number of physiological actions on the cell, all of which are excitatory. These include inhibition of potassium currents and elevations of intracellular calcium. Recent investigations have elucidated physiological roles of NPY within the taste bud (*20*). VIP and SST are less explored. Though evidence exists for the expression of these peptides, nothing is yet known of their receptor expression or physiological actions resulting from receptor activation.

Of these neurotransmitters and neuropeptides, recent investigations in our laboratory suggest that two may serve as inhibitory pathways within the taste bud – Neuropeptide Y and GABA. The remainder of the chapter will summarize data on these two signaling agents and conclude with putative manners in which these inhibitory pathways could interact with sweet and bitter signaling within the taste bud.

Neuropeptide Y acts as an inhibitory paracrine signaling agent in the taste bud.

The discovery of the neuropeptide cholecystokinin in taste receptor cells and its excitatory physiological actions (*19*) raised the possibility that other neuropeptides may play yet undiscovered roles in peripheral taste processing. Neuropeptide Y, a member of the brain-gut family of peptides that also includes CCK and VIP, was a logical candidate for investigation since it is also widely expressed throughout the nervous system and, like CCK, is implicated in feeding behavior.

A subset of taste receptor cells express the neuropeptide NPY.

Like CCK, immunocytochemical investigation demonstrated that NPY is expressed by a subset of taste receptor cells. Using a polyclonal NPY antibody, several immunopositive cells were observed within each taste bud. These cells displayed reaction product that was evenly distributed throughout the cytoplasm with clear round nuclei (Figure 1). Positive cells were observed in fungiform, foliate and circumvallate papillae, as well as taste buds located in the nasoincisor ducts. All taste buds displayed some immunopositive cells.

To verify the expression of NPY in TBCs, RT-PCR experiments were performed on total RNA isolated from individually harvested circumvallate taste

CV   FOL

NID   AT

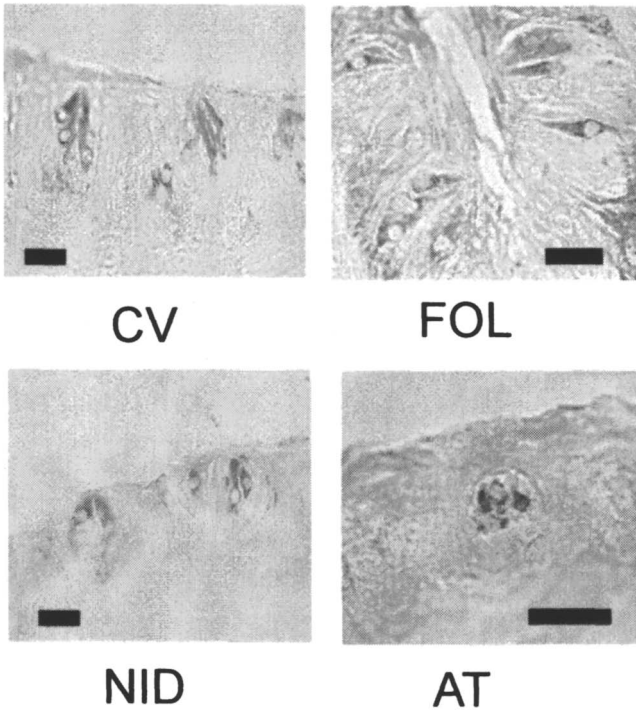*Figure 1. Photomicrographs of taste buds containing NPY immunopositive taste bud cells from circumvallate (CV), foliate (FOL), and fungiform papillae (AT) of the rat tongue and from the nasoincisor ducts (NID). Calibration bars represent 20 microns. (Reproduced from reference 20. Copyright 2005 Proc. Natl.Acad. Sci.)*
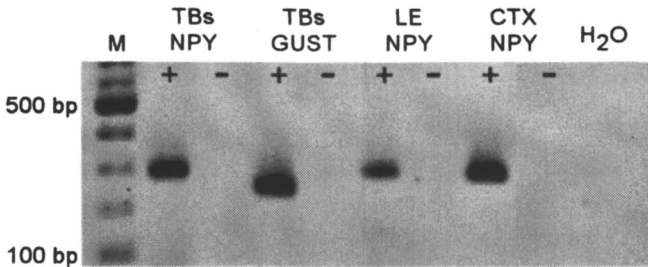

Figure 2. RT-PCR results. (Reproduced from reference 20. Copyright 2005 Proc. Natl.Acad. Sci.)

buds. Single buds were pooled (20 to 50), lysed, and total RNA was extracted. A previously published (19) primer set specific for the rat NPY gene was used: [forward primer, 5'-GCT AGG TAA CAA ACG AAT GGG G-3'; reverse primer, 5'-CAC ATG GAA GGG TCT TCA AGC-3"] that produced an expected product size of 288 bp was used. RT-PCR experiments included positive control primer sets for gustducin (GUST, expected product size, 231 bp), a G protein highly expressed in many TBCs, and β-actin to verify the integrity of the extracted RNA. RT-PCR conditions for the NPY primer set were optimized on RNA extracted from cerebral cortex, which was used as a positive control tissue. All experiments were performed with parallel negative control experiments that either omitted the reverse transcriptase enzyme (RT-) or template (H_2O control). These control experiments yielded expected results. PCR product derived from taste bud template using NPY primers was sequenced to confirm its identity. Results are illustrated in Figure 2. Bands of expected size for positive control tissues, GUST in TBs, and NPY mRNA in rat cortex, were observed. Bands for PCR products indicative of NPY mRNA were observed in two experimental samples, pure TB as well as lingual epithelium containing TBs. PCR with (+) or without (-) inclusion of reverse transcriptase is illustrated for each primer set.

These data suggest that some taste receptor cells express the neuropeptide NPY. Since physiological actions of exogenous CCK on taste receptor cells were previously observed, similar techniques were employed to investigate possible actions of NPY on taste receptor cells. Our working hypothesis is that neuropeptides may be playing neuromodulatory roles within the taste bud.

NPY inhibits the electrical activity of taste receptor cells.

If neuropeptides play neuromodulatory roles within the taste bud, putative physiological actions of NPY on taste receptor cells might involve changes in the electrical excitability of the cell. To begin to investigate what role NPY may play in the taste bud, possible physiological actions of NPY on taste receptor cells were tested with patch clamp recordings on dissociated cells. In preliminary examinations, no major actions were noted on many of the ionic currents endogenous to taste receptor cells including voltage-dependent sodium current, outward potassium current, or chloride current. However, when the inwardly-rectifying potassium current (K_{IR}) was examined, a reversible enhancement of this current was noted in the presence of exogenous application of NPY in the nanomolar concentration range (Figure 3). This action was concentration dependent over the range of 1 to 500 nM. Cells that did not respond to NPY, when pooled, produced no response (101 $\pm$ 1.1%, n=66). These observations suggest that NPY responses are likely dependent upon the expression or absence of NPY receptors. The magnitude of the peak current
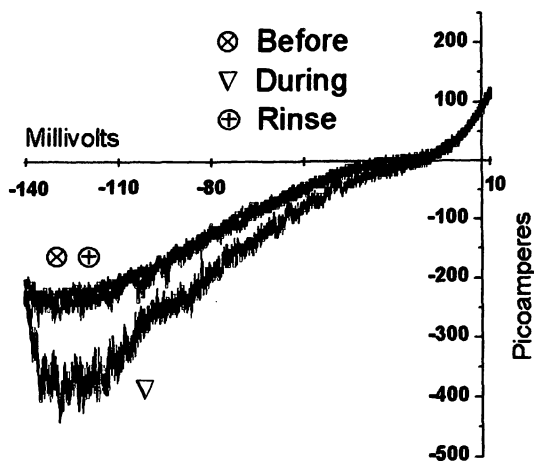
Figure 3. Patch clamp recordings of inwardly rectifying potassium current from a posterior taste receptor cell before, during, and after application of exogenous NPY. (Reproduced from reference 20. Copyright 2005 Proc. Natl.Acad. Sci.)

increased by up to 30% and was reversible with washout of the peptide. Since the K_{IR} current helps to set the resting potential of taste receptor cells (*22*) its enhancement would act to hyperpolarize the resting potential of the cell by allowing to move closer to the potassium equilibrium potential. Interestingly, the action of NPY is opposite to that observed for CCK.

NPY inhibition acts through NPY-1 receptors.

Although responses of an excitable cell to a neuropeptide such as NPY would be predicted to be mediated by a corresponding neuropeptide receptor, peptides could alternatively produce responses mediated by taste receptors (e.g. some peptides produce a bitter taste and hence could be detected by a member of the T2R family). To both verify neuropeptide receptor mediation and to begin identification of NPY receptor subtype, a pharmacological approach was taken to investigate the NPY response. Of the six members of the NPY receptors family, the NPY-1 receptor subtype was targeted since it is the most commonly expressed. Both an NPY-1 receptor antagonist, BIBP3226 [(R)-N 2-(diphenylacetyl)-N-[(4-hydroxy-phenyl)methyl]- D-argininamide], and a NPY-1 receptor agonist, [Leu31, Pro34]-NPY, were employed. BIBP3226 was observed to significantly decrease the response produced by either 10 or 100 nM NPY (p < 0.005).

To further confirm involvement of the NPY receptor subtype, [Leu31, Pro34]-NPY was tested. At 10 nM, 7 of 21 (33%) tested cells responded to this NPY-1 receptor agonist and at 100 nM 13 of 36 (36%) tested cells responded. These percentages are similar to those produced by NPY itself. If either concentration was preceded by exposure to 1 μM BIBP3226 only 7 or 8% responded (10 and 100 nM $p < 0.03$, $p < 0.02$, respectively;), suggesting that the NPY-1 receptor antagonist could successfully block the response of to a NPY-1 receptor agonist. Collectively, these data strongly imply NPY responses to be mediated by the NPY-1 receptor subtype.

NPY and NPY-1 receptors operate in a paracrine manner.

The consistency of these patch-clamp results suggests NPY operates within the taste bud via NPY-1 receptors. Next, the presence of NPY-1 receptors was confirmed with immunocytochemistry. Using an antibody specific for the NPY-1 receptor subtype on taste receptor cells, both basal cells and elongate taste receptor cells were observed to be immunopositive. The presence of NPY-1 receptor immunopositive cells corroborates the pharmacological results. The significance of immunopositive basal cells within the taste bud and lingual epithelium has yet to be pursued.

An essential question pertaining to how NPY may operate in the taste bud is the relationship of NPY-expressing cells to those cells expressing the NPY-1 receptor. For example such a relationship could be expected to be either autocrine, where the same subsets of taste receptor cells within the taste bud express both NPY and NPY-1 receptor, or paracrine, where these two signaling molecules are expressed in non-overlapping populations within the bud. To test for these possibilities, fluorescent double label immunocytochemistry was performed using antibodies against NPY and NPY-1 receptor. The results of these experiments on taste buds within rat foliate and crimumvallate papillae have provided a consistent answer to this question. In any taste bud where both NPY and NPY-1 receptor expressing cells were observed, the cells were always non-overlapping. This provides strong support to a paracrine signaling role for NPY within the taste bud.

NPY co-localization studies.

To examine whether the peptides CCK, VIP, and NPY are expressed in discrete non-overlapping subpopulations of taste receptor cells or whether they are expressed in similar subsets of cells, double labeling immunocytochemistry experiments were performed. In separate experiments, two primary antibodies,

each directed against a different peptide, was visualized using the fluorescent immunocytochemistry using Cy3 or FITC fluroprobes (see *20* for details).

Results of double labeling experiments with antibodies against NPY and CCK demonstrated two patterns of immunopositive cells. Most cells displayed immunoreactivity for both antigens whereas a smaller subset of labeled cells displayed immunoreactivity for CCK alone. Single-labeled NPY cells were rarely observed. In a quantitative analysis, 68% of all the CCK-containing cells co-expressed NPY whereas 95% of all the NPY-expressing cells co-expressed CCK. Similar experiments were conducted to the neuropeptides NPY and VIP. Results of the NPY:VIP combination were strikingly similar to those produced in the NPY:CCK experiments. Two major types of labeled subsets of taste receptor cells emerged from these experiments. Most labeled cells demonstrated fluorescence to both fluroprobes demonstrating double label for both NPY and VIP whereas fewer labeled taste receptor cells were immunopositive for VIP alone. Cells were rarely observed that labeled for NPY without co-incident VIP labeling. In these experiments, about 59% of all the VIP-expressing cells co-expressed NPY whereas 98% of the NPY-expressing cells co-expressed NPY. To complete the examination of co-expression patterns, a final series of experiments with double-labeling immunocytochemistry was performed using two primary antibodies directed against CCK or VIP. With this combination the majority of cells displayed immunofluorescence to both peptides. Additionally, single-labeled taste receptor cells, though fewer in number, for either CCK or VIP were also observed. These double labeled cells represent 76% of all the CCK-expressing cells and 73% of all the VIP-expressing cells. A quantitative illustration of the overlapping distribution pattern of the expression of these neuropeptides is presented in Figure 4.

Summary of NPY in the taste bud.

The combined results of these multiple approaches studying NPY in taste receptor cells suggest that NPY is expressed within the taste bud, in addition to previously observed CCK and VIP expression. NPY appears to operate opposite to that of CCK—it is inhibitory rather than excitatory; it operates in a paracrine rather than autocrine manner. Moreover, these two peptides seem to arise from the same pre-synaptic cell, suggesting they have divergent post-synaptic actions both in terms of directionality and effect.

Gamma amino butyric acid (GABA)

A second inhibitory pathway within the taste bud involves the classic neurotransmitter, γ-amino butyric acid (GABA). Prior to our investigations,
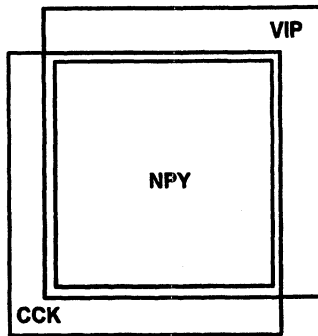
Figure 4. Overlapping expression pattern of three neuropeptides within taste receptor cells. (Reproduced from reference 20. Copyright 2005 Proc. Natl.Acad. Sci.)

little was known regarding GABA in the taste bud. An early study, using immunocytochemical techniques, had reported localization of GABA and a GABA transporter, GAT3, to subsets of taste receptor cells in rat circumvallate papillae (*12*). However, no studies were available to demonstrate any physiological data that could suggest a functional role for GABA among taste receptor cells. The present studies were designed to characterize in greater detail both the expression pattern of GABA, to explore the subtypes of GABA receptors expressed within the taste bud, and to examine any putative physiological actions on taste receptor cells. As well, co-localization studies of GABA or GABAergic markers with other signaling agents in the taste bud helped to provide inferential evidence for the role of GABA in sweet and bitter transduction cascades.

Some taste receptor cells in rat taste buds are GABAergic.

Using immunocytochemistry with an antibody specific to GABA, GABAergic taste receptor cells in posterior taste buds could be demonstrated. Subpopulations of taste receptor cells in foliate and circumvallate papillae of the rat tongue were observed to be immunopositive (Figure 5) and most cross sections of taste buds contained immunopositive cells. These cells were often located in the center of the bud. Immunoreactivity was observed to be evenly distributed across the cytoplasm. Immunopositive cells typically had large round or oval nuclei that were situated in the middle of the cell. These observations help to confirm those of Obata et al. (*12*).
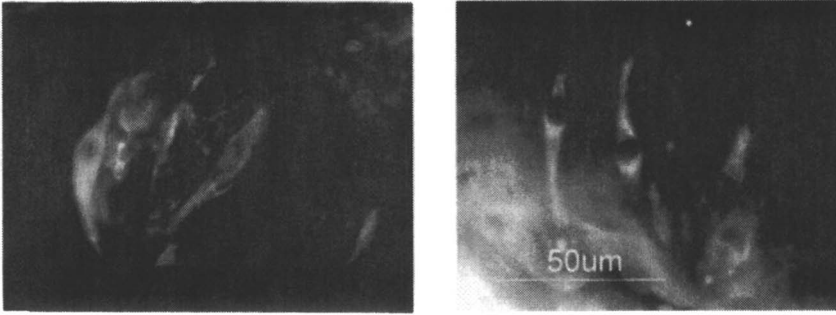
Figure 5. Localization of GABA-immunoreactive (left) and GAD65/67 immunoreactive (right) taste receptor cells in rat posterior taste buds.

To further corroborate that some taste receptor cells are GABAergic, expression of GABA's key synthetic enzyme, glutamic acid decarboxylase (GAD), was examined using both western blotting and immunocytochemical techniques. Total protein extracts from taste papillae of anterior and posterior regions of rat tongue were separated based on molecular weight using PAGE electrophoresis, transferred to a PVDF membrane, and reacted with an antibody directed against GAD that recognizes both its 65 and 67 kD isoforms (GAD65/67). Immunoreactive bands of the appropriate molecular weight were observed. This same antibody was also employed for immunocytochemical investigation of taste buds. In these experiments, immunopositive cells were observed that displayed similar morphology to the previously observed GABAergic cells. Collectively, these data strongly support expression of GABA in a subset of taste receptor cells within the taste bud.

GABA inhibits the electrical activity of taste receptor cells via GABA$_A$ and GABA$_B$ receptors.

Experiments to test for physiological actions of GABA on taste receptor cells were designed based on its well known physiological actions on neurons mediated by its well characterized receptors. GABA exerts inhibitory actions on neurons through either ionotrophic GABA$_A$ or metabotrophic GABA$_B$ receptors. GABA$_A$ receptors are chloride channels whereas GABA$_B$ receptors often couple to inwardly rectifying potassium currents via G-proteins. Patch clamp experiments were designed to test for each of these possible effects.

Using isolated taste receptor cells from rat foliate and circumvallate papillae with whole cell or perforated patch recordings, chloride currents were isolated

using pharmacology and ion substitution (*e.g.*, *8*) and tested with a ramp command potential protocol. In rat taste receptor cells, chloride currents are small compared to other currents, such as its potassium currents, with maximal magnitudes typically less than 200 pA. Exogenous application of GABA enhanced both the outward and inward portions of the chloride current (*e.g.* Figure 6). With focal application of GABA over the tested range of 30 μM to 2 mM, concentration dependent enhancement of chloride currents were observed and were reversible with washout of GABA. $GABA_A$ receptor-specific agonists muscimol and isoguvacine were also tested. Both were able to induce elevation of current amplitudes in a concentration-dependent manner for both outward and inward portions of the chloride current.

Unlike $GABA_A$ receptors, $GABA_B$ receptors are metabotropic, coupling to G proteins and modulating potassium channels to mediate their long term effects. In most cases this modulation acts on inwardly rectifying potassium channels that produce the K_{IR} current. The K_{IR} current has been previously characterized in rat taste receptor cells (*22*). It contributes to the resting potential and helps to stabilize the membrane potential. When applied focally, GABA, ranging from 500 μM to 2 mM, effectively elevated the current magnitude of the inwardly rectifying portion of the K_{IR} current. Baclofen, a synthetic analog of GABA, is a potent $GABA_B$ receptor-specific agonist. When applied at 500 μM, baclofen produced similar effects on K_{IR} in approximately 30% of taste receptor cells recorded. Both GABA responses and baclofen responses on K_{IR} displayed receptor desensitization, a general attribute of G-protein coupled receptors. Significant reduction of response amplitude was observed when either GABA or baclofen was applied repeatedly.

Since $GABA_B$ receptors couple to G-proteins in order to exert their effects, an additional test of their participation involved treatment of taste receptor cells with GDP-βS. This nonhydrolyzable GTP analog inactivates G proteins by irreversibly binding to the alpha subunit. GDP-βS (2 mM) completely abolished baclofen's enhancing effects on K_{IR} in all of the 19 cells tested. Additionally, bacolfen's action on taste receptor cells was tested in the presence of the $GABA_B$ receptor specific antagonist, CGP 35348. In these experiments, the baclofen's enhancement on Kir current was effectively abolished. Nine of ten tested cells responded to baclofen alone but subsequently failed to respond to baclofen in the presence of CGP 35348.

To verify the expression of GABA receptors in taste receptor cells, immunocytochemistry and western blotting approaches were tested for the presence of either $GABA_A$ and $GABA_B$ receptors. The $GABA_A$ receptor is a composed of multiple subunits categorized into seven classes: α_{1-6}, β_{1-4}, δ, ϵ, π, and θ. Functional $GABA_A$ receptors contain at least one α, one β, and one δ subunit isoform. Of the alpha subunits, the $\alpha 1$ and $\alpha 3$ subunits are the most common. In western blotting experiments using antibodies to either the $\alpha 1$ or the
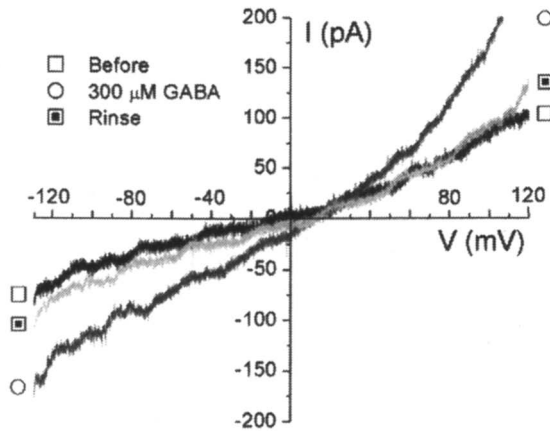
Figure 6. GABA's enhancement of chloride currents recorded from taste receptor cells.

α3 subunit, bands were observed to only the α1 subunit in membrane protein extracts of both anterior and posterior taste papillae. This receptor was observed to be expressed in a subset of taste receptor cells using immunocytochemistry with the same antibody whereas no immunopositive cells were observed when the α3 antibody was tested. Forebrain sections served as positive control for each immunocytochemical experiment. Alpha-1 immunopositive cells were elongate with processes extending from apical end to basement membrane and contained a clear circular or oval nuclear region.

The GABA$_B$ receptor belongs to a particular group of G-protein coupled receptors which operate via heterodimer formation. The two subunits of this receptor are termed R1 and R2. Immunocytochemistry using an antibody directed against the R1 subunit revealed a subset of immunopositive cells within the taste bud. These cells were spindle-shaped and demonstrated a morphology similar to GABA-, GAD 65/67-, or GABA$_A$-α1 subunit–immunoreactive cells.

GABA's role in the taste bud is likely paracrine cell to cell communication.

The morphological features of GABA and GAD expressing taste receptor cells, such as their spindle shape and round nucleus, suggest that these cells resemble those expressing molecules of gustatory signaling cascades, such as gustducin. Since GAD65/67 immunocytochemistry is a more reliable marker of GABAergic cells than is GABA immunocytochemistry (which requires glutaraldehyde fixation), double labeling immunocytochemistry experiments

were performed using GAD65/67 and antibodies directed against taste receptor cells markers α-gustducin, neural cell adhesion molecule (NCAM), or PGP 9.5.

Overall, taste receptor cells expressing the protein markers α-gustducin, NCAM, or PGP9.5 represent distinctive cell populations in taste buds yet these cells all share morphological similarities to the GABAergic taste receptor cells. NCAM is expressed in a subset taste receptor cells in rat circumvallate papillae that are separate from those expressing α-gustducin. It is suggested that the NCAM expressing taste receptor cells synapse directly with the afferent nerve fiber. Most of these cells have elongate shape, relatively narrow nuclear region, and express serotonin. Many PGP 9.5 expressing taste receptor cells express in a subset of taste receptor cells that demonstrate NCAM-immunoreactivity. These PGP 9.5 taste receptor cells are thought to form synapses with afferent nerves but do not display serotonin immunoreactivity.

There was virtually no overlap of GAD 65/67 immunoreactive cells with either NCAM- or PGP9.5-immunoreactive taste receptor cells. In contrast, over half the GAD65/67-immunopositive cells also displayed α-gustducin immunoreactivity. This result implies that GABAergic taste receptor cells are unlikely to be the chemosensory cells that make direct synaptic contacts with gustatory afferent nerve fibers inside the taste bud and hence its primary function could be cell-to-cell communication within the bud. It also implies that GABA and serotonin are likely to be two separate populations of cells in taste buds since serotonin co-localizes with NCAM. On the other hand, co-localization of GAD65/67 and α-gustducin immunoreactive taste receptor cells suggests some GABAergic taste receptor cells are capable of transducing taste signals such as sweet or bitter. Therefore GABAergic taste receptor cells may be involved in transduction of signals belonging to multiple taste modalities.

$GABA_A$ or $GABA_B$ receptors could be localized either pre-synpatically or post-synaptically. In double labeling experiments using GAD 65/57 antibody and an antibody directed against $GABA_B$ R1 subunit, GAD 65/57 immunoreactive taste receptor cells appeared as a separate group of cells from the one exhibiting immunoreactivity to the $GABA_B$ receptor R1 subunit, indicating a paracrine role of GABA mediated by the $GABA_B$ receptor subtype in the taste buds.

Summary of GABA in the taste bud.

GABA is a classic inhibitory neurotransmitter distributed widely throughout the nervous system. Our data now strongly imply that GABA plays a role in cell to cell communication within the taste bud. Subsets of taste receptor cells express this neurotransmitters and subsets of taste receptor cells respond to this transmitter in an inhibitory fashion mediated by either $GABA_A$ or $GABA_B$ class

of receptors. Although the relationship of GABA and GABA receptor cells isn't completely elucidated, it is clear that for at least $GABA_B$ the relationship is paracrine. GABA is probably expressed in cells without synapses to the afferent nerve.

Discussion

Our laboratory has elucidated two previously unrecognized inhibitory signaling pathways within the taste bud that are likely playing important roles in processing of taste information via paracrine cell-to-cell communication. These agents—NPY and GABA—are only two among many newly discovered signaling molecules within the taste bud. The plethora of such transmitters and peptides suggest that cell-to-cell communication may be a critical feature of how taste buds inform the central nervous system of the identity and intensity of chemical stimuli in the oral cavity. After tastants are recognized by their corresponding receptors, downstream signal transduction events may include not only activation of an individual taste receptor but also both convergent and divergent forms of information transfer among the cells of the bud. This processing could include signaling of information from taste receptor cells without synapses to the afferent nerve to taste receptor cells that have such synapses. Additionally, other processes such as lateral inhibition among cells with differing tastant quality could help to sharpen the discharge of the afferent nerve.

Why are there so many neurotransmitters and neuropeptides expressed within the taste bud?

The seemingly large number of neurosignaling agents expressed within the relatively small structure of a single taste bud at first glance presents itself as both superfluous and enigmatic. For the moderate number of cells involved (50 to 100), the expression of more than a dozen signaling agents appears to create an unnecessarily complicated network. Moreover, the gustatory system needs to encode relatively few qualities (sweet, sour, salty, bitter, and umami). Coupled with the emerging observations that single taste receptor cells appear to express receptors for only a single taste quality, why design a system where signaling agents outnumber the encoded qualities by more than three to one? Some of this confusion is alleviated when the notion of co-transmission is considered.

Co-transmission of neurotransmitters and neuropeptides is a common motif of signaling molecules found from invertebrates to vertebrates. Neurotransmitters are sometimes accompanied by a second signaling agent, a neuropeptide, that is co-released from the same pre-synaptic terminal. Both

neurotransmitter and neuropeptide produce their neurophysiological effects via activation of their corresponding receptors postsynaptically. Although co-transmission may operate in a variety of manners, most commonly the neuropeptide is released at higher stimulus intensity where it plays a neuromodulatory to amplify the actions of the classic transmitter. Many other scenarios are also possible. The pattern of expression of signaling agents and their corresponding receptors produces a fixed "hard-wired" circuit within a neuronal network.

Co-transmission confers advantages to a neural network. One is that it extends dynamic flexibility to signaling possibilities. For example, the time course of communication can be altered. The slow signals set up by neuropeptides can adjust the gain of fast signals. This, in turn, can alter the intrinsic properties of networks dynamically and transform effects of fast synaptic actions. Additionally, it may increase sharpness of a signal or it may influence adaptation.

Taste receptor cells vary widely in different intrinsic membrane properties, in distribution ion channels, both voltage and ligand–gated. Alterations in balance of these conductances through the process of neuromodulation can modify firing properties. That taste receptor cells express neurotransmitter receptors and stimulation of these receptors produces patent physiological actions (*e.g.*, 5, 6, 9) requires a revisitation of peripheral gustatory signal processing. The most conservative biological explanation is that the pattern of neurotransmitter expression and neurotransmitter receptor expression defines the convergent and divergent pathways that represent a "hard wiring" of the system that "stores" information processing pathways (*23, 24*). The functional consequences of transmitter complexity are that multiple dimensions are encoded simultaneously. Salient questions are what produces neurotransmitter release (*i.e.*, the neurotransmitter expressing taste receptor cell) and what subsequent subsets of taste receptor cells are then modulated (*i.e.*, neurotransmitter receptor expressing taste receptor cells). There is some suggestive evidence at present that modulation could relate to sweet and bitter transduction pathways.

How do the known list of neurotransmitters and neuropeptides relate to sweet and bitter sensations within the taste bud?

If the plethora of signaling agents within the taste bud act to enhance coding of information related to taste quality and intensity by creating hard-wired pathways between defined sets of individual taste receptor cells, then it should be possible to elucidate correlative pathways between receptors and signaling agents. At present data are insufficient to assign roles of particular neurotransmitters to specific taste qualities. However, co-localization studies with signaling molecules, helps to make logical predictions.

Peptide co-localization studies with gustducin and T1R2 along with the physiological studies of the peptides help to set up some logical predictions as to how the expression of CCK and NPY might relate to sweet and bitter transduction schemes. The peptides CCK, VIP, and NPY show large overlap in their expression patterns (20). NPY-expressing taste receptor cells, the smallest in number, are virtually 100% overlap with CCK and VIP. Since CCK and VIP show little overlap with T1R2 expression, it is predicted that NPY will similarly show little overlap with T1R2. As well, since members of the T1R family co-express, NPY may co-express little with any member of this family.

On the other hand, these overlapping peptide expressing taste receptor cells do show overlap with gustducin expressing taste receptor cells. Since the T2R expressing cells are a subset of the gustducin expressing cells, if is perhaps probable that the peptide expressing cells also co-express T2R receptors. This notion is supported by the observation that CCK-expressing taste receptor cells respond well to bitter stimuli (25). Although co-locaization of peptide-expressing taste receptor cells with T2R receptors still requires experimental verification, the notion remains plausible. If NPY acts to inhibit surrounding cells, the NPY-1 receptor expressing cells, it is intriguing to speculate that these may be T1R-expressing cells. Hence NPY could potentially represent a mechanism for bitter responsive taste receptor cells to inhibit sweet responsive cells.

At present the relationship of GABA to sweet and bitter transduction mechanisms is less clear. In preliminary experiments, GABA-expressing taste receptor cells appear to co-localize with peptide-expressing taste receptor cells. This would suggest GABA could play an inhibitory role in the taste bud in concert with NPY. However, further studies will be required before reasonable hypothesis regarding the inhibitory role of GABA in processing taste quality information within the taste bud can be proposed.

References

1. Baryshnikov, S.G.; Rogachevskaja, O.A.; Kolesnikov, S.S. *J. Neurophysiol.* **2003**, *90*, 3283-3294.
2. Bigiani, A.; Delay, R.J.; Chaudhari, N.; Kinnamon, S.C.; Roper, S.D. *J. Neurophysiol.* **1997**, *77*, 3048-3059.
3. Caicedo, A.; Jafr, M,S; Roper, S.D. *J. Neurosci.* **2000**, *20*, 7978-7985.
4. Chaudhari, N.; Yang, H.; Lamp, C.; Delay, E.; Cartford, C.; Than, T.; Roper, S. *J. Neurosci.,* **1996**, *16*, 3817-3826.
5. Herness, S.; Chen, Y. *NeuroReport,* **1997**, *8*, 3257-3261.
6. Herness, M.S.; Chen, Y. *J. Membr. Biol.,* **2000**, *173*, 127-138.
7. Kaya, N.; Shen, T.; Lu, S.G.; Zhao, F.L.; Herness, S. *Am. J. Physiol.* **2004**, *286*, R649-R658.

8. Herness, M.S.; Sun, X.-D. *J. Neurophysiol.,* 1999, *82,* 260-271.
9. Herness, S.; Zhao, F.L.; Lu, S.G.; Kaya, N.; Shen, T.; Sun, X.-D. *J. Physiol. (Lond.),* 2002, *543,* 601-614.
10. Kim, D.-J.; Roper, S.D. 1995. *J. Comp. Neurol.,* 1995, *353,* 364-370.
11. Lin, W.; Kinnamon, S.C. *J. Neurophysiol.,* 1999, *82,* 2061-2069.
12. Obata, H.; Shimada, K.; Sakai, N.; Saito, N. *Molec. Brain Res.,* 1997, *49,* 29-36.
13. Ogura, T. *J. Neurophys,* 2002, *87,* 2643-2649.
14. Finger, T.; Danilova, V.; Barrows, J.; Bartel, D.L.; Vigers, A.J.; Stone, L.; Hellekant, G.; Kinnamon, S.C. *Science,* 2005, *310,* 1495-1499.
15. Chaudhari, N.; Landin, A..; Roper, S.D. *Nat. Neurosci.,* 2000, *3,* 113-119.
16. Nelson, G.; Chandrashekar, J.; Hoon, M.A.; Feng, L.; Zhao, G.; Ryba, N.J.P.; Zuker, C.S. *Nature,* 2002, *416,* 199-202.
17. Lu, S.G.; Herness, S. *Neurosci. Lett.,* 2003, *351,* 157-160.
18. Herness, M.S. *Neurosci.,* 1989, *33,* 411-419.
19. Herness, S.; Zhao, F.L.; Lu, S.G.; Kaya, N.; Shen, T. *J. Neurosci., 2002,* 22, 10018-10029.
20. Zhao, F.L.; Shen, T.; Kaya, N.; Lu, S.G.; Cao, Y.; Herness, S. *Proc. Natl. Acad. Sci. (USA),* 2005, *102,* 11100-11105.
21. Cao, Y.; Zhao, F.; Herness, M.S. *Chem. Sens.,* 2006, *31,* A22.
22. Sun, X.-D.; Herness, S. *Am. J. Physiol.* 1996, *271,* C1221-C1232.
23. Brezina, V.; Weiss, K.R. *Trends Neurosci.* 1997, *20,* 538-543.
24. Dumont, J.E.; Dremier, S.; Maenhaut, C. *Am. J. Physiol.* 2002, *283,* C2-C28.
25. Lu, S.G.; Zhao, F.L.; Herness, S. *Neurosci. Lett.* 2003, *351,* 157-160.

Chapter 15

cAMP: A Role in Sweet Taste Adaptation

Nirupa Chaudhari[1] and Sue C. Kinnamon[2]

[1]Department of Physiology and Biophysics, University of Miami Miller
School of Medicine, Miami, FL 33136
[2]Department of Biomedical Sciences, Colorado State University,
Fort Collins, CO 80523

Sweet taste transduction is initiated by sugars or synthetic
sweeteners binding to the G protein coupled receptor,
T1R2+T1R3, and activating G protein(s) and downstream
signaling effectors. Recent genetic and functional studies
implicated phospholipase C (PLCβ2) and Ca^{2+} release from
intracellular stores in sweet transduction. Considerable
evidence suggests that cAMP also plays a role in the sweet
response. Initial observations pointed to cAMP as the second
messenger, because sweet stimuli modulate cAMP levels in
taste tissue, and because membrane permeant cAMP strongly
influences the physiological response of taste buds to sweet
stimuli. Further, enzymes that regulate cAMP levels, adenylyl
cyclases (ACs) and phosphodiesterases (PDEs) and gustducin,
a G protein that can activate PDEs, are all expressed in many
sweet-sensitive taste cells. And, loss of gustducin impairs
sweet responses. Yet, the precise role of cAMP in sweet taste
remains unclear. Here, we review evidence that cAMP is
produced as a direct consequence of receptor activation, that
cAMP directly depolarizes mammalian taste cells, and that
cAMP-dependent Protein Kinase (PKA) likely underlies
adaptation to the sweet response. We suggest a model in
which the role of α-gustducin is to keep cAMP levels low to
prevent chronic adaptation of sweet-sensitive taste cells.

Introduction

Sweet taste transduction involves binding of sweet ligands (sugars, synthetic sweeteners, D-amino acids, and some sweet proteins) to G protein-coupled taste receptors of the T1R class[1]. T1R3, the third member of this class to be identified, was cloned from the murine *sac* locus. *Sac* "taster" strains show increased preferences and neural responses to sucrose and saccharin compared to "nontaster" strains [2-7]. When co-transfected in cell culture, T1R3 and the related GPCR, T1R2, form heterodimeric receptors that can be activated by sucrose and many synthetic sweeteners[4]. In contrast, T1R3+T1R1 heterodimers can be activated by L-amino acids, not by sugars or sweeteners[8,9]. In mouse, T1R3 is abundantly expressed all taste fields, while T1R2 is more prevalent in palate and vallate taste buds, and T1R1 is more abundant in fungiform taste buds[10]. Nevertheless, all three T1Rs are expressed to some extent in all taste fields.

In analogy to the thoroughly investigated steps in bitter transduction, ligand binding to sweet receptors is thought to result in $G\beta\gamma$ activation of the phospholipase, PLCβ2, producing the second messengers, IP$_3$ and diacylglycerol (DAG) and eventually releasing Ca^{2+} from intracellular stores[11]. The cation channel, TRPM5[12-15], is an essential component of sweet, bitter and umami transduction, although its exact role in the transduction cascade remains unclear. Knockout of either PLCβ2 or TRPM5 severely impacts sweet transduction[15], although a recent study shows that mice retain some sweet sensitivity after knockout of TRPM5[16].

Despite the recent emphasis on phosphoinositide signaling, a longstanding literature suggests that cAMP also plays a significant role in sweet transduction. Taste cells express several adenylyl cyclases (ACs)[17], phosphodiesterases (PDEs)[18], G proteins capable of activating ACs and PDEs[18-20], and direct cyclic nucleotide modulated ion channels[21,22]. In fact, *cAMP was originally proposed to be the main second messenger in sweet transduction*, based on the observation that sucrose and saccharin stimulated AC activity in rat anterior tongue epithelium[23]. Direct measurements of cAMP in circumvallate or fungiform taste buds showed that both sucrose and/or synthetic sweeteners increase cAMP levels[24-26].

Source of the cAMP signal

Synthesis of cAMP could be a direct consequence of receptor activation, perhaps by $G\alpha_s$ subunits stimulating AC. Conversely, cAMP synthesis could be secondary to the PLC-mediated increase in intracellular Ca^{2+}. Several ACs have

been identified in taste cells, including AC8, which is stimulated by Ca^{2+} [26]. To determine if the sucrose-evoked cAMP was Ca^{2+} dependent, paired halves of CV epithelium were stimulated with 500 mM sucrose + 0.3 mM IBMX, or with 0.3 mM IBMX alone. Sucrose stimulation resulted in a substantial accumulation of cAMP, compared with the control (Figure 1, left panel; [26]), as has been documented previously[26]. We then repeated this stimulation paradigm under conditions where Ca^{2+} was lacking in the extracellular milieu or when release of intracellular Ca^{2+} was blocked with a PLC inhibitor (Figure 1, middle and right panels; [26]). Depleting Ca^{2+} did not eliminate the sucrose-stimulated cAMP accumulation, suggesting that cAMP is produced directly as a consequence of receptor activation rather than downstream of Ca^{2+} signaling.

Physiological role of cAMP in taste

Pharmacological agents that modulate cAMP strongly influence taste cell and neural responses to sweeteners. Using loose patch recording from hamster taste buds in situ, we showed that membrane permeant analogs of cAMP, as well as IBMX and forskolin (each of which increase cAMP), elicited action potentials in sweet responsive taste buds[27] (Figure 2). Further, whole-cell patch clamp recordings showed that both synthetic sweeteners and membrane permeant cAMP analogs depolarized sweet-sensitive taste cells by blocking voltage-gated K^+ currents and a resting K^+ conductance (Figure 3 and[28]). Although the molecular identity of this K^+ channel has not been determined, we expect it would act in concert with TRPM5 to depolarize taste cells in response to sweet stimuli.

In the loose-patch recording configuration, responses to sucrose and the synthetic sweetener, NC01, persist in the presence of a membrane permeant PKA inhibitor, and even *increase* (Figure 4 and[29]). We interpret the results (Figures 2, 3, 4) to indicate that sweetener-evoked cAMP elevation has two sequential effects. First, cAMP directly modulates a membrane K^+ conductance to depolarize taste cells. Subsequently, cAMP produces adaptation by activating PKA, and phosphorylating signaling proteins. This phosphorylation could occur at several levels including the taste receptor itself, or various effectors of the PLC signaling pathway. Indeed, PKA-mediated phosphorylation is known to inhibit both $PLC\beta2$ and IP_3R3[30,31], both integral components of the phosphoinositide signaling pathway in taste cells. Phosphorylation-mediated suppression would develop slowly and with a delay. In contrast, the neural response to sweet compounds typically begins within seconds.
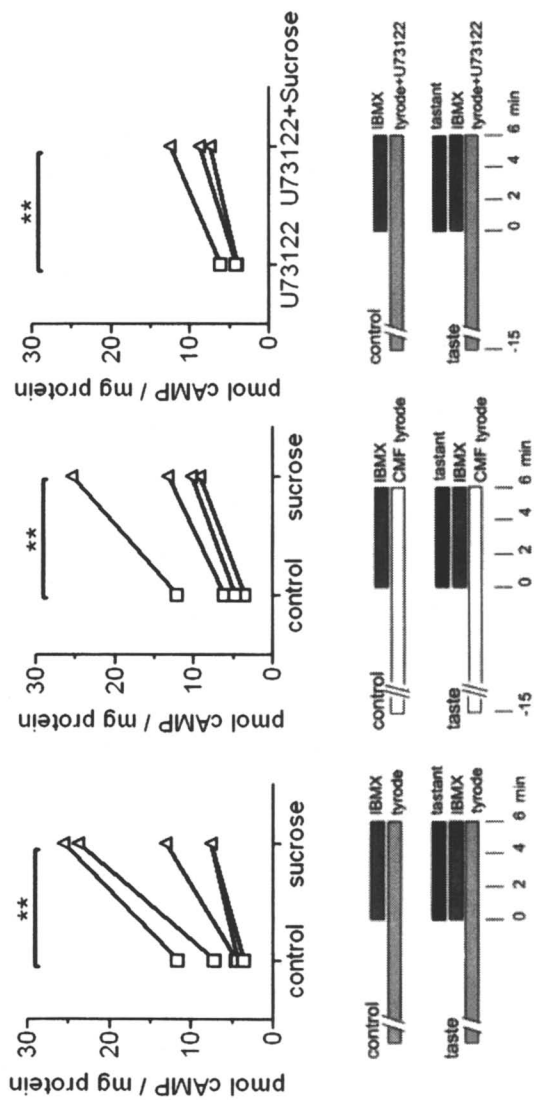
Figure 1. Sucrose stimulates cAMP accumulation in normal Tyrode's (left), Ca^{2+}/Mg^{2+} free (CMF) Tyrode's (middle), or in the presence of a PLC inhibitor, U73122 (right). (Adapted, with permission, from reference 26. Copyright 2006 The American Physiological Society.)
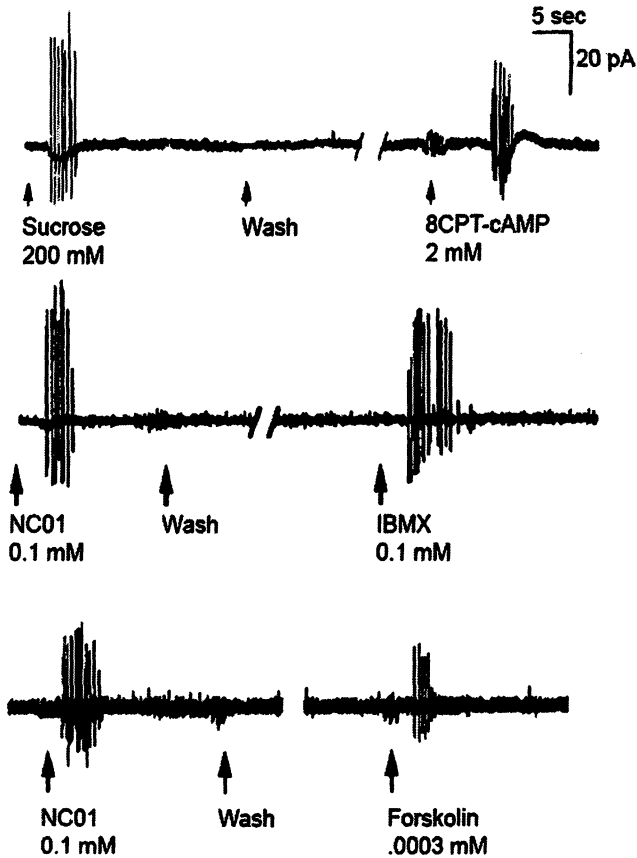
Figure 2. Loose patch recordings from hamster taste buds in situ showed that membrane permeant cAMP analogs (top) and agents that increase intracellular cAMP (middle, bottom) elicit action potentials in sweet responsive taste buds. (Adapted, with permission, from reference 27. Copyright 1993 The American Physiological Society.)
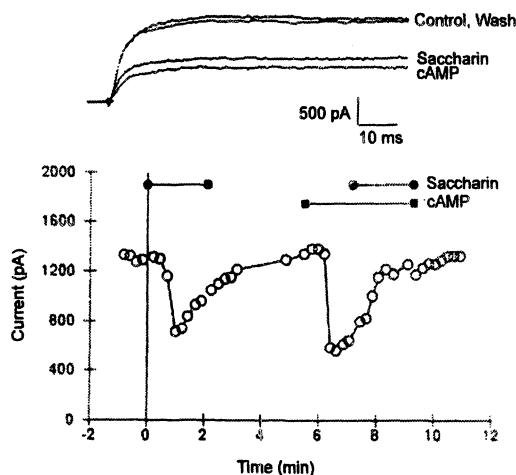
Figure 3. Whole-cell voltage clamp recording from a hamster fungiform taste cell showed that cAMP and saccharin both inhibited a tonically-active voltage-gated K⁺ current. The effect of cAMP and sweetener was not additive. (Adapted, with permission, from reference 28. Copyright 1996 The American Phsiological Society.)
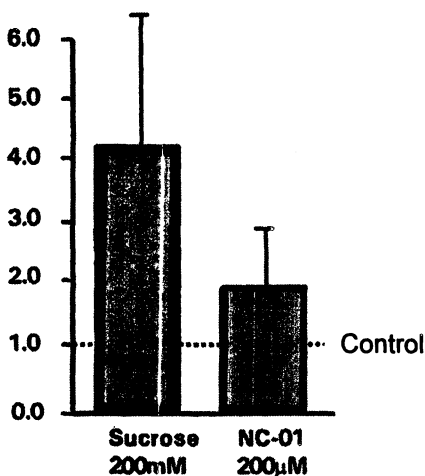
Figure 4. The PKA inhibitor H-89 increases the frequency of action potentials elicited by sucrose and the synthetic sweetener NC01. Sweetener-elicited frequency in the presence of inhibitor is shown normalized to frequency in absence of inhibitor. (Adapted, with permission, from reference 29. Copyright 2000 The American Physiological Society.)
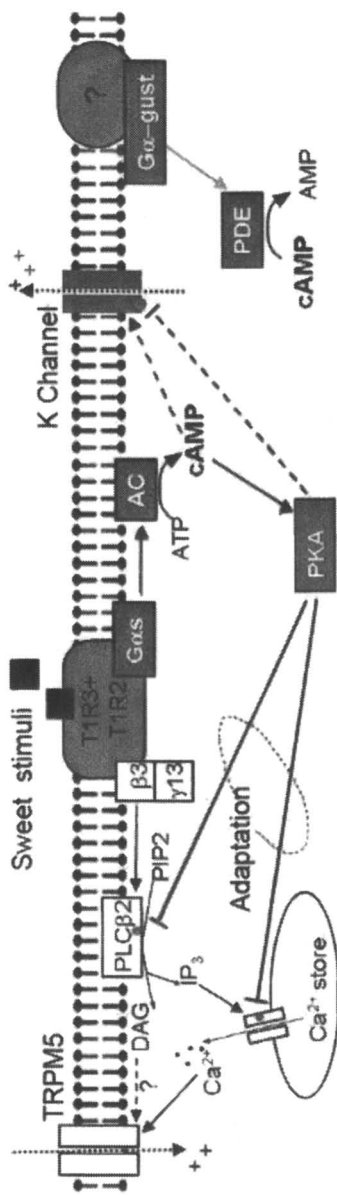
Figure 5. The proposed role of cAMP in sweet taste adaptation. α-gustducin, tonically active through an undefined receptor, keeps cAMP levels low at rest. In the absence of gustducin, increased basal levels of cAMP keep PKA activity elevated. This phosphorylates sweet taste signaling proteins, causing the taste cell to be chronically adapted to sweet stimuli.

Role of α-gustducin in sweet taste

α-Gustducin knockout mice are compromised to sweet stimuli[32], yet the mechanism for this has never been explained, especially considering the apparent centrality of the PLC signaling pathway[15]. Further, biochemical measurements have generally shown that sweet stimuli elevate, rather than decrease cAMP levels. The knockout effect for sweet appears to be predominantly limited to fungiform and palatal taste fields, where T1Rs and α-gustducin are expressed in the same taste cells[10,33]. Yet, even in these anterior taste fields, biochemical measurements have shown that sweet stimuli increase cAMP levels[25].

We present a model (Figure 5), based on new preliminary data obtained from circumvallate taste buds, that α-gustducin knockout mice have elevated *resting* levels of cAMP[34]. If this situation holds up for anterior taste fields, it would suggest that the role of α-gustducin is tonically to activate PDE, keeping basal cAMP levels low. If cAMP-dependent phosphorylation normally mediates adaptation, as we propose here, then taste buds in α–gustducin knockout mice would be in a chronically adapted state and unable to respond normally to sweet stimuli. Further studies, measuring cAMP levels in individual taste cells of gustducin knockout mice, will be required to verify if this model explains the gustducin knockout effect on sweet taste.

References

1. Hoon, M. A.; Adler, E.; Lindemeier, J.; Battey, J. F.; Ryba, N. J.; Zuker, C. S. *Cell* **1999**, *96*, 541-551.
2. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. *Nat Genet* **2001**, *28*, 58-63.
3. Montmayeur, J. P.; Liberles, S. D.; Matsunami, H.; Buck, L. B. *Nat Neurosci* **2001**, *4*, 492-498.
4. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S. *Cell* **2001**, *106*, 381-390.
5. Sainz, E.; Korley, J.N.; Battey, J.F.; Sullivan, S.L. *J. Neurochem* **2001**, *77*, 896-903.
6. Bachmanov, A.A.; Li, X.; Reed, D.R.; Ohmen, J.D.; Li, S.; Chen, Z.; Tordoff, M.G.;deJong, P.J.;Wu, C.; West, D.B.; Chatterjee, A.; Ross, D.A.; Beauchamp, G.K. *Chem. Senses* **2001**, *26*, 925-933.
7. Kitagawa, M.; Kusakabe, Y.; Miura, H.; Ninomiya, Y.; Hino, A. *Biochem Biophys Res Commun* **2001**, *283*, 236-242.
8. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc Natl Acad Sci U S A* **2002**, *99*, 4692-4696.

9. Nelson, G.; Chandrashekar, J.; Hoon, M. A.; Feng, L.; Zhao, G.; Ryba, N. J.; Zuker, C. S. *Nature* **2002**, *416*, 199-202.

10. Stone, L. M.; Barrows, J.; Finger, T. E.; Kinnamon, S. C. *Chemical Senses* **2006**, *in press.*

11. Huang, L.; Shanker, Y. G.; Dubauskaite, J.; Zheng, J. Z.; Yan, W.; Rosenzweig, S.; Spielman, A. I.; Max, M.; Margolskee, R. F. *Nat Neurosci* **1999**, *2*, 1055-1062.

12. Perez, C. A.; Huang, L.; Rong, M.; Kozak, J. A.; Preuss, A. K.; Zhang, H.; Max, M.; Margolskee, R. F. *Nat Neurosci* **2002**, *5*, 1169-1176.

13. Hofmann, T.; Chubanov, V.; Gudermann, T.; Montell, C. *Curr Biol* **2003**, *13*, 1153-1158.

14. Liu, D.; Liman, E. R. *Proc Natl Acad Sci U S A* **2003**, *100*, 15160-15165.

15. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. *Cell* **2003**, *112*, 293-301.

16. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili, Z.; Perez, C. A.; Shigemura, N.; Yoshida, R.; Mosinger, B., Jr.; Glendinning, J. I.; Ninomiya, Y.; Margolskee, R. F. *Chem Senses* **2006**, *31*, 253-264.

17. Abaffy, T.; Trubey, K. R.; Chaudhari, N. *Am J Physiol Cell Physiol* **2003**, *284*, C1420-C1428.

18. McLaughlin, S. K.; McKinnon, P. J.; Spickofsky, N.; Danho, W.; Margolskee, R. F. *Physiol Behav* **1994**, *56*, 1157-1164.

19. Kusakabe, Y.; Yasuoka, A.; Asano-Miyoshi, M.; Iwabuchi, K.; Matsumoto, I.; Arai, S.; Emori, Y.; Abe, K. *Chem Senses* **2000**, *25*, 525-531.

20. McLaughlin, S. K.; McKinnon, P. J.; Margolskee, R. F. *Nature* **1992**, *357*, 563-569.

21. Kolesnikov, S. S.; Margolskee, R. F. *Nature* **1995**, *376*, 85-88.

22. Misaka, T.; Kusakabe, Y.; Emori, Y.; Arai, S.; Abe, K. *Ann N Y Acad Sci* **1998**, *855*, 150-159.

23. Striem, B. J.; Pace, U.; Zehavi, U.; Naim, M.; Lancet, D. *Biochem J* **1989**, *260*, 121-126.

24. Striem BJ, Naim M, and Lindemann B. *Cell Physiol Biochem* **1991**, *1*, 46-54.

25. Nakashima, K.; Ninomiya, Y. *Cell Physiol Biochem* **1999**, *9*, 90-98.

26. Trubey, K. R.; Culpepper, S.; Maruyama, Y.; Kinnamon, S.C.; Chaudhari, N. *Am J Physiol Cell Physiol* **2006**, *291*, C237-C244.

27. Cummings, T. A.; Powell, J.; Kinnamon, S. C. *J Neurophysiol* **1993**, *70*, 2326-2336.

28. Cummings, T. A.; Daniels, C.; Kinnamon, S. C. *J Neurophysiol* **1996**, *75*, 1256-1263.

29. Varkevisser, B.; Kinnamon, S. C. *J Neurophysiol* **2000**, *83*, 2526-2532.

30. Giovannucci, D. R.; Sneyd, J.; Groblewski, G. E.; Yule, D. I. *J Korean Med Sci* **2000**, *15 Suppl*, S55-S56.

31. Liu, M.; Simon, M. I. *Nature* **1996**, *382*, 83-87.

32. Wong, G. T.; Gannon, K. S.; Margolskee, R. F. *Nature* 1996, *381*, 796-800.
33. Danilova, V.; Damak, S.; Margolskee, R. F.; Hellekant, G. *Chem Senses* 2006, *31*, 573-80.
34. Kinnamon, S.; Trubey, K.; Margolskee, R.F..; Chaudhari, N. *Amer. Chem. Soc.* 2006, *Ann. Mtg. Paper # 918342*. (abstract).

Chapter 16

Saccharin: Artificial Sweetener, Bitter Tastant, and Sweet Taste Inhibitor

Marcel Winnig[1], Christina Kuhn[1], Oliver Frank[2], Bernd Bufe[1], Maik Behrens[1], Thomas Hofmann[2], and Wolfgang Meyerhof[1]

[1]Department of Molecular Genetics, German Institute of Human Nutrition Potsdam-Rehbruecke, Arthur-Scheunert-Allee 114–116, 14558 Nuthetal, Germany
[2]Institute of Food Chemistry, University of Münster, Corrensstrasse 45, 48149 Münster, Germany

Like all other sweet tasting compounds reported to date, saccharin activates the sweet taste receptor TAS1R2/TAS1R3. Its threshold of activation is in the sub-mM range and the receptor responses saturate at 1-3 mM. In the same concentration range saccharin also activates the human bitter taste receptors TAS2R43 and TAS2R44. They likely mediate saccharin's bitter aftertaste that many subjects complain. At concentrations above 3 mM, saccharin antagonizes activation of TAS1R2/TAS1R3 by itself and other sweeteners. Apparently, saccharin binds to two sites, a high-affinity agonist-binding site and a low-affinity allosteric site. While only the former is occupied at low agonist concentrations leading to receptor activation, the latter becomes occupied at higher agonist concentrations causing receptor inhibition. Thus, we suggest that with rising concentrations the sensory properties of saccharin are impaired by a disproportionate increase in its bitter taste at the expense of its sweet taste.

Introduction

The taste system provides the organisms with essential information about their food. Humans, like other mammals, detect and distinguish the five basic taste modalities salty, sour, umami, sweet, and bitter. Each modality is assumed to hold a specific subtask. Whereas sweet taste monitors carbohydrate-rich food and activates attractive neural pathways that stimulate intake, bitter taste serves as a warning system that activates repulsive behaviors protecting organisms from ingesting spoiled or toxic food. Sweet taste is elicited by the class C G protein-coupled receptors TAS1R2 and TAS1R3 with typical large N-terminal "venus-fly-trap modules" and short C-termini (*1-3*). They function as dimers, the TAS1R2/TAS1R3 heterodimer being a general sweet taste receptor for numerous natural and artificial ligands including mono- and disaccharides, D- and L-amino acids, peptides, proteins, metal ions, sulfamates, sulfonyl amides, and isovannillyl compounds, and the TAS1R3 homodimer being a low affinity receptor for some saccharides (*2-4*). Apparently various binding sites of the sweet taste receptor allow the interactions with so many different ligands (*5-8*). Bitter taste is initiated through the interactions of bitter substances with members of the TAS2R family, G protein-coupled receptors with short N- and C-termini, which are encoded by ~25 genes in humans.(*9-12*).

The use of high potency artificial sweeteners is constantly increasing as weight-conscious subjects and diabetics use these compounds to reduce their calorie or sugar intake (*13, 14*). However, the sweet taste of the two commonly used sulfonyl amide sweeteners, saccharin and acesulfame K, is accompanied by a lingering bitter after taste that increases with higher concentrations, thereby limiting their use (*15, 16*). We here show by functional expression of the recombinant receptors that the two sulfonyl amide sweeteners activate specific TAS2R bitter taste receptors and inhibit at high concentrations the sweet taste receptor heteromer. We conclude that both actions contribute to the off-taste of sulfonyl amides.

Materials and Methods

We transfected HEK293T cells stably expressing the chimeric G protein Gα16gust44 with cDNA constructs (150 ng per well) for hTAS2R bitter taste receptors or TAS1R2/TAS1R3 sweet taste receptor. 24 h after transfections cells were stained with Fluo-4-AM (Molecular Probes). After administration of tastants we analyzed the cells for changes of fluorescence by calcium imaging experiments in an automated fluorometric system, FLIPR (Molecular Devices) or by the single cell calcium imaging technique. We established dose response

curves by performing quadruplicates in at least two independent experiments. Calcium signals were corrected for the response of mock transfected cells and the data normalized to the fluorescence of cells prior to the stimulus ($\Delta F/F=(F-F0)/F0$). We calculated concentration-response curves and EC_{50} values with SigmaPlot by nonlinear regression using the function $f=((a-d)/(1+(x/EC_{50})^{nH})+d)$.

Results and Discussion

Identification of TAS2R Bitter Taste Recptors for Sulfonyl Amide Sweeteners

We used the sulfonyl amide sweeteners, saccharin and acesulfame K, to challenge HEK293T-Gα16Gust44 cells individually transfected with cDNAs of the 25 human TAS2R bitter taste receptors (Figure 1) tagged with the somatostatin receptor 3 plasma membrane targeting sequence at their N-termini and with the herpes simplex virus glycoprotein D epitope at the C-termini (*10*).
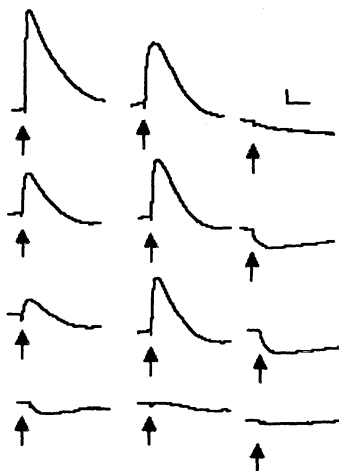
Figure 1. Heterologous expression of hTAS2R43 and hTAS2R44. Calcium responses of cells transfected with DNA for hTAS2R43 (left) or hTAS2R44 (middle) or of mock-transfected cells (right) elicited by 10 μM aristolochic acid, 10 mM saccharin, 10 mM acesulfame K or vehicle (from top to Bottom). Arrows denote the application of compounds. Scale bars, horizontal, 100 s; vertical ΔF/F = 0.1.

Cells expressing hTAS2R43 or hTAS2R44 concentration dependently responded to this treatment with elevated Ca^{2+} levels, while mock-transfected cells or cells transfected with any other hTAS2R DNA did not (*17*). hTAS2R44 showed higher affinities for saccharin and acesulfame K (EC_{50} values, 1.1 ± 0.01 mM and 2.5 ± 0.02 mM, respectively) than hTAS2R43 (EC_{50} values, 1.7 ± 0.02 mM and >10 mM, respectively). Human TAS2R44-mediated signals displayed also ~twofold higher amplitudes, suggesting that it contributes stronger to the bitter taste of sulfonyl amides than hTAS2R43. Both receptors are also activated by the purely bitter compound aristolochic acid with much higher affinity (EC_{50} values, 81 ± 0.8 nM for hTAS2R43 and 455 ± 5.3 nM for hTAS2R44), whereas various other sweeteners, bitter or umami compounds failed to activate them (Figure 2). Moreover, the inhibitor lactisole did not diminish sulfonyl amide-
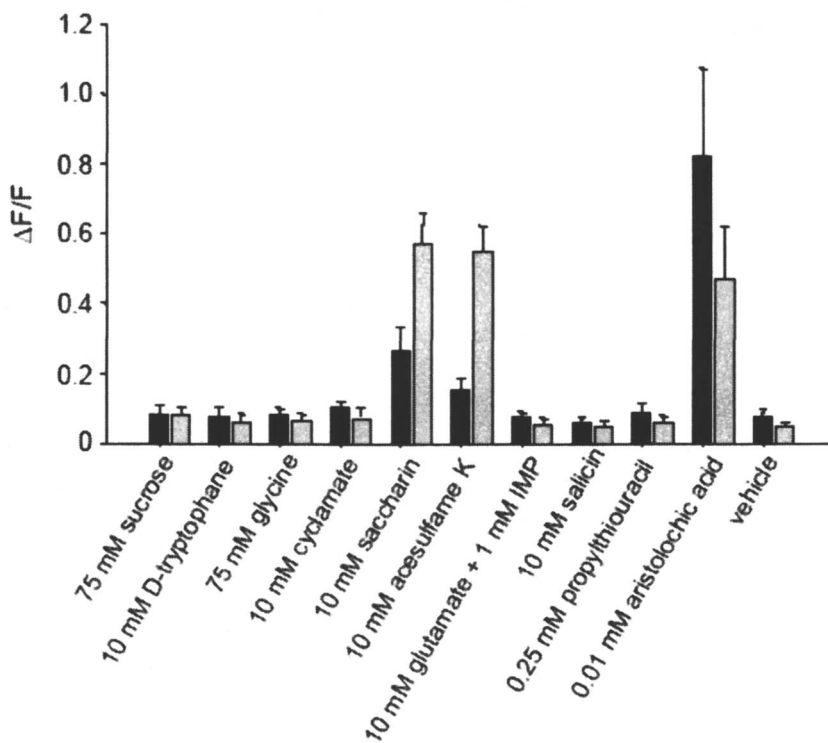
*Figure 2. Calcium responses of hTAS2R43 (black) or hTAS2R44 (grey)
expressing cells to various taste compounds.*

234

induced responses from hTAS2R43 and hTAS2R44 at concentrations known to block sweet taste perception in subjects or responses from recombinant TAS1R2/TAS1R3 (not shown). Together, these results indicate that hTAS2R43 and hTAS2R44 are true bitter taste receptors and not contributing to the sweet taste of the sulfonyl amides. The data are in line with previous psychophysical studies which suggested a common receptor mechanism for the bitter taste of saccharin and acesulfame K (*15, 16*).

We performed adaptation and cross-adaptation experiments to further examine the role of hTAS2R43 and hTAS2R44 for bitter tasting of sulfonyl amides (Figure 3). Adaptation refers to the decline in taste responses of subjects
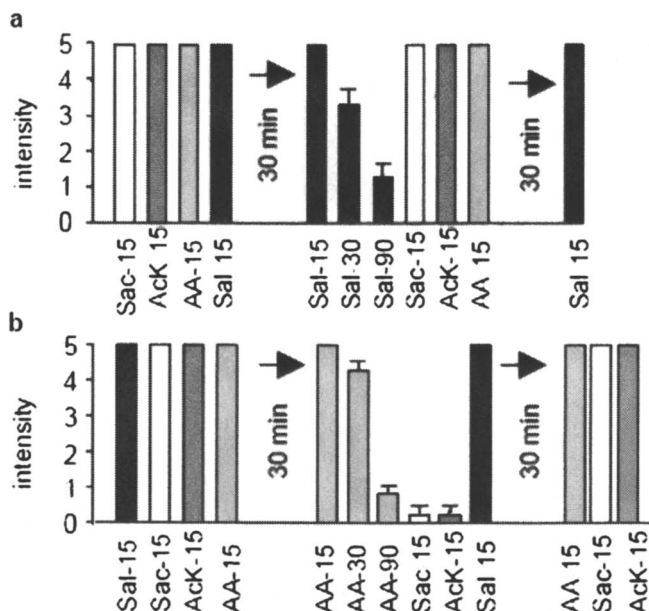
Figure 3. Adaptation and cross-adaptation of subjects' taste responses to various tastants. a, Adaptation to the bitterness of the hTAS2R16 agonist salicin (Sal). No cross-adaptation is seen with TAS2R43/hTAS2R44 agonists.
b, Adaptation to the bitterness of the hTAS2R43/hTAS2R44 agonist aristolochic acid (AA). Cross-adaptation is observed with the other hTAS2R43/hTAS2R44 agonists, saccharin (Sac) and acesulfame K (AcK) but not with the hTAS2R16 agonist salicin. Figures indicate the time in seconds that tastant were kept in the mouth before intensity rating was done. The phases of the experiment were separated by 30 min.

seen after prolonged stimulation with a taste stimulus (18, 19). It has successfully been employed to determine whether taste stimuli elicit the same or different signaling mechanisms (10, 20, 21).

We investigated adaptation and cross-adapatation behaviors of the three hTAS2R43 and hTAS2R44 agonists saccharin, acesulfame K and aristolochic acid and the hTAS2R16 agonist salicin (17). After 15 s subjects rated, at appropriate concentrations, all four compounds as equally intense bitter on an arbitrary scale of 0 to 5. However, the bitterness of all four compounds declined over a time period from 15 to 90 s from 5 to ~1. When subjects with declined responses to the hTAS2R16 agonist salicin as first stimulus tasted any of the hTAS2R43/hTAS2R44 agonists directly thereafter they reported unaltered bitterness of these compounds (Figure 3a). Similarly, subjects with declined bitter responses after prolonged tasting of any of the three hTAS2R43/hTAS2R44 agonists as first stimulus reported normal bitterness of salicin when given as the second stimulus (Figure 3b). When, however, subjects with decreased bitter responses to any of the hTAS2R43/hTAS2R44 agonists given as first stimuls were subsequently given another hTAS2R43/hTAS2R44 agonist as second stimulus, they showed largely diminished responses also to the second treatment (Figure 3b). Adaptation to taste responses was reversible. These results indicate that subjects adapted to the bitter taste of all compounds.

Adaptation was apparently receptor-specific as the bitterness of such compounds that activate different receptors was unaltered, whereas the bitterness of all compounds that activate the same receptor was diminished. The data further suggest that aristolochic acid and the sulfonyl amide sweeteners signal through the same mechanisms and that salicin signals through a different mechanism. Human TAS2R43 and hTAS2R44 were identified by in situ hybridization in taste receptor cells of human circumvallate papillae (not shown). As the TAS2R receptors are assumed to occur in the same set of taste receptor cells and use the same intracellular signaling cascade, we conclude that in vivo hTAS2R16 mediates the salicin response and hTAS2R43 and hTAS2R44 mediate the bitterness of aristolochic acid and sulfonyl amide sweeteners.

Recently, individual differences were seen among 65 subjects in the bitter responses to saccharin and acesulfame K, which were not correlated to propylthiouracil tasting. Although this observation has not been followed up so far, it is interesting to note here that intense genetic variability has been observed for the TAS2Rs (22). The best-studied example is the TAS2R38 gene. This has been identified to determine the ability to taste phenylthiocarbamide and other thioamides (23, 24). Single nucleotide polymorphisms (SNPs) are present at three positions in the hTAS2R38 gene specifying five haplotypes, referred to as PAV, PVI, AAV, AAI and AVI depending on the amino acids present in the three positions (24). These haplotypes give rise to receptor variants that, when expressed in cell lines, differ in their responses to various thioamides . Whereas

the PAV variant is a very sensitive receptor for these compounds, the AVI variant is totally insensitive and the other variants are of intermediate sensitivities (*23*). Moreover, receptor sensitivity measured *in vitro* correctly predicted the sensitivity of subjects for tasting phenylthiocarbamide (*23*). Thus, in this case, a clear correlation of genotype and phenotypical tasting a particular bitter compound is seen. It has also been shown that the two known hTAS2R16 variants 172N and 172K differ in their sensitivities for various β-glucopyranosides and that these differences have behavioral consequences for humans (*25, 26*). SNPs also occur in the hTAS2R43 and hTAS2R44 genes, namely 6 and 11, respectively (*22*). Although the functional consequences of this variability have not been addressed so far, a genetic basis for differences in saccharin and acesulfame K tasting appears likely.

Inhibition of the sweet taste receptor hTAS1R2/hTAS1R3 by saccharin and acesulfame K

As a high potency sweetener saccharin is commonly employed to sweeten food and beverages at low concentrations. In marked contrast, high concentrations of saccharin taste mostly bitter and show reduced sweetness (*27*). This resulted from the property of saccharin to reduce its own sweetness and the sweetness of other compounds at high concentrations. When such high concentrations of saccharin are rinsed from the mouth bitterness declines while an intense sweet sensation is initiated, the sweet "water-taste". A detailed examination of these effects revealed that induction of sweet "water-taste" by a compound is associated with its ability to inhibit sweet taste (*27*). It was also ruled out that cognitive suppression of sweetness by bitterness or adaptation account for the observed effects.

To elucidate whether these effects were mediated by intrinsic properties of the human sweet taste receptor we characterized the hTAS2R2/hTAS2R3 dimer in HEK293T-Gα16Gust44 cells by monitoring calcium levels in response to bath application of sweet tasting compounds (*27*). Saccharin and acesulfame K showed bell shaped concentration-response relations at hTAS1R2/hTAS1R3 (Figure 4a). Both compounds exerted maximal responses at a concentration of ~3 mM, while higher concentrations caused diminished responses. At 60 mM, the highest concentration tested, responses declined by ~75% indicating that high concentrations of the sulfonyl amide sweeteners cause receptor inhibition. This effect was receptor specific as the same concentrations of another sweetener, cyclamate, elicited normal, sigmoid dose-response relations with no inhibition of hTAS1R2/hTAS1R3 (Figure 4a). Moreover, also the bitter taste receptor hTAS2R44 elicited normal calcium responses when stimulated with high concentrations of the sulfonly amides lacking any sign of inhibition (Figure
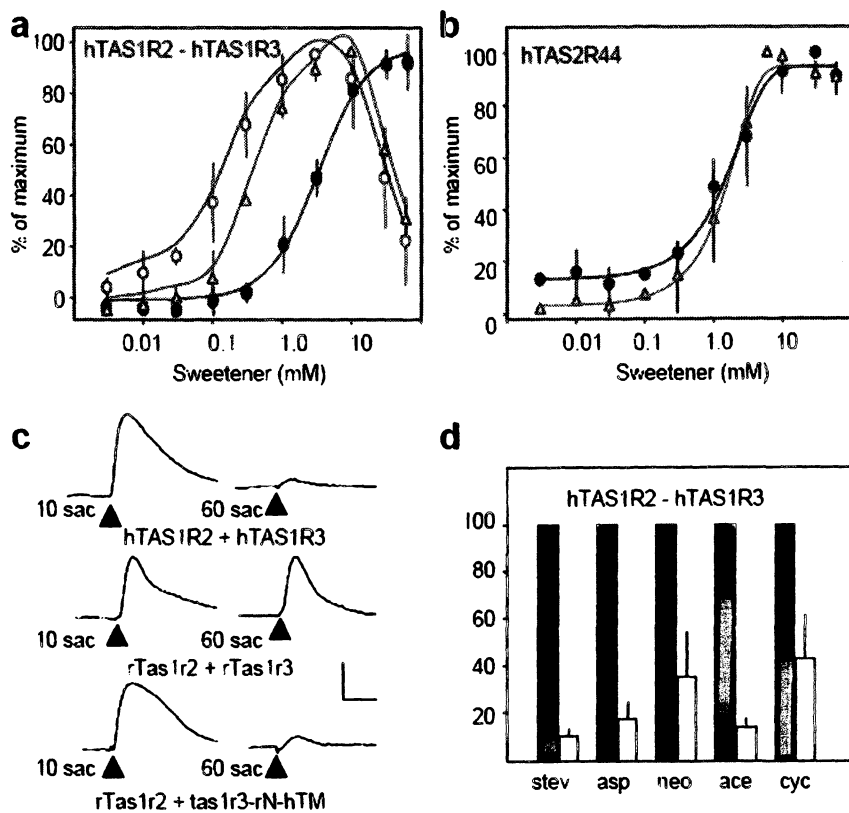
*Figure 4. Functional properties of the sweet receptor hTAS1R2/hTAS1R3 and
the bitter receptor hTAS2R44. a, Dose-response curves of the effect of saccharin
(open circles), acesulfame K (triangles) or cyclamate (filled circles) on cells
expressing the human sweet taste receptor. b, Dose-response curves of the effect
of saccharin (circles), or acesulfame K (triangles) on cells expressing the
human bitter receptor hTAS2R44. c, Calcium responses of cells expressing the
indicated sweet taste receptors that have been challenged (arrowheads) with 10
or 60 mM saccharin (sac). Scale bars, horizontal, 1 min; vertical, 2000 light
units. d, Calcium responses of cells expressing hTAS1R2/hTAS1R3 to 1 mM
stevioside (stev), 5 mM arspartame (asp), 1 mM neohesperdin dihydrochalcone
(neo), 5 mM acesulfame K or 5 mM cyclamate (cyc) with (white bars) or without
(black bars) 60 mM saccharin. (Reproduced with permission from reference 27.
Copyright 2006 authors.)*

4b). Apparently, the inhibitory effects of the sulfonyl amides was specific for the human sweet taste receptor as the rat rTas1r2/rTas1r3 mediated normal calcium signals at high concentrations (Figure 4c). However, a chimeric sweet taste receptor composed of rat Tas1r2 and a subunit comprising the rat N-terminal extracellular domain of rTas1r3 fused to the heptahelical domain of human hTAS1R3 was also inhibited by high concentrations of saccharin (Figure 4c). In addition saccharin inhibited the calcium responses elicited by the other sweet tasting compounds, stevioside, aspartame, neohesperidin dihydrochalcone, acesulfame K, and cyclamate (Figure 4d). Together, these results clearly show that the inhibitory cellular response to high concentrations of saccharin or acesulfame K was mediated specifically by the human hTAS1R2/hTAS1R3. Moreover, they reveal that the inhibitory effect was mediated through the heptahelical region of hTAS1R3.

To mimic the induction of sweet "water-taste"in the cellular assays we washed off high concentrations of saccharin or acesulfame K with buffer from HEK293T-Gα16Gust44 cells expressing hTAS1R2/hTAS1R3 during calcium imaging (27). While administration of 50 mM saccharin or 60 mM acesulfame K to the cells *per se* elicited no or only a small signal, the wash-off induced robust calcium resoponses. Similar results were also obtained when the sweet inhibitor lactisole was washed off the cells. The specificity of the wash out effect was verified by the observation that it is not seen in mock-transfected cells or in cells expressing the hTAS2R44 bitter taste receptor.

The actions of saccharin or acesulfame K on the hTAS1R2/hTAS1R3 dimer are best explained by assuming an allosteric receptor model with two binding sites for the sulfonyl amide sweeteners. At low concentrations the compounds bind preferentially to a high-affinity agonist binding site causing receptor activation. At high concentration they also bind to the low-affinity allosteric site leading to receptor inhibition. Thus, the properties of hTAS1R2/hTAS1R3 offer a molecular basis for the dimished sweet taste perception in subjects at high concentrations of sulfonyl amide sweeteners. In our model sweet "water-taste" is elicited by the preferential removal of these sweeteners from the allosteric site.

Conclusions

We have investigated the interactions of the sulfonyl amide sweeteners saccharin and acesulfame K with taste receptors. Our results demonstrate that the two compounds activate the sweet taste receptor hTAS1R2/hTAS1R3 and the bitter taste receptors hTAS2R43 and hTAS2R44 in an *in vitro* receptor assay at overlapping concentration ranges. Our results also show that both compounds at higher concentrations occupy an allosteric inhibitory site of hTAS1R2/hTAS1R3. The interactions of sulfonyl amide sweeteners with their cognate

taste receptors likely account for their increased bitterness at the expense of sweetness at higher concentrations thereby producing off tastes impacting on the sensory properties of saccharin and acesulfame K. Detailed understanding of the interactions between tastants and their receptors may help designing taste-active compounds with improved sensory properties.

Acknowledgement

References

1. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili, Z.; Varadarajan, V.; Zou, S.; Jiang, P.; Ninomiya, Y.; Margolskee, R. F., *Science* **2003**, *301*, (5634), 850-853.
2. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S., *Cell* **2001**, *106, (3)*, 381-390.
3. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E., *Proc. Nat.l Acad. Sci. U S A* **2002**, *99, (7)*, 4692-4696.
4. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N. J. P.; Zuker, C. S., *Cell* **2003**, *115*, 255-266.
5. Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Osman, R.; Margolskee, R. F.; Max, M., *J. Biol. Chem.* **2005**, *280, (15)*, 15238-15246.
6. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L. A.; Benard, L. M.; Osman, R.; Max, M.; Margolskee, R. F., *J. Biol. Chem.* **2005**, *280, (40)*, 34296-34305.
7. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Margolskee, R. F.; Max, M., *J. Biol. Chem.* **2004**, *279, (43)*, 45068-45075.
8. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X., *Proc. Natl. Acad. Sci. U S A* **2004**, *101, (39)*, 14258-14263.
9. Matsunami, H.; Montmayeur, J. P.; Buck, L. B., *Nature* **2000**, *404, (6778)*, 601-604.
10. Bufe, B.; Hofmann, T.; Krautwurst, D.; Raguse, J. D.; Meyerhof, W., *Nat. Genet.* **2002**, *32, (3)*, 397-401.
11. Adler, E.; Hoon, M. A.; Mueller, K. L.; Chandrashekar, J.; Ryba, N. J.; Zuker, C. S., *Cell* **2000**, *100, (6)*, 693-702.
12. Chandrashekar, J.; Mueller, K. L.; Hoon, M. A.; Adler, E.; Feng, L.; Guo, W.; Zuker, C. S.; Ryba, N. J., *Cell* **2000**, *100, (6)*, 703-711.

13. Hinson, A. L.; Nicol, W. M., *Food Addit. Contam.* **1992**, *9, (6)*, 669-680.
14. Ilback, N. G.; Alzin, M.; Jahrl, S.; Enghardt-Barbieri, H.; Busk, L., *Food Addit. Contam.* **2003**, *20, (2)*, 99-114.
15. Schiffman, S. S.; Reilly, D. A.; Clark, T. B., 3rd, *Physiol. Behav.* **1979**, *23, (1)*, 1-9.
16. Horne, J.; Lawless, H. T.; Speirs, W.; Sposato, D., *Chem. Senses* **2002**, 27, (1), 31-38.
17. Kuhn, C.; Bufe, B.; Winnig, M.; Hofmann, T.; Frank, O.; Behrens, M.; Lewtschenko, T.; Slack, J. P.; Ward, C. D.; Meyerhof, W., *J. Neurosci.* **2004**, 24, (45), 10260-10265.
18. Bartoshuk, L. M.; McBurney, D. H.; Pfaffmann, C., *Science* **1964**, 143, 967-968.
19. Pfaffmann, C., *Actual Neurophysiol.* **1965**, 6, 85-97.
20. Keast, R. S.; Breslin, P. A., *Chem. Senses* **2002**, 27, (2), 123-131.
21. Froloff, N.; Lloret, E.; Martinez, J. M.; Faurion, A., *Chem. Senses* **1998**, 23, (2), 197-206.
22. Kim, U.; Wooding, S.; Ricci, D.; Jorde, L. B.; Drayna, D., *Hum. Mutat.* **2005**, 26, (3), 199-204.
23. Bufe, B.; Breslin, P. A.; Kuhn, C.; Reed, D. R.; Tharp, C. D.; Slack, J. P.; Kim, U. K.; Drayna, D.; Meyerhof, W., *Curr. Biol.* **2005**, 15, (4), 322-327.
24. Kim, U. K.; Jorgenson, E.; Coon, H.; Leppert, M.; Risch, N.; Drayna, D., *Science* **2003**, 299, (5610), 1221-1225.
25. Soranzo, N.; Bufe, B.; Sabeti, P. C.; Wilson, J. F.; Weale, M. E.; Marguerie, R.; Meyerhof, W.; Goldstein, D. B., *Curr. Biol.* **2005**, 15, (14), 1257-1265.
26. Hinrichs, A. L.; Wang, J. C.; Bufe, B.; Kwon, J. M.; Budde, J.; Allen, R.; Bertelsen, S.; Evans, W.; Dick, D.; Rice, J.; Foroud, T.; Nurnberger, J.; Tischfield, J. A.; Kuperman, S.; Crowe, R.; Hesselbrock, V.; Schuckit, M.; Almasy, L.; Porjesz, B.; Edenberg, H. J.; Begleiter, H.; Meyerhof, W.; Bierut, L. J.; Goate, A. M., *Am J Hum. Genet.* **2006**, 78, (1), 103-111.
27. Galindo-Cuspinera, V.; Winnig, M.; Bufe, B.; Meyerhof, W.; Breslin, P. A., *Nature* **2006**.

Chapter 17

Permeation of Amphipathic Sweeteners into Taste-Bud Cells and Their Interactions with Post-Receptor Signaling Components: Possible Implications for Sweet-Taste Quality

M. Naim[1], M. E. Shaul[1], A. I. Spielman[2,3], L. Huang[3], and I. Peri[1]

[1]Institute of Biochemistry, Food Science and Nutrition,
Faculty of Agricultural, Food and Environmental Quality Sciences,
The Hebrew University of Jerusalem, Rehovot, Israel
[2] College of Dentistry, New York University, 245 East 24th Street,
New York, NY 10012
[3]Monell Chemical Senses Center, 3500 Market Street,
Philadelphia, PA 19104

The sweetness of sugar is regarded by humans as the optimal sensation; that of alternative non-sugar sweeteners possesses an inferior sweet quality, which limits their use in low-calorie foods. These sweeteners may produce bitter, metallic or cooling sensations, as well as lingering sweet aftertaste. The molecular basis for these undesirable sensations is poorly understood. Although various sweeteners may stimulate the same G-protein-coupled taste receptors (GPCRs), inferior sweet-taste quality is uniquely related to some non-sugar sweeteners. This chapter presents data indicating that such tastants may rapidly permeate taste cells under physiological conditions *in vivo* and interact with downstream signaling components such as signal-termination-related kinases *in vitro*. The implications of these properties for sweet-taste quality are discussed.

Our intake of foods containing refined sugars and high-caloric-density constituents has increased significantly in the last century *(1)*, and has been linked to metabolic disorders such as diabetes and obesity, coined 'diabesity' *(2)*, considered to be the main threats to human health in the 21st century *(3)*. In response to such health hazards, for the last four decades chemical studies have been exploring alternative low-calorie sweeteners with high sweet potency *(4-8)*. The resulting synthetic (and some natural) non-sugar sweeteners include a large collection of diverse compounds such as sulfamates, flavonoids, oximes, amino acids, peptides, proteins, guanidines and terpenoids.

The nutritional justification for sweeteners as alternatives to sugars has led to increased consumption of low-calorie soft drinks and other low-calorie foods. However, the sweet taste of sugars, especially that of sucrose, is regarded as pure with optimal sensation in humans, whereas many non-sugar sweeteners possess inferior sweet quality. Psychophysical sensory studies in humans using the multi-dimensional similarity (MDS) analysis *(9)* have clearly shown that the sweetness of a variety of non-sugar sweeteners may be located at a different site in the sweet MDS map from that of sugar sweeteners. Indeed, they may produce bitter or metallic sensations, as well as lingering sweet aftertaste *(9)*. A further factor that in many cases makes non-sugar sweeteners inferior to sugars is their temporal properties. Time-intensity relationship studies have indicated that, compared to sucrose, it takes a longer time for the sensation of a non-sugar sweetener to reach maximal sweet-taste intensity, and more time (sometimes minutes) for the sweetness to be extinguished (lingering aftertaste) *(4,10-12,)*. As a result, when water is being tasted after tasting some of these sweeteners, water becomes sweet, i.e., "water sweet aftertaste" *(12,13)*. The lingering aftertaste phenomenon is also known to occur with a variety of bitter stimuli.

Delayed sweet-taste termination following the tasting of non-sugar sweeteners is not unique to humans. It has also been shown to occur in Old World monkeys during behavioral studies *(14)* and during electrophysiological recordings of taste nerves *(15)*. Some evidence suggests that the phenomenon is peripheral: electrophysiological recordings of taste nerves at the periphery *(15)* have indicated "taste persistence" in response to stimulation by non-sugar sweeteners. Furthermore, a delay in inositol 1,4,5-trisphospahte (IP$_3$) accumulation in taste cells following stimulation by some bitter tastants known to possess lingering taste has also been reported *(16)*. These results suggest that the delay in taste-signal termination induced by some non-sugar sweeteners and bitter tastants is at the periphery, at the taste-cell level. Nevertheless, the molecular basis for the "slow taste onset" and "lingering aftertaste" phenomena is still not known, even though it has significant implications with respect to the acceptance of a variety of food products.

The existence of G-protein-coupled receptors (GPCRs) in taste cells for both sweet and bitter tastes, as well as for umami taste, is now well established *(17-24)*. Apparently, both sugar and non-sugar sweeteners stimulate the same sweet receptors *(19, 26)*. Nevertheless, the lingering aftertaste phenomenon is uniquely related to non-sugar sweeteners. One may hypothesize that while the receptors

may be the same, modifications in cellular-signal activation and/or signal termination may be responsible for the changes in temporal properties induced by non-sugar sweeteners. In the following we discuss the hypothesis that one reason for the lingering aftertaste produced by non-sugar sweeteners and bitter tastants is their ability to permeate taste cells and interact with signal-termination components located downstream of the taste GPCRs.

Permeation of Amphipathic Tastants into Taste-Bud Cells

For amphipathic (i.e., containing both hydrophobic and hydrophilic domains) tastants to interact directly with membrane-transduction components located downstream of the GPCRs, they need to be able to permeate the membrane and translocate to the cytosolic side of the cell. In contrast to sugars, the chemical structures of non-sugar sweeteners (as well as bitter tastants) are very diverse, often amphipathic, and therefore they are putative membrane-permeant compounds. A variety of membrane-permeant amphipathic compounds that affect GPCR-signal-transduction-related pathways and specific downstream transduction components are routinely used for research or in clinical practice as activators or inhibitors of downstream signaling. Among them are direct G-protein activators such as the peptides mastoparan and melittin *(27-30)*; the diterpene forskolin, a direct activator of adenylyl cyclase *(31, 32)*; various xanthines such as IBMX and theophylline inhibitors of phosphodiesterases (PDE) *(33, 34)*; and the aromatic-isoquinoline derivatives such as H-89 *(35, 36)* and H-7 *(37)*, which specifically inhibit protein kinase A (PKA) and protein kinase C (PKC), respectively.

Permeation through the plasma membrane of living cells may include passive diffusion, i.e., translocation of a solute across a membrane down its electrochemical gradient according to Fick's law without the participation of a transport protein *(38)*. Diffusion has a low temperature coefficient and usually occurs with small, neutral molecules that are soluble in the lipid membrane. In facilitated diffusion, translocation of a solute across a membrane also occurs down its electrochemical gradient but is catalyzed by a transport protein which may obey Michaelis-Menten relationships. Biological substrates that follow this mechanism are typically charged or larger than the size of glycerol *(39)*. Active transport occurs when net transport of a solute across a biological membrane moves from a low to high electrochemical potential. This transport is characterized by the following: the solute is not chemically modified, saturable steady-state kinetics is observed, substrate specificity is restricted and an input of metabolic energy is required. Active-transport processes involve a variety of molecular mechanisms, for which energy may be derived from light, oxidoreduction, ATP hydrolysis, or a pre-existing solute gradient. Obviously, the transport reactions involved in ATP synthesis are localized in the mitochondria, which use an H^+ electrochemical gradient for energy coupling. Mammalian cells utilize a Na^+ gradient generated by a Na^+/K^+ ATPase to accommodate solute-Na^+ symporter or antiporter *(40)*.

Our previous experiments, utilizing the auto-fluorescence properties of some amphipathic tastants, indicated that the sweetener saccharin and the bitter tastants quinine and the cheese-derived cyclo(Leu-Trp) peptide can translocate through multilamellar lipid vesicles (MLV)(liposomes), apparently due to their hydrophobic or amphipathic properties *(41)*. Under the experimental conditions (pH 6.8 to 7.25), quinine was a slightly cationic (pKa 8.52) tastant with a significant portion in an uncharged form. The cyclo(Leu-Trp) peptide is uncharged under these conditions *(42)*. Saccharin is a strong anion (pKa 1.8) under these conditions, but appears to be more lipophilic than would be inferred from its dissociation constant *(43)*. Using their autoflourescence, confocal microscopic studies and HPLC analyses indicated that the same tastants also rapidly permeate the cells of isolated taste-bud sheets derived from rat circumvallate (CV) papillae *(41)*.

The following experiments were designed to further characterize saccharin permeation into taste-bud cells using the above preparation. CV taste-bud sheets were prepared by means of collagenase treatments *(41, 44)*. They were then incubated for 30 s with increased concentrations of saccharin, washed, the cells were permeabilized by freeze-thaw processes *(44)* and the intracellular content of saccharin was determined by HPLC as previously described *(41)*. As shown in Figure 1A, the saccharin permeation rate fits Michaelis-Menten kinetics, resulting in a K_d of approximately 52 mM with a maximal (V_{max}) accumulation rate of 5.5 mM/s, similar to the value we found previously *(41)*. This permeation into the taste-bud cells appeared to be against the "concentration gradient" (see discussion below).

Next, we aimed at revealing whether saccharin permeation into taste-bud cells requires the use of metabolic energy, i.e., ATP. Since a main force for ensuring a desirable ATP level in the cell depends on maintaining the proton gradient across the mitochondrial membrane needed for oxidative phosphorylation, we first used the uncoupling ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which is expected to abolish this proton gradient and inhibit oxidative phosphorylation, and thus the availability of cellular ATP *(45)*. Saccharin permeation into taste-bud cells was significantly reduced when cells were preincubated for 5 min with CCCP (Figure 1B). Nevertheless, it should be noted that CCCP, a proton ionophore that collapses the mitochondrial potential, may also change the plasma membrane's potential *(46)*. If a given solute is charged, changes in membrane potential may affect its permeation independently of cellular ATP availability. Consequently, we next used oligomycin, which inhibits ATP synthase, an enzyme located on the internal mitochondrial membrane, and thus inhibits the formation of ATP from ADP without affecting membrane potential. In addition, since saccharin is a strong anion under the experimental conditions used, the effect of pentachlorophenol (PCP), an electron uncoupler and a potent anion-transport inhibitor *(47, 48)*, was tested. Figure 1B shows that, in contrast to CCCP, preincubation of taste-bud cells with oligomycin for 30 min or with PCP for 40 min does not affect saccharin permeation (glucose was removed from the assay buffer to abolish or significantly reduce ATP formation via glycolysis).
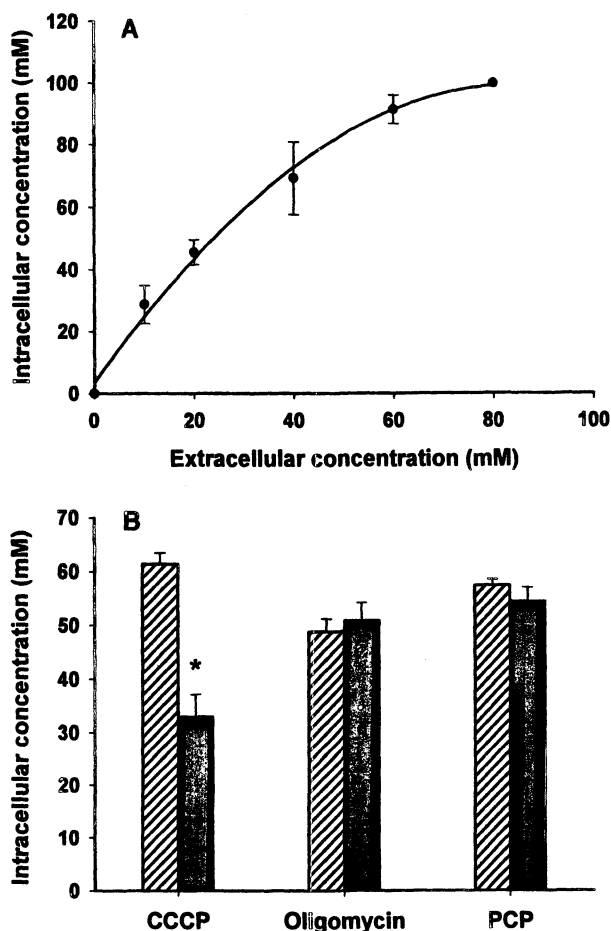
Figure 1. Permeation of saccharin into taste-bud cells. **A.** Permeation into
taste-bud cells during 30 s of incubation as a function of extracellular
concentrations of sodium saccharin. Results are means ± SEM of two replicates
for each data point. Each point represents the responses of four rats. Saccharin
permeation fits Michaelis-Menten kinetics (R^2 = 0.99, p < 0.001), K_d = 52.2 mM,
V_{max} = 5.54 mM/s. **B.** The effect of carbonyl cyanide m-chlorophenyl hydrazone
(CCCP), oligomycin and pentachlorophenol (PCP) on saccharin permeation
into taste-bud cells. Taste-bud sheets were preincubated with (black bars) and
without (hatched bars) CCCP (50 μM), oligomycin (10 μg/mL) or PCP (500
μM) for 5, 30 or 40 min, respectively. Then, sheets were incubated for 30 s with
saccharin (30 mM) and the intracellular content was determined as previously
(41). Results are means ± SEM of three to four replicates for each data point.
Each point represents four rats. * indicates significant inhibition of saccharin
permeation (p < 0.05).

Therefore, it is likely that saccharin permeated the cells without the involvement of metabolic energy.

The likelihood that saccharin permeation does not involve an active carrier may be partly supported by the rates at which saccharin permeated the taste cells. The estimated volume of a taste cell is about 0.25 pL (41), suggesting that the accumulated rate of saccharin into a taste cell was 5.54 mM/s (around 10^9 molecules/cell per second). We estimated the surface area of a taste cell to be about 300 μm^2, but due to the large diversity in the number of channels that are present per square micrometer in different cells (49), it is difficult to estimate the number of putative "saccharin channels" in one taste cell. However, if one speculates between 1000 and 10,000 "saccharin channels" per cell, the permeation rate would be between 10^5 and 10^6 molecules/s for a single channel. These rates are higher than the uptake rates of 10^2 to 10^4 molecules/s which are known for solutes being taken up by various active carriers (glucose transporters, Na^+-K^+-ATPase, Cl^-/HCO_3^- exchanger) and lower than those found for passive diffusion flux (10^8/s), known for water or ions via the model pores of gramicidin (49). Therefore, the permeation of saccharin via putative "saccharin channels" may be classified as facilitated diffusion (38) which does not use metabolic energy for permeation even though shows Michaelis-Menten kinetics.

Could it be that our amphipathic tastants are able to form pores as they do in lipid vesicles (41)? This remains to be determined. Nevertheless, independent of the mechanism involved, it appears that such high permeation rates of saccharin into taste cells may not be achievable with active carriers; this would support a diffusion process which does not depend on metabolic energy.

If this is indeed the case, how does saccharin permeate the taste-bud cell against a concentration gradient? Our localization experiments (41, 50) suggested the presence of saccharin attached to cytosolic organelles. This may suggest the binding of saccharin, as well as other amphipathic tastants, to cytosolic organelles (e.g., the nucleus), and perhaps to cytosolic proteins (41). The latter observation suggested that such binding actually reduces the concentration of these tastants in the cytosol and thus permeation occurs along the concentration gradient, not against it.

In the aforementioned studies with isolated taste-bud sheets (41), it is likely that not only the apical mucosal side (where tastants normally interact with sensory cells), but also the serosal side of the taste-bud cells was exposed to saccharin and other tastants. Therefore, we further investigated whether amphipathic tastants can permeate taste cells via the apical oral route under physiological conditions. The auto-fluorescence that some sweet and bitter amphipathic tastants possess was used to monitor tastant permeation into taste cells by two different procedures (51). First, in-situ confocal-microscopy imaging of an intact CV papilla surgically removed without collagenase treatment (thus, avoiding exposure of the basolateral side) indicated the dynamics of tastant permeation [using the sweeteners saccharin and D-tryptophan and the bitter tastants caffeine, cyclo(Leu-Trp), naringin and quinine] into CV papilla cells located around the circular inner trench where most of the CV taste buds are found.

Addition of the membrane-impermeant quencher, KI, to the tastant-permeated CV buds had no effect on tastant fluorescence, indicating the presence of such tastants inside the cytosol or on the cytosolic side of the membrane rather than their adsorption to the extracellular surface of the cells.

Table I. Estimated permeation of amphipathic tastants into CV taste-bud cells via the apical side

Tastant	Extracellular conc. (mM) during oral stimulation	Efflux (%) during collagenase trt.	Intracellular conc. (mM) after oral stimulation
D-Tryptophan	30	86* ± 9.5	6.4** ± 1.0
Quinine	2	75* ± 5.6	5.5** ± 0.8
Cyclo(Leu-Trp)	2	64* ± 3.0	0.96** ± 0.1
Caffeine	10	70* ± 2.5	13.7* ± 3.6

Oral stimulation was conducted for 90 s; collagenase treatment (trt) for 25 min. Data are means ± SEM of four to nine replicates, each derived from one or two rats.
* and ** indicate significant values at $p < 0.03$ and $p < 0.001$, respectively.
Reproduced from reference 51. Used by permission from The American Physiological Society.

In a second series of experiments (Table I), to further verify that tastants indeed permeate taste-bud cells under physiological conditions and to quantify the intracellular content of each tastant, the oral cavity of anesthetized rats was stimulated on-and-off with tastant solutions for 90 s, followed by immediate animal sacrifice. Tongues were then removed, and the CV taste-bud sheets were prepared by collagenase treatment *(41)*. Cells were then washed, permeabilized as described earlier and the intracellular content of each tastant was determined by HPLC *(51)*: millimolar levels of these tastants were found inside the taste cells following this oral stimulation (Table I). One might assume that some of the tastants would be likely to leave the cells (e.g., by MDR1, *(52)*) during the collagenase treatment after the 90-s oral stimulation. The efflux of tastants, estimated from additional experiments, during the enzymatic treatment was 64 to 86% *(51)*.

Together, the *in-situ* confocal laser-scanning imaging and HPLC analyses provide evidence that these and perhaps additional (although not necessarily all) amphipathic tastants rapidly permeate taste-bud cell membranes under physiological conditions (*in vivo*), either through the taste-bud pore or through the tight junctions, and that such permeation also occurs in non-sensory lingual epithelial cells. Nevertheless, one would anticipate no significant modifications in

cell volume or ion gradients due to the permeated millimolar concentration of tastants under physiological conditions.

Inhibition of Signal-Termination-Related Kinases by Membrane-Permeant Sweet Tastants

GPCR signaling often desensitizes rapidly as a consequence of receptor phosphorylation by two families of protein kinases *(53-56)*. One possibility is second messengers-dependent kinases (e.g., PKA and PKC): this is termed heterologous desensitization. The others are dependent on GPCRs (homologous desensitization), such as GPCR kinases (GRKs). In contrast to the second-messenger-dependent kinases, GRKs discriminate between the inactive and agonist-activated states of the receptor (catalytically activated by stimulated receptors) *(55, 56)*, and specifically phosphorylate the agonist-activated form of GPCRs.

Cellular-transduction experiments indirectly suggest the presence of PKA and PKC in taste cells *(36, 57)*. Recently, two studies were published on the presence of GRKs in taste-bud cells *(51, 58, 59)*. Our recent study *(51, 59)*, using reverse transcription-polymerase chain reaction (RT-PCR), suggested the presence of GRK2, GRK3, GRK5 and GRK6 along with T2R4 and T1R3 in cells of isolated taste-bud sheets of rat CV papillae (Figure 2). The same GRKs were found to be expressed in non-sensory epithelium but as expected, no PCR products for the T2R4 and T1R3 receptors were observed in the latter. Furthermore, antibodies against GRK2, GRK5 and GRK6 (with weak staining of GRK3) yielded clear, positive immunostaining in the 10-μm frozen sections of the CV papilla *(51)*. However, only GRK5 was clearly stained in the CV taste-bud cells; GRK2 and GRK6 were notably stained in the surrounding epithelium but their presence in the taste cells remains questionable. These immunostaining experiments cannot provide evidence for T2R4 and T1R3 receptor expression in the same subpopulation of taste cells that contains the GRKs. However, since GRK5 appeared to be expressed in almost all taste-bud cells, it is likely that it co-expresses in cells expressing taste GPCRs. A study in mouse *(58)*, using immunohistochemistry of the CV papilla, suggested the differential distribution of GRK2, GRK3 and GRK5 in the CV papilla while only GRK2 was present in taste-bud cells. It remains to be determined whether the discrepancy between these two studies results from the different species used (rats vs. mice) or the different methodologies for GRK detection. Nevertheless, these results warrant further investigation to verify that GRK5, and possibly other GRKs, are co-expressed and functionally coupled with T1R2/T1R3 receptors.

Subsequently, the effect of tastants on GRK2- and GRK5-mediated rhodopsin phosphorylation, a well-studied *in-vitro* model for GPCR phosphorylation *(55)*, was investigated in the same study *(51)*. Because an isolated
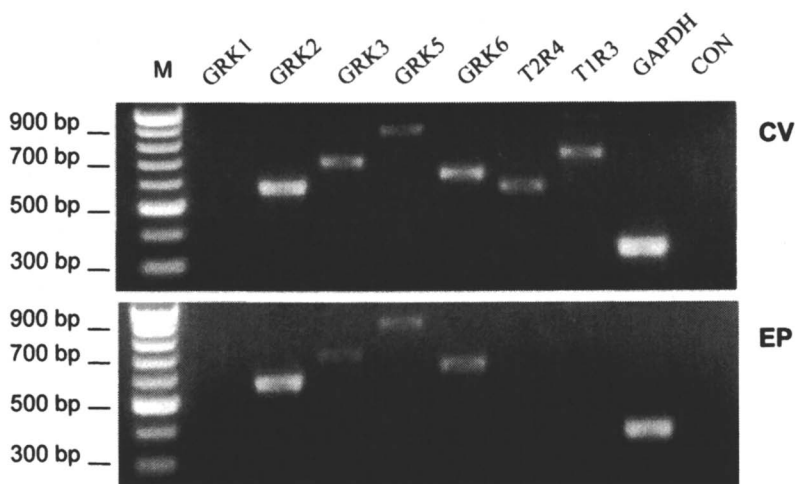
*Figure 2. RT-PCR analysis of GRK1, GRK2, GRK3 GRK5, GRK6, T2R4 and
T1R3 mRNA in circumvallate taste-bud sheets (CV) and non-sensory epithelium
(EP). cDNA was synthesized from CV and EP RNA and then amplified by PCR
using specific primers for GRK1, GRK2, GRK3, GRK5, GRK6, T2R4 or T1R3;
GAPDH was used as an internal reference gene. CON designates parallel PCR,
omitting the RT step and using GRK2 primers. Reproduced from reference 51.
Used with permission by The American Physiological Society.*

taste-receptor protein was not yet available, rhodopsin, the vision GPCR, was
used as a model. The ability of tastants to affect the incorporation of ^{32}P from [γ-^{32}P]ATP into rhodopsin by GRK2 and GRK5 and into casein by PKA was
monitored. As shown in Figure 3, the amphipathic non-sugar sweeteners
cyclamate, saccharin, neohesperidin dihydrochalcone (NHD) and D-tryptophan,
with diverse chemical structures, significantly inhibited the phosphorylation of
rhodopsin by GRK2 and GRK5 and of casein by PKA *in vitro*. Their effects
depended on the types of kinases being tested. Acesulfame K did not affect
kinase activity under the experimental conditions. The tastant concentrations
needed to stimulate these kinases under the experimental conditions were at the
millimolar level, higher than the micromolar levels usually used with other
kinase inhibitors *in vitro* and *in vivo* clinically *(60)* or experimentally in non-
taste systems *(61)*. Moreover, these tastants do not appear to be very specific
since they inhibited PKA as well as the GRKs, and may very well inhibit
additional kinases. Nevertheless, the range of tastant concentrations applied in
this study *(51)* matches that used in sensory and biochemical studies *(9, 15, 19,
33, 62, 63)* and the tastant levels found inside taste-bud cells within seconds
after their extracellular application (Table I). Therefore, their physiological
significance as kinase inhibitors in taste cells may result from their almost
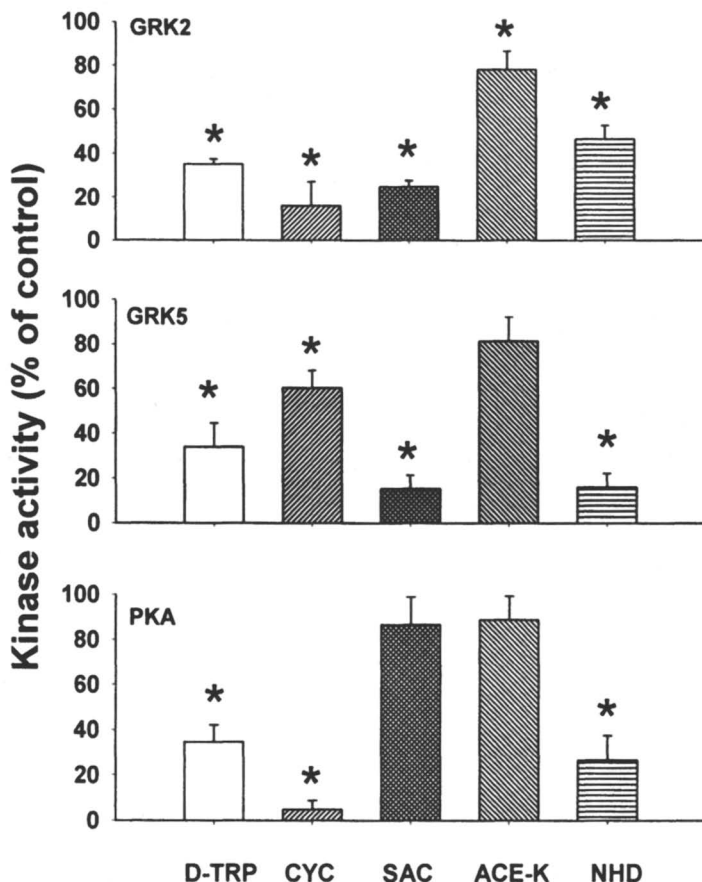
*Figure 3. Amphipathic tastants inhibit GRK2- and GRK5-induced
phosphorylation of rhodopsin and PKA-induced phosphorylation of casein.
GRK2 or GRK5 with rhodopsin or PKA with casein were incubated with
[γ-³²P]ATP for 15 min with the following sweeteners: D-tryptophan (D-TRP,
20 mM), sodium cyclamate (CYC, 20 mM), sodium saccharin (SAC, 20 mM),
acesulfame K (ACE-K, 3.5 mM) and neohesperidin dihydrochalcone (NHD,
1.25 mM). Results (% of kinase activity obtained during incubation without
tastants) are the means and SEM of four independent experiments.
*Significantly (at least at the p < 0.05 level) lower value than controls
incubated without tastants. (Adapted with permission from reference 51.
Copyright 1985 The American Physiological Society.)*
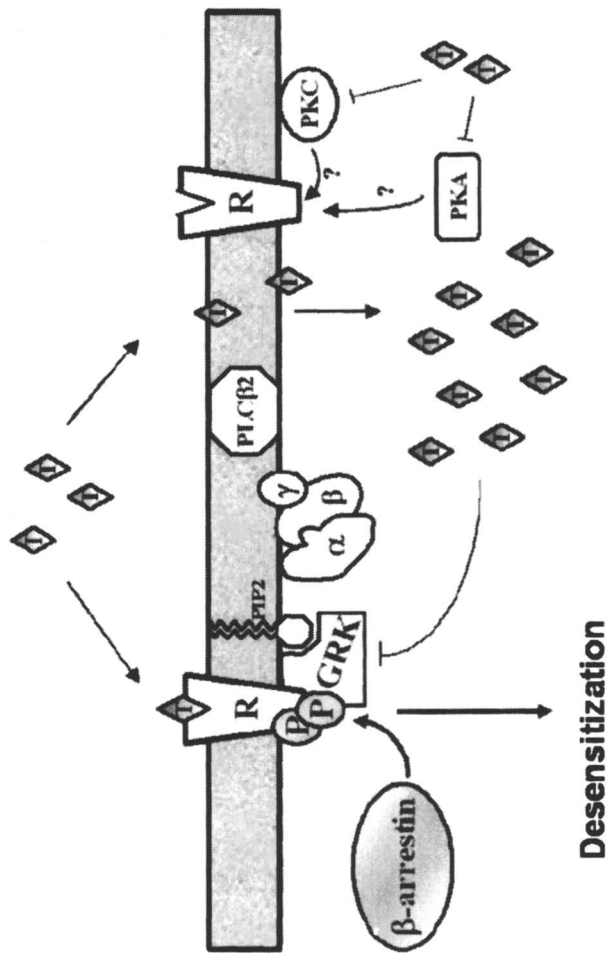
Desensitization

Figure 4: Hypothetical pathways for the desensitization of sweet-taste receptors. Non-sugar sweeteners (T) stimulate receptors (R) from the extracellular side to activate G-proteins (α, β, γ). βγ-subunits, in turn, activate PLCβ2. Receptor-mediated signaling may be desensitized by G-protein-coupled receptor kinases (GRKs) which phosphorylate R and promote the binding of arrestin proteins to the receptor, uncoupling the receptor from the G-proteins. Alternatively, R may be phosphorylated and desensitized by second-messenger-dependent kinases, e.g., protein kinase A (PKA) or protein kinase C (PKC). Intracellular inhibition of these receptors' phosphorylation, e.g., directly by the membrane-permeant Ts, would be expected to inhibit desensitization and therefore delay taste-signal termination. Modified from reference (55) and based on reference (51).

immediate access to the cytosolic side of those cells, and thus to GRKs, which are present in taste tissue and to PKA or PKC, which have been indirectly shown to be active in taste cells (36).

Interestingly, the same tastants have also been recently shown to act on the melatonin and α_2-adrenergic receptors (both are GPCRs) to reduce cellular cAMP in *Xenopus laevis* melanophores (50). Since both of these receptors appear to occur in taste-bud cells (50, 64), such tastants may further inhibit PKA activity in those cells due to the reduction in cAMP.

These results led to a new hypothesis which may partly explain the lingering aftertaste produced by non-sugar sweeteners and bitter tastants (Figure 4). According to this model, amphipathic tastants stimulate sweet-taste GPCRs located on the extracellular surface of taste cells, and concomitantly permeate to the cytosolic side of the cell membrane or to the cytosol under physiological conditions. Thus, such tastants have access to direct interaction with GRKs or with other receptor-related kinases (e.g., PKA, PKC). Inhibition of GRK- and/or PKA/PKC-induced phosphorylation of GPCRs then leads to a delay in signal termination, and therefore may extend the taste response (i.e., lingering).

In conclusion, the cellular events occurring in taste cells upon stimulation by sweeteners are very diverse and complex. The present results suggest that following taste stimulation, direct interactions of some of these sweeteners with downstream transduction components occur. This may explain some of the remarkable differences in the sweet taste quality of different sweeteners and calls for further investigation of such intracellular events.

Acknowledgements

References

1. Grande, F. Sugars in cardiovascular disease. In *Sugars in Nutrition;* Sipple, H. L.; McNutt, K. W. Eds.; Academic Press: New York, 1974; pp. 401-437.
2. Shafrir, E. *Diabetes Metab* 1996, 22, 122-131.
3. Zimmet, P.; Alberti, K. G. M. M.; Shaw, J. *Nature* 2001, 414, 782-787.
4. DuBois, G. E.; Crosby, G. A.; Stephenson, R. A.; Wingard, R. E. J. *J. Agric. Food Chem.* 1977, 25, 763-772.
5. DuBois, G. E.; Walters, D. E.; Kellog, M. S. The rational design of ultra-high-potency sweeteners. In *Sweet-Taste Chemoreception;* Mathlouthi, M.;

Kanters, J. A.; Birch, G. G. Eds.; Parthenon Press: London, 1993; pp. 237-267.

6. Nofre, C.; Tinti, J. M. Sweetening agents: United States Patent 4,645,678, 1987.

7. Nofre, C.; Tinti, J. M.; Chatzopoulos-Ouar, F. *Chemical Abstracts* **1988**, *109*, 190047k.

8. Tinti, J. M.; Nofre, C. New high-potency sweeteners. In *The Contribution of Low- and Nonvolatile Materials to the Flavor of Foods;* Pickenhagen, W.; Ho, C.-T.; Spanier, A. M. Eds.; Allured Publishing Corp.: Carol Stream, IL, 1996; pp. 77-93.

9. Schiffman, S. S.; Reilly, D. A.; Clark, T. B. r. *Physiol. Behav.* **1979**, *23*, 1-9.

10. Birch, G. G.; Latymer, Z.; Hollaway, M. *Chem. Senses* **1980**, *5*, 63-78.

11. Larson-Powers, N.; Pangborn, R. M. *J. Food Sci.* **1978**, *43*, 41-46.

12. Naim, M.; Dukan, E.; Zehavi, U.; Yaron, L. *Chem. Senses* **1986**, *11*, 361-370.

13. Bartoshuk, L. M. Is sweetness unitary? An evaluation of the evidence for multiple sweets. In *Sweetness;* Dobbing, J. Ed.; Springer-Verlag: London, 1987; pp. 33-46.

14. Rogatka, H.; Naim, M.; Zehavi, U. *Chem. Senses* **1985**, *10*, 269-277.

15. Hellekant, G. The Physiology of Thaumatin. In *Thaumatin;* Witty, M.; Higginbotham, J. D. Eds.; CRC Press: Boca Raton, FL, 1994; pp. 99-113.

16. Spielman, A. I.; Yan, W.; Krizhanovsky, V.; Rosenzweig, S.; Yamamoto, T.; Naim, M. Modulation of two second messengers in bitter taste transduction of agriculturally relevant compounds. In *Chemistry of Taste, Mechanisms, Behaviors, and Mimics;* Given, P.; Paredes, D. Eds.; American Chemical Society: Washington, D.C., 2002; Vol. ACS Symposium Series 825; pp. 18-31.

17. Bachmanov, A. A.; Li, X.; Reed, D. R.; Ohmen, J. D.; Li, S.; Chen, Z.; Tordoff, M. G.; de Jong, P. J.; Wu, C.; West, D. B.; Chatterjee, A.; Ross, D. A.; Beauchamp, G. K. *Chem Senses* **2001**, *26*, 925-933.

18. Bufe, B.; Hofmann, T.; Krautwurst, D.; Raguse, J. D.; Meyerhof, W. *Nat. Genet.* **2002**, *32*, 397-401.

19. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J. P.; Zuker, C. S. *Cell* **2001**, *106*, 381-390.

20. Montmayeur, J. P.; Liberles, S. D.; Matsunami, H.; Buck, L. B. *Nat Neurosci* **2001**, *4*, 492-498.

21. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. *Nat. Genet.* **2001**, *28*, 58-63.

22. Adler, E.; Hoon, M. A.; Mueller, K. L.; Chandrashekar, J.; Ryba, N. J. P.; Zuker, C. S. *Cell* **2000**, *100*, 693-702.

23. Chandrashekar, J.; Mueller, K. L.; Hoon, M. A.; Adler, E.; Feng, L.; Guo, W.; Zuker, C. S.; Ryba, N. J. P. *Cell* **2000**, *100*, 703-711.

24. Chaudhari, N.; Landin, A. M.; Roper, S. D. *Nat. Neurosci.* **2000**, *3*, 113-119.

25. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc Natl Acad Sci U S A* **2002**, *99*, 4692-4606.

26. Nelson, G.; Chandrashekar, J.; Hoon, M. A.; Feng, L.; Zhao, G.; Ryba, N. J.; Zuker, C. S. *Nature* **2002**, *416*, 199-202.

27. Higashijima, T.; Burnier, J.; Ross, E. M. *J. Biol. Chem.* **1990**, *265*, 14176-14186.

28. Higashijima, T.; Uzu, S.; Nakajima, T.; Ross, E. M. *J. Biol. Chem.* **1988**, *263*, 6491-6494.

29. Wilson, S. P. *FEBS Lett.* **1989**, *247*, 239-241.

30. Jasani, B.; Kreil, G.; Mackler, B. F.; Stanworth, D. R. *Biochem. J.* **1979**, *181*, 623-632.

31. Bouhelal, R.; Guillon, G.; Homburger, V.; Bockaert, J. *J. Biol. Chem.* **1985**, *260*, 10901-10904.

32. Seamon, K. B.; Padgett, W.; Daly, J. W. *Proc. Natl. Acad. Sci.* **1981**, *78*, 3363-3367.

33. Rosenzweig, S.; Yan, W.; Dasso, M.; Spielman, A. I. *J. Neurophysiol.* **1999**, *81*, 1661-1665.

34. Hube, F.; Lee, Y. M.; Rohrig, K.; Hauner, H. *Horm Metab Res.* **1999**, *31*, 359-362.

35. Chijiwa, T.; Mishima, A.; Hagiwara, M.; Sano, M.; Hayashi, K.; Inoue, T.; Naito, K.; Toshioka, T.; Hidaka, H. *J Biol Chem* **1990**, *265*, 5267-5272.

36. Varkevisser, B.; Kinnamon, S. C. *J. Neurophysiol.* **2000**, *83*, 2526-2532.

37. Quick, J.; Ware, J. A.; Driedger, P. E. *Biochem Biophys Res Commun.* **1992**, *187*, 657-663.

38. Griffith, J. K.; Sanson, C. E. The Transporter: Facts book. In *FactsBook Series*; Academic Press: San Diego, 1998; pp. 500.

39. Mitchell, P. *J. Bioenerg.* **1973**, *4*, 63-91.

40. Hirayama, B. A.; Lostao, M. P.; Panayotova-Heiermann, M.; Loo, D. D.; Turk, E.; Wright, E. M. *Am. J. Physiol. Gastrointest. Liver Physiol.* **1996**, *270*, G919-G926.

41. Peri, I.; Mamrud-Brains, H.; Rodin, S.; Krizhanovsky, V.; Shai, Y.; Nir, S.; Naim, M. *Am. J. Physiol. Cell Physiol.* **2000**, *278*, C17-C25.

42. Minamiura, N.; Matsumura, Y.; Yamamoto, T. *J. Biochem.* **1972**, *72*, 841-848.

43. Ziesenitz, S. C.; Siebert, G. *Z. Ernahrungswiss.* **1988**, *27*, 155-169.

44. Striem, B. J.; Naim, M.; Lindemann, B. *Cell. Physiol. Biochem.* **1991**, *1*, 46-54.

45. Jarvis, S. M. Assay of membrane transport in cells and membrane vesicles. In *Membrane transport;* Baldwin, S. A. Ed.; Oxford University Press: Oxford, 2000; pp. 1-20.

46. Guzman-Grenfell, A. M.; Bonilla-Hernandez, M. A.; Gonzales-Martinez, M. T. *Biochim. Biophys. Acta* **2000**, *1464*, 188-198.

47. Krischer, S. M.; Eisenmann, M.; Bock, A.; Mueller, M. J. *J. Biol. Chem.* **1997**, *272*, 10601-10607.
48. Ruklisha, M.; Ionina, R. *Proc. Biochem.* **2000**, *35*, 841-848.
49. Hille, B. *Ion Channels of Excitable Membranes*, 3rd Edition ed.; Sinauer Assoc., Inc.: Sunderland, Massachusetts, 2001.
50. Zubare-Samuelov, M.; Peri, I.; Tal, M.; Tarshish, M.; Spielman, A. I.; Naim, M. *Am. J. Physiol. Cell Physiol.* **2003**, *285*, C1255-C1262.
51. Zubare-Samuelov, M.; Shaul, M. E.; Peri, I.; Aliluiko, A.; Tirosh, O.; Naim, M. *Am. J. Physiol. Cell Physiol.* **2005**, *289*, C483-C492.
52. Jakob, I.; Hauser, I. A.; Thevenod, F.; Lindemann, B. *Am. J. Physiol. Cell Physiol.* **1998**, *274*, C182-C191.
53. Bohm, S. K.; Grady, E. F.; Bunnett, N. W. *Biochem. J.* **1997**, *322*, 1-18.
54. Hamm, H. E.; Gilchrist, A. *Curr. Opin. Cell. Biol.* **1996**, *8*, 189-196.
55. Pitcher, J. A.; Freedman, N. J.; Lefkowitz, R. J. *Annu. Rev. Biochem.* **1998**, *67*, 653-692.
56. Gainetdinov, R. R.; Premont, R. T.; Bohn, L. M.; Lefkowitz, R. J.; Caron, M. G. *Ann. Rev. Neurosci.* **2004**, *27*, 107-144.
57. Avenet, P.; Hofmann, F.; Lindemann, B. *Nature* **1988**, *331*, 351-354.
58. Masuho, I.; Tateyama, M.; Saitoh, O. *Chem. Senses* **2005**, *30*, 281-290.
59. Naim, M.; Zubare-Samuelov, M.; Peri, I.; Shaul, M. *FASEB J* **2004**, *18*, A313 (abst 213.22).
60. Sasaki, Y.; Suzuki, M.; Hidaka, H. *Pharamacol. Therap.* **2002**, *93*, 225-232.
61. Vera, J. C.; Reyes, A. M.; Velasquez, F. V.; Rivas, C. I.; Zhang, R. H.; Strobel, P.; Slebe, J. C.; Nunez-Alarcon, J.; Golde, D. W. *Biochemistry* **2001**, *40*, 777-790.
62. Wong, G. T.; Gannon, K. S.; Margolskee, R. F. *Nature* **1996**, *381*, 796-800.
63. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. P. *Cell* **2003**, *112*, 293-301.
64. Herness, S.; Zhao, F. L.; Kaya, N.; Lu, S. G.; Shen, T.; Sun, X. D. *J. Physiol (Lond.).* **2002**, *543*, 601-614.

Chapter 18

We Are What We Eat, but Why? Relationships between Oral Sensation, Genetics, Pathology, and Diet

Derek J. Snyder[1], Valerie B. Duffy[2], Susan E. Marino[3], and Linda M. Bartoshuk[4]

[1]Neuroscience, Yale University, New Haven, CT 06520
[2]Allied Health Sciences, University of Connecticut, Storrs, CT 06269
[3]Pharmacy Practice, University of Florida, Gainesville, FL 32710
[4]Community Dentistry & Behavioral Science, Center for Smell and Taste, University of Florida, Gainesville, FL 32710–3628

We live in different taste worlds thanks to genetic and pathological influences. Individuals are born with varying numbers of fungiform papillae (i.e., structures that house taste buds), and those with the most fungiform papillae (i.e., supertasters) experience the most intense taste sensations. For example, supertasters experience roughly three times the sweetness experienced by those with the fewest fungiform papillae (i.e., nontasters). Since fungiform papillae receive touch and pain as well as taste innervation, supertasters also perceive the most intense sensations from oral tactile stimuli (e.g., fats) and oral irritants (e.g., chili peppers). Taste sensation is vulnerable to damage from multiple sources, including dental work, ear infection, head injury, and the use of certain drugs (e.g., antibiotics). Because taste normally inhibits non-taste oral sensations centrally, taste damage can intensify these sensations via disinhibition. These alterations affect food/beverage palatability and thus have commercial and health implications.

The sense of taste plays a significant role in food choice, as it signals the presence of biologically important molecules in the mouth (e.g., salt, sugars, toxins), guides their acceptance or rejection, and initiates their metabolism. Although we generally experience foods and beverages as a complex mixture of taste, tactile, and olfactory cues, taste appears to be the primary modality used to distinguish food vs. non-food items.   As such, an understanding of taste sensation at multiple levels is critical to the development of novel foods, drugs, and other ingested products. While great strides have been made recently in the molecular biology and physical chemistry of taste cues, the sensory and behavioral consequences of taste activation remain important research targets, mainly because they are often measured improperly and thus misunderstood. In this chapter, we identify several long-standing misconceptions regarding human taste perception. By resolving these errors, we hope to convey the influence of oral sensation on long-term health.

The Tongue Map is Widely Accepted, but False

Many texts addressing the sense of taste claim that specific modalities are perceived best on specific regions of the tongue; the typical arrangement has sweet on the tip, bitter at the base, and salty and sour on the edges. This "tongue map" originated from a mistranslation (1) of a German paper published at the beginning of the 20th century (2). Hänig found small differences in the thresholds for the four basic tastes (i.e., sweet, salty, sour, bitter) around the perimeter of the tongue. To Hänig, these differences suggested four discrete receptor mechanisms for taste, an idea that seems obvious today but was markedly ahead of its time.

Years later, Boring reexamined Hänig's data, but he plotted the reciprocals of the threshold values (i.e., 1/threshold) as measures of sensitivity. The resulting plot showed a maximum sensitivity for sweetness at the tip of the tongue and a maximum sensitivity for bitterness at the rear. To make matters worse, Boring failed to label the ordinate on his plot, leaving hapless readers unaware that the observed differences in regional sensitivity were actually very small. As a result, readers interpreted minimum sensitivity as no sensation, and the tongue map was born (3). Modern data confirm that the tongue map is false by showing that all four taste qualities are perceived wherever there are taste buds (4), yet this map persists.

A Relationship between Oral Anatomy and Taste Perception

While differences in sensitivity across the tongue are bogus, individual differences in tongue anatomy are not. In particular, marked variation occurs in

the density of fungiform papillae, the structures that house taste buds on the anterior tongue. Miller and his colleagues (5) showed that blue dyes fail to stain fungiform papillae, which remain pink against the blue background of the rest of the tongue, thus facilitating quantification. Figure 1 shows a line drawing of the tongues of a supertaster and a nontaster, demonstrating that the supertaster has significantly more fungiform papillae. Under higher magnification, taste pores (i.e., the conduits to taste buds) appear as blue dots against the pink background of the fungiform papilla. Although supertasters have slightly more taste buds per fungiform papilla, the difference in fungiform papilla density accounts for most of the anatomical variation observed (6).
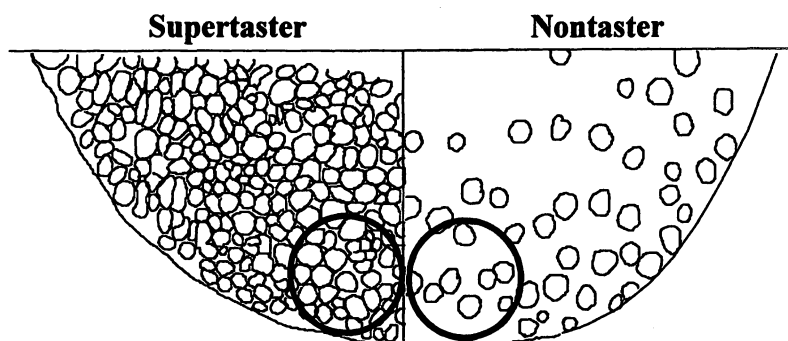
Figure 1.   Line drawings of fungiform papillae on the anterior tongue of a supertaster and a nontaster.   The circles indicate templates (6 mm diameter) used for counting.   This supertaster has 34 fungiform papillae partially or wholly within the template; some supertasters have as many as 60. The nontaster has only 8.   Note that fungiform papilla diameter decreases with rising density (6, 7).

Taste Blindness and PTC/PROP Genetics

The discovery of taste blindness paved the way for the study of individual differences in oral sensation. This phenomenon was first observed serendipitously by the industrial chemist A.L. Fox; while synthesizing phenylthiocarbamide (PTC), a colleague noticed a bitter taste in the air while Fox tasted nothing. Further testing revealed that PTC tastes bitter to most (i.e., tasters) but tasteless to some (i.e., nontasters) (8). Family studies suggested that taste blindness is genetically mediated, with tasting dominant and nontasting recessive (e.g., (9)). Anthropological studies suggested that taste blindness

varies by sex (i.e., females tend to be tasters) and race (i.e., Caucasians tend to be nontasters) (see (6) for the application of modern statistics to these early data). Meanwhile, evaluations of compounds of similar chemical structure to PTC associated its bitter taste with the N-C=S functional group. One of the members of this chemical family, 6-*n*-propylthiouracil (PROP), became the test stimulus of choice for taste blindness studies, both because it lacks the sulfurous odor of PTC (10) and because its clinical use to suppress thyroid function provides toxicity data.

Miller discovered the connection between tongue anatomy and PTC/PROP blindness. Rodents do not show taste blindness to PTC/PROP, but various mouse strains can be classified into "nontasters" or "tasters" of the bitterness of sucrose octaacetate, and taster mice have significantly more taste buds in the circumvallate papillae found on the rear of the tongue (11). Miller and Reedy (5) extended this finding to humans when they found that taste bud counts on fungiform papillae correlated significantly with the perceived intensities of some tastants (including PROP). The development of improved psychophysical scaling methods that permit accurate comparisons of suprathreshold PROP bitterness across subjects (12) revealed that some tasters (i.e., supertasters) experience much more intense bitterness than do others (i.e., medium tasters). Supertasters have the highest numbers of fungiform papillae and taste buds (6).

We speculated that supertasters might carry two dominant alleles for taste blindness, nontasters might carry two recessive alleles, and medium tasters might carry one dominant and one recessive allele. The discovery of a gene on chromosome 7 (TAS2R38) mediating PTC threshold sensitivity (13) proved this hypothesis wrong. On average, those carrying two dominant alleles for TAS2R38 find PROP slightly more intense than do those carrying only one dominant allele, but the difference is quite small. In other words, the density of fungiform papillae plays a critical role in taste blindness by amplifying the effects of TAS2R38 allele expression, as supertasters appear to be tasters who have a high density of fungiform papillae (14, 15).

Oral Pathology

Sensory information from the tongue is carried by several afferent nerves. The chorda tympani (CT), a branch of the facial nerve, carries taste information from the anterior, mobile tongue; the trigeminal nerve carries pain, tactile, and temperature information from the same region. Multimodal information (i.e., taste, touch, pain, temperature) is carried from the posterior tongue by the glossopharyngeal nerve (GL). Each of these nerves projects ipsilaterally into the central nervous system, but there are bilateral inhibitory interactions among their

central targets. These interactions contribute to the sensory alterations produced by taste damage (e.g., (16)). For example, unilateral CT damage intensifies taste perception on the contralateral rear of the tongue (i.e., GL) (17) and intensifies oral burn perception on the contralateral anterior tongue (i.e., trigeminal) (18).

Damage to nerves mediating taste is fairly common because of their anatomical paths. CT passes through the pterygomandibular space (i.e., between the pterygoid muscles and the lower jaw) with the lingual and inferior alveolar branches of the trigeminal nerve; the inferior alveolar nerve carries pain information from the lower teeth and is often targeted in dental anesthesia, which can damage any of these nerves. CT also passes through the middle ear, so it is exposed to pathogens during ear infections (i.e., otitis media) and upper respiratory infections. Before reaching the brainstem, CT traverses a long, bony passage, so it may be pinched or severed by head injury. CT and the greater superficial petrosal nerve (another branch of the facial nerve which conveys taste from the palate) join at the brainstem to form the nervus intermedius; this combined nerve and GL can be damaged by vestibulocochlear tumors (i.e., acoustic neuromas) or the surgery to remove them. GL is also vulnerable during tonsillectomy. Damage at these loci is believed to account for many clinical reports of taste loss and phantom oral sensations (e.g., dysgeusia, burning mouth syndrome) (4, 17, 19-22).

One noteworthy consequence of oral pathology is that the relationship between PROP bitterness and fungiform papillae density is imperfect (23), mainly because such damage may render a genetic and anatomical supertaster unable to taste PROP. Meanwhile, a small percentage of individuals carry two recessive alleles for TAS2R38 and cannot taste PTC/PROP, but have a high density of fungiform papillae. These groups raise the question: Are supertasters those who taste PROP as most bitter, or are they those with the most fungiform papillae? In hindsight, had we observed the anatomical variation before the perceptual, we would probably have defined supertasting in terms of anatomy, as we feel that the most salient feature of supertasting is the high intensity of oral sensation experienced across many stimuli. In practice, we have found it most useful to measure the contributions of multiple taste-related variables (e.g., PROP bitterness, quinine bitterness, sucrose sweetness, fungiform papilla density, TAS2R38 expression, oral pathology) to health outcomes (e.g., (24)).

Many Intensity Scales Fail to Produce Valid Group Comparisons

To show that individual differences in suprathreshold taste sensation are real, we must first be certain that the tools we use to measure sensory intensity do so accurately. The history of these tools reveals much about how they are used (and misused) today.

Measures of Human Experience

Labeled Scales Arose as a Practical Response to Specific Problems

We seem to communicate our sensory experiences easily: "There is a slight bitter taste in the wine; there is a strong sweet taste in the lemonade." Thus, many of the scales crafted to measure these sensations (e.g., Likert, 9-point, visual analogue) were labeled with the same descriptors used in everyday conversation. For example, an early 9-point category scale designed to measure taste sensations labeled alternate points: 1 = none, 3 = slight, 5 = moderate, 7 = strong, 9 = extreme (25). Scales like these were often created in response to specific research needs; Likert scales were designed for the measurement of attitudes (26), while the Natick 9-point scale was designed to help the Army assess preferences for field rations (27).

Today, measurement scales labeled with intensity descriptors are used widely throughout the medical, scientific, and consumer disciplines. Category scales rank sensations according to their perceived intensity, but the numbers used do not have ratio properties (i.e., a rating of "8" is greater than a rating of "4", but not necessarily twice as great). However, we can adjust the spacing of labels to confer ratio properties (28-31). The Labeled Magnitude Scale (LMS) accomplishes this task for oral sensations; it is a 100-point scale with several anchors spaced empirically: 0 = no sensation, 1.4 = barely detectable, 6 = weak, 17 = moderate, 35 = strong, 53 = very strong, 100 = strongest imaginable oral sensation (31). The Visual Analog Scale (VAS) is another labeled scale with ratio properties in which intermediate descriptors are removed, leaving a line labeled with the minimum and maximum sensations of interest (e.g., see (32)).

Interestingly, the relative spacing of intensity descriptors on the LMS is essentially the same as that found across most sensory modalities (33); one could imagine these descriptors printed on an elastic ruler that stretches to fit large domains (e.g., pain) or compresses to fit smaller ones (e.g., flavor of teas). S.S. Stevens captured this idea ((34), page 633): "Mice may be called large or small, and so may elephants, and it is quite understandable when someone says it was a large mouse that ran up the trunk of the small elephant." As this quotation illustrates, words like "large" and "small" have no absolute meaning until we know the domain to which they are applied.

Magnitude Estimation Arose from Measurement Theory

As a conceptual parallel to labeled scales, measurement theorists were also concerned with the quantification of human experiences. Stevens revolutionized psychophysics in the 1950s by introducing direct scaling methods with ratio

properties, the most popular of which is magnitude estimation (35). Stevens' methodology was applied to the chemical senses beginning in the 1960s (e.g., (36-39)), with impressive results. For example, magnitude estimation showed that the sweetness of sucrose grows with concentration much faster than does the bitterness of quinine (40). Magnitude estimation was also used to show the rules of cross-adaptation: Common qualities cross-adapt (e.g., NaCl adaptation abolishes the saltiness of other salts (41)), dissimilar qualities do not (e.g., quinine adaptation does not reduce the sweetness of sucrose (42)), and apparent quality alterations occurring with cross-adaptation are artifacts of water taste (e.g., quinine adaptation makes a sucrose solution taste sweeter because it makes the water solvent taste sweet (42)).

Invalid Comparisons: Causes, Consequences, and Corrections

Labeled scales and magnitude estimation: invalid across-group comparisons

Consider what happens when two people call a sucrose solution "strong" using a labeled scale or assign the number "10" using magnitude estimation; we cannot conclude that those two people are experiencing the same sweetness. Borg and Teghtsoonian addressed this problem with their range theory, which assumes that maximum sensory intensity is the same for virtually all modalities and all subjects (29, 43). If this assumption were true, then across-subject comparisons could be accomplished by rating sensations on a scale where the top boundary is maximum sensation. But the assumption is false; for example, individual differences in taste perception prove that range theory is not correct, as anatomical variation in fungiform papilla density makes maximum taste intensity much greater for supertasters than for nontasters (33). This systematic difference in perception of taste intensity across these groups makes comparisons across them with labeled scales or magnitude estimation invalid. Note that labeled scales and magnitude estimation are valid for making within-subject comparisons or for making across-group comparisons when members of the groups have been randomly assigned.

Improper scaling leads to invalid comparisons and erroneous conclusions

When studies using labeled scales fail to consider differences in the absolute intensities denoted by the labels, one cannot determine if comparisons across individuals or groups are accurate. To our dismay, this problem occurs in most published studies that use labeled scales to make across-group comparisons. Paradoxically, researchers make across-group comparisons because they believe interesting differences exist, but those very differences are likely to make scale labels mean different things to the groups being compared.

REVERSAL ARTIFACT
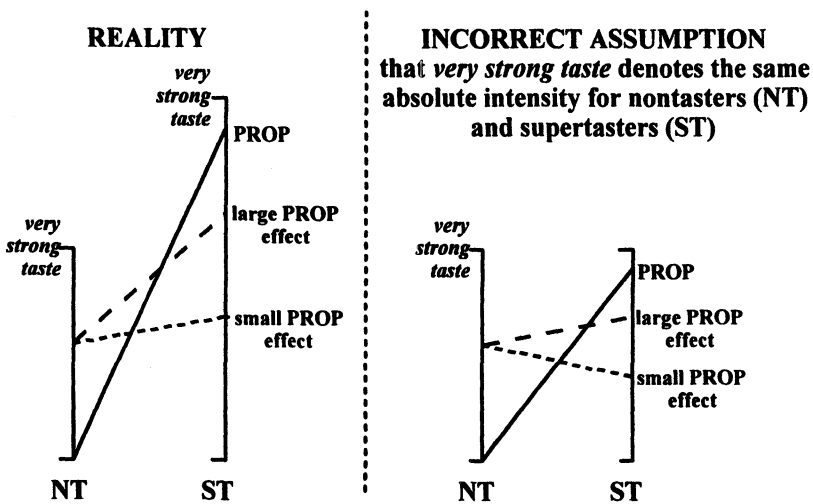
Figure 2. Actual differences between nontasters and supertasters of PROP are shown on the left. The consequences of assuming that a "very strong taste" is equally intense to both groups are shown on the right. See text for details.

Figure 2 illustrates the logic of invalid comparisons, using taste as an example. The left side of the figure shows a summary of the kinds of differences demonstrated between nontasters and supertasters; supertasters live in a more intense taste world than do nontasters and so a taste sensation that a supertaster would describe as a "very strong taste" is actually more intense than the experience that a nontaster would describe as a "very strong taste." The differences in perception of the bitter taste of PROP are indicated on the graph on the left. A stimulus that shows a large PROP effect (e.g., quinine) is indicated by the larger dashes; a stimulus that shows a smaller PROP effect (e.g., NaCl) is indicated by the smaller dashes. The right side of figure 2 shows what happens when the label "very strong taste" is treated as if it denotes the same average intensity to nontasters and supertasters. Reality is distorted. The PROP effect is still present, but it is diminished. For any stimulus with a large enough PROP effect (i.e., the difference across nontasters and supertasters must be larger than the difference between the intensities denoted by "very strong taste") the effect will still be present but the size of the effect will appear to be diminished. Moreover, any difference that is smaller than the difference between labels will appear to go in the wrong direction. This phenomenon is known as a reversal artifact, and it is a problem that unfortunately appears in the PTC/PROP literature (e.g., (44-48)).

Labeled scales and magnitude estimation have been used to make comparisons incorrectly for many years, and many investigators have commented on this error (e.g., (12, 33, 49-53)). Of particular note, Aitken observed when he originally introduced the VAS that it could not be used to make comparisons across subjects (54). Despite these numerous warnings, invalid scaling comparisons have flourished in many fields of study. One possible explanation for the ubiquity of invalid comparisons is that labeled scales were not regarded highly by measurement theorists. Stevens, for example, was interested in refining his psychophysical law and cared little about individual or group differences. As a result, the misuse of psychophysical scales may have escaped the attention of those best poised to correct it.

Magnitude matching: Non-oral standards enable oral sensory comparisons

Although his views on sensory maxima proved wrong, Borg was on the right track. The key to making valid across-subject comparisons came from studies on cross-modality matching (55, 56). We can match the intensities of stimuli from unrelated modalities, as illustrated by the following example: A concentrated NaCl solution and a whisper clearly do not match in intensity, but neither do a very dilute NaCl solution and a piercing scream; if these extremes do not match, an intermediate NaCl solution and an intermediate sound must. Extending this logic, if taste and hearing are unrelated, auditory stimuli can serve as a standard and taste stimuli can be rated relative to sound. This procedure, known as magnitude matching (57-59), enabled us to identify those who experience the most bitterness from PTC/PROP (60, 61).

The General Labeled Magnitude Scale (gLMS) permits magnitude matching

As noted above, we find it useful to think of labeled scales as elastic; the LMS can be stretched to fit the taste world of a supertaster or compressed to fit the taste world of a nontaster. Since the relative spacing among intensity descriptors is fixed, might stretching the LMS to its maximum produce a labeled scale that encompasses all sensory experience, thus allowing valid comparisons of oral sensory intensity? To generalize the LMS for experiences beyond oral sensation, we replaced its top anchor with the label "strongest imaginable sensation *of any kind*." This scale is now known as the general LMS (gLMS).

The top anchor of the gLMS functions as a standard, so it must remain unrelated to oral sensation to ensure valid comparisons of chemosensory function. Because taste is rarely the strongest sensation experienced in life, we assume that it is unrelated to those modalities that are strongest, so the "strongest imaginable sensation of any kind" should be, on average, equal for nontasters, medium tasters, and supertasters. Thus magnitude matching (using magnitude

estimation) and the gLMS should produce similar differences in bitterness among taster groups, and they do; both methods provide valid comparisons of oral sensation (62).
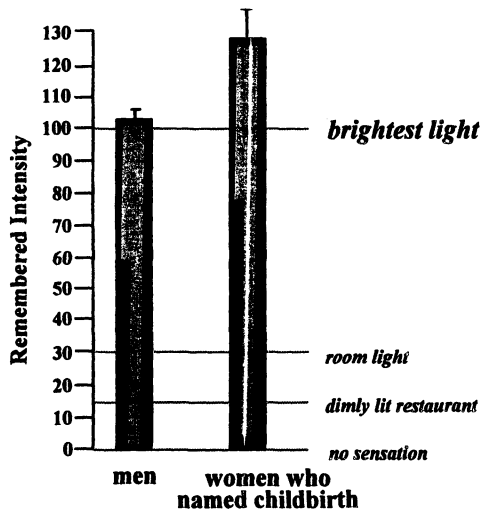
Figure 3. Remembered intensities of the strongest pain ever experienced by men and by women who named childbirth as their strongest pain. Ratings are expressed (i.e., normalized) relative to the brightest light ever seen. Ratings for the remembered brightness of a well-lit room and a dimly-lit restaurant are provided as well (52).

Because the gLMS can be used to assess sensations from multiple sensory domains, the intensity of one stimulus can be expressed relative to any other stimulus (i.e., magnitude matching). When the top of the gLMS is related to a sensation of interest, comparisons using raw gLMS ratings are invalid. For example, when lecture attendees used the gLMS to rate the strongest pain of any kind that they had ever experienced, many subjects rated that pain near the top of the scale, so different kinds of pain could not be compared. However, different kinds of pain could be compared by expressing pain ratings relative to an unrelated standard: "brightest light ever seen." That is, using an alternate standard, gLMS data were used for magnitude matching because the top anchor of the gLMS was an inappropriate standard for the comparisons of interest. With "brightest light ever seen" as the standard, a sex difference was observed (Figure 3): Women who rated childbirth as their most intense pain rated it 20% more intense than the brightest light they had seen; men rated their most intense pain nearly equal to the brightest light (52). The pain scale of these women is

stretched relative to that of men (e.g., (63)). This example illustrates the logical problem of using a scale labeled in terms of the sensation of interest; differences across subjects for the maximal sensation cannot be revealed.

The gLMS also shows promise as the basis for a hedonic scale. Building on the empirical finding that sensory and hedonic intensity labels are similarly spaced (28, 64, 65), a bipolar hedonic scale was created by extending two gLMSs in opposite directions from a common midpoint: "neutral." "Strongest imaginable disliking" anchors one end, and "strongest imaginable liking" anchors the other (66-79). This scale has proven useful for measuring food preferences. Later in this chapter, we will note that food preferences increase overall with body mass index, suggesting that the obese experience greater palatability from foods than do the non-obese (16). We are able to assess these differences because we assume that obese and thin subjects have many affective experiences in common (e.g., hearing a favorite song, loss of a loved one).

The search for appropriate standards continues

The standards used in the laboratory often require cumbersome and expensive equipment. Because scale labels rely on memories of perceived intensity, remembered sensations have been proposed as standards for magnitude matching. Table 1 lists remembered sensations rated by subjects in our ongoing questionnaire study (e.g., (52)), while Figure 4 shows how they can be used to quantify differences in taste sensation. Remembered sensations are especially useful in that they familiarize subjects with the idea of rating sensations of all kinds on a common scale, they result in a snapshot of a subject's sensory world, and they serve as a screening tool for subjects who do not understand the scaling task (e.g., loudness ratings should show an order effect: whisper < conversation < loudest sound ever heard). Although the precise relationship between real and remembered intensity is unclear (e.g., (80)), remembered oral sensations appear to reflect effects seen with actual stimuli (81). Including both real and remembered sensations as standards allows us to confirm our conclusions across a variety of assumptions.

The term "imaginable" has been added to the top label on a variety of scales (e.g., "strongest imaginable pain"). The use of this term appears to have been fueled by the hope that somehow we all imagine the same maximum even if our experiences vary. Fast (75) showed that the most intense imaginable sensation is highly correlated with the most intense sensation ever experienced. Thus "imaginable' confers no benefit to psychophysical scales.

Scale labels on the gLMS serve as an internal standard only when subjects use them outside the narrow context of the stimulus at hand. For example, when we study differences between nontasters and supertasters, it is critical that subjects rate taste stimuli relative to all experience, not just the taste experiences

Table 1. Remembered Sensations Used for Magnitude Matching

Brightness of a well-lit room
Brightness of a dimly-lit restaurant
Brightest light you have seen
Loudness of a whisper
Loudness of a conversation
Loudest sound you have heard
Warmth of warm bread in your mouth
Strongest smell of a flower (e.g., lilac, rose)
Sweetness of a coke
Bitterness of celery
Strongest saltiness experienced
Strongest sweetness experienced
Strongest sourness experienced
Strongest bitterness experienced
Strongest oral burn experienced (e.g., chili peppers)
Strongest oral pain experienced (e.g., toothache)
Strongest pain of any kind experienced (name type of pain)
Strongest sensation of any kind experienced (name type of sensation)
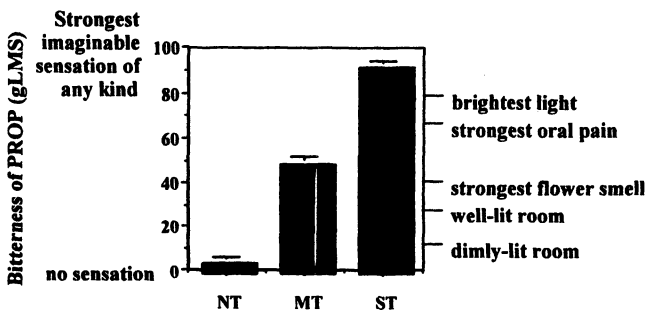
Figure 4. Ratings (gLMS) of filter papers impregnated with ~1.6 mg PROP. Supertasters (ST) were defined as the top 25% of respondents, nontasters (NT) as the bottom 25%. Ratings for remembered sensations reflect means for all subjects (N = 1348).

they have had. To prevent confusion, perhaps all labels should be abandoned except for those at the ends of the scale. The resulting scale – a line denoting the distance from "no sensation" to the "strongest sensation of any kind ever experienced" – is essentially a VAS encompassing all sensory modalities; we have proposed calling it the general/global VAS (gVAS) (12).

Individual Differences in Taste Perception Extend to Food-Related Sensations, Dietary Preferences, and Health Risk

Taste and Trigeminal Sensations

Even before we discovered supertasters, PTC/PROP nontasters and tasters showed important sensory differences. The bitter tastes of saccharin, KCl, sodium benzoate, and potassium benzoate are more intense for tasters (61, 82), as are the tastes of a multitude of sweeteners (Figure 5) (83). (Note, however, that psychophysical functions vary for each sweetener, consistent with the idea that sweeteners are perceptually distinct and utilize more than one binding site.) Some of these findings hinted at broader effects on diet: Differences in the tastes of $CaCl_2$ and casein fueled the discovery that some cheeses are more bitter and less palatable to tasters (84, 85). Ultimately, it became clear that virtually all tastes become more intense with increasing PROP bitterness (Figure 6) (86).
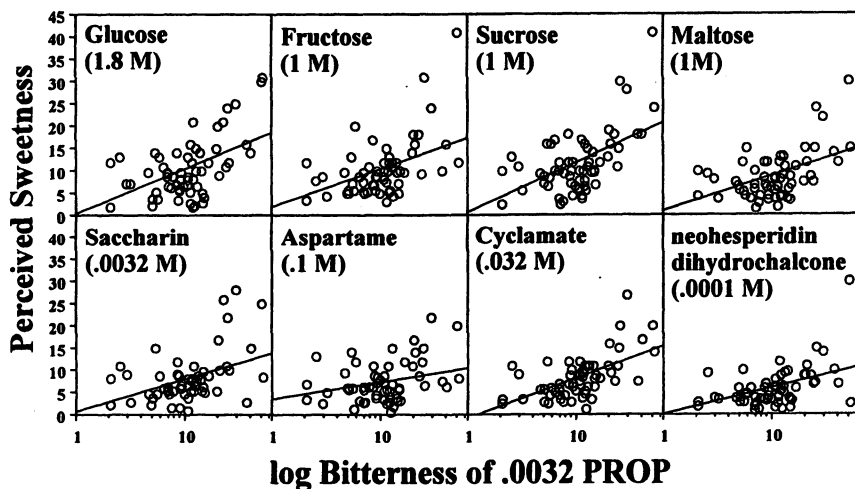
Figure 5. Sweetness of 8 sweeteners vs the log of PROP bitterness (magnitude estimates normalized to an auditory standard (87)).
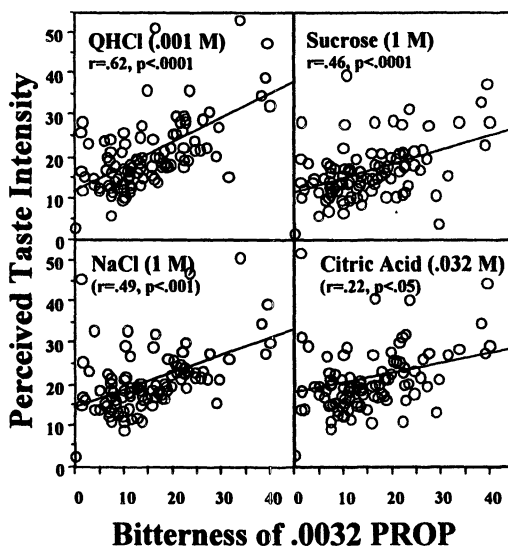
Figure 6. Similar to Figure 5; Taste intensities of quinine hydrochloride (QHCl), sucrose, NaCl, and citric acid vs. PROP bitterness (86).

Fungiform papillae are dually innervated, with 75% percent of nerve fibers arising from the trigeminal nerve (which carries pain, touch, and temperature cues) and 25% arising from the chorda tympani (which carries taste cues) (88). Chorda tympani nerve fibers form synapses with taste receptor cells within the taste bud, but trigeminal fibers appear to show functional segregation: Pain afferents form basket-like clusters surrounding taste buds (89-91), while tactile fibers innervate the papilla more diffusely to interact with tactile end-organs (92-96). Thus, increased fungiform papilla density is associated with elevated taste and trigeminal input. Supporting this view, two-point thresholds on the tongue correspond to the distance between fungiform papillae (97), resulting in elevated tactile acuity among supertasters (98). This dual innervation accounts for the elevated perceived intensities of oral irritants (e.g., alcohol, capsaicin) and thickeners (e.g., fats) observed among supertasters in Figure 7 (71, 99-106): They have the most fungiform papillae and thus the greatest oral sensory capacity.

Retronasal Olfaction

Odors reach olfactory receptors on the roof of the nasal cavity via two pathways. Sniffing through the nostrils (i.e., orthonasal olfaction) causes turbulence in the nasal cavity and allows a puff of air to pass through the olfactory cleft to the olfactory epithelium. Alternatively, chewing and
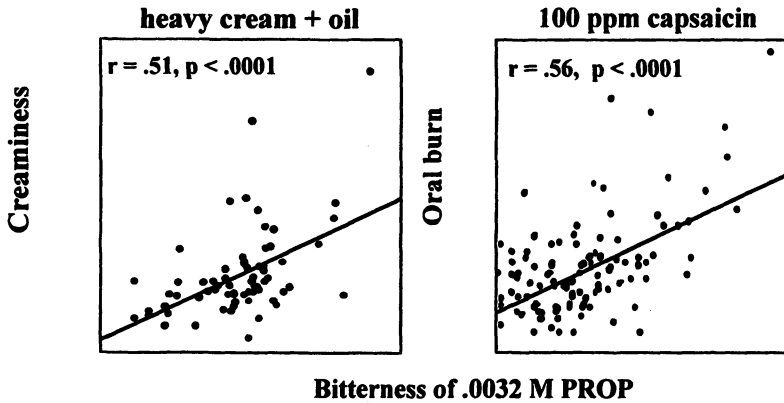
Figure 7. Magnitude estimates of oral touch (i.e., creaminess) produced by heavy cream + oil (left) (100) and oral burn produced by capsaicin (right) (101) vs. PROP bitterness. Data are normalized to the salty taste of NaCl. Note that these associations are conservative estimates of reality because the intensity of NaCl varies with PROP status (see Figure 6).

swallowing movements drive food volatiles in the mouth behind the palate and into the nasal cavity (i.e., retronasal olfaction), where they rise to the olfactory epithelium. Flavor is a composite sensation incorporating taste, oral somatosensory, and olfactory cues arising from the mouth; orthonasal olfaction is perceptually localized to the nose and retronasal olfaction is perceptually localized to the mouth.

Localization of retronasal cues to the mouth was long believed to involve oral touch alone (107), but recent experiments indicate that taste plays an important role as well. PROP intensity influences the perceived intensity of retronasal olfaction, as supertasters perceive more intense retronasal cues than do nontasters (Figure 8, top) (108-110). Further evidence for the role of taste in retronasal olfaction comes from experiments involving anesthesia of the chorda tympani; these studies indicate that taste loss compromises both the intensity and localization of flavor cues (109, 111). Clinical reports parallel these findings: Patients with surgical damage to the chorda tympani report a loss of flavor from foods, but orthonasal olfaction remains intact (112). Also, childhood ear infections (i.e., otitis media) can damage the chorda tympani as it traverses the middle ear, resulting in functional taste loss (113); recent data indicate that retronasal olfaction is lost as well (Figure 8, bottom) (114). As orthonasal and retronasal input appear to be processed in different regions of the brain (115), these experiments suggest that taste cues help to identify the route by which odorants reach the olfactory system. The food industry has capitalized on this idea for many years (e.g., (116)); flavor intensity is often enhanced by increasing the concentration of a congruent taste stimulus (e.g., fruit flavor + sweet taste).
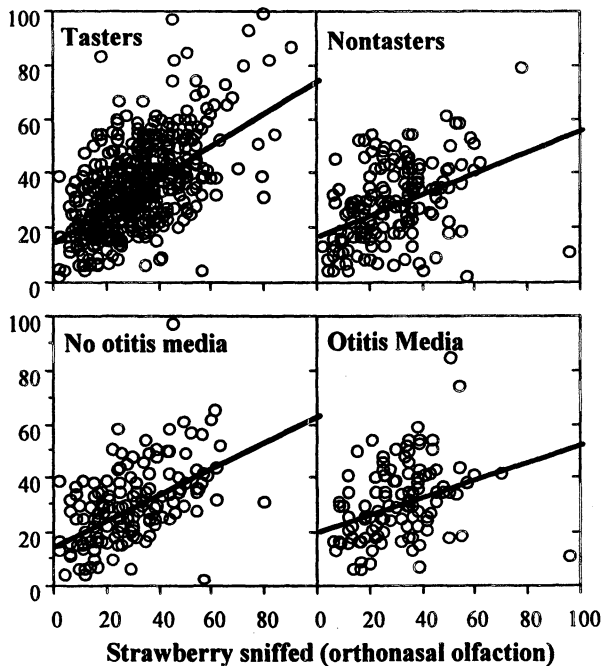
Figure 8. Retronasal perception of sampled strawberry candy vs. orthonasal perception of sniffed strawberry candy, (gLMS). The correlations for tasters (r = 0.58) and nontasters (r = 0.42) are significantly different (p = 0.01), as are the correlations for no history of otitis media (r = 0.53) vs. history of moderate/severe otitis media (r = 0.33; p = 0.05).

Food Hedonics and Health

Oral sensory variation naturally leads to variation in preferences for foods, beverages, and other oral stimuli (e.g., tobacco). These hedonic effects in turn affect behaviors that carry long-term health significance (e.g., dietary choices); aging and pathology modulate these effects and may bring emergent changes.

Cancer risk rises with increased PROP perception

In the 1960s, Milunicová reported more female cancers (e.g., breast, ovary, cervix) in tasters of PTC (117), and subsequent reports suggested that tasters may foment risk by consuming fewer foods containing cancer-preventive phytochemicals (118). We tested this hypothesis on male subjects undergoing colonoscopy as part of a colon cancer prevention program (119); among older men in the sample, we found that greater PROP bitterness associated with more

colon polyps and lower vegetable intake. In addition, subjects with more polyps had higher body mass indices (BMI) than did those with fewer polyps. Both low vegetable intake and adiposity are potential risk factors for colon cancer (e.g., (120, 121)), and both are associated with PROP status (e.g., (78)).

Cardiovascular disease is associated with low PROP intensity

Supertasters perceive more intense sensations from fats in foods (e.g., (100, 102, 122)) and they show reduced preference (100) and intake (122) for high-fat foods. As such, supertasters have superior cardiovascular profiles; female supertasters are at special advantage in this regard (122, 123).

Alcohol and tobacco use are associated with nontasting

For many years reports have suggested that the inability to taste PROP is associated with a higher rate of alcoholism (e.g., (124, 125)). More recently, studies with subjects recruited independently of alcohol status support this association: Nontasters show the greatest alcohol intake (15, 79), presumably because they experience the greatest sweetness and least bitterness from alcoholic beverages (23). These findings suggest that supertasters are protected against alcoholism due to its noxious bitter taste. Similarly, nontasters are most likely (126) and supertasters, least likely to smoke (127). These findings suggest that supertasting may confer protection against alcohol and tobacco use due to the unpleasant oral sensations (i.e., bitterness, irritation) associated with these products.

Aging brings dramatic shifts in oral sensation and preference

Aging probably contributes to diet-related health risk through complex interactions with PROP genetics, sex hormones, and pathology (e.g., (73, 128, 129)). Age-related changes in sex hormones confer diminished bitter sensation, particularly at menopause (e.g., (97)), leading to increased preference and intake measures for bitter beverages, fruits, and vegetables in aged females (130, 131). Concurrent disinhibition of oral somatosensory cues contributes to increased intensity from capsaicin (132), NaCl (70), and high-fat foods (133). These changes may explain broader alterations in food behavior and hedonics observed with advancing age.

Burning mouth syndrome (BMS) is a particularly extreme consequence of aging; bitter diminishes after menopause in women. BMS is characterized by severe oral pain in the absence of obvious pathology; it primarily affects postmenopausal women (134). BMS has often been described as psychogenic, but several reports led us to believe that it may be caused by taste damage. BMS

pain usually abates during eating, but it resumes soon afterward (sometimes with greater intensity), suggesting a link between taste and oral pain. Oral anesthesia usually causes BMS pain to intensify (135), implying oral disinhibition; consistent with our anesthesia data, nearly 50% of BMS patients experience taste phantoms (136). Indeed, psychophysical tests revealed that BMS patients show CT loss, particularly for bitter stimuli; many could not recognize the bitterness of concentrated quinine on the anterior tongue (21, 137). In addition, the intensity of the peak oral pain experienced with BMS showed strong correlation with fungiform papillae density, revealing that BMS is a disorder of supertasters. We suggest that BMS is an oral pain phantom akin to other sensory phantoms like tinnitus and phantom limb. BMS results because taste normally inhibits oral pain (e.g., (18)) and CT damage releases that inhibition, leading to chronic oral pain. Consistent with this view, BMS pain can be treated with agonists to the inhibitory neurotransmitter GABA, such as clonazepam (138). Presumably, GABA agonists restore lost inhibition from absent taste cues, thereby suppressing the phantom pain.

Nontasters are most likely to become obese, but supertasters with taste damage are also at risk...

Fischer (139) related PROP status to body types described by Kretschmer and Sheldon (140): Those with the lowest PROP and quinine thresholds (i.e., tasters) tend to be ectomorphs (i.e., thin), while those with the highest thresholds (i.e., nontasters) tend to be endomorphs (i.e., heavier). Multiple studies support this association in adults (67, 141-149); in studies where psychophysical methodology permitted separation of medium tasters and supertasters, supertasters had the lowest body mass indices (BMI), presumably because they avoid sweet-fat foods (78, 150-156). Results have been less consistent for children: Early work suggested that taster children have lower BMIs (157), but more recent studies in children have failed to show a relationship between PROP status and weight (158-160).

Nevertheless, environmental factors during childhood may influence long-term body mass. Middle ear infection (i.e., otitis media; OM) is a common disease of childhood that, if sufficiently severe, may damage CT. As we have described, taste damage elevates oral sensations elsewhere in the mouth, and adults with a history of severe OM show reduced taste sensation on the anterior tongue, increased taste perception on the posterior tongue, and increased oral touch and irritation (161). Given that CT loss also compromises retronasal olfaction, severe OM may cause the intensity (e.g., (133)) and salience of fats (e.g., (162, 163)) to rise gradually over time, thereby altering food behavior by shifting the balance of flavor cues. We have recently discovered that supertasters over age 30 with a history of severe OM have significantly elevated BMIs. In addition, their food preferences vary in a sex-specific manner that

reflects CT damage and trigeminal disinhibition (129, 164, 165): Unlike other male supertasters (e.g., (67, 141)), these men show avidity for bitter and high-fat foods; supertasting women normally show reduced sweet preferences with age (73), but these women fail to do so. Tonsillectomy, which may damage GL, appears to exacerbate the effects of OM: Preliminary data show that adults with histories of otitis media and tonsillectomy show reduced taste, elevated high-fat food preferences, and higher BMIs (166).

...or are they?

The idea that elevated preferences for sweet foods promote obesity makes intuitive sense, as observed by Pangborn and Simone in 1958: "In the mind of the layman, sugar and sweets are 'fattening' and most overweight individuals have a 'sweet tooth'" (167). While this view has persisted for many years, experimental data tell a different story: Several researchers (including Pangborn and Simone) have failed to find an association between sweet food preferences and BMI, and at least one study concluded that obese subjects show blunted liking for sweet (168). Meanwhile, a number of sensory studies have found that sweetness perception remains stable across variations in body weight (168-171). In contrast, studies on fat preference support the commonsense view that the obese like high-fat foods more than do normal-weight individuals (172-174).

Many of these studies were performed with scales that produce invalid group comparisons, which makes this question worth revisiting. Recent data collected with the sensory and hedonic gLMS support earlier findings on fat preference but challenge those for sweet (16): Obese individuals perceive less sweetness than do the non-obese, and when perceived sweetness is taken into account, the obese show higher sweet preferences (Figure 9). In addition, maximum and minimum liking scores for food rise with BMI, meaning that relative to other pleasures, food pleasure is more intense among the obese. Because the conventional hedonic scales used in earlier work treat maximum food liking as if it were equally intense for everyone, real differences in sweet liking between the obese and non-obese were obscured or reversed.

Summary

In an earlier era, it was easy to believe that we all experience roughly the same sensations from foods and beverages. The discovery of taste blindness in the 1930s was a curiosity that seemed to affect only a small group of chemically related bitter compounds; moreover, little was known about how chemosensation is damaged by disease or injury. However, the development of psychophysical tools that permit valid comparisons revealed a very different world. We now know that genetic and pathological factors result in large differences in oral
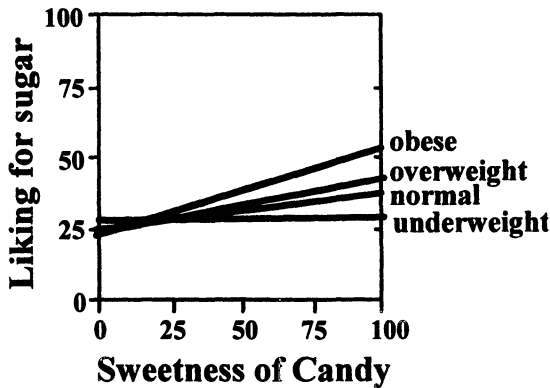
Figure 9. Regression lines for plots of the remembered liking for sugar vs. sweetness of butterscotch candy (N=3740) for underweight (BMI < 18.5), normal (BMI = 18.5-24.9), overweight (BMI = 25-29.9), and obese (BMI ≥ 30) subjects. Correlation coefficients (i.e., slopes of the regression lines) for underweight and normal subjects are significantly less than for obese subjects (p < 0.01) (16). These plots permit us to control for sweetness intensity, revealing that for a given level of sweetness, preference rises with BMI (from (16)).

sensory perception that influence behavior and disease risk. More recently, the human genome project has led to the identification of a variety of genes affecting taste and olfaction, and advances in neuroscience have led to increased understanding of the causes and consequences of chemosensory damage. In short, we have emerged from a time when human chemosensation seemed strictly a quality-of-life issue to one in which taste and olfaction are acknowledged as widespread contributors to overall health status.

References

1. Boring, E. G. *Sensation and perception in the history of experimental psychology*; Appleton: New York, 1942.
2. Hänig, D. P. *Philosophiche Studien* **1901**, *17*, 576-623.
3. *Genetic and pathological taste variation: What can we learn from animal models and human disease?*; Bartoshuk, L. M., Ed.; John Wiley and Sons: New York, 1993; Vol. Ciba Foundation Symposium 179.
4. Yanagisawa, K.; Bartoshuk, L. M.; Catalanotto, F. A.; Karrer, T. A.; Kveton, J. F. *Physiology and Behavior* **1998**, *63*, 329-335.
5. Miller, I. J.; Reedy, F. E. *Physiology and Behavior* **1990**, *47*, 1213-1219.
6. Bartoshuk, L. M.; Duffy, V. B.; Miller, I. J. *Physiology and Behavior* **1994**, *56*, 1165-1171.

7. Cohen, Z. D.; Bartoshuk, L. M.; Fast, K.; Duffy, V. B. *Chemical Senses* **1999**, *24*, 604.
8. Fox, A. L. *Science News Letter* **1931**, *9*, 249.
9. Snyder, L. H. *Ohio Journal of Science* **1932**, *32*, 436-440.
10. Fischer, R.; Griffin, F. *Experientia* **1961**, *17*, 1-7.
11. Miller, I. J.; Whitney, G. *Neuroscience Letters* **1989**, *100*, 271-275.
12. Bartoshuk, L. M.; Fast, K.; Snyder, D. *Current Directions in Psychological Science* **2005**, *14*, 122-125.
13. Kim, U. K.; Jorgenson, E.; Coon, H.; Leppert, M.; Risch, N.; Drayna, D. *Science* **2003**, *299*, 1221-1225.
14. Bartoshuk, L.; Davidson, A.; Kidd, J.; Kidd, K.; Speed, W.; Pakstis, A.; Reed, D.; Synder, D.; Duffy, V. *Chemical Senses* **2005**, *30*, A87.
15. Duffy, V. B.; Davidson, A. C.; Kidd, J. R.; Kidd, K. K.; Speed, W. C.; Pakstis, A. J.; Reed, D. R.; Snyder, D. J.; Bartoshuk, L. M. *Alcoholism: Clinical and Experimental Research* **2004**, *28*, 1629-1637.
16. Bartoshuk, L. M.; Duffy, V. B.; Hayes, J. E.; Moskowitz, H.; Snyder, D. J. *Philosophical Transactions of the Royal Society: Biological Sciences* **2006**, *361*, 1137-1148.
17. Lehman, C. D.; Bartoshuk, L. M.; Catalanotto, F. C.; Kveton, J. F.; Lowlicht, R. A. *Physiology and Behavior* **1995**, *57*, 943-951.
18. Tie, K.; Fast, K.; Kveton, J.; Cohen, Z.; Duffy, V. B.; Green, B.; Prutkin, J. M.; Bartoshuk, L. M. *Chemical Senses* **1999**, *24*, 609.
19. Catalanotto, F. A.; Bartoshuk, L. M.; Östrum, K. M.; Gent, J. F.; Fast, K. *Chemical Senses* **1993**, *18*, 461-470.
20. Kveton, J. F.; Bartoshuk, L. M. *Laryngoscope* **1994**, *104*, 25-29.
21. Grushka, M.; Bartoshuk, L. M. *The Canadian Journal of Diagnosis* **2000**, *17*, 99-109.
22. Bartoshuk, L. M.; Snyder, D. J.; Grushka, M.; Berger, A. M.; Duffy, V., B,; Kveton, J. F. *Chemical Senses* **2005**, *30 (suppl 1)*, i218-i219.
23. Lanier, S. A.; Hayes, J. E.; Duffy, V. B. *Physiology & Behavior* **2005**, *83*, 821-831.
24. Dinehart, M. E.; Hayes, J. E.; Bartoshuk, L. M.; Lanier, S. L.; Duffy, V. B. *Physiology & Behavior* **2006**, *87*, 304-313.
25. Kamen, J. M.; Pilgrim, F. J.; Gutman, N. J.; Kroll, B. J. *Journal of Experimental Psychology* **1961**, *62*, 348-356.
26. Likert, R. *Archives of Psychology* **1932**, *140*, 5-55.
27. Peryam, D. R.; Pilgrim, F. J. *Food Technology* **1957**, *11*, 9-14.
28. Moskowitz, H. R. *Journal of Food Quality* **1977**, *1*, 195-228.
29. Borg, G. In *Psychophysical Judgment and the Process of Perception*; Geissler, H. G., Petzold, P., Eds.; VEB Deutscher Verlag der Wissenschaften: Berlin, 1982, p 25-34.
30. Gracely, R. H.; McGrath, P.; Dubner, R. *Pain* **1978**, *5*, 19-29.
31. Green, B. G.; Shaffer, G. S.; Gilmore, M. M. *Chemical Senses* **1993**, *18*, 683-702.

32. Hetherington, M. M.; Rolls, B. J. In *Feeding and Drinking*; Toates, F. M., Rowland, N. E., Eds.; Elsevier Science Publishers (Biomedical Division): New York, 1987, p 77-109.

33. Bartoshuk, L. M.; Duffy, V. B.; Fast, K.; Green, B. G.; Prutkin, J. M.; Snyder, D. J. *Food Quality and Preference* 2002, *14*, 125-138.

34. Stevens, S. S. *The American Journal of Psychology* 1958, *71*, 633-646.

35. Stevens, S. S. *American Journal of Psychology* 1956, *69*, 1-25.

36. Bartoshuk, L. M. *Perception and Psychophysics* 1968, *3*, 69-72.

37. Engen, T. *Reports from the Psychological Laboratory, The University of Stockholm* 1961, *106*, 1-13.

38. McBurney, D. H. *Journal of Experimental Psychology* 1966, *72*, 869-873.

39. Stevens, S. S. *Perception and Psychophysics* 1969, *6*, 302-308.

40. Bartoshuk, L. M. *Physiology and Behavior* 1975, *14*, 643-649.

41. McBurney, D. H.; Lucas, J. A. *Psychonomic Science* 1966, *4*, 301-302.

42. McBurney, D. H.; Bartoshuk, L. M. *Physiology and Behavior* 1973, *10*, 1101-1106.

43. Teghtsoonian, R. *American Journal of Psychology* 1973, *86*, 3-27.

44. Schifferstein, H. N. J.; Frijters, J. E. R. *Chemical Senses* 1991, *16*, 303-317.

45. Drewnowski, A.; Henderson, S. A.; Shore, A. B. *American Journal of Clinical Nutrition* 1997, *66*, 391-397.

46. Drewnowski, A.; Henderson, S. A.; Shore, A. B.; Barratt-Fornell, A. *Physiology and Behavior* 1997, *62*, 649-655.

47. Smagghe, K.; Louis-Sylvestre, J. *Appetite* 1998, *30*, 325-339.

48. Yackinous, C.; Guinard, J. X. *Physiology and Behavior* 2001, *72*, 427-437.

49. Narens, L.; Luce, R. D. *Theory and Decision* 1983, *15*, 247-260.

50. Biernat, M.; Manis, M. *Journal of Personality and Social Psychology* 1994, *66*, 5-20.

51. Birnbaum, M. H. *Psychological Methods* 1999, *4*, 243-249.

52. Bartoshuk, L. M.; Duffy, V. B.; Chapo, A. K.; Fast, K.; Yiee, J. H.; Hoffman, H. J.; Ko, C.-W.; Snyder, D. J. *Food Quality and Preference* 2004, *15*, 617-632.

53. Snyder, D. J.; Fast, K.; Bartoshuk, L. M. *Journal of Consciousness Studies* 2004, *11*, 40-57.

54. Aitken, R. C. B. *Proceedings of the Royal Society of Medicine* 1969, *62*, 989-993.

55. Stevens, J. C. *Journal of Experimental Psychology* 1959, *57*, 201-209.

56. Stevens, J. C.; Marks, L. E. *Proceedings of the National Academy of Sciences* 1965, *54*, 407-411.

57. Stevens, J. C.; Marks, L. E. *Perception and Psychophysics* 1980, *27*, 379-389.

58. Marks, L. E.; Stevens, J. C. In *Aging in the 1980's: Psychological issues*; Poon, L. W., Ed.; American Psychological Association: Washington, 1980, p 592-598.

59. Marks, L. E.; Stevens, J. C.; Bartoshuk, L. M.; Gent, J. G.; Rifkin, B.; Stone, V. K. *Chemical Senses* **1988**, *13*, 63-87.

60. Hall, M. J.; Bartoshuk, L. M.; Cain, W. S.; Stevens, J. C. *Nature* **1975**, *253*, 442-443.

61. Bartoshuk, L. M. *Science* **1979**, *205*, 934-935.

62. Bartoshuk, L. M.; Duffy, V. B.; Green, B. G.; Hoffman, H. J.; Ko, C.-W.; Lucchina, L. A.; Marks, L. E.; Snyder, D. J.; Weiffenbach, J. *Physiology & Behavior* **2004**, *82*, 109-114.

63. Dionne, R. A.; Bartoshuk, L. M.; Mogel, J.; Witter, J. *Trends in Pharmacological Sciences* **2005**, *26*, 125-130.

64. Moskowitz, H. R.; Chandler, J. W. In *Sensory Properties of Foods*; Birch, G. G., Brennan, J. G., Parker, K. J., Eds.; Applied Science Publishers Ltd.: London, 1977, p 189-211.

65. Schutz, H. G.; Cardello, A. V. *Journal of Sensory Studies* **2001**, *16*, 117-159.

66. Bartoshuk, L. M.; Duffy, V. B.; Fast, K.; Green, B.; Kveton, J.; Lucchina, L. A.; Prutkin, J. M.; Snyder, D. J.; Tie, K. *Appetite* **1999**, *33*, 228-229.

67. Duffy, V. B.; Fast, K.; Cohen, Z.; Chodos, E.; Bartoshuk, L. M. *Chemical Senses* **1999**, *24*, 545-546.

68. Duffy, V. B.; Phillips, M. N.; Peterson, J. M.; Bartoshuk, L. M. *Appetite* **2001**, *37*, 137-138.

69. Chapo, A. K.; Bartoshuk, L. M.; Peterson, J. M.; Phillips, M. N.; Duffy, V. B. *Chemical Senses* **2001**, *26*, 1072.

70. Chapo, A. K.; Phillips, M. N.; Ilich, J. Z.; Duffy, V. B. *The Gerontologist* **2001**, *41*, 83.

71. Phillips, M. N.; Bartoshuk, L. M.; Peterson, J. M.; Duffy, V. B. *Chemical Senses* **2001**, *26*, 1040-1041.

72. Snyder, D. J.; Duffy, V. B.; Fast, K.; Hoffman, H. J.; Ko, C. W.; Weiffenbach, J. M.; Bartoshuk, L. M. *Chemical Senses* **2001**, *26*, 1050.

73. Snyder, D. J.; Duffy, V. B.; Fast, K.; Weiffenbach, J. M.; Bartoshuk, L. M. *Appetite* **2001**, *37*, 164.

74. Bartoshuk, L. M.; Duffy, V. B.; Fast, K.; Green, B. G.; Snyder, D. J. In *Food Selection from Genes to Culture*; Anderson, H., Blundell, J., Chiva, M., Eds.; Danone Institute: Paris, France, 2002, p 173-187.

75. Fast, K.; Green, B. G.; Bartoshuk, L. M. *Appetite* **2002**, *39*, 75.

76. Reed, D. R.; Li, X.; Chen, Z.; Mascioli, K.; Bachmanov, A. A.; Beachamp, G. K.; Tordoff, G. K.; Max, M.; Margolskee, R. F.; Bartoshuk, L. M.; Duffy, V. B. *Obesity Research* **2002**, *10*, 67.

77. Duffy, V. B.; Peterson, J. M.; Dinehart, M. E.; Bartoshuk, L. M. *Topics in Clinical Nutrition* **2003**, *18*, 209-220.

78. Duffy, V. B.; Lucchina, L. A.; Bartoshuk, L. M. In *Genetic Variations in Taste Sensitivity: Measurement, Significance and Implications*; Prescott, J., Tepper, B. J., Eds.; Dekker: New York, 2004, p 195-228.

79. Duffy, V. B.; Peterson, J. M.; Bartoshuk, L. M. *Physiology & Behavior* **2004**, *82*, 435-445.

80. Algom, D. In *Psychophysical Approaches to Cognition*; Algom, D., Ed.; North-Holland: New York, 1992, p 441-513.

81. Stevenson, R. J.; Prescott, J. *Acta Psychologica* **1997**, *95*, 195-214.

82. Bartoshuk, L. M.; Rifkin, B.; Marks, L. E.; Hooper, J. E. *Chemical Senses* **1988**, *13*, 517-528.

83. Gent, J. F.; Bartoshuk, L. M. *Chemical Senses* **1983**, *7*, 265-272.

84. Marino, S.; Bartoshuk, L. M.; Monaco, J.; Anliker, J. A.; Reed, D.; Desnoyers, S. *Chemical Senses* **1991**, *16*, 551.

85. Anliker, J. A.; Bartoshuk, L. M.; Ferris, A. M.; Hooks, L. D. *Americal Journal of Clinical Nutrition* **1991**, *54*, 316-320.

86. Ko, C. W.; Hoffman, H. J.; Lucchina, L. A.; Snyder, D. J.; Weiffenbach, J. M.; Bartoshuk, L. M. *Chemical Senses* **2000**, *25*, 639-640.

87. Bartoshuk, L. M.; Fast, K.; Karrer, T. A.; Marino, S.; Price, R. A.; Reed, D. A. *Chemical Senses* **1992**, *17*, 594.

88. Silver, W. L.; Finger, T. E. In *Smell and Taste in Health and Disease*; Getchell, T. V., Doty, R. L., Bartoshuk, L. M., Snow, J. B., Eds.; Raven Press: New York, 1991, p 97-108.

89. Nagy, J. I.; Goedert, M.; Hunt, S. P.; Bond, A. *Neuroscience* **1982**, *7*, 3137-3151.

90. Finger, T. E. *Chemical Senses* **1986**, *11*, 135-144.

91. Whitehead, M. C.; Beeman, C. S.; Kinsella, B. A. *American Journal of Anatomy* **1985**, *173*, 185-201.

92. Hilliges, M.; Astback, J.; Wang, L.; Arvidson, K.; Johansson, O. *Anatomical Record* **1996**, *245*, 621-632.

93. Spassova, I. *Journal of Anatomy* **1974**, *118*, 1-9.

94. Toyoshima, K.; Miyamoto, K.; Itoh, A.; Shimamura, A. *Cell and Tissue Research* **1987**, *250*, 237-239.

95. Watanabe, L. S. *Anatomical Science International* **2004**, *79*, 55-61.

96. Zahm, D. S.; Munger, B. L. *Brain Research Reviews* **1985**, *9*, 147-186.

97. Prutkin, J. M.; Duffy, V. B.; Etter, L.; Fast, K.; Gardner, E.; Lucchina, L. A.; Snyder, D. J.; Tie, K.; Weiffenbach, J.; Bartoshuk, L. M. *Physiology and Behavior* **2000**, *69*, 161-173.

98. Essick, G. K.; Chopra, A.; Guest, S.; McGlone, F. *Physiology & Behavior* **2003**, *80*, 289-302.

99. Karrer, T.; Bartoshuk, L. M.; Conner, E.; Fehrenbaker, S.; Grubin, D.; Snow, D. *Chemical Senses* **1992**, *17*, 649.

100. Duffy, V. B.; Bartoshuk, L. M.; Lucchina, L. A.; Snyder, D. J.; Tym, A. *Chemical Senses* **1996**, *21*, 598.

101. Snyder, D. J.; Lucchina, L. A.; Duffy, V. B.; Bartoshuk, L. M. *Chemical Senses* **1996**, *21*, 673.

102. Tepper, B. J.; Nurse, R. J. *Physiology and Behavior* **1997**, *61*, 949-954.

103. Prutkin, J. M.; Fast, K.; Lucchina, L. A.; Bartoshuk, L. M. *Chemical Senses* **1999**, *24*, 243.

104. Prescott, J.; Swain-Campbell, N. *Chemical Senses* **2000**, *25*, 239-246.

105. Prescott, J.; Johnstone, V.; Munro, P. *Chemical Senses* **2001**, *26*, 800.

106. Prescott, J.; Bartoshuk, L. M.; Prutkin, J. M. In *Genetic Variations in Taste Sensitivity: Measurement, Significance and Implications*; Prescott, J., Tepper, B. J., Eds.; Dekker: New York, 2004, p 89-104.

107. Hollingworth, H. L.; Poffenberger, A. T. *The Sense of Taste*; Moffat, Yard and Company: New York, NY, 1917.

108. Duffy, V. B.; Chapo, A. K.; Hutchins, H. L.; Bartoshuk, L. M. *Chemical Senses* **2003**, *28*, A33.

109. Snyder, D. J.; Dwivedi, N.; Mramor, A.; Bartoshuk, L. M.; Duffy, V. B. In *Society of Neuroscience Abstract*; Society for Neuroscience: San Diego, CA, 2001; Vol. 27, p AA-2, Program No. 727.11.

110. Bartoshuk, L. M.; Christensen, C. M.; Duffy, V.; Sheridan, K.; Small, D. M.; Synder, D. *Chemical Senses* **2005**, *30*, A236.

111. Fast, K.; Tie, K.; Bartoshuk, L. M.; Kveton, J. F.; Duffy, V. B. *Chemical Senses* **2000**, *25*, 614-615.

112. Bull, T. R. *Journal of Laryngology and Otology* **1965**, *79*, 479-493.

113. Bartoshuk, L. M.; Chapo, A. K.; Duffy, V. B.; Grushka, M.; Norgren, R.; Kveton, J.; Pritchard, T. C.; Snyder, D. J. *Chemical Senses* **2002**, *27*, A52.

114. Collins, S. P.; Snyder, D. J.; Catalanotto, F.; Bartoshuk, L. M. *Chemical Senses* **2006**, *31*, A38.

115. Small, D. M.; Jones-Gotman, M. *Chemical Senses* **2001**, *26*, 1034.

116. Noble, A. C. *Trends in Food Science and Technology* **1996**, *7*, 439-444.

117. Milunicová, A.; Jandová, A.; Skoda, V. *Human Heredity* **1969**, *19*, 398-401.

118. Drewnowski, A.; Henderson, S. A.; Barratt-Fornell, A. *Drug metabolism and disposition* **2001**, *29*, 535-538.

119. Basson, M. D.; Bartoshuk, L. M.; Dichello, S. Z.; L., P.; Weiffenbach, J.; Duffy, V. B. *Digestive Diseases and Sciences* **2005**, *50*, 483-489.

120. Giovannucci, E. *Gasteroenterology Clinics of North America* **2002**, *31*, 925-943.

121. Rafter, J. J. *British Journal of Nutrition* **2002**, *88 Suppl 2*, S219-224.

122. Hutchins, H. L.; Healy, N. A.; Duffy, V. B. *Chemical Senses* **2003**, *28*, 15.

123. Duffy, V. B.; Hutchins, H.; Allen, G. J.; Pescatello, L. S. *Chemical Senses* **2002**, *27*, A23.

124. Pelchat, M. L.; Danowski, S. *Physiology and Behavior* **1992**, *51*, 1261-1266.

125. DiCarlo, S. T.; Powers, A. S. *Physiology and Behavior* **1998**, *64*, 147-152.

126. Fischer, R.; Griffin, F.; Kaplan, A. R. *Medicina Experimentalis* **1963**, *9*, 151-167.

127. Snyder, D. J.; Davidson, A. C.; Kidd, J. R.; Kidd, K. K.; Speed, W. C.; Pakstis, A. J.; Cubells, J. F.; O'Malley, S. S.; Bartoshuk, L. M. In *Society for Research on Nicotine and Tobacco* Prague, Czech Republic, 2005.

128. Chapo, A. K.; Bartoshuk, L. M.; Brownbill, R.; Hutchins, H. L.; Ilich, J.; Duffy, V. B. In *International Congress of Dietetics* Chicago, IL, 2004.

129. Snyder, D. J.; Duffy, V. B.; Chapo, A. K.; Hoffman, H.; Bartoshuk, L. M. *Chemical Senses* **2003**, *28*, 12.

130. Chapo, A. K.; Bartoshuk, L. M.; Duffy, V. B. *Chemical Senses* **2003**, *28*, 36.

131. Snyder, D. J.; Duffy, V. B.; Hoffman, H. J.; Ko, C. W.; Bartoshuk, L. M. *Chemical Senses* **2002**, *27*, A19.

132. Bartoshuk, L. M.; Caseria, D.; Catalanotto, F.; Dabrila, G.; Duffy, V. B.; Lucchina, L. A.; Nadoolman, W.; Sasaki, C.; Snyder, D. J.; Wolfe, J. *Chemical Senses* **1996**, *21*, 578.

133. Chapo, A. K.; Bartoshuk, L. M.; Ilich, J. Z.; Duffy, V. B. *Chemical Senses* **2002**, *27*, A19.

134. Grushka, M.; Sessle, B. J. *The Clinical Journal of Pain* **1987**, *2*, 245-252.

135. Ship, J. A.; Grushka, M.; Lipton, J. A.; Mott, A. E.; Sessle, B. J.; Dionne, R. A. *Journal of the American Dental Association* **1995**, *126*, 842-853.

136. Grushka, M.; Sessle, B. J.; Howley, T. P. *Chemical Senses* **1986**, *11*, 485-498.

137. Bartoshuk, L. M.; Grushka, M.; Duffy, V. B.; Fast, K.; Lucchina, L. A.; Prutkin, J. M.; Snyder, D. J. *Chemical Senses* **1999**, *24*, 609.

138. Grushka, M.; Epstein, J.; Mott, A. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **1998**, *86*, 557-561.

139. Fischer, R.; Griffin, F.; Rockey, M. A. *Perspectives in Biology and Medicine* **1966**, *9*, 549-577.

140. Maher, B. A.; Maher, W. B. *Journal of Abnormal Psychology* **1994**, *103*, 72-77.

141. Dabrila, G. M.; Bartoshuk, L. M.; Duffy, V. B. *Journal of the American Dietetic Association* **1995 (Supplement)**, *95*, A64.

142. Duffy, V. B.; Bartoshuk, L. M.; Peterson, J. M.; Phillips, M. N. *Chemical Senses* **2001**, *26*, 1115.

143. Lucchina, L. A. Ph.D., University of Connecticut, 1995.

144. Lucchina, L.; Bartoshuk, L. M.; Duffy, V. B.; Marks, L. E.; Ferris, A. M. *Chemical Senses* **1995**, *20*, 735.

145. Tepper, B. J.; Ullrich, N. V. *Physiology & Behavior* **2001**, *75*, 305-312.

146. Tepper, B.; Ullrich, N. *Appetite* **1999**, *33*, 234-235.

147. Tepper, B. J. *Appetite* **1999**, *32*, 422.

148. Tepper, B. J. *Chemical Senses* **2003**, *28*, E60.

149. Tepper, B. J. In *Genetic Variation in Taste Sensitivity*; Prescott, J., Tepper, B. J., Eds.; Marcel Dekker, Inc.: New York, 2004, p 155-177.

150. Duffy, V. B.; Bartoshuk, L. M. *Journal of the American Dietetic Association* **2000**, *100*, 647-655.

151. Duffy, V. B. *Appetite* **2004**, *43*, 5-9.

152. Duffy, V. B.; Hutchins, H. L.; Creighton, J.; Pescatello, L. S. *Journal of the American Dietetic Association-Supplement 1* **2003**, *103*, A-11.

153. Duffy, V. B.; Hurchins, A. K.; Chapo, A. K.; Bartoshuk, L. M. *Chemical Senses* **2003**, *28*, E15.

154. Duffy, V. B.; Weingarten, H. P.; Bartoshuk, L. M. *Chemical Senses* **1995**, *20*, **688**.

155. Peterson, J. M.; Bartoshuk, L. M.; Duffy, V. B. *Journal of the American Dietetic Association* **1999**, *99 (Supplement 1)*, A-28.

156. Looy, H.; Weingarten, H. P. *Physiology and Behavior* **1992**, *52*, 75-82.

157. Johnston, F. E.; Hertzog, K. P.; Malina, R. M. *American Journal of Physical Anthropology* **1966**, *24*, 253-255.

158. Keller, K. L.; Steinman, L.; Nurse, R. J.; Tepper, B. *Chemical Senses* **1999**, *24*, 546.

159. Keller, K. L.; Tepper, B. J. *Chemical Senses* **2002**, *27*, A42-A43.

160. Keller, K. L. *Obesity Research* **2004**, *12*, 904-912.

161. Bartoshuk, L. M.; Duffy, V. B.; Reed, D.; Williams, A. *Neuroscience and Biobehavioral Reviews* **1996**, *20*, 79-87.

162. Johnson, S. L.; McPhee, L.; Birch, L. L. *Physiology and Behavior* **1991**, *50*, 1245-1251.

163. Lucas, F.; Sclafani, A. *Physiology and Behavior* **1989**, *46*, 403-412.

164. Snyder, D. J.; Duffy, V. B.; Bartoshuk, L. M. In *Pangborn Sensory Science Symposium* Yorkshire, UK, 2005.

165. Snyder, D. J.; Duffy, V. B.; Chapo, A. K.; Cobbett, L. E.; Bartoshuk, L. M. *Obesity Research* **2003**, *11 (Suppl)*, A147.

166. Chapo, A.; Alex, J.; Coelho, D.; Duffy, V. B.; Snyder, D.; Bartoshuk, L. *Chemical Senses* **2005**, *30*, A88.

167. Pangborn, R. M.; Simone, M. *Journal of the American Dietetic Association* **1958**, *34*, 924-928.

168. Grinker, J.; Hirsch, J.; Smith, D. V. *Journal of Personality and Social Psychology* **1972**, *22*, 320-325.

169. Frijters, J. E. R.; Rasmussen-Conrad, E. L. *Journal of General Psychology* **1982**, *107*, 233-247.

170. Enns, M. P.; Van Itallie, T. B.; Grinker, J. *Physiology and Behavior* **1979**, *22*, 999-1003.

171. Thompson, D. A.; Moskowitz, H. R.; Campbell, R. C. *Journal of Applied Physiology* **1976**, *41*, 77-83.

172. Pangborn, R. M.; Bos, K. E.; Stern, J. S. *Appetite* **1985**, *6*, 25-40.

173. Drewnowski, A.; Kurth, C. L.; Rahaim, J. E. *American Journal of Clinical Nutrition* **1991**, *54*, 635-641.

174. Mela, D. J.; Sacchetti, D. A. *American Journal of Clinical Nutrition* **1991**, *53*, 908-915.

Chapter 19

Perception and Acceptance of Sweeteners

Jeannine F. Delwiche[1] and Amanda R. Warnock[1,2]

[1]Department of Food Science and Technology, The Ohio State University, Columbus, OH 43210
[2]Unilever, 271 Jones Hill Road, West Haven, CT 06516

This study examined the possibility that variation in acceptability of sweeteners is due more to variation across individuals in sensitivity to non-sweet tastes rather than sensitivity to sweetness *per se*. Thirty individuals assessed 13 sweeteners, rating sweet, sour, salty, bitter and metallic intensities, as well as liking and acceptance. Results indicated that bitter intensity and sweetener type were the two largest factors contributing to liking and acceptance. Sensitivity to PROP did not contribute significantly to liking or acceptance.

Until recently, cumulative biochemical and electrophysiological research suggested two models of sweet taste transduction[1]. Several such studies with a variety of rodents indicated that while carbohydrate sweeteners were transduced by a cAMP second messenger system, artificial sweeteners and amino acids were transduced by an IP3 second messenger system[2-9]. In contrast, more recent findings indicate there is only one receptor[10, 11] and one signaling pathway [12] involved in the perception of sweetness. These most recent findings contradict psychophysical research findings that routinely suggest more than a single mechanism is involved in the perception of sweeteners[13-17].

This apparent contradiction between the recent neurophysiological findings and human perception can be explained by hypothesizing that some sweeteners are activating other taste receptors in addition to the sweet receptors. Finding suggest that there are 20-30 bitter receptors[18-21] and extreme variability in the

perception of bitter compounds across individuals is well documented (see [22] for a review). It is possible that individuals have similar variability in their sensitivity to the non-sweet tastes associated with certain sweeteners.

Of course, differences in liking and acceptance of particular sweeteners are driven by more than differences in perceived intensity alone. Acceptance of any taste or flavor (or any other perceptual experience for that matter) is logically driven by a minimum of three factors. The first is *sensitivity* because in order to accept or reject something, one must first be able to perceive it. The second is *familiarity*. It is well-known that the valence of the affect (positive or negative) of previous exposure(s) will shape the affect elicited by subsequent exposure. In other words, if prior exposure to the sensation is associated with a pleasant experience, such as a subsequent feeling of satiety, it will be more likely to elicit a positive affect, and in turn liking of the sensation, when it is experienced later. If instead the prior exposure to the sensation is associated with an unpleasant experience, such as a feeling of nausea following the sensation, it will be more likely to elicit negative affect, and in turn dislike of the sensation, when it is experienced later. Of course, the culture in which one lives will determine which experiences will become familiar. A third factor that impacts liking and acceptance is *personality*. While some individuals actively seek out new experiences and new sensations, others prefer to limit their contact with the unknown and prefer to limit their exposure to new sensations. Such traits will impact an individual's liking and acceptance of sensations.

The perceptual differences between various sweeteners are obvious when used by consumers. A brief search of the internet on November 5, 2006 revealed half a dozen non-commercial sites where individuals expressed a preference for the taste of one artificial sweetener over others. This study examined the hypothesis that individual differences in liking and acceptance of sweeteners is due to variation across individuals in sensitivity to the taste qualities of non-sweet tastes (bitter, sour, and metallic) of some sweeteners. Thirty individuals assessed thirteen sweeteners for perceived intensity, liking, and acceptance.

Materials and Methods

Stimuli

Aqueous solutions were made from thirteen types of sweeteners, selected from several chemical categories (*carbohydrates*: sucrose, glucose, and fructose; *proteins/amino acids*: thaumatin, aspartame, d-tryptophan, and glycine; *terpenoids*: stevioside; *N-sulfonylamides*: acesulfame-K and sodium saccharin; *halogenated sugar*: sucralose; *sugar alcohol*: xylitol; and *sulfamate*: sodium cyclamate). Concentrations of each sweetener was set to be the same intensity as

200 mM sodium chloride as determined by Guinard et al.[23] for most (aspartame, acesulfame-K, cyclamate, d-tryptophan, sucrose, glucose, thaumatin, xylitol, glycine and saccharin) and bench top testing for the compounds not included in that study (sucralose, fructose, stevioside). Specifically, the concentrations were as follows: 401 mM sucrose, 1120 mM glucose, 15 mM d-tryptophan, 0.0023 mM thaumatin, 930 mM xylitol, 5.21 mM sodium saccharin, 2.89 mM aspartame, 2380 mM glycine, 0.745 mM stevioside, 29.1 mM sodium cyclamate, 1.21 mM sucralose, 600 mM fructose and 0.038 mM acesulfame potassium. All solutions were made with Millipore™ polished water (Millipore RiOs™ 16 and Milli-QR Gradient, Millipore Corporation, Bedford, MA) between 24 hours and 5 days prior to testing.

Before subjects began assessing intensities, the subjects were presented with reference solutions for sweet (292 mM sucrose), sour (5.2 mM citric acid), salty (125 mM sodium chloride), bitter (0.032 mM quinine sulfate) and metallic (250 mg ferrous sulfate tablets per liter). In addition, to encourage the subjects to rate a single solution with more than one taste quality, a "sweet + bitter" reference containing 292 mM sucrose and 0.032 mM quinine sulfate was included. In the final session, individuals' sensitivity to 6-n-propyl-3-thiouracil (PROP) was determined by panelists' ratings of PROP bitterness intensity at several concentrations: 0.055 mM, 0.174 mM, 0.55 mM, 1.74 mM and 5.50 mM (6-n-propyl-3-thiouracil, Sigma Chemical, St. Louis, MO).

Panelists

Thirty paid volunteer subjects (19 female, 11 male; 18 - 45 years of age) were recruited in accordance with the approval of procedures by The Ohio State University Office of Responsible Research Practices. All subjects gave informed consent before participating. No qualifying criteria were used.

Procedure

Each panelist attended four sessions. All sessions were conducted in computer equipped sensory testing booths and data was collected using Compusense® five version 4.6 software (Compusense Inc, Guelph, Ontario, Canada). In all sessions, samples were presented as 20 mL aliquots in 1 oz plastic cups (Solo Plastic Souffles, P100, Solo Cup Company, Baltimore MD) labeled with random 3-digit codes. Samples were counterbalanced across panelists and blocked so that each panelist received all solutions once before receiving any of the solutions a second time. In sessions 2 and 3 only, panelists were instructed to rinse for 30 seconds between assessments while a 30-second

countdown appeared on-screen. Depending on the particular session (see details below), the rinse was either water (Millipore™ polished) or concentrated sucrose (814 mM).

In the first session, panelists rated both overall liking and overall acceptability of each of the thirteen sweeteners in duplicate, for a total of 54 assessments. Overall liking was rated on the 9-point hedonic scale[24], ranging from "1 = dislike extremely" to "9 = like extremely." Overall acceptability was rated on a 7-point scale that ranged from "not acceptable at all" to "completely acceptable." Half of the subjects rated all stimuli for acceptability before proceeding to rate the stimuli for liking, while the remaining half rated all stimuli for liking before proceeding to rate the stimuli for acceptability. As they were hedonic assessments, the participants did not receive any specific training before beginning.

In the sessions 2 and 3, the panelists rated, in replicate, perceived intensity of sweet, sour, salty, bitter, and metallic taste of the thirteen sweeteners and a water blank on the generalized LMS scale[25] for a total of 28 assessments. Before assessing the sweeteners, panelists were familiarized with the taste qualities by sampling and rating labeled reference solutions (described above). The difference between session 2 and session 3 was the rinse solution. In session 2 the rinse solution was Millipore™-polished water while in session 3 the rinse was a concentrated sucrose solution (814 mM). It is well documented that the intensities of taste qualities are often perceived as less intense when present in a mixture. For example, a mixture of quinine and sucrose is less sweet than an equal concentration of sucrose tasted alone, and less bitter than an equal concentration of quinine tasted alone[26]. However, after adaptation to one of these, the perceived intensity of the other mixture component will return to its unmixed intensity level, a phenomenon known as release from suppression[27]. As the focus of this research was non-sweet tastes, this condition was intended to accentuate non-sweet taste intensities, the premise being that increasing the intensity of these non-sweet tastes would allow for more accurate assessment. Half of the panelists completed session 2 (water rinse) before completing session 3 (concentrated sucrose rinse) while the remaining panelists completed the sessions in the reverse order.

In the fourth and final session, panelist sensitivity to PROP was determined following the protocol of Delwiche et al.[22]. Panelists rated the perceived bitterness of 5 concentrations of PROP in duplicate on the generalized LMS scale. Also in this final session, panelists rated the perceived loudness intensity of a series of tones (0, 20, 35, 50, 65 and 80 decibels) twice on the generalized LMS scale[25]. The tones were played for 1 second at 4000 Hz. Tones were presented to the right ear of each panelist via a headset attached to the AS208 audiometer from Interacoustics (Denmark). These tone intensity ratings were used to account for differences in scale usage, as described below.

Statistical Analysis

The loudness ratings of the tones collected in the last session were used following the protocols developed in Delwiche et al[22] to reduce discrepancies due to differences in scale usage. Using natural breaks in the bitterness intensity ratings of 1.74 mM PROP, subjects were broken into three groups, with 10 hypo-tasters, 17 tasters, and 3 hyper-tasters (often called non-tasters, tasters, and super tasters, as in [28, 29, 30]).

Two-way repeated measures ANOVAs on sweeteners (all 13) and rinse (water vs. sucrose) were performed on ratings for each taste quality (sweet, sour, salty, bitter, metallic). In addition, a one-way repeated measures ANOVA across compounds (13 sweeteners and the water blank) was conducted on ratings of each taste quality from the water-rinse session. Scheffé's post-hoc tests were used when appropriate. Repeated measures ANOVAs were conducted with Statistica 7 (Statsoft Inc. Tulsa, OK).

Additionally, two linear models of the data were created, the dependent variables being ratings of overall liking in one and ratings of acceptability in the other. The independent variables in both were the intensity ratings of the sweet, sour, salty, bitter and metallic taste qualities from the water rinse condition, the PROP status (non-taster, taster, or hyper-taster) and the sweetener. The categorical variables (PROP status and sweetener) were included in the model by means of dummy coding [31]. Linear models were created with SPSS 14.0 (SPSS, Inc. Chicago, IL).

Results

Despite the fact that concentrations were selected so that all sweeteners would have the same sweet intensity, a significant difference in sweetness was found across compounds (one-way ANOVA, $p < 0.001$ – see Table I). In fact, when sweeteners were compared to water (with one-way ANOVA), no significant difference in sweetness was found between water, acesulfame-K, glycine, and stevioside (Scheffé's, $p < 0.05$ – see Table I). However, these differences in the means do not make clear the huge individual differences in perceived sweetness of the compounds. For example, ratings for acesulfame K showed the greatest variation, ranging from barely detectable to strongest imaginable while glucose, which showed the least variation, ranged from weak to strong. As expected, sweetness ratings were lower after the sucrose rinse than after the water rinse (two-way ANOVA, $p < 0.001$). While the concentrated sucrose rinse significantly reduced sweetness for fructose, sucralose and sucrose (Scheffé's, $p < 0.05$), it did not for the other sweeteners (Scheffé's, $p > 0.05$), resulting in a significant interaction between rinse and sweeteners (two-way

ANOVA, p<0.001). Since the average sweetness of acesulfame K, glycine, and stevioside was not rated as sweeter than water (Scheffé's, p > 0.05), it is not surprising that the concentrated sucrose rinse did not significantly lower their ratings. However, it is more difficult to explain why the concentrated sucrose rinse did not significantly suppress the sweetness of aspartame, glucose, xylitol, saccharin, d-tryptophan, sodium cyclamate and thaumatin without hypothesizing the existence of more than one perceptual mechanism for sweetness. These findings do, nevertheless, correspond with earlier findings[15-17] indicating that sweeteners do not cross-adapt symmetrically or uniformly.

Table I. Mean Intensity of Sweetener Taste Qualities

Compound	Sweet	Sour	Salty	Bitter	Metallic
Water	0.33[a]	0.96[a]	0.43[a]	2.07[a]	2.54[a]
AceK	0.43[a]	0.42[a]	0.29[a]	2.16[a]	2.88[a]
Aspartame	18.93[bcd]	0.91[a]	0.72[a]	1.45[a]	0.42[a]
Cyclamate	25.12[cd]	2.51[a]	4.11[ab]	3.14[a]	1.56[a]
D-tryptophan	19.18[bcd]	1.52[a]	0.87[a]	20.05[c]	2.97[a]
Fructose	28.04[cd]	3.69[a]	0.98[ab]	2.54[a]	1.63[a]
Glucose	24.26[cd]	2.37[a]	0.56[a]	1.35[a]	2.30[a]
Glycine	6.78[ab]	24.14[b]	5.82[b]	1.97[a]	3.00[a]
Saccharin	24.91[cd]	2.39[a]	0.79[a]	14.53[bc]	1.39[a]
Stevioside	14.00[abc]	2.07[a]	1.63[ab]	10.12[abc]	0.18[a]
Sucralose	30.98[d]	0.98[a]	0.32[a]	1.96[a]	0.74[a]
Sucrose	27.48[cd]	0.71[a]	0.22[a]	0.84[a]	0.88[a]
Thaumatin	25.70[cd]	1.59[a]	2.44[ab]	8.51[ab]	4.69[a]
Xylitol	25.48[cd]	4.95[a]	1.03[ab]	0.94[a]	0.86[a]
AOV p-values	<0.001	<0.001	<0.001	<0.001	0.411

NOTE: Means in a column with the same superscript are not significantly different (Scheffé's, p > 0.05). Significant p-values are in **bold**.

One-way ANOVAs also found significant differences across sweeteners for sourness, saltiness and bitterness (p < 0.05, Table I), although none was found for metallic taste (p > 0.05, Table I). As with the sweetness intensities, tremendous variation across panelists was found in assessments of the other attributes as well. For sour, salty, bitter, and metallic taste qualities, the rinse condition (water vs. concentrated sucrose) did not significantly alter ratings (two-way ANOVAs, p>0.05), nor were interactions between rinses and sweeteners significant (two-way ANOVAs, p>0.05). The non-sweet tastes of the sweeteners did seem to be somewhat increased by rinsing with concentrated sucrose, which may have become significant with the testing of additional

panelists or at other sweetener concentration, suggesting there was some release from suppression. However, rinsing with concentrated sucrose certainly did not clarify assessments of non-sweet tastes. Due to its differential impact on the perception of sweetness across sweeteners, it added an unwarranted level of complexity to the dataset; these assessments were not used in any additional analyses.

Table II. Significance of Variables in Linear Models

Variable	Liking (p-values)	Acceptance (p-values)
Sweet	0.997	0.214
Sour	0.138	0.128
Salty	0.866	0.474
Bitter	**0.007**	**0.013**
Metallic	0.839	0.098
Sweetener	**<0.001**	**<0.001**
PROP Status	0.678	**0.030**
Adjusted R-squared	**0.327**	**0.350**

Values in bold were significant (p<0.05).

The first linear model showed that two variables significantly contributed to ratings of overall liking (see Table II): sweetener compound (p<0.001) and bitter intensity rating (p=0.007). The second model, using ratings of overall acceptability as the dependent variable, showed three independent variables made significant contributions (see Table II): sweetener compound (p<0.001), bitter intensity rating (p=0.013) and PROP status (p=0.030). Individuals who were PROP hypo-tasters had a larger negative regression coefficient than either tasters or hyper-tasters, suggesting that hypo-tasters are more likely to rate sweetener acceptability as low than tasters or hyper-tasters. This finding is fairly surprising as one might predict that since hypo-tasters are generally less sensitive to bitter tastes, they would also be less sensitive to the bitterness of certain sweeteners and thus more accepting of them. However, it is not entirely clear how the panelists interpreted "Acceptability," as there were a few panelists who paradoxically gave ratings for a particular sweetener of both "completely acceptable" and "dislike extremely." Perhaps they assumed as all sweeteners were safe and therefore acceptable. Or perhaps they recognized certain sweeteners and, while they personally disliked it, recognized that others found it acceptable. However, as the rinsing protocol was much more lax during session 1, the most likely explanation is that the presentation order had a profound impact on these hedonic assessments.

Discussion

The large individual differences in intensity ratings for the sweeteners that remained even after centralization of the means for all rated attributes were striking. On average, the acesulfame K solution was rated so low so as to be found not significantly different in sweetness than water, while one individual rated the solution as being the strongest imaginable sweetness. While there was also large variability across individuals in the perceived intensities of the non-sweet attributes, they were not as large as the variations in sweetness. In addition, it is important to note that adaptation to a high concentration sucrose solution did not significantly reduce the perceived sweetness of several sweeteners. Thus, it still remains difficult to reconcile the most recent neurophysiological findings on sweetness with the human perception of sweetness.

The results of the general linear models suggest the perception of bitterness and the sweetener type were the two largest factors contributing to overall liking of a sweetener. Since the concentration levels of the sweeteners were selected to be similar, greater variation in non-sweet tastes was expected, which may explain why sweetness did not contribute significantly to the models of overall liking or acceptance. Despite the fact that metallic taste is a common complaint associated with certain sweeteners, it also failed to contribute significantly to the models of overall liking or acceptance.

While PROP status did not contribute significantly to the linear model of overall liking, it did contribute significantly to the model of acceptability. As mentioned above, the hypo-tasters were less accepting of the sweeteners they rated higher in bitterness than were tasters and hyper-tasters. It is possible that hyper-tasters not only perceive more bitterness than do the hypo-tasters, but also more sweetness, which in turn suppressed the additional bitterness. In fact, several studies have shown that hyper-tasters are more sensitive to all tastes, not just bitterness (e.g., [28, 29, 36, 38-40]).

The adjusted R-squared values of both models are relatively low. Clearly, the variables considered do not fully account for the variability across individuals in the liking and acceptance of sweeteners. One variable not measured which has been shown both to differ across sweeteners[32-36] and to impact liking[37] is the onset and off-times of each sweetener. In addition, as mentioned earlier, liking and acceptance is also influenced by familiarity and personality factors, which also went unmeasured. Thus, it is not surprising that the adjusted R-squared values were not higher. Nonetheless, it is striking that bitterness and type of sweetener are as effective as they are at predicting liking. Also worth noting is that consumer acceptance of sweeteners are better predicted from perceived bitterness than from perceived sweetness, as was hypothesized.

References

1. Herness, M. S.; Gilbertson, T. A., Cellular mechanisms of taste transduction. *Annual Review of Physiology* **1999**, *61*, 873-900.
2. Bernhardt, S. J.; Naim, M.; Zehavi, U.; Lindemann, B., Changes in IP3 and cytosolic Ca^{2+} in response to sugars and non-sugar sweeteners in transduction of sweet taste in the rat. *The Journal of Physiology* **1996**, *490 (Pt 2)*, 325-336.
3. Cummings, T. A.; Powell, J.; Kinnamon, S. C., Sweet taste transduction in hamster taste cells: evidence for the role of cyclic nucleotides. *Journal of Neurophysiology* **1993**, *70, (6)*, 2326-2336.
4. Cummings, T. A.; Daniels, C.; Kinnamon, S. C., Sweet taste transduction in hamster: sweeteners and cyclic nucleotides depolarize taste cells by reducing a K+ current. *Journal of Neurophysiology* **1996**, *75, (3)*, 1256-1263.
5. Striem, B. J.; Pace, U.; Zehavi, U.; Naim, M.; Lancet, D., Sweet tastants stimulate adenylate cyclase coupled to GTP-binding protein in rat tongue membranes. *The Biochemical Journal* **1989**, *260, (1)*, 121-126.
6. Striem, B. J.; Naim, M.; Lindemann, B., Generation of cyclic AMP in taste buds of the rat circumvallate papilla in response to sucrose. *Cellular Physiology and Biochemistry* **1991**, *1, (1)*, 46-54.
7. Tonosaki, K.; Funakoshi, M., Cyclic nucleotides may mediate taste transduction. *Nature* **1988**, *331, (6154)*, 354-356.
8. Uchida, Y.; Sato, T., Changes in outward K+ currents in response to two types of sweeteners in sweet taste transduction of gerbil taste cells. *Chemical Senses* **1997**, *22, (2)*, 163-169.
9. Uchida, Y.; Sato, T., Intracellular calcium increase in gerbil taste cell by amino acid sweeteners. *Chemical Senses* **1997**, *22, (1)*, 83-91.
10. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E., Human receptors for sweet and umami taste. *Proceedings of the National Academy of Sciences of the United States of America* **2002**, *99*, 4692-4696.
11. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S., Mammalian sweet taste receptors. *Cell* **2001**, *106, (3)*, 381-390.
12. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. P., Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* **2003**, *112, (3)*, 293-301.
13. Breslin, P.; Beauchamp, G.; Puch, E., Monogeusia for fructose, glucose, sucrose, and maltose. *Perception and Psychophysics* **1996**, *53*, 327-341.
14. Faurion, A.; Saito, S.; Mac Leod, P., Sweet taste involves several distinct receptor mechanisms. *Chemical Senses* **1980**, *5, (2)*, 107-121.

294

15. Lawless, H. T.; Stevens, D. A., Cross adaptation of sucrose and intensive sweeteners. *Chemical Senses* **1983**, *7, (3/4)*, 309-315.

16. McBurney, D. H., Gustatory cross adaptation between sweet-tasting compounds. *Perception & Psychophysics* **1972**, *11, (3)*, 225-227.

17. Schiffman, S. S.; Cahn, H.; Lindley, M. G., Multiple receptor sites mediate sweetness: evidence from cross adaptation. *Pharmacology Biochemistry and Behavior* **1981**, *15, (3)*, 377-88.

18. Adler, E.; Hoon, M. A.; Mueller, K. L.; Chandrashekar, J.; Ryba, N. J. P.; Zuker, C. S., A novel family of mammalian taste receptors. *Cell* **2000**, *100*, 693-702.

19. Caicecdo, A.; Roper, S. D., Taste receptor cells that discriminate between bitter stimuli. *Science* **2001**, *291*, 1557-1560.

20. Chandrashekar, J.; Mueller, K. L.; Hoon, M. A.; Adler, E.; Feng, L.; Guo, W.; Zuker, C. S.; Ryba, N. J. P., T2Rs function as bitter taste receptors. *Cell* **2000**, *100*, 703-711.

21. Matsunami, H.; Montmayeur, J.-P.; Buck, L. B., A family of candidate taste receptors in human and mouse. *Nature* **2000**, *404*, 601-604.

22. Delwiche, J. F.; Buletic, Z.; Breslin, P. A. S., Covariation in individuals' sensitivities to bitter compounds: Evidence supporting multiple receptor/transduction mechanisms. *Perception and Psychophysics* **2001**, *63, (5)*, 761-776.

23. Guinard, J.-X.; Hong, D. Y.; Zoumas-Morse, C.; Budwig, C.; Russell, G. F., Chemoreception and perception of the bitterness of isohumulones. *Physiology & Behavior* **1994**, *56, (6)*, 1257-1263.

24. Lawless, H. T.; Heymann, H., *Sensory Evaluation of Food: Principles and Practices*. Chapman & Hall: New York, 1998.

25. Bartoshuk, L. M.; Duffy, V. B.; Green, B. G.; Hoffman, H. J.; Ko, C. W.; Lucchina, L. A.; Marks, L. E.; Snyder, D. J.; Weiffenbach, J. M., Valid across-group comparisons with labeled scales: The gLMS versus magnitude matching. *Physiology & Behavior* **2004**, *82, (1)*, 109-114.

26. McBurney, D. H.; Bartoshuk, L. M., Interactions between stimuli with different taste qualities. *Physiology & Behavior* **1973**, *10, (6)*, 1101-1106.

27. Lawless, H., The taste of creatine and creatinine. *Chemical Senses and Flavour* **1979**, *4, (3)*, 249-258.

28. Bartoshuk, L. M.; Duffy, V. B.; Miller, I. J., PTC/PROP tasting: anatomy, psychophysics, and sex effects. *Physiology & Behavior* **1994**, *56, (6)*, 1165-1171.

29. Bartoshuk, L. M.; Duffy, V. B.; Reed, D.; Williams, A., Supertasting, earaches and head injury: genetics and pathology alter our taste worlds. *Neuroscience and Biobehavioral Review* **1996**, *20, (1)*, 79-87.

30. Prescott, J.; Soo, J.; Campbell, H.; Roberts, C., Responses of PROP taster groups to variations in sensory qualities within foods and beverages. *Physiology and Behavior* **2004**, *82, (2-3)*, 459-469.

31. Aiken, L. S.; West, S. G., *Multiple regression: Testing and interpreting interactions*. Sage Publications: London, 1991.

32. Ayya, N.; Lawless, H. T., Quantitative and qualitative evaluation of high-intensity sweeteners and sweetener mixtures. *Chemical Senses* **1992**, *17, (3)*, 245-259.

33. Calvino, A. M.; Iglesias, M. P.; Tamasi, O. P., Sensory analysis of the sweetener potency by time-intensity curves. *Alimentaria* **2000**, *311*, 77-82.

34. Calvino, A.; Garrido, D.; Garcia, M., Potency of sweetness of aspartame, D-tryptophan and thaumatin evaluated by single value and time-intensity measurements. *Journal of Sensory Studies* **2000**, *15, (1)*, 47-64.

35. Cardello, H. M. A. B.; Silva, M. A. A. P. d.; Damasio, M. H., Sensory characterization of sweeteners by descriptive analysis and time-intensity analysis. *Alimentaria* **2003**, *346*, 23-30.

36. Portmann, M. O.; Serghat, S.; Mathlouthi, M., Study of some factors affecting intensity/time characteristics of sweetness. *Food Chemistry* **1992**, *44, (2)*, 83-92.

37. DuBois, G., Challenges in the reproduction of sucrose taste with high-potency sweeteners. In *Science for the Food Industry of the 21st Century: Supercritical Fluids, Membranes and other Advanced Technologies for Low Calorie, Healthy Food Alternatives*, Yalpani, M., Ed. ATL Press, Inc.: Mount Prospect, IL, 1993; pp 25-36.

38. Bartoshuk, L. M.; Cunningham, K. E.; Dabrila, G. M.; Duffy, V. B.; Etter, L.; Fast, K. R.; Lucchina, L. A.; Prutkin, J. M.; Snyder, D. J., From sweets to hot peppers: Genetic variation in taste, oral pain, and oral touch. In *Tastes & Aromas*, Bell, G. A.; Watson, A. J., Eds. University of New South Wales Press Ltd: Sydney, 1999; pp 12-22.

39. Pelchat, M. L.; Danowski, S., A possible genetic association between PROP-tasting and alcoholism. *Physiology & Behavior* **1992**, *51, (6)*, 1261-1266.

40. Tepper, B. J.; Nurse, R. J., Fat perception is related to PROP taster status. *Physiology & Behavior* **1997**, *61, (6)*, 949-954.

41. Delwiche, J. F.; Buletic, Z.; Breslin, P. A. S., Relationship of papillae number to bitter intensity of quinine and PROP within and between individuals. *Physiology & Behavior* **2001**, *74*, 1-9.

Chapter 20

Interindividual Differences of Taste Sensitivity in Humans and Hamsters: Multiple Receptor Sites for Single Organic Molecules

Annick Faurion

CNRS/INRA, UMR mixte INRA-Université PARIS 11 Sud NeuroBiologie Sensorielle, nopa, B325, Domaine de Vilvert, F-78352 Jouy en Josas, France

Measuring taste sensitivity in groups of human subjects for series of single molecules used as sapid stimuli and/or measuring taste nerve responses in hamsters reveals the strong discriminative power of the taste peripheral system. In the human, different molecular structures are always perceived as different tastes. Simultaneously, inter-individual differences of quantitative responses (sensitivity) are great, both in humans and in hamsters that are not inbred. These results were obtained under carefully controlled conditions, suppressing olfactory information and training subjects to each stimulus, which ensured a high level of intra-subject and intra-stimulus reproducibility. These data suggest that several weakly specific receptor sites code for each stimulus and these sites should be structurally, at least partially, different from subject to subject. Recent results of molecular biology point to the corollary interpretation that different receptors should bind and code every ligand. The inventory of nucleotide polymorphisms, which are the source of inter-individual receptor diversity, remains to be undertaken.

Sweeteners are numerous (hundreds) and display a large diversity of molecular structure, suggesting a variety of sweet tastes and a variety of mechanisms for detecting and discriminating sweet molecules. Inter-individual differences of sensitivity have been known and reported since 1935 by Blakeslee and Salmon (1) who determined thresholds the distributions of which were widespread in the population for most organic substances tested and up to 10^{13} in concentration for

© 2008 American Chemical Society

some of them. A second indication of inter-individual differences of sensitivity came from the discovery of the reduced sensitivity to phenyl-thiocarbamide (PTC) in some individuals by Fox in 1930 (2). This inter-individual difference was shown to be a heritable trait by Snyder (3) and further worked out by Kalmus (4) who demonstrated that only homozygous twins, but not siblings, had a similar sensitivity. Then, Lugg (5) showed, with several hundreds of subjects, a multimodal rather than bimodal distribution of thresholds. This should have prepared scientists to consider multiple factors for inter-individual differences of sensitivity. However, in the 1970s and even later, it was not yet clear that subjects perceived tastes differently and most investigators continued averaging subject responses. Moreover, it was not usual to consider that every molecule although grouped with other ones in one category (e.g., the sweet group etc.) would elicited a unique distinct taste. The first study showing that perceived intensities for sweet taste are significantly different depending on the individual and the molecule was published in 1980 (6).

Aristotle (7) described a continuum for taste, which was a one-dimension continuum, where sweet (with no reference to sugar as sugar was not known yet) and bitter were at opposite ends; Linnaeus (1753) defined a series of "tastes" including "wet" and "mucous"; Chevreul (8) discriminated taste from olfaction. Authors progressively suppressed words from the taste descriptor list of Linnaeus and the "four tastes" (i.e., sweet, sour, salty and bitter) were the last descriptors which remained; Kiesow, in 1898 (9) described a bi-dimensional taste continuum behind the four words used as milestones. In 1914, Cohn (10) claimed he could categorize 4000 chemicals into only four categories. In opposition to the theory of Cohn, Henning in 1916 (11) replied in a subtle dissertation in favour of the continuous aspect of taste. He developed the idea that sapid stimuli could not be actually categorized but rather constituted a three dimensional continuous taste space. He localized the prototypic semantic references sweet, sour, salty, bitter as dots, landmarks or milestones within the continuous taste space constituted by chemicals. Due to a misinterpretation of his text illustrated by the image of the famous tetrahedron, he was further quoted, *a contrario,* as the father of the "four tastes theory". Other developments concerning the first theorization about four tastes in the 19th century may be found in Erickson (12).

Pfaffmann (13) working in 1939 on single taste fibres in the rat chorda tympani taste nerve wrote: "there is evidence that certain other substances may also stimulate more than one fibre, so that if a wide variety of agents were used, each fibre might be found to have a chemical *spectrum* which overlapped those of other fibres".

Erickson, in 1963 (14), showed that the quantitative activity of each single neuron of a collection of neurons constituted a unique pattern, which was different for each of the tested stimuli. It is this very pattern of activity across neurons that codes for the taste, as a "signature" of each molecule. The array of neurons potentially coding for taste quality allows an infinite number of different patterns explaining the huge discriminating power of the taste system. The author

furthermore considered this pattern constituted both the entry signal identified with the stimulus and the *"read out"* (15) of the taste system for signalling the sapid molecule.

In spite of these first timid and sparse scientific contributions indicating a complex variety of the taste perceptions among human beings, - and human beings never agree on the denomination of tastes - the layman's vocabulary continues to be limited to the use of four words for the description of hundreds of sweet compounds, thousands of bitter compounds and even those compounds which elicit tastes that do not match with any of sweet, sour, salty or bitter.

The huge cognitive power of words maintained a majority of tenants in favour of four unique basic, primary tastes, versus authors in favour of a continuous variety of taste sensations possibly experienced. Such a radical divergence of opinions strongly motivated us to look further into this controversy. Rather than subjective qualitative descriptions, we used quantitative evaluation of perceived intensities to compare subjects' sensations elicited by a wide range of taste stimuli. We probed the question......would these stimuli nicely range into cultural semantic categories or would physiological data significantly depart from language as in a world of unlimited and unarticulated sensations?

Should we discover many varieties of sensations for each subject and a diversity of sensations across various subjects, we would contribute to understanding receptor mechanisms, at the very level of the interface between the external medium and the inside of our self. We expected that inter-individual differences in sensitivity for stimuli molecules may lead to an understanding of the number and nature of receptor sites in individuals for every molecule.

In the mean time, the food industry has been struggling at the replacement of sucrose by non-caloric sweeteners for nutritional or cost-reduction reasons. Although the motivation was there, the conclusion 30 years later is that this aim has not yet been reached and no one knows how to replace sucrose harmoniously. Is taste really such a simple sense?

The aim of this chapter is to summarize some aspects of a wealth of data presently still under examination for looking for multiple receptor sites with the recently available molecular biology approach. Three main lines directed our research, which were:

1. approaching taste receptors by measuring the effect of tastants on the living organism,
2. quantifying similarities/dissimilarities between molecules from the point of view of their interacting properties with the taste receptor system (i.e., biological similarities). For that purpose, we made use of the inter-individual differences of quantitative sensitivity:
   - in the human, using quantitative measurement of <u>individual</u> sensitivity to each molecule
   - in the animal, quantifying chorda tympani taste nerve responses in <u>each</u> animal, after selecting a species with genetics variability instead of inbred animals

3. identifying structural and energy properties on the van der Waals surface of molecules to map receptor sites (Froloff: 16,17).

In the design of our studies aiming at the evaluation of inter-individual differences of perceived intensities, or, of differences of nerve response amplitudes, great care was taken to ensure individual reproducibility. Thus, great care was given to the development of experimental conditions which are reproducible over time.

Experimental

Pre-Selection of Tastants

Stimuli were chosen according to their ability to elicit taste in humans and/or hamsters. First, 182 candidate molecules were rapidly tested on laboratory staff members and on hamsters and Wistar rats (with chorda tympani taste nerve recordings in these rodents). Among the 182 stimuli extracted from the literature (see Beets (18) for a review), 123 elicited taste in the human subjects. Included were 7 PTC – PROP related chemicals containing the –N-C=S- functional group. In the hamster, 92 compounds elicited responses in the chorda tympani taste nerve. We observed that the hamster is a PROP non-taster, giving only minute neural responses to concentrations at the limits of solubility for four PROP type compounds. The Wistar rat, also found to be a PROP non-taster, was tested for 49 substances and found responsive to 22. Our aim was to collect rectangular matrices of data, where subjects are represented as rows and stimuli as columns for both humans (psychophysics) and hamsters (electrophysiological recording of the uncut CT taste nerve). In such a matrix of data, each figure located at {line x, column y} is a statistically validated evaluation, after training, of the sensitivity of one given subject for one given compound. This collection of data was used to calculate the correlation between paired stimuli across subjects, i.e. to evaluate the covariance of the effect of compounds on the taste system, indicating their relative similarity from the point of view of *their interaction with the peripheral receptors*. Independently, the covariance of results on paired subjects was also evaluated looking for a quantitative measurement of inter-individual differences of taste sensitivity. When working at supra-threshold levels, concentrations were chosen below half maximum, or below the inflection point of a dose/response curve. The body of data developed includes findings on 101 tastants in studies on 178 human subjects and 108 hamsters, which were partitioned in several experimental blocks.

In the Human, Thresholds were evaluated for 43 Stimuli, in "Experiment I" including 19 Stimuli and 61 Subjects and in "Experiment II" including 19 other Stimuli and 31 Subjects. 38 stimuli and 19 Subjects were common to both studies. At Supra-Threshold Levels, "Experiment I" gathered 21 Stimuli and up to 71 Subjects, "Experiment II" included 20 Stimuli and 46 Subjects; a matrix of 26 Stimuli and 27 Subjects was common to both studies.

In the Hamster Chorda Tympani Recordings were performed in Experiment I including 49 Hamsters and 41 stimuli and in Experiment II including 59 hamsters and 51 stimuli. 54 stimuli as a whole were studied in humans and 70 in the hamster.

These data were collected between 1985 and 1991 and were partially published (19).

The High Discriminative Power of the Taste System: Evaluation of Human Taste Sensitivity (Detection Threshold and Supra-Threshold Sensitivity)

Experimental procedures:

In humans, the protocol employed was based on a paired comparison task associated to a staircase procedure. The exact protocol of Dixon (20) was used including the calculation of a p_{50} taken as the definition of threshold. A modification was brought so as to take account of a geometric progression of the stimulus concentration instead of an arithmetic progression as originally employed by Dixon. Several caveats were worked out:

Starting concentration:

This indication comes from Dixon himself: the starting test concentration should be at the level of the expected result; hence, we averaged results from the subject's previous session, which gave the starting concentration of the next session. Another caveat from Dixon is also that the ratio of concentrations presented should be approximately equal to the standard deviation of the data.

Randomization:

Several tests were run simultaneously for each subject, inter-mingling the concentrations presented for different tests so that presentations were randomized for the subject.

Sterile solutions:

In preliminary studies, it appeared that threshold evaluation could give reproducible data, provided no bacteria would grow up in the solutions. Subjects are earnestly looking at threshold level for any tiny perception, which should correspond to the stimulus diluted in the solvent, not to any other source of stimulation for taste or olfactory systems. Therefore, the experiments included preparing solutions in sterile conditions (UV illuminated water and sterilized glassware) and keeping them sterile throughout the experimental session. Solutions were made each day and checked every evening, after experimental sessions, on PCA culture medium. Data obtained with accidentally contaminated solutions were discarded.

Temperature control at 0.1°C:

It also appeared that subjects could possibly associate the "higher" temperature to the stimulus, then "recognize" the stimulus from the reference owing to the difference of temperature instead of taste. It was therefore necessary to control differences in stimuli temperatures in every pair. This could be technically achieved at the level of 0.1°C, which was not sufficient since the trigeminal temperature sense is so sensitive that subjects are able to make out differences of temperature as low as a few hundredths of a Celsius degree. To escape this bias, the (computer controlled) solutions delivery was organized by programming so that residual temperature differences <0.1°C were randomized among water and stimulus across all pairs.

Suppression of retronasal olfaction:

Since the ultimate goal of the work described herein is one of understanding of taste receptor structures, it was of the utmost importance to avoid any information that would not result from taste receptors. Hence, a specific device was built for each subject, in order to suppress retro-nasal olfaction, blowing an adjustable airflow of about 200 l/h in the nostrils whenever tasting and responding, for preventing the odorized air contained in the oral cavity to reach the upward nasal cavity through the choanae. Finally, 4-6 up and down tests were run for each molecule and each subject at every session, 3-4 sessions were run per week per subject during 15 weeks/semester and subjects participated in one or two semesters. The whole test was computer driven through a laboratory made automatic diluter and delivering system, which also carried out the calculation of threshold data after Dixon's formula.

Stimuli

The following 54 stimuli were used: 2-Nitrobenzoic acid (2NBA), 3-Nitrobenzoic acid (3NBA), 3-Aminobenzoïc acid (ABZ), Aspartame (ASP), 2(3-Hydroxy-4-methoxyphenyl)-1,3-benzodioxane (BZX), Caffeine or 1,3,7-trimethylxanthine (CAF), Creatine (CRE), Cyclamate Na or cyclohexane sulphamic acid (CYC), Neohesperidin Dihydrochalcone (DHC), D-Leucine (DLEU), D-Phenylalanine (DPHE), D-Threonine (DTHR), D-Tryptophan (DTRP), Dulcin (DUL), D-Valine (DVAL), Fructose (FRU), Glycine (GLC), Glucose (GLU), Glycyrrhizic acid (GLY), Guanosine 5'-monophosphate (GMP), 4-10-dimethyl-1,2,3,4,5,10,-hexahydrofluorene-4-6-dicarboxilic acid (HFL), Glutamic acid (HG), tartaric acid (HTAR), L-glutamic acid, diethyl ester (LGDE), L-Leucine (LLEU), L-Phenylalanine (LPHE), L-Threonine (LTHR), L-Tryptophan (LTRP), L-Valine (LVAL), methyl chlorinated acesulfam derivative (MCH),

chlorinated acesulfam derivative (MCL), Methyl-α-D-mannopyranoside (MMP), Acesulfame-K (MOD), Monosodium Glutamate (MSG), Sodium chloride (NAC), Naringin (NAR), Niacinamide (NIA), 3-Nitrobenzenesulfonic acid (NSA), 1-Propoxy-2amino 4-nitrobenzene (PAN), Sodium pentobarbital (PB), Perillartine or perillaldehyde antioxime (PER), Picric acid (PIC), Quinine (QUI), Saccharin (SAC), Cyclo-octyl sulphamate (SCOS), Sorbitol (SOR), Stevioside (STV), Sucrose (SUC), 2,4,6-Tribromo-3-carbamoylphenylpropionic acid (TBB), Tetracycline (TCY), Thaumatin (THA), Trans-4-hydroxy-L-proline (THP), Theophyllin (TOF), αα-Trehalose (TRH).

Threshold Evaluations

Threshold evaluation corresponds to the detection of ligands at concentrations which are too low for taste quality identification.

Learning phase and data selection:

Thresholds collected each day were averaged for each subject individually. From day to day, the threshold average and its standard deviation diminished for 3, 4 or 5 sessions until reaching a minimum value, which remained stable during further repetitions. Two periods could hence be distinguished: a familiarization phase and a data collection phase. The average calculated on stable values after familiarization was about 5-10 times lower than the first session average. The training by familiarization was not transferable from one stimulus to other ones, but was observed for each new chemical stimulus introduced in the experiment. Matrices of data gathered the number of Dixon's tests, the average and standard deviation for each subject and each stimulus collected in the stable period after familiarization. Then, rectangular data matrices to be treated by paired statistical tests or by multidimensional analyses gathered the average value obtained in several sessions after learning for each subject and each stimulus.

Group Thresholds:

Group thresholds for 43 tastants ranked from 10^{-2} to 10^{-8}M. Results are summarized in Figure 1. It is noteworthy that lower thresholds did not correspond systematically to bitter compounds. Individual thresholds usually spread, for each stimulus, in a range from 8 to 50 fold in concentration with a coefficient of variation from 0.5 to 1 depending on the stimulus. For some tastants, the distribution of individual thresholds was clearly not Gaussian. A few extraneous subjects were sometimes discarded or non parametric statistics used. These few extraneous subjects are prototypical subjects for the study of hypogeusia to given compounds.
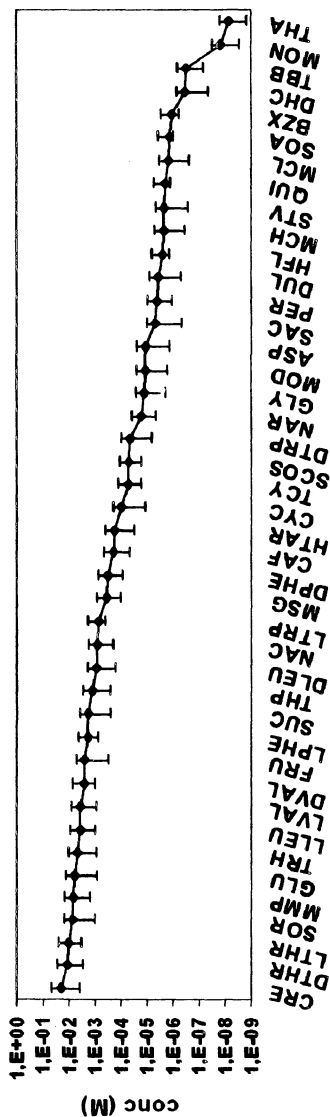
Figure 1. Group detection thresholds for a series of 43 tastants. 27-60 Human Subjects, m± sem; reproducible data obtained after the familiarization period (ordinates: Molar Concentration, log scale).

Individual thresholds: comparing Subjects:

Subjects' thresholds for paired tastants were not predictable. Subject A's threshold may be significantly lower than subject B's threshold for one stimulus and the reverse can be observed for another compound of similar quality. Figure 2 exemplifies with two subjects the concept of non co-variation across subjects. The threshold of subject "vilo" is significantly lower than the threshold of subject "bou" for thaumatin and the threshold of "vilo" is significantly higher than the threshold of "bou" for saccharin. Looking at other molecules would show other unpredictable data. Taste sensitivity is unpredictable from one subject to another one, for one molecule to another one. The differences of sensitivity are consistent and no group average would represent the reality of each individual. The width of these differences is to be quantified.

Every subject exhibited a different profile of sensitivity for a series of stimuli and every stimulus demonstrated a different profile of thresholds across all subjects (Figure 3). These differences were quantified by correlation coefficients calculated either subject-wise or stimulus-wise.

Comparing Tastants:

Figure 4, in which each dot represents a human subject, shows a fair reproducibility of individual data averaged after the familiarization period (Pearson $r = 0.88$ for saccharin). In this work, repeated threshold measurements do co-vary.
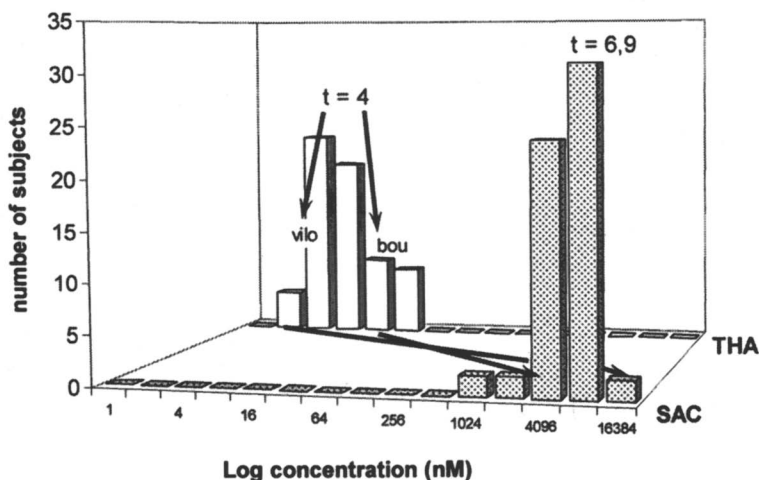
Figure 2. distribution of individual detection thresholds for thaumatin (THA) and saccharin (SAC). Abscissae: Log molar concentration; t: Student t test, p<0.01.
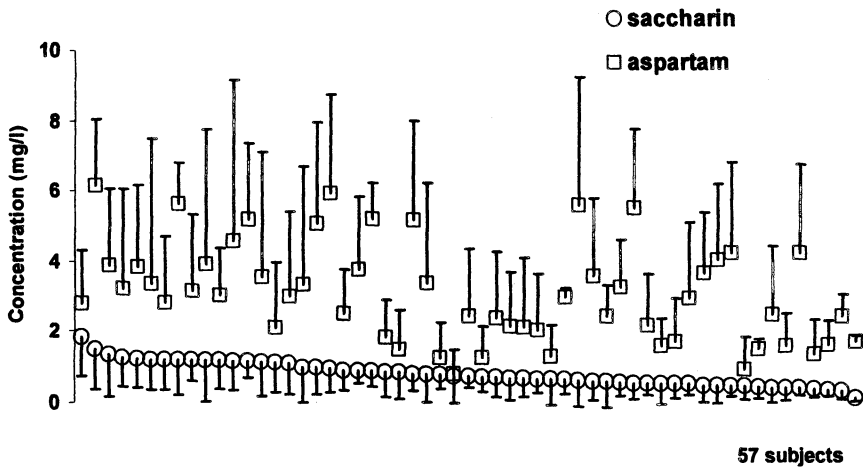
Figure 3. Individual human detection thresholds: profile for two sweet eliciting stimuli and 57 subjects. M±sd

Provided all caveats are observed (sterile condition, learning phase respected, etc.), the difficulty of recording thresholds acknowledged by Pangborn (21), including learning, can be overcome. The same figure shows low correlations between different compounds indicating that the thresholds for the considered compounds do not co-vary across subjects. For example, no similarity is observed between the two sweeteners aspartame and saccharin (r=0.45). Since data for both saccharin and aspartame are reproducible, this means that at threshold level, the chemoreception system involved is not identical for aspartame and saccharin across all subjects.
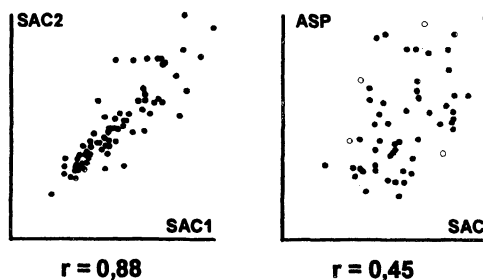
Figure 4. Individual Human detection thresholds. Coordinates: concentration. ASP: aspartame, SAC: saccharin. Each dot is a subject, left, reproducibility: data from the last session and the previous one for each subject; right, difference between stimuli: plotting the sensitivity to ASP as a function of the sensitivity to SAC exhibits the non covariance of thresholds for aspartame and saccharin.

Figure 5 shows a matrix of multiple pair comparisons involving 20 tastants: only 54 out of 190 correlations were above 0.3 and the highest correlation was found between sucrose and saccharin: r = 0.58. In a second experiment, another matrix of data including 19 compounds also showed a very small number of correlations with only 38 correlations out of 171 reaching 0.3. The low level of correlation indicates a great discrimination between tastants and suggests a variety of mechanisms of receptor-ligand interactions.

A continuous Taste Space of Organic Molecules:

Other parameters than the Pearson r correlation coefficient can be used to assess and quantify similarities. The χ^2 metric is the most robust and can fit with non Gaussian data. Factor analysis of correspondences, based on the χ^2 distance, helps describing a taste space in which proximity between tastants indicates a certain level of similarity of these tastants; proximity between molecules results from individual sensitivities co-varying across all subjects. Figure 6 shows a representation of the data obtained on 38 compounds (data collected after the period of familiarization to each single molecule) on 19 subjects. At first sight, the taste space seems relatively "organized", when considering only the first factorial plane with axes 1 x 2, which represents 34% of the information (variance) within the matrix of data. Bitter tasting compounds seem to be localized on one side of the figure and sweet tasting compounds on the other side of the figure. But (i) these subgroups are overlapping and (ii) the space generated by this collection of data is very multidimensional: at least 10 dimensions are needed to represent 70% of the reality of the relationships between compounds. Looking at further factors (the weight of information associated to each of which is still well above the level of statistical significance) shows that stimuli are further discriminated. Two dots projected nearby one another on the first factorial plane may be separated on the third dimension and so on. Every compound is so different from another one that there is as much difference between two sweeteners as between a sweetener and a bitter compound. In the lower part of the figure, bitter and sweet compounds seem to be intermingled in the geometric projection because they are all far apart, on different dimensions, as shown by the low level of correlations (*vide supra*). Hence, the taste space appears to be multidimensional and continuous. In this space, subjects can be mathematically represented (data not shown): they are all dispersed, covering the whole continuum, according to their relative sensitivity to each compound. Every one sees the taste space from his own point of view.

The hierarchical classification of Figure 7 shows a continuous range of similarity for sweeteners arranged into two sub-continua (upper and lower part of the figure). Sweet taste eliciting substances are dispersed, intermingled with some substances tasting differently. The loose group including thaumatin, glycyrrhizic acid, stevioside, neohesperidin dihydrochalcone gathers some tastants eliciting a so-called liquorice after-taste in some subjects, if considered from the point of view of taste quality. D-leucine and acesulfame-K do not share the same after taste.

```
SAC
DTRY  0,44
SOR   0,41 0,49
ASP   0,45 0,46 0,36
CYC   0,34 0,29           0,45
MOD   0,40 0,40 0,34 0,42
FRU   0,34 0,56 0,39           0,30 0,30
GLU                  0,37      0,30 0,37
DUL   0,36 0,27           0,34      0,40 0,30
SUC   0,58 0,49 0,51 0,42 0,32 0,51      0,30

DLEU            0,33           0,39      0,35      0,30
GLY
THA   0,33                0,34      0,32 0,36
STV   0,41                          0,37 0,36
DHC                                      0,50
MSG
NAC        0,42 0,35                0,42      0,47
CRE             0,40                          0,43
CAF                                     -0,29 0,30

      SAC DTRY SOR  ASP  CYC  MOD  FRU  GLU  DUL  SUC  DLEU GLY  THA  STV  DHC  MSG  NAC  CRE  CAF
      0,83 0,86 0,87 0,86 0,80 0,77 0,74 0,84 0,87 0,77 0,85 0,86 0,85 0,80 0,82 0,92 0,85 0,67 0,89
```

Figure 5. Human detection thresholds: inter stimuli paired correlation calculated across 61 Subjects for 19 stimuli. Only correlation > 0.3 are indicated. Lower line: intra-stimulus correlations calculated on the data from the last two sessions showing reproducibility with r about 0.8. Triangular matrix: interstimuli correlations are very low as a whole. A group of sweet taste eliciting compounds (including saccharin, aspartame, sorbitol, cyclamate, acesulfam K (MOD) fructose, glucose, sucrose etc.) can be noticed. Glycyrrhizzic acid (GLY), Thaumatin (THA), Stevioside (STV) and neohesperidin dehydrochalcone (DHC), which also elicit tsweet tastes are not correlated to this group, neither is DLEU (D-leucine).

Figure 6. Human detection thresholds. Multidimensional analysis (Factor Analysis of Correspondences) showing on axes 1 and 2 (upper part) that the taste space seems relatively organized with sweet and bitter opposed on the first axis but the space is very multidimensional. Lower part: bitter and sweet tastants look intermingled on a further projection. On axis 4, cyclohexyl sulphamate and cyclooctyl sulphamate, two chemically similar compounds, are clearly discriminated by the taste peripheral system. Taste exhibits a high power of discrimination and compounds surrounded by squares (sweet) or circles (bitter) are examples of different varieties of sweet or bitter tastes. The number of statististically validated dimensions is above ten.

Even at threshold level, data reproducibility is high. Interestingly, sub-groups of stimuli, obtained with purely quantitative evaluations, may tentatively be identified, which are relevant to the quality that will be perceived at supra-threshold level. We can hypothesize that the receptors involved at threshold level may be the receptors that present the best affinity for these ligands. As a consequence, the receptors engaged in binding at threshold level should be less overlapping than at supra-threshold level.

Supra-Threshold level: Quantitative Evaluation of Iso-Intense Concentrations of a series of Organic Compounds in a series of Individual Subjects

Thresholds have demonstrated a high diversity of sensitivity between subjects and a high diversity of the effect of various compounds on the taste system. Are

Thaumatin — THA 1, THA 2
Glycyrrhizic — GLY 2, GLY 1
Stevioside — STV 1, STV 2
Neohesperidin Dihydrochalcone — DHC 1, DHC 2
D Leucine — DLEU, DLEU
(MOD 2, MOD 1)
Acesulfam K — MSG 1
Glutamate Na — MSG 2
Creatine — CRE 2, CRE 1
Cafein — CAF 2, CAF 1
Sorbitol — SOR 2, SOR 1
NaCl — NAC 2, NAC 1
Na Cyclamate — CYC 1, CYC 2
Glucose — GLU 1, GLU 2
Dulcin — DUL 2, DUL 1
D Tryptophane — DTRY, DTRY
Aspartame — ASP 2, ASP 1
Fructose — FRU 1, FRU 2
Sucrose — SUC 1, SUC 2
saccharin — SAC 2, SAC 1

sweet

Figure 7. Hierarchical classification calculated on the factors of the Factor Analysis of Correspondences (19 compounds X 61 subjects). The last and previous last sessions were used in the data set to compare the long distances in between compounds to the short distances within compound (corresponding to Pearson r of about 0.8). The vertical line defines groups of compounds in this continuum.

these large differences between the biological effects of various molecules also observed at supra-threshold level?

In order to measure taste sensitivity at supra-threshold level, the same protocol of Dixon was used comparing the perceived intensity of a variable concentration of the stimulus to a reference supposed to be equally different from all stimuli, namely NaCl. The concentration of the reference (29 mM) was chosen to be clearly perceived by subjects but low enough to enable working in the lower part of the linear dose response function for all compounds. Paired samples including this reference and the variable concentration of the stimulus were compared repeatedly by the subject who had to answer the forced choice question "which is stronger?". Applying the protocol and Dixon's formula led to the evaluation of the

concentration perceived iso-intense to the reference of 29 mM NaCl for each chemical. These concentrations will be further referred to here as iso-intense concentrations. Results were obtained from two independent experiments including different subjects and two groups of partially overlapping stimuli.

Again, an effect of familiarization to the compound within the first few sessions was recorded (Figure 8), showing a decrease in the concentration needed to obtain the iso-intense sensation together with a decrease of the variance. Simultaneously, magnitude estimates increased.
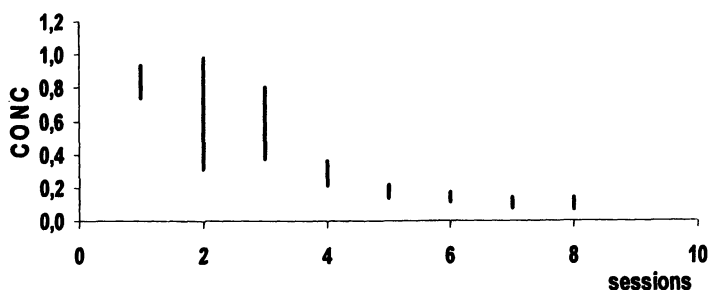
Figure 8. Pattern of familiarization during successive sessions of evaluation of iso-intense concentrations at supra-threshold level by one single subject (jou). The reference was previously learned, the stimulus (guanosine 5'monophosphate) was novel to the subject at first session. Data showed that the iso-intense concentration decreased during the first 4 sessions, then remained constant indicating the performance of receptors was reached. This learning was not transferable from molecule to molecule: the learning had to be performed for each stimulus prior to collecting reproducible data.

A Wide Range from Low to High Similarities:

The highest inter-individual variance corresponded to dulcin, a sweet compound and a structural analogue of the bitter ethoxy-phenyl thiocarbamide which discriminates PTC tasters from non-tasters. Comparing stimuli two by two, showed that the distribution of inter-stimuli correlation coefficients was widely spread between -0.5 to +0.9 whereas the intra-stimuli correlations (reproducibility) were all high (Figure 9). There appears the difference with data recorded at threshold. Correlations can reach a higher level than at threshold.

Correlations between tastants calculated across subjects showed, for example, that acesulfame-K was fairly similar to sucrose ($r = 0.8$) whereas sodium cyclamate was not ($r = 0.5$). Not all correlations between compounds classified within the same quality were high. Instead, a continuous variation was observed from no similarity to high similarity between sweet compounds (Figure 10).
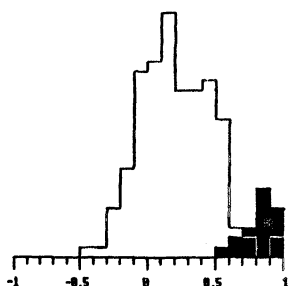
Figure 9. Intra-stimulus (black) and inter-stimuli (white) paired correlations calculated across subjects at supra-threshold level (iso-intense concentrations).
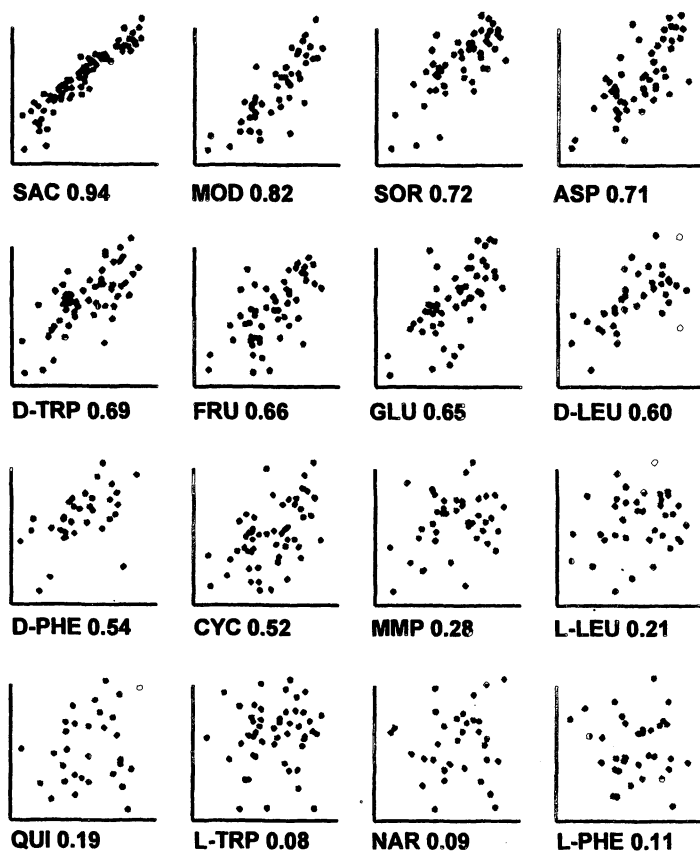
Figure 10. Correlations between paired stimuli including sucrose (ordinates) calculated across subjects on is- intense concentrations measured by reference to NaCl, 1.7 g/l (extract of a matrix of 190 pairs of stimuli, experiment I).

Compared to threshold level, where only 25% of Pearson r values are above 0.3 and where no tight correlation was found (the highest being 0.6), similarity distances are actually quantitative at supra-threshold levels, spanning the full range from zero to about 0.9. The correlation index appears to be a valid parameter to quantify similarity between tastants.

Understanding taste mechanisms from inter-individual differences of sensitivity at threshold and supra-threshold levels.

The Distance between Stimuli compared at Threshold and at Supra-Threshold levels gives an indication on the Number of different Receptor Sites involved and on their Affinity.

The difference between low threshold correlations and supra-threshold correlations, which happen to be higher, is a significant finding. More numerous receptors with lower affinities for each compound should contribute to the taste signal when concentration increases. (This hypothesis matches well with the case of non-tasters of 6-n-propyl-thiouracil –PROP-, who experience a taste for this compound, provided the concentration is increased.) In these conditions, the overlap between sets of receptors activated by stimuli would be higher at supra-threshold than at threshold level explaining a higher level of correlation. A question remains here to be discussed: what are the consequences of these higher inter-stimuli correlations on the qualitative similarities? on the discrimination power of the taste system? Is it lower at the higher supra-threshold concentrations than at lower concentrations?

The Distance between Stimuli eliciting Sweet and Bitter Taste:

As at threshold levels, Factor Analysis of Correspondences exhibits at suprathreshold levels a continuous taste space with no gap between sweet and bitter tastants. The distance between sweet compounds and bitter compounds, considered as a whole, did not increase (Figure 11), although we could have expected the contrary. The taste space remains the same multidimensional continuum in both experiments that were conducted at supra-threshold levels on numerous stimuli.

Groups of Interest:

It is also possible to define subgroups of compounds within the same quality category based on correlations observed. They are visualized on the factor analysis display (Figure 11), which uses the χ^2 metric and also in the hierarchical classification based on the factors on the analysis (Figure 12). For example, Glycyrrhizic acid is associated to the third dimension and is distinguished from stimuli such as sucrose, saccharin, sorbitol, acesulfame-K, D-tryptophan (Figure
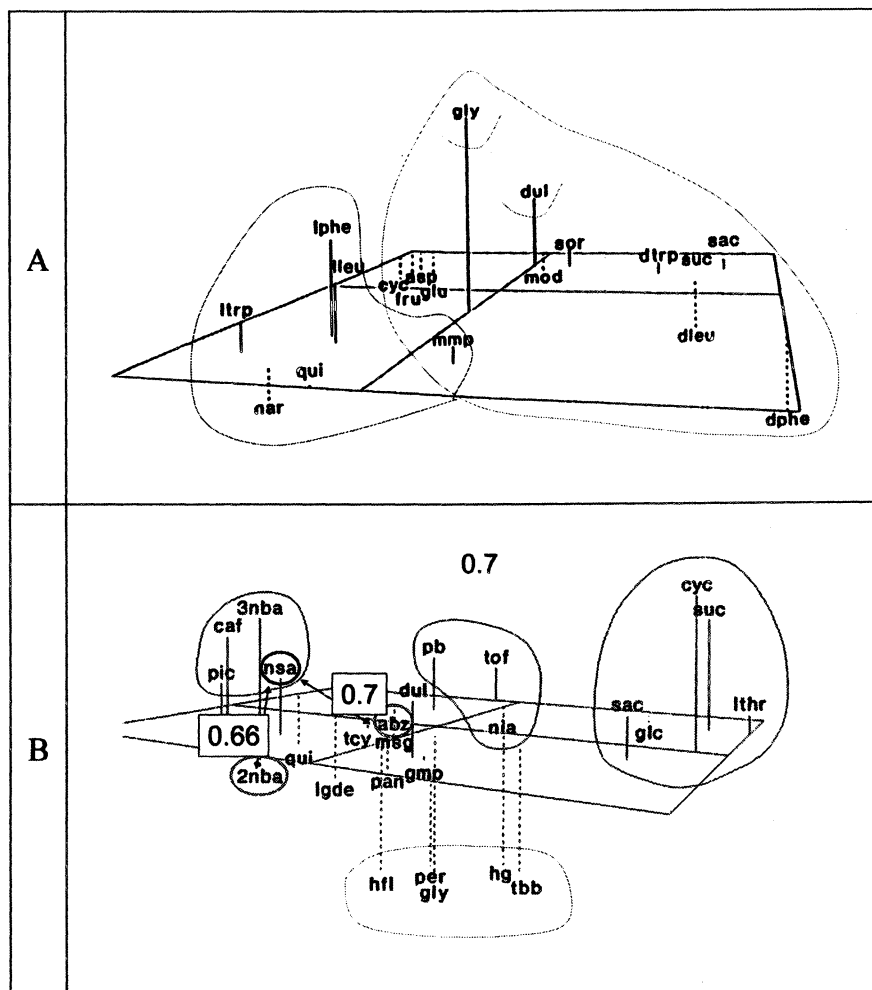
*Figure 11. Factor Analysis of Correspondences: supra-threshold experiments I
(A) and II (B and C, two different views rotating around the 3rd axis). Examples of
correlations: r GLY-TBB: 0.6, TBB-2NBA: 0.7, 2NBA-DUL: 0.7, DUL-PER: 0.7;
SUC-DUL: 0.5, SUC-PER: 0.6, SUC-TBB: 0.5, SUC-2NBA: 0.4; SAC-ABZ: 0.7,
SAC-NSA (sweet-bitter): 0.4, NSA-ABZ (bitter-sweet): 0.8, NSA-2NBA (bitter-
sweet): 0.66; CYC-CAF: 0.13, SIC-PIC: 0.22. Continued on next page.
(See color insert in this chapter.)*
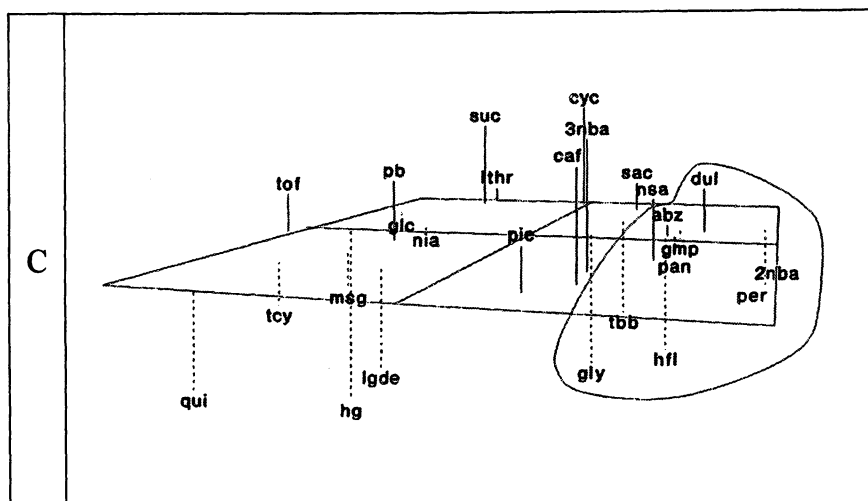
Figure 11. Continued. (See color insert in this chapter.)

11A). Glycyrrhizic acid is the marker sharing with thaumatin, neohesperidin dihydrochalcone and stevioside a so-called liquorice taste in some subjects (see also Figure 6, threshold). Dulcin is different from the group of compounds ranged with sucrose and from the loose group of compounds associated with glycyrrhizic acid. D-amino acids are arranged according to their similarity to sucrose with D-phenylalanine or D-leucine far from sucrose, whereas D-alanine is nearer (Threshold, Figure 6), which means more similar. This is true from the qualitative point of view, as well for both humans and hamsters (22). Qualitatively, the ranking of D-aminoacids makes sense. The second experiment at supra-threshold levels, with other stimuli partly overlapping the preceding ones, confirms these results (Figure 11B) and shows that dulcin is loosely associated to the sub-group (Figure 11C) constituted by 3-aminobenzoic acid, 1-propoxy-2-amino-4-nitrobenzene, 2-nitrobenzoic acid (r = 0.7). This third new group presents two interesting characteristics: these stimuli do not elicit responses on the hamster chorda tympani and, although they elicit sweet taste, they are eventually correlated to structural analogues that elicit bitter taste to humans.

Some Sweet and Bitter Compounds are Correlated with the same Relative "Strength" as the correlations found in between Sweet Compounds or in between Bitter Compounds:

We can find pairs of bitter and sweet compounds relatively correlated. For example, 3-aminobenzoic acid (ABZ) is sweet and 3-nitrobenzenesulfonic acid (NSA) is bitter. These compounds are correlated at the level of r = 0.7. Also, 2-nitrobenzoic acid (2NBA) is sweet and 3-nitrobenzenesulfonic acid (NSA) is bitter
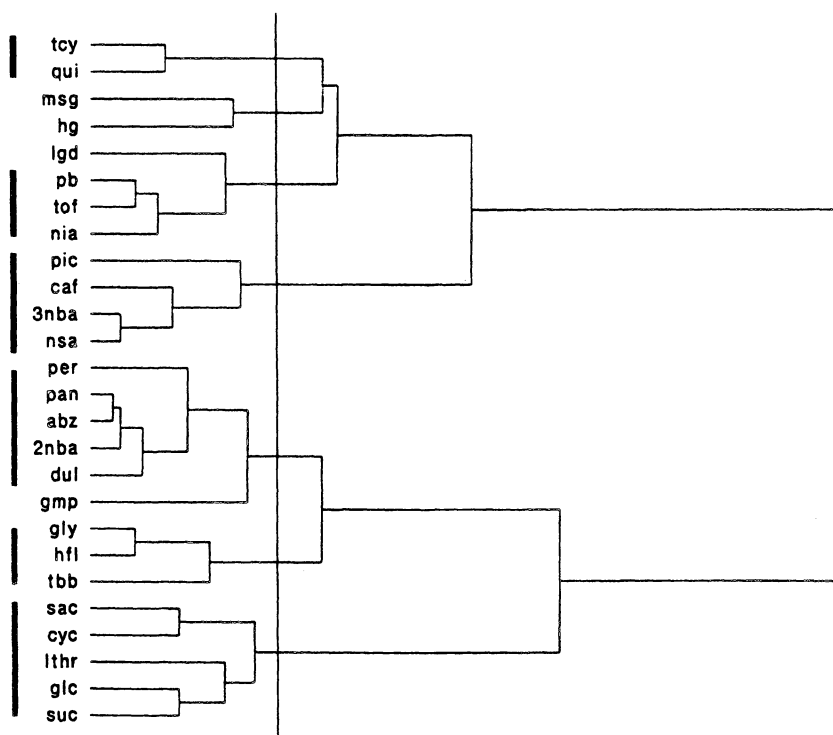
Figure 12. Hierarchical classification calculated on the factors of the factor analysis of correspondences (supra-threshold, experiment II). Using the correlation coefficients, subgroups can be confirmed and validated (upper part: bitter eliciting compounds, lower part: sweet taste eliciting compounds). The highest correlation coefficient is only 0.8 (PAN-ABZ). Notice the relatively long branches of the loose "SUC" group.

with correlation of r = 0.66. Although these correlations are loose, they are of the same level as correlations calculated between sweeteners (DUL-2NBA: 0.7; ASP-SUC: 0.7; FRU-SUC: 0.66) or between bitter compounds (CAF-QUI: 0.6). Either we shall consider that there is no similarity between any pair of sweeteners of we shall have to consider that we may disclose as much similarity between two sweeteners as between one bitter and one sweet tastant. The level of relative similarity between sweeteners being comparable to the level of relative similarity between sweet and bitter compounds suggests that a common group of receptors detects all these organic compounds. This means that some of the receptors contributing to signaling sweet taste-eliciting compounds may also contribute signaling bitter taste-eliciting compounds. That would explain why MMP is sweet and bitter but only sweet to some subjects or only bitter to some other subjects.

Similarly, the taste of L-valine is sweet and/or bitter. Moreover some subjects can declare acesulfame-K is bitter or naringin is sweet.

T1R and T2R receptors

Arguments supporting this idea recently came from molecular biological investigations showing, for example (1), that saccharin or acesulfame-K may bind to T2R receptors that are supposed to code for bitter compounds (23) whereas the response to saccharin elicited sweet taste is first thought to be coded by T1R receptors (24). And also (2) that glutamate is supposedly detected by the hetero-dimeric umami receptor T1R1-T1R3 which shares the T1R3 subunit in common with the heterodimeric sweetener receptor T1R2-T1R3 (25). Physiological and behavioral arguments have been raised to suggest common mechanisms of signaling for sweeteners and glutamate. However, the actual taste of glutamate has no similarity with sweet taste and hence, more than one receptor should be envisaged for cooperatively coding the specific umami taste (26, 27). In both these cases, a notion has emerged: to signal the presence of a single chemical, a cooperation of receptors may be necessary. No receptor individually will code for the quality perceived. Should these different receptors be different proteins coded by different genes or different receptor proteins resulting from nucleotide polymorphisms or several loci for different compounds on one single receptor will be further discussed.

How will the system code for the category of perception?

Categories are not clear-cut:

Firstly, we should consider that although it is clear that quinine is bitter and sucrose is sweet, since they are references, categories are not clear-cut. Numerous examples are known of compounds which are sweet but not sweet like sucrose and even not pleasant (for example, pure D-phenylalanine).

No Descriptors for Taste Perception. Where do these Four Words come from?

Some compounds like D-threonine are tasty but impossible to describe in terms of either sweet or bitter tastes. The same is true for glutamate, at least in subjects clearly perceiving the specific taste of glutamate (28). The taste of D-threonine is unique; the taste of L-glutamate is unique, among other examples. The only word to describe these tastes is the name of the compound. For glutamate, a word meaning "good taste" in Japanese was introduced (29) at the beginning of the 20th century, by the chemist Ikeda who identified glutamate as the source of this very taste. It is by no way a word belonging to the traditional culture of Japan to designate the taste of MSG.

Indeed, do we have descriptors for taste sensations? No more than for odor sensations. In the same way as we name an odor from its source (the odor of the rose, etc.), the name of the sapid chemical remains the best word to evoke the corresponding qualitative perception that no word describes.

Let us examine the semantic status of the "four words" used in taste. Indeed, salty is no descriptor of the perception, just the adjective built on the name of the prototype: sodium salt or "salt". Similarly, in many languages, "sugared" is used instead of sweet, sweet having a strong connotation of both touch and positive hedonic valence. "Sugared" is a prototype. Both sugar and salt are pure chemicals present in the kitchen and in common use as training references. Acids are also present in the kitchen and commonly used and could be also a cultural prototypic reference. However, acid taste is not so well known. Acid (e.g., the taste of H^+ in HCl) is masked in the common weak acids found in the kitchen by the anion contributing another taste and another odor than the mere taste of the proton. The result is that nobody knows the true taste of H^+ at pH above those that produce a somato-sensory pungency. Often naïve subjects propose that acid be the taste of lemon, they say that "acid is pungent and "tastes" like lemon". Pungent is no taste. Is the "lemon" note a taste or an odor?

For bitter taste, the semantic status is probably different: usually subjects say bitter for a hedonic negative perception. Only those subjects trained in laboratories know that bitter "is" the taste of quinine. The layman confuses the bitter taste of quinine and the acid taste of HCl in 50% of cases. Thus the consumer answers at chance level and does not know either acid or bitter taste as laboratory members or professionals of the aroma industry do.

Saying that a solution is sweet does not "describe" our perception but rather refers to the fact that it is more or less sugar-like. Is it possible to describe our perception? Those who are using panels and profiling techniques well know how difficult it is to gather words and converge to a consensus using terms to describe "notes". Measuring the similarity of action of different stimuli in humans clearly points to differences among subjects. When given a pair of tastants, some subjects will claim both compounds are more similar compared to other paired tastants whereas other subjects may claim the contrary. Discrepancies may be found also when expressing the perceived taste for some chemicals: for example, a small proportion of subjects will perceive the methyl α-D-mannopyranoside purely sweet whereas others will describe it purely bitter. At the same time, a majority perceives it both sweet and bitter. Similarly L-valine is more bitter or more sweet, depending on the subject. As a further example of inter-individual differences, the author recently collected data on a subject experiencing only sweet taste for naringin, the common grapefruit bitterant.

What are the Physiological Reasons for Not Having Taste Descriptors?

Besides salt and sugar, the taste of pure chemicals is seldom tested. First, food introduced in the mouth is interpreted in terms of taste whereas it also elicits

somatosensory and olfactory sensations. Second, all taste sensations which we experience result from mixtures. When suppressing retro-nasal olfaction in the laboratory, the sensation experienced by subjects is first low and then increases with familiarization across successive sessions and days of experimentation. At first, subjects usually do not pay attention to taste sensations elicited by unknown pure chemicals. But, it comes with training. The first reason for not having words for tastes might be a lack of familiarity with tastes that are masked within a multi-sensory perception. Further, if we do agree neither on the similarities between pure chemical compounds, or on the description of the taste elicited by these compounds (e.g. MMP: methyl □-D-mannopyranoside), it means that there is no natural consensus on taste perceptions. Each one experiences a different taste for one and the same tastant. If there is no natural consensus on taste perceptions, there is no reason why any culture would have developed words for describing these taste perceptions. How can we reconcile the fact that we have no taste sensation descriptor, and the existence of four words to first address taste? We have seen that three of these four words are prototypes and the fourth one, bitter, probably refers to a hedonic category ("it is disgusting, it is bitter"). That we do not have words for describing our taste sensations does not allow reducing taste perception to only four items. Let us compare with the continuum of color vision: there are "greens" and "blues" according to the wavelength; colors at the frontier between these two categories can be classified as green by some subjects or blue by others, though all know very well what the words blue and green mean. These subjects are not making a mistake; they simply do not experience the same similarities among slightly different colors. These differences of perceptions are due to the relative proportion of their three visual pigments. The same happens for taste, except that the space is far more multidimensional (a mathematical finding, emerging from the degree of non co-variance of data across subjects and compounds) indicating we have far more than three independent channels for signaling taste compared to the three pigments of color vision. Categories of taste and categories of colors are not clear-cut.

What do dimensions of the taste space stand for?

The number of taste space dimensions as extracted from experiments which include a few dozens of tastants that may seem huge but represent in fact so few of the sapid molecules, is well above 10 and obviously underestimated. These independent peripheral channels of information, by definition, represent the receptors that signal the presence of molecules by an adequate binding. What is their biophysical reality? Are they receptor proteins, receptor sites, combinations of given receptor proteins and coupled G proteins and transduction pathways?

Comparatively, with recent literature contributions from molecular biological investigators, we can now sum up 3 T1R, and more than 20 T2R receptors (30). We can add their variants including all combinations of nucleotide polymorphisms (31, 32). These numbers are in concordance with the dimension of the taste space

obtained with physiological data. However, there are probably other still unknown taste receptors. The neurotransmitter GABA elicits responses on the hamster chorda tympani (*vide infra*) and antagonists of glutamate receptors inhibit the chorda tympani response to glutamate and to 5'guanosine monophosphate but not to NaCl (Vandenbeuch et al., article in preparation).

Intensity Measurement and Qualitative Similarity Evaluation lead to the Same Taste Space Depicted:

To compare with intensity measurements (6), Schiffman (33) asked subjects to measure the subjective qualitative similarity between paired tastants and showed the taste space built on these data was continuous. Hence, a subjective, qualitative analysis converges with objective intensity measurements onto the same result: a unique taste for all tastants and a continuous variation across all tastants.

It is wonderful to see that quantified sensitivity data lead to the same taste space as subjective quality similarity data. In both studies, somehow, the same tool was used, the interface translating the external chemical world of tastants into internal physiological signals interpreted by the brain. This means that both kinds of information reflect the coding mechanism of chemicals. The same neurons carry both qualitative and quantitative information. The quality of the stimulus being coded as a spatial pattern of activated neurons, the increased intensity will appear as an increased frequency of spikes in some neurons and an increased number of neurons recruited. Hence, the quality will vary with intensity. Each neuronal image is a code and the code is different from one stimulus to another one, depending on the activated receptors. However, for a given stimulus, the code will also vary with the concentration (34) as lower affinity receptors may be recruited.

The Superiority of a Discriminating System upon a Categorizing System.

How to code into categories is not actually the problem to be solved for the benefit of living organisms and four categories are not sufficient to represent taste. Just as red can be used to designate many different varieties of colors from purple or maroon to nearly pink or nearly orange, sweet can be used for chemicals eliciting a variety of different taste qualities from nearly bitter (D-phenylalanine) to unique (D-threonine) to sugar-like (sucralose). It is especially important to remember that our four words of sweet, sour, salty and bitter are far from providing a comprehensive classification system. Ishii and O'Mahony (35) showed the natural number of dimensions needed to classify 13 tastants was usually more than four, most often seven or even more. Researchers recently added a word for the glutamate taste in a demand for "identifying" not describing a sensation; on the other hand, "licorice" can be considered as a taste name for the prototypic glycyrrhizic acid eliciting a very different taste from the taste of sucrose. D-threonine, which elicits a taste with no name associated, produced ¼ of quotations

of each of the four words proposed, sweet, bitter, salt and acid in 100 subjects: these percentages represent the chance level and correspond to the use "at random" of an inadequate system of description. Once we have shown that, suppressing retro-nasal olfaction, all tastants are discriminated by the taste system, we can understand that slightly different tastes correspond either to different molecules or to different associations of molecules.

What is important for the living organism is to discriminate the taste of different compounds and attach a cognitive image to a sensory image. From a teleological point of view, taste is not designed to distinguish the bitter taste of toxic compounds from the useful sweet taste of energy carrying compounds. Many toxic compounds are not bitter and many bitter compounds are not toxic. Sweet is not the only source of energy and there are natural sweet tastants that are not carrying energy (e.g., thaumatin, glycyrrhizic acid, stevioside, etc.). Another source of energy is fat and fat has not been considered a tastant during either the 19th or 20th centuries. It remains a concept that is still to be demonstrated (36). It is important to be able to recognize a given sensory image among many, should they result from pure or mixtures of chemicals. Furthermore, experiments have shown that animals depleted in a given compound that is necessary in their diet, are able to look for it specifically. This behavior shows the animal is able to make use of a complex amount of information. Namely, the recognition of a sensory image, which was memorized and attached to a sensation of internal welfare, furthermore associated to the memory of the consumption of a given food. Moreover, the delay between memorization of taste and the internal body state may be very long. Learning and plasticity, associated to the ability for recognizing sensory taste images among a great variety of them, seem to be involved, rather than predetermined behavior for one of the so-called taste qualities.

It is particularly important for an omnivorous organism which can eat anything new (a "novel" taste), hence anything unknown and maybe somehow toxic, to be cautious, to learn and recognize the relevant taste. Novel and unfamiliar taste is often interpreted as "bad" taste, a reaction called neophobia (Rozin, 1976 (37), Fischler (38)). Among myriads of different sensory images produced in our neurons for myriads of mixtures of molecules hitting our taste receptors, it is important to identify the very special neuronal activation pattern corresponding to the compound or the mixture that is generated (15, 34).

What is important is to recognize the variety of sensory images that can be produced by a food that revealed to be positively a nutrient for the organism from the sensory images corresponding to its rotten counterpart, the ingestion of which can produce a visceral malaise. In most cases olfaction and taste will cooperate but it is good to know that taste by itself is able to perform such a task.

Hence, taste appears to be a system with a high discriminative power which probably developed for minutely discriminating what should be eaten from what should not, independent from any taste category. A mere deviance from a familiar taste is an indication that bacteria may infect the nutrient. It is important to memorize all the varieties of the different tastes of a given nutrient, which are

acceptable, and to detect unknown differences indicating it might not be acceptable for health. As tastes are culinary culture dependent, these mechanisms of recognition are of course plastic and learned. The only "hardware" characteristic is an extremely good power of discrimination.

Neophobia, the support of self-conservative behavior often extinguishes when the ingestion of the "new" taste was not followed by visceral malaise. The new taste is no more suspect and the period of familiarization to the new taste also gives opportunity to digestive enzymes to adapt to the novel food.

In this respect, taste as a discriminating system is "more useful" to the individual than a categorizing system built on too few prototypes. Taste semantics can be improved to the benefit of communication in groups of cooks, wine tasters, food industry panels, etc. using mainly metaphors. However necessary for the group, semantics remains poor and limited compared to the number of sensory images that can be built thanks to taste signaling neurons.

The high discriminative power of the taste system: Taste coding at the periphery, the hamster chorda tympani taste nerve.

Given that a complex taste space is observed with psychophysics, can we demonstrate that such a complex taste space also exists at the peripheral level? Is this complexity a matter of receptors or could it be brain processing? What could happen between coding at the entry of the system and the "read out" (15), which, of course provides the basis for the cognitive identification of the source of the taste. Recording CT nerve responses to series of numerous tastants may give an answer.

Method

At least three nerve pairs convey taste information. They are the chorda tympani, the glossopharyngeal, and the superior branch of the laryngeal nerves. We should also consider nerves innervating the soft palate contributing to taste in probably more than 70% of human subjects and the trigeminal lingual nerve transporting also chemical information together with somato-sensory sensitivity. Among these taste nerves, chorda tympani nerves are very interesting because their sensitivity to mechanical and thermal stimuli is low. A method to compare the coding of different compounds at the level of taste nerves, just after the information has been transduced by receptors and prior to any brain processing, is to look at the chorda tympani responses in the animal. The integrated amplitude of the response will depend on the stimulus nature and on the concentration applied to the tongue. This information seems very rough compared to single unit recordings but looking at quantitative responses in a certain number of animals for the same collection of stimuli applied on each of them leads to data equivalent to those that were reported for humans earlier in this paper. As for human psychophysics, these "integrated" or "averaged" responses reflect the quantitative response of each individual animal to

each stimulus. They convey all the information that helps in discriminating stimuli. Contrary to human psychophysics, data are recorded just after being transduced in sensory cells. An interesting characteristic is the precise and reproducible quantitative aspect of these responses associated to a clear discrimination between animals or between profiles of tastants across animals. The kind of information we are looking for needs, of course, an animal with genetically determined inter-individual differences of sensitivity as in human beings. Techniques for gathering such data imply never averaging responses across animals but, on the contrary, extracting the differences of relative response amplitudes recorded for various stimuli in a series of animals. The degree of co-variation of the response amplitudes measured for stimuli across 60 animals can thus be calculated.

Hamsters are not inbred animals. When working on preferences using two-bottle preference tests, for example, the average for a group may be null due to their variety of preferences. A possible interpretation might be that the compound was not perceived. Although chorda tympani recordings assess the taste of a compound, some do prefer, some do reject and their behavior can vary with the repetition. It appeared with electrophysiological recordings that this difference of preferences is doubled by inter-individual differences of sensitivity that we could make use of, as we did with inter-individual differences of sensitivity in human psychophysics.

Just as in human psychophysics, a familiarization effect appeared at the level of chorda tympani responses for repeated stimulus application of stimuli (39). Hence, we took great care to overcome the "learning" period in every animal recorded and to check the reproducibility of the amplitude of responses throughout the experiment. All stimuli were repeated and the reproducibility of the amplitude of responses was assessed for each animal and for each stimulus. Stimuli were used in low concentrations, in the lower part of the linear dose-response function but clearly above threshold in order to obtain really quantitative responses.

Stimuli:

Two experiments were run independently; 58 stimuli (two groups of 41 and 51, respectively) were applied in a continuous flow rate in two sets of experiments on 49 and 59 hamsters, respectively. The stimuli employed in this study are as follows: ABZ: 3-aminobenzoic acid, AMC: ammonium chloride, AMG: ammonium glutamate, ARL: arecoline, ASC: ascorbic acid, ASP: aspartame, ATRO: atropine, BALA: beta alanine, BET: betaine, CAC: calcium chloride, CAG: calcium glutamate, CAR: carbinoxamine, COT: nicotinic acid, CYC: cyclamate Na, DABE: D-alanine, tert-butyl ester, DAL: D-alanine, DHIS: D-histidine, DLAAA: DL-aminoadipic acid, DLEU: D-leucine, DLHCA: DL-homocysteic acid, DPHE: D-phenylalanine, DSER: D-serine, DTHR: D-threonine, DTRP: D-tryptophan, DUL: dulcin, DVAL: D-valine, FRU: fructose, GABA: γ-aminobutyric acid, GLU: glucose, GLY: glycine, GMP: 5'guanosine monophosphate, disodium salt, GMPA: 5'guanosine monophosphate acid, HCl 2.7: hydrochloric acid @ pH 2.7, HCL 2.3: hydrochloric acid @ pH 2.3, HG: glutamic

acid, KET: ketamine, LAA: L-aspartic acid, LAB: L-alanine, tert-butyl ester, LAL: L-alanine, LGDE: L-glutamic acid, diethyl ester, LHIS: L-histidine, LPRO: L-proline, LSER: L-serine, LTHR: L-threonine, LVAL: L-valine, MAGC: magnesium chloride, MAGG: magnesium glutamate, MGL: methyl α-D-glucopyranoside, MMA: hydroxymandelic acid, MMP: methyl α-D mannopyranoside, MMZ: methimazole, MOD: acesulfame-K, MSG: monosodium L-glutamate, NAC: sodium chloride, NIC: nicotine, NIP: nipecotic acid, NSA: 3-nitrobenzene sulfonic acid, PTU: n-propylthiouracil, QUI: quinine, SAC: saccharin, STV: stevioside, SUC: sucrose, TAU: taurine, TDO: taurodeoxycholic acid, TGS : trichlorogalactosucrose (sucralose), THP: trans-hydroxy-L-proline, TIA: thiamine, TRO: taurocholic acid, URE: urea, URZ: urazole, VMA: vanylmandelic acid.

Inter-individual Differences of Sensitivity at Neural Level in the Hamster:

Figures 13 and 14 show responses to compounds reproduced at least twice in each animal and the intra-animal or intra-stimulus correlation is usually above 0.8. By comparison, the inter-animal correlation is usually much less, as calculated by correlation coefficients.

A Continuum of Tastants:

As in supra-threshold quantitative psychophysics, we obtained a continuous range of correlations (from <0 to 0.8). The correlation quantifies similarity between molecules as they are "seen" by the taste system (Figure 15).

Subgroups of Tastants of Interest:

As in humans, correlation acts as an evaluation of the similarity between compounds compared by pairs (Figures 15 and 16) and the similarities obtained make sense with data on generalization of conditioned taste aversion used to identify the qualitative taste similarities experienced by the animal. But only 137 out of the 820 correlations calculated between the 41 stimuli were above 0.3. This proportion of correlations above 0.3 reaches 18% in this first experiment and 5% out of 1275 paired stimuli, in the second experiment. All stimuli are well discriminated, the majority of compounds are dispersed in the taste space and only a few groups appear. This very small proportion of noticeable inter-stimulus correlations confirms the high power of discrimination of the peripheral taste system. In this respect, these results are similar to supra-threshold psychophysics results in humans.

The lay out of factor analysis (Figure 17) exhibits a continuous display of stimuli. The dimension of such a space is above 10 (10 dimensions represent only 73% of the data variance in experiment II including 51 stimuli and 60 hamster CT nerves). This means that the stimuli that seem grouped together are possibly further discriminated. Discrimination can only be underestimated.
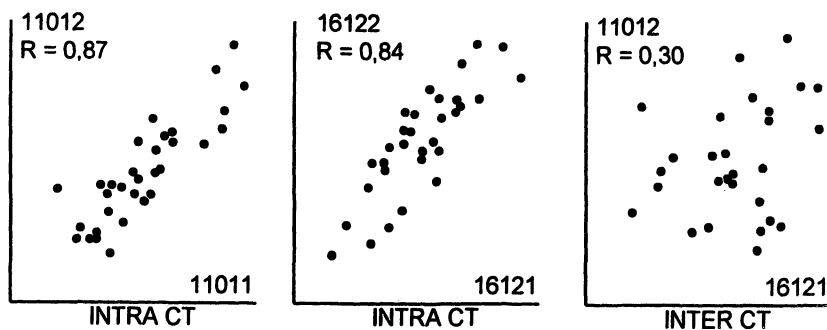
Figure 13. Comparison of response amplitude within and between hamsters for a series of stimuli. Intra-nerve and inter-nerve correlations. Left: the same series of stimuli is presented twice (first time in abscissae, second time in ordinates) in random order to the same animal, each dot represents the CT response to one stimulus, the correlation r: 0.87 represents intra-nerve reproducibility. Center: idem for a second animal, r = 0.84. Right: comparison of the responses obtained in the two different animals for the same series of stimuli: r = 0.30. This part of the figure illustrates the differences of sensitivity between two nerves, i.e. between two animals.
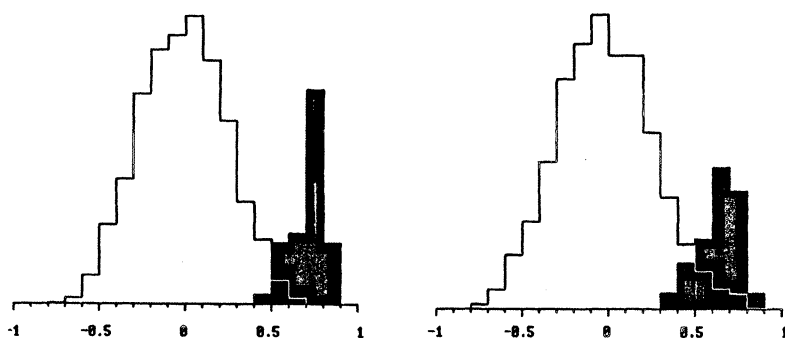
Figure 14. Distribution of correlation coefficients between and within hamster nerves and stimuli. Left: high correlations calculated within nerves (black) on the profile of responses to stimuli indicate intra-animal reproducibility; low correlations calculated between nerves (white) indicate inter-animal differences of sensitivity. Right: idem, correlation within stimuli (black) and between stimuli (white) calculated on the profiles of responses across animals.
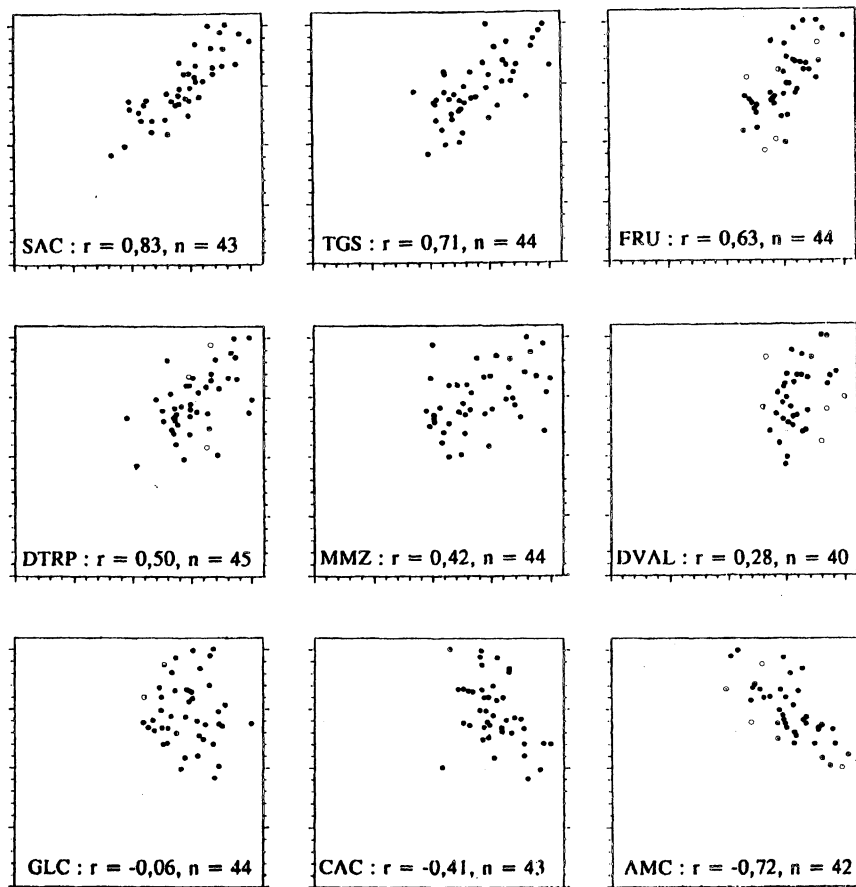
Figure 15. Correlations between sucrose and a few compounds calculated on the responses of the series of hamsters (extract of results of chorda tympani responses in hamsters of the first experiment). Each dot represents one hamster and the correlation is calculated between responses to sucrose and to a given stimulus across 49 animals. (Extract of the data including 41 tastants leading to 820 pairs). The correlation coefficient appears to be quantitative index of similarity between compounds.

	SUC	TGS	MOD	SAC	FRU	MGL	DUL	DPHE	DLEU	DTRP	DVAL
TGS	71										
MOD	73	51									
SAC	83	70	76								
FRU	63	53	49	63							
MGL	70	58	78	64	64						
DUL	67	59	61	61		42					
DPHE	62	42	44	43	46	46	46				
DLEU	55	47	43	43	35	39	58	65			
DTRP	50	41	44	50	34	44	49	76	73		
DVAL							35	51	40	59	
GLC								41		45	53
MMZ	48	59	48	54	40	46					
GLU					38						
MSG											

Figure 16. Correlation coefficients calculated between stimuli on the results of chorda tympani responses across hamsters of the first experiment. Upper Part: extract. Lower Part: total matrix of correlations exhibiting very few correlations above 0.3.

*Figure 17. Factor analysis of correspondences,Hamster experiment I. Figures:
correlation coefficients. (See color insert in this chapter.)*

Similarities/dissimilarities between the Hamster and the Human Taste Space:

Analyses of similarities in both animal experiments show that the hamster shares a part of its taste space with the human taste space. Namely, sweeteners clustered with sucrose (Figure 16) and the D-amino acids. However, L-amino acids do not elicit responses on the hamster chorda tympani. And none of thaumatin, neohesperidin dihydrochalcone and glycyrhizzic acid elicit CT responses in hamster while only high concentrations of stevioside do.

None of the compound set including 2-NBA, PAN, TBB constituting a third group of sweetness eliciting compounds, which is distinct from the "sucrose group" for humans did elicit any response on hamster CT nerve. ABZ elicited only acid responses in the hamster. Only dulcin, which is correlated partly to elements of the sucrose group and partly to elements of this third group was also able to stimulate hamsters.

An intriguing contribution comes from a group of compounds including arecoline, carbinoxamine, nicotinic acid, ketamine, urazole, thiamine and D- and L-histidine. Although all these compounds are acids from the chemical point of view, it does not seem that their acidic properties are the basis of their grouping since they are all correlated below 0.3 with either HCl (pH2.3) or HCl (pH 2.7). Hence, the anion in these compounds probably elicits in the hamster a specific taste apart from acidity. The interesting point is that none of these compounds elicits any clear

taste for humans apart from acidity. The question may be raised of genes knocked down to pseudo-genes in the taste system of humans. For olfaction comparatively, similar cases are already well documented.

Hierarchical classification showed two main groups in both experiments. In other words, sweet versus "others" but these "other" stimuli are not grouped according to what would be expected from a qualitative categorization into four tastes. There are far more than 4 groups. Moreover, carefully looking at sweet taste showed that some compounds tasting sweet to man and sugar-like to the animal fall outside the group including sucrose, e.g.: L-serine, D-serine, L-threonine, L-alanine. Conversely, some compounds fall inside, which are not sweet to humans. As examples, urea, betaine, methimazole (methimazole was introduced in both experiments, showing the same result twice, on two different groups of animals). In all cases, the distance is nevertheless high. Values above 0.7 were exceptionally recorded (for sucrose-saccharin, saccharin–acesulfame-K), among 2095 pairs of stimuli.

What did the animal experiment teach us?

As well as humans, hamsters actually exhibit high inter-individual differences of taste sensitivity and a high power of discrimination between molecules. Laboratory rats or mice that are inbred animals, if used in such an experiment, would not have shown such inter-individual differences. The hamster appears to be a good model for the human in the respect that it shares a similar level of inter-individual differences of sensitivity.

Some parts of the hamster taste space look like the human taste space. This is the case, concerning, for example, sugars, D-amino acids and some others of the sweeteners. On the contrary, some stimuli do not stimulate the hamster. Among them are molecules eliciting bitter as a whole, together with the sweet tasting sodium cyclamate, aspartame and the sweet proteins. Some compounds are arranged among sweet eliciting compounds in the hamster (e.g., methimazole, MMZ), which are bitter for humans. Members of a group of molecules, which are non-tasting for humans seem to share a common taste property for the hamster (Figure 18).

Quantitative human psychophysics and the analysis of hamster chorda tympani responses both led to similar results. Diamant already showed in humans (40) that the quantitative evaluation of taste perception was linearly related to the chorda tympani response of the same person. And indeed, this demonstration stongly supports using quantitative psychophysics. Thus, quantitative psychophysics reflects the peripheral information as it is generated at receptor level.

Above all, it seems that there is no difference between the structures of the hamster taste space and the human taste space. Both exhibit an equivalent number of dimensions, both are continuous.
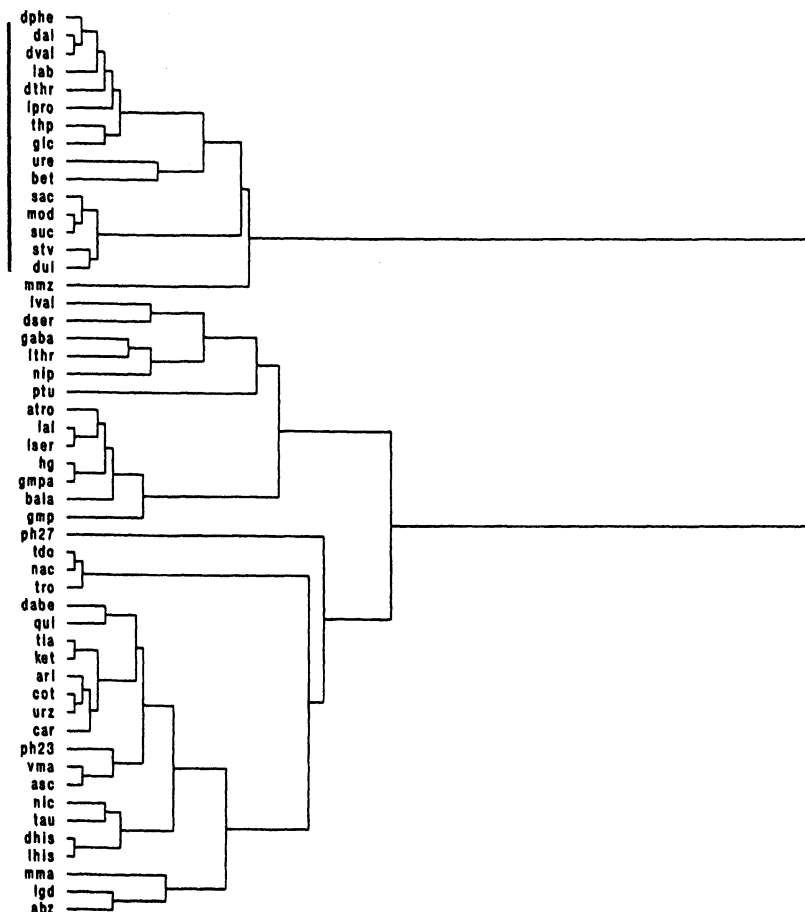
Figure 18. Hierarchical classification, Hamster experiment II. The bar indicates sweet taste eliciting compounds in humans except for "URE" (urea) and "BET" (betaine). Notice that D-serine, L-serine, L-threonine which are sweet to humans are associated to stimuli which are not sweet in humans, among which GABA (LVAL is sweet and bitter). Notice the group gathering TIA, KET, ARL, COT, URZ, CAR (see text). As in humans who taste it bitter, DABE (D-alanine, tert-butyl ester) is grouped with quinine.

Interpretation in terms of Receptors:

We have to explain how the taste system is able to discriminate every single molecule from another one. Both for humans and hamsters, the statistical analysis of subsets of chemicals included in our experiments shows that the number of dimensions increases rapidly from 4 to 12-20 stimuli, then tends to plateau: the number of receptors is limited and does not increase above a certain number. Hence, the high power of discrimination does not result from a number of receptors as wide as the number of stimuli. To account for the extreme discrimination power of taste, we can suggest the hypothesis of the cooperation of a finite number of these receptors, leading to a huge number of different combinations. Whether we have to invoke multiple receptor sites or multiple receptor proteins, or both, to explain the inter-individual differences and the discrimination of every single chemical is to be discussed.

Only one receptor would not explain non covariance of sensitivities across subjects. Neither would one receptor protein, including several receptor sites, each interacting with different sweet molecules (41-43). To function independently the proteins serving as receptors should be independently coded by different genes, so that they can be distributed in various relative amounts in subjects.

A certain number of single nucleotide polymorphisms (SNPs) was shown in T1R1, T1R2, T1R3 (31, 32) and in T2R genes (44). We should consider the existence of different proteins resulting from the expression of both alleles of one single gene. In the case of heterozygous subjects, two proteins could be coded by the two alleles of one and the same gene (45). Thus, the presence of SNPs would then increase the number of functional receptors. Furthermore, results in the literature indicate that the two proteins T1R2 and T1R3 (46-48) combine to form a functional heterodimeric receptor for sweet eliciting compounds (49, 50). These findings authorize a greater number of receptors corresponding to a variety of combinations of SNPs on both monomers, a system that would contribute to non covariance of sensitivities to various "sweet" eliciting compounds. More recent work tends to show that sucrose may bind to only one of the subunits (45) suggesting also some independent binding functions for T1R2 and T1R3 monomers.

It would be interesting to test statistically (with modeling techniques) which number of SNPs on various receptors might lead, by combination, to a sufficient number of different proteins to account for the high discriminative power of the "sweet" taste system. Would T1R2 and T1R3, in the case of sweet eliciting chemicals, be enough? Or would we need supplementary receptors for coding sweetness as a whole? Moreover, it has been shown that saccharin and acesulfame-K can activate both the so-called "sweetener receptor" T1R2/T1R3 and "bitterant receptors" T2R (23), demonstrating the non specificity of binding. Again, the number of independent receptors is increased. The non specificity of receptors is also demonstrated by one given receptor protein accepting several different tastants (51).

Are these independent receptors listed above sufficient to account for a continuous display of different taste sensitivities? Do we know all of taste receptors when including T1Rs and T2Rs?

The mathematical independence found between sensitivities to compounds across Sucjects tend to support the idea of several distinct physical entities coded in each individual to signal the whole range of sweet tasting compounds. Otherwise, sensitivities would be different from one subject to another one, but not necessarily non co-variant. Hence, different SNPs in one single receptor (namely T1R2-T1R3) across subjects does not seem sufficient to account for these physiological results, whereas different SNPs in a limited number of receptor proteins would fit the non covariance of data.

Towards a Correlation between Phenotype and Genotype:

It is very interesting to go back to initial data and consider every correlation diagram between paired stimuli where each dot represents a subject. In order to be able to calculate any valid correlation, a few subjects had to be discarded since they presented a sensitivity 3, 4 or 5 standard deviations above the group mean for one or a few of the tested stimuli. These subjects are indeed now very interesting for looking at the SNPs they might exhibit with respect to the peculiarity of their profile of sensitivity. Could a pattern of SNPs across a certain number of receptors explain a specific individual profile of sensitivity to a collection of tastants?

Specificity of receptors:

It would be tempting to think that sweet tasting molecules interact with T1R receptors and bitter tasting molecules with T2R receptors. Now if some compounds taste bitter for one subject and sweet for another one (whereas these very subjects sense other sweeteners or bitter compounds as others do), it is difficult to understand how only T1R in one subject and only T2R in another one would accept one and the same molecule. The correlations calculated between paired sweet and bitter tastants indicate a continuous distribution. This continuity argues for a commitment of a pool of receptors with relative and globally low specificity for all organic molecules. The relative contribution of each receptor might be different in each individual. A single pool of receptors concerned would explain the continuity between the pure sweet taste of sucrose to the pure bitter taste of quinine. This is in no way a mono-dimensional continuum but a space the dimension of which depends on the number of independent receptor proteins involved. The problem of categorizing tastes is still easy to solve in the same way as in color vision. Depending on the relative amount of different taste receptors, subjects might classify MMP sweet or bitter, just as the relative proportion of visual pigments have causes subjects classify turquoise with blues or with greens.

332

Concerning the number and specific nature of "taste" receptors, it has probably been a mistake to consider as receptors only those proteins that were exclusively present within taste cells and to exclude proteins that were also present in other tissues. Receptors might be ubiquitous, serving different functions in different tissues as do taste signaling proteins in the intestinal cells and olfactory receptors in the sperm (52, 53). The example of responses to GABA, glutamate, nicotine, nipecotic acid, etc. in the taste chorda tympani nerve, the demonstration that antagonists of brain glutamate receptors can reduce the peripheral chorda tympani taste responses to glutamate give weight to this hypothesis. Receptors for neurotransmitters or immunological receptors might be present at the apex of taste cells and bind tastants eventually with a lower affinity. The half molar concentrations of sucrose used in everyday life situations illustrate "low affinity" binding for most tastants. The alternative to this hypothesis is that variants of these receptors could be the taste receptors, binding with lower affinities both tastants and gaba and glutamate and nicotine, etc. applied on the tongue.

Considering non-specific receptors, disregarding "taste qualities" will be a heuristic approach for identifying ligands corresponding to receptors. We hope this short essay will have documented the idea that qualities are neither so few nor so clear-cut. On the other hand, several low specific receptors will give no clear-cut categorization of chemicals. A key for explaining the coding of taste and, said more precisely, the coding of multiple taste sensory images.

A strong conclusion: if semantics may be useful for communicating, it is important to get out of it to study physiological mechanisms.

Acknowledgements

References

1. Blakeslee, A. F.; Salmon, T. N. *Proc. Nat. Acad. Sci. USA* **1935**, *21*, 84-90.
2. Fox, A. L. *Proc. Nat. Acad. Sci. USA* **1932**, *18*, 115-120.
3. Snyder, L. H. *Science* **1931**, *74*, 151-152.
4. Kalmus, H. *Handbook of Sensory Physiology*; Beidler,L.M.,ED.; Chemical Senses Part 2; Springer Verlag; New York, US, 1971, Vol.4, pp 165-179.
5. Lugg, J. W. H. *Ann. Hum. Genet.* **1966**, *29*, 217-230.
6. Faurion, A.; Saito, S.; Mac Leod, P. *Chemical Senses* **1980**, *5*, 107-121.
7. Aristote. Chapitre 10 422b, in *De Anima, Livre II*, Chapter 10, pp. 10-15.
8. Chevreul, M. E. *Journal de Physiologie Expérimentale et Pathologique* **1824**, *4*, 127-131.
9. Kiesow, F. *Archives Italiennes de Biologie* **1898**, *30*, 399-425.

10. Cohn, G. *Pharmazeutische Zentralhalle* **1914**, *55*, 735-747.

11. Henning, H. *Zeitschrift fr Psychologie* **1916**, *74*, 203-219.

12. Erickson, R. P. *Neuroscience and Biobehavioral Reviews* **1984**, *8*, 105-127.

13. Pfaffmann, C. *Journal of Physiology* **1939**, *96*, 41-42.

14. Erickson, R. P.; Doetsch, G. S.; Marshall, D. A. In: *Olfaction and Taste I*, Zotterman, Y., Ed., Pergamon, 1963, p 213.

15. Erickson, R. P. *American Scientist* **1984**, *72*, 233-241.

16. Froloff, N.; Faurion, A.; Mac Leod, P. *Chemical Senses* **1996**, *21*(4), 425-446.

17. Froloff, N.; Lloret, E.; Martinez, J.-M.; Faurion, A. *Chemical Senses* **1998**, *23*(2), 197-206.

18. Beets, M. G. J. *Stucture-Activity Relationships in Human Chemoreception;* Applied Science: London, UK, 1978.

19. Faurion, A. In *Sweet Taste Chemoreception;* Mathlouthi, M., Ed.; Elsevier Applied Science: London, UK, 1993; pp 291-351.

20. Dixon, W. J.; Massey, F. In: Introduction to Statistical Analysis, MacGraw Hill: New York, 1960, 394.

21. Pangborn, R. M. In *Flavour '81;* Schreier, P., Ed.; Walter de Gruyter: Berlin, DE, 1981; pp 3-32.

22. Nowlis, G. H.; Frank, M. E.; Pfaffmann, C. *Journal of Comparative and Physiological Psychology* **1980**, *94*, 932-942.

23. Kuhn, C.; Bufe, B.; Winnig, M.; Hofmann, T.; Frank, O.; Behrens, M.; Lewtschenko, T.; Slack, J. P.; Ward, C. D.; Meyerhof, W. *J.Neurosci.* **2004**, *24*, 10260-10265.

24. Montmayeur, J. P.; Liberles, S. D.; Matsunami, H.; Buck, L. B. *Nat. Neurosci.* **2001**, *4*, 492-498.

25. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696.

26. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili, Z.; Varadarajan, V.; Zou, S.; Jiang, P.; Ninomiya, Y.; Margolskee, R. F. *Science* **2003**, *301*, 850-853.

27. Maruyama, Y.; Pereira, E.; Margolskee, R. F.; Chaudhari, N.; Roper, S. D. *J. Neurosci.* **2006**, *26*, 2227-2234.

28. Lugaz, O.; Pillias, A. M.; Faurion, A. *Chemical Senses* **2002**, *27*, 105-115.

29. Ikeda, K. *Journal Tokyo Chem. Soc.* **1909**, *30*, 820-836.

30. Meyerhof, W. *Rev. Physiol. Biochem. Pharmacol.* **2005**, *154*, 37-72.

31. Kim, U. K.; Wooding, S.; Riaz, N.; Jorde, L. B.; Drayna, D. *Chemical Senses* **2006**, *31*, 599-611.

32. Winnig, M.; Bufe, B.; Meyerhof, W. *BMC Neuroscience;* 2005, 6:22.

33. Schiffman, S. S.; Sennewald, K.; Gagnon, *J. Physiol. Behav.* **1981**, *27*, 51-59.

34. Yamamoto, T.; Kawamura, Y. *Physiology & Behavior* **1972**, *9*, 559-563.

35. Ishii, R.; O'Mahony, M. *Chemical Senses* **1987**, *12*, 37-51.

36. Laugerette, F.; Passilly-Degrace, P.; Patris, B.; Niot, I.; Febbraio, M.; Montmayeur, J. P.; Besnard, P. *J. Clin. Invest.* **2005**, *115*, 3177-3184.

37. Rozin, P. In *Advances in the Study of Behavior;* Rosenblatt, J. S., Ed.; Academic Press: New-York, US, 1976; 6, pp 21-76.

38. Fischler, C. *L'Homnivore;* Odile Jacob: Paris, FR, 2000.

39. Berteretche, M. V.; Boireau-Ducept, N.; Pillias, A. M.; Faurion, A. *Appetite* **2005**, *45*, 324-333.

40. Diamant, H.; Oakley, B.; Strom, L.; Wells, C.; Zotterman, Y. *Acta Physiologica Scandinavica* **1965**, *64*, 67-74.

41. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Margolskee, R. F.; Max, M. *J. Biol. Chem.* **2004**, *279*, 45068-45075.

42. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L. A.; Benard, L. M.; Osman, R.; Max, M.; Margolskee, R. F. *J. Biol. Chem.* **2005**, *280*, 34296-34305.

43. Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Osman, R.; Margolskee, R. F.; Max, M. *J. Biol. Chem.* **2005**, *280*, 15238-15246.

44. Bufe, B.; Breslin, P. A.; Kuhn, C.; Reed, D. R.; Tharp, C. D.; Slack, J. P.; Kim, U. K.; Drayna, D.; Meyerhof, W. *Curr. Biol.* **2005**, *15*, 322-327.

45. Nie, Y.; Vigues, S.; Hobbs, J. R.; Conn, G. L.; Munger, S. D. *Curr. Biol.* **2005**, *15*, 1948-1952.

46. Bachmanov, A.; Li, X.; Reed, D. R.; Ohmen, J. D.; Li, S.; Chen, Z.; Tordoff, M. G.; de Jong, P. J.; Wu, C.; West, D. B.; Chatterjee, A.; Ross, D. A.; Beauchamp, G. K. *Chemical Senses* **2001**, *26*, 925-933.

47. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. *Nat. Genet.* **2001**, *28*, 58-63.

48. Li, X.; Inoue, M.; Reed, D. R.; Huque, T.; Puchalski, R. B.; Tordoff, M. G.; Ninomiya, Y.; Beauchamp, G. K.; Bachmanov, A. *Mamm. Genome* **2001**, *12*, 13-16.

49. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696.

50. Nelson, G.; Chandrashekar, J.; Hoon, M. A.; Feng, L.; Zhao, G.; Ryba, N. J.; Zuker, C. S. *Nature* **2002**, *416*, 199-202.

51. Behrens, M.; Brockhoff, A.; Kuhn, C.; Bufe, B.; Winnig, M.; Meyerhof, W. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 479-485.

52. Bezençon, C.; le Coutre, J.; Damak, S. *Chemical Senses* **2007**, *32*, 41-49.

53. Parmentier, M.; Libert, F.; Schurmans, S.; Schiffman, S.; Lefort, A.; Eggerickx, D.; Ledent, C.; Mollereau, C.; Gerard, C.; Perret, J. *Nature* **1992**, *355*, 453-455.

Chapter 21

The "Bitter-Sweet" Truth of Artificial Sweeteners

C. T. Simons[1], C. Adam[1], G. LeCourt[1], C. Crawford[1], C. Ward[1],
W. Meyerhof[2], and J. P. Slack[1]

[1]Global Research and Development Center, Givaudan Flavors Corporation,
Cincinnati, OH 45216
[2]German Institute of Human Nutrition, Potsdam-Rehbruecke,
14558, Nuthetal, Germany

For some consumers, an unpleasant aftertaste is thought to underlie the rejection of artificially sweetened foods and beverages. As consumers become more informed regarding health and wellness issues, the consumption of artificially sweetened foods is expected to grow. Thus, understanding the negative characteristics associated with artificial sweeteners is crucial. However, this is not an easy task due to consumer differences in artificial sweetener sensitivity and difficulties in characterizing the artificial aftertaste. Recent evidence suggests that consumer sensitivity may be linked to differences in the expression of T2R genes. We will present the results of our studies on genetic variability and saccharin and ACEK bitterness sensitivity in humans. Furthermore, we characterized and differentiated the sensations elicited by natural and artificial sweeteners. Using trained panelists with proven sensitivity to artificial sweetener aftertastes, we generated sensory and temporal profiles that described and differentiated the aftertaste associated with each of the natural and artificial sweeteners. Finally, by linking aftertaste sensitivity to the liking of naturally and artificially-sweetened soft drinks, we were able to reveal the sensory attributes that contribute to the decreased liking of the artificial sweeteners studied. A discussion of possible approaches aimed at attenuating or masking the off-tastes of artificial sweeteners will be presented.

Introduction

Emerging health and wellness trends have led to a more sophisticated consumer in which increased knowledge and awareness of dietary health implications influences the foods and beverages they choose to consume. Consequently, there has been increased effort by the food and beverage industry to deliver products that meet the consumer's health expectations while simultaneously maintaining sensory quality. Among the recent products released by the food and beverage industry are those fortified with functional ingredients as well as those containing reduced levels of fat, salt, and/or sugar. Unfortunately, despite their healthy benefits, many of these products have achieved only limited market success due to a perceived lack of sensory quality. One particular area, the diet beverage sector, has enjoyed significant sales success. Indeed, global diet beverage sales have increased 44% over the last 9 years (*1*). Despite this success, a significant proportion of the population refuses to consume diet beverages due to the perception of a negative aftertaste that is associated with the use of artificial sweeteners. In an effort to better understand this negative aftertaste, we used a multidisciplinary approach to first delineate the mechanism subserving its detection and then to characterize the sensory properties associated with its perception. Finally, using a variety of technologies including high-throughput screening and the generation of reaction flavors, we sought to identify unique ingredients that would mitigate or reduce the perceived negative attributes associated with artificial sweeteners.

Sensitivity to Artificial Sweetener Aftertaste

Estimates suggest that between 15-35% of the population would consume more artificially sweetened products if the negative aftertaste attributed to artificial sweeteners could be reduced (*2*). These figures imply that a significant proportion of the population is sensitive to the aftertaste evoked by one or more of the various artificial sweeteners commonly used in the food and beverage industry. We therefore developed a sensitive sensory methodology to screen and identify individuals who are sensitive to aspartame, acesulfame K and/or sucralose offtastes.

Sensitivity—Human Sensory Testing

Over 100 panelists participated in the initial screenings for each of the artificial sweeteners. Aqueous solutions of sucrose and artificial sweetener were prepared according to Table 1. Concentrations of artificial sweetener were chosen to approximate the sweetness intensity of the corresponding sucrose solution. All solutions were prepared in deionized water and presented to the panelists at room temperature.

Table 1. Concentrations of sucrose, aspartame, ecesulfame K or sucralose used to screen subjects for sensitivity to artificial sweetener aftertaste.

Natural sweetener	Artificial sweetener	Concentration
Sucrose (3 %, 4 %, 5 %)	Aspartame	0.15 %
		0.20 %
		0.25 %
	Acesulfame K	0.0158 %
		0.0186 %
		0.0275 %
	Sucralose	0.005 %
		0.006 %
		0.008 %

To ascertain aspartame sensitivity, panelists were given three sucrose solutions and three aspartame solutions in a randomized order. In six replications conducted over two days, panelists were asked to rank the six solutions in order of increasing aftertaste. If panelists were insensitive to the aftertaste of the aspartame, then the rank order of solutions would be expected to be random (figure 1 top). If, however, a panelist was sensitive to the aftertaste of aspartame, the sucrose solutions would be expected to be ranked as having the least amount of aftertaste whereas the aspartame solutions would be expected to be ranked as having the most perceived aftertaste. Moreover, the least concentrated aspartame solution (0.15%) would be expected to have less aftertaste than the more concentrated aspartame solution (0.25%; figure 1 bottom). From these replicated evaluations, R-indices could be calculated and used to identify panelists capable of discriminating aspartame from sucrose. A similar protocol was used for evaluating acesulfame K and sucralose sensitivity.

As expected, sensitivity to the offtaste of the artificial sweeteners was highly variable (figure 2). Nearly 60% of the screened population were unable to reliably discriminate aqueous sucrose solutions from aspartame and were therefore deemed insensitive. The remaining 40% displayed a bimodal distribution of sensitivity; 8% were able to discriminate all three levels of aspartame from sucrose and were deemed highly sensitive whereas 22% of the panelists discriminated at least one of the three aspartame levels (figure 2). Similar findings were observed for acesulfame K and sucralose; 65% and 75% of panelists were insensitive to acesulfame K and sucralose, respectively. The remaining panelists displayed some degree of sensitivity to artificial sweetener aftertaste. For acesulfame K, 27% of panelists were classified as highly sensitive

Rank in order of increasing aftertaste

Insensitive

| Sucrose 4% | Apm 0.20% | Sucrose 5% | Apm 0.15% | Apm 0.25% | Sucrose 3% |

Sensitive

| Sucrose 4% | Sucrose 5% | Sucrose 3% | Apm 0.15% | Apm 0.20% | Apm 0.25% |
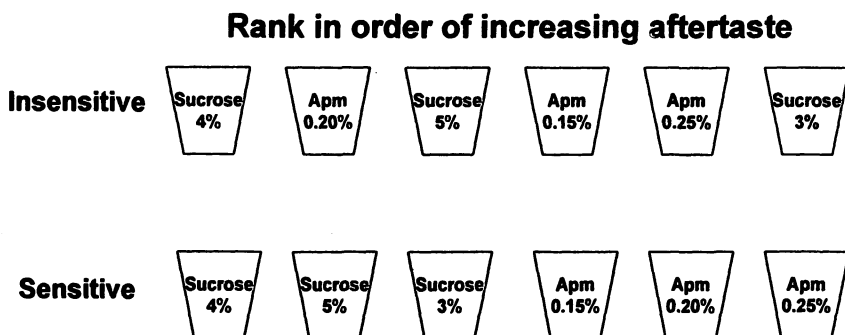
Figure 1. Expected distribution of sucrose and aspartame solutions for panelists that are insensitive (top) and sensitive (bottom) to the aftertaste of aspartame.
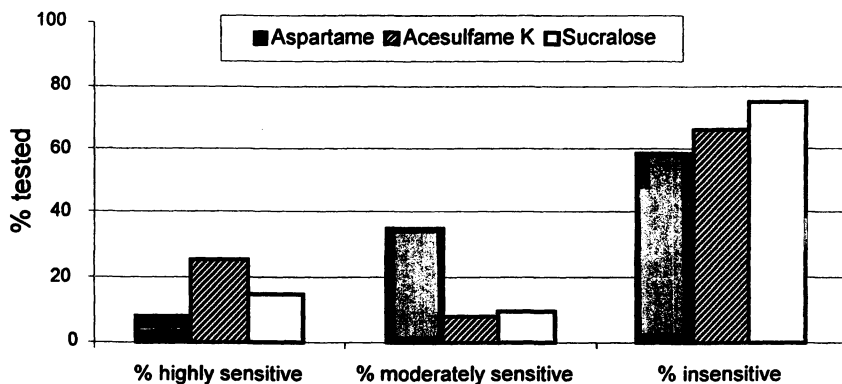
Figure 2. Distribution of panelist sensitivity to the aftertaste evoked by aspartame, acesulfame K and sucralose. Bars show the percentage of screened panelists who were identified as highly sensitive (able to discriminate all levels of artificial sweetener from sucrose), moderately sensitive (able to discriminate at least one level of artificial sweetener from the corresponding level of sucrose) and insensitive (unable to discriminate any level of artificial sweetener from sucrose).

and 8% as moderately sensitive whereas for sucralose, 15% and 10% of panelists were classified as highly or moderately sensitive, respectively (figure 2). It is of interest to note that 6 panelists were screened for sensitivity to all three artificial sweeteners. None of these panelists were highly sensitive to more than one artificial sweetener. In fact, three subjects were insensitive to all artificial sweeteners and three others were highly sensitive to one artificial sweetener and moderately sensitive to a second. These results are in agreement with what is to be expected if the sensitivity to artificial sweeteners has a genetic underpinning. Indeed, we found substantial variability across the population with no one displaying sensitivity to all three sweeteners. These findings suggest that different genes are responsible for controlling aftertaste sensitivity to aspartame, acesulfame K and sucralose.

Sensitivity—Genetic Basis

Much of the variation in sensitivity to chemical stimuli is thought to have a genetic basis. For instance, recent studies have shown that phenylthiocarbamide (PTC) sensitivity can be explained by the presence of five haplotypes of the human TAS2R38 (hTAS2R38) gene that encodes a member of the T2R bitter taste receptor family (*3, 4*). Recently, we identified two additional genes in the bitter T2R family, hTAS2R43 and hTAS2R44, that respond to saccharin and acesulfame K in addition to its cognate bitter ligand, aristolochic acid (*5*). Similar to what was observed with PTC, we reasoned that polymorphisms within hTAS2R43 and/or hTAS2R44 could affect sensitivity to saccharin and acesulfame K (*5*). Initial sensory studies were implemented to ascertain the relative sensitivity of a cross-section of panelists to the bitter aftertaste of saccharin. Simultaneously, we established a functional cellular assay by inducing the expression of hTAS2R43 and hTAS2R44 in human embryonic kidney (HEK) cells to correlate receptor and perceptual saccharin sensitivity. HEK cells expressing either hTAS2R43 or hTAS2R44 were loaded with a fluorescent calcium-sensitive dye and fluorescence changes in response to saccharin administration were used to measure receptor activity. Dose response curves were generated for hTAS2R43 and hTAS2R44 using saccharin concentrations ranging from 0.05-50 mM (figure 3a).

Human TAS2R43 and hTAS2R44 displayed similar sensitivity to saccharin; EC50's were 1.7 and 1.1 mM, respectively. Correspondingly, we asked 64 human subjects to rate the perceived bitterness intensity of various saccharin solutions. In three separate sessions, subjects were given each of 10 solutions, ranging from 0-100 mM saccharin, in random order and asked to first rank the solutions from least bitter to most bitter and then assign intensity ratings using a 100-point line scale. The ratings obtained from each replication were averaged for each panelist at each of the 10 saccharin concentrations. As anticipated, there was an enormous degree of variability observed in the bitterness intensity
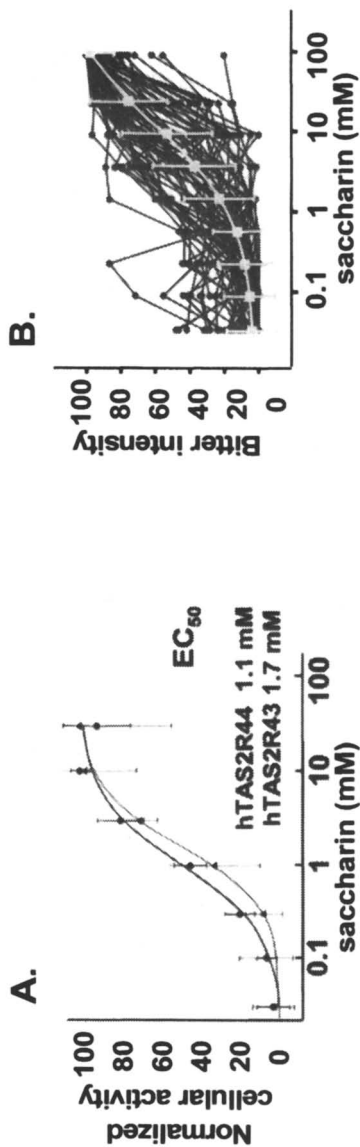
Figure 3. Saccharin-evoked bitterness dose response curves. A. Dose response curve of saccharin obtained in hTAS2R43 (dotted line) and hTAS2R44 (solid line), respectively. Graph shows the normalized fluorescence ratio of stimulated to unstimulated HEK cells. B. Human sensory dose response curves generated from 64 panelists. Note the variability in human bitterness scores obtained from the various saccharin solutions. The grey curve represents the average ± S.D. bitterness rating obtained from each saccharin solution. The concentration of saccharin that elicited a bitterness intensity rating of 50 (half-maximal) was 10 mM.

ratings (figure 3b). Some panelists had very steep dose response curves suggesting a high degree of saccharin bitterness sensitivity whereas others were extremely flat, implying an almost complete lack of sensitivity to saccharin bitterness. Across all panelists, the concentration of saccharin that elicited a half-maximal bitterness intensity rating was 10 mM (figure 3b), nearly 10-fold higher than the EC50 obtained *in vitro*. Several factors may contribute to this. Firstly, rating taste intensity is inherently variable and an inability to accurately assign ratings can alter the slope of the dose response curve. Secondly, *in vitro*, saccharin has unfettered access to the T2R receptors that are expressed on the cell surface. In human sensory testing, saccharin molecules can only interact with the taste receptor by first entering the pore of a taste bud and may also require traversing a mucous plug which often sits within the pore (*6, 7*). Thus, as compared to *in vitro* testing, higher concentrations of stimuli are often needed to elicit a taste sensation in humans.

Results from this study suggest that the bitter aftertaste of saccharin is mediated by the T2R receptors hTAS2R43 and hTAS2R44 and that significant variation most likely exists within these genes leading to the dramatic differences in sensitivity observed presently. Further studies have been initiated to link saccharin bitterness sensitivity to specific polymorphisms found within the hTAS2R43 and hTAS2R44 genome. It is enticing to speculate that genetic variation within hTAS2R43 and hTAS2R44 leading to increased bitterness sensitivity might explain the unwillingness of some consumers to consume foods and beverages sweetened with saccharin or acesulfame K. If this hypothesis bears out, then the need to specially tailor flavor formulations for different groups of consumers becomes a crucial avenue by which the food and beverage industry can increase the palatability of artificially sweetened foods.

Description of Artificial Sweetener Aftertaste

Results from the *in vitro* and human sensory screenings suggest that bitterness is a primary attribute that differentiates artificial from natural sweeteners. Despite these findings, anecdotal evidence suggests that other descriptors are used to describe the aftertaste associated with artificial sweeteners. For instance, aspartame and acesulfame K are also described as metallic and plastic whereas sucralose is described as lingering or having a swimming pool taste. It is possible that all of these descriptors are used to describe different types of bitter sensation. Human (*8*) and rodent (*9*) psychophysical studies have demonstrated perceptual distinctions between compounds described as bitter. Alternatively, other perceptual elements may exist in addition to bitterness that contribute to the sensory profile elicited by artificial sweeteners. To address this, we used a trained descriptive panel to evaluate and describe the aftertaste evoked by several natural and artificial sweeteners.

342

Descriptive Analysis

Eleven panelists were screened for sensitivity to aspartame, acesulfame K and sucralose offtaste. Six of the panelists were sensitive to aspartame, nine were sensitive to acesulfame K, and 6 were sensitive to sucralose; these panelists were used subsequently to evaluate the aftertaste of cola-flavored carbonated soft drinks containing natural or artificial sweeteners. Each panelist has undergone extensive training in descriptive analysis techniques and has served on the Givaudan trained descriptive panel for 10 or more years.

Over a 1 week training period, panelists identified ten attributes that described the aftertaste of naturally and artificially sweetened colas. Following the training session, panelists were given samples of the colas containing a natural (sucrose or high fructose corn syrup) or artificial (aspartame, aspartame/acesulfame K blend, sucralose) sweetener in randomized order and asked to rate the perceived intensity of each attribute. Averaged data were subjected to Principal Components Analysis such that the products and attributes could be co-visualized (figure 4). The first two principal components explained nearly 67% of the cola variance.
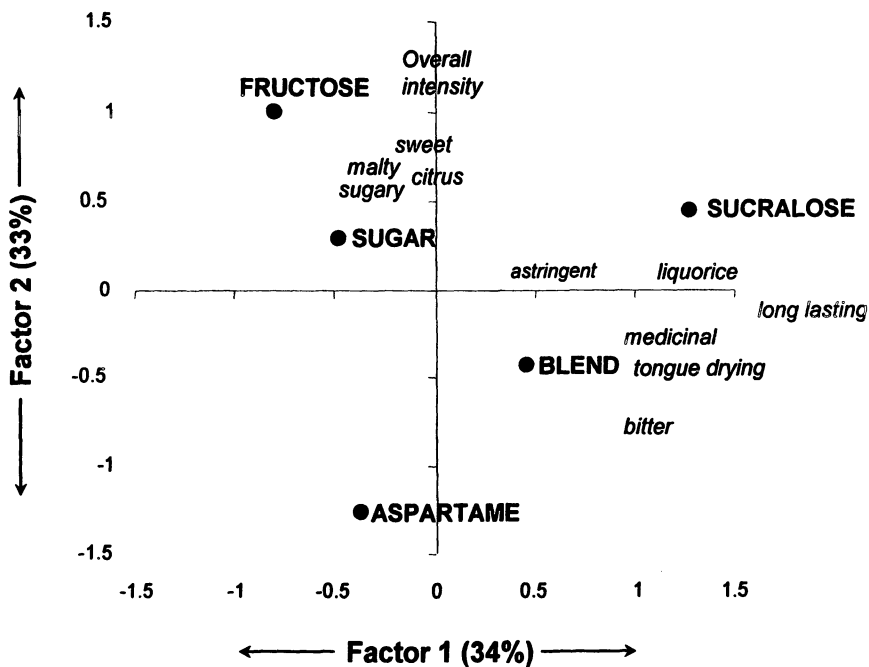
Figure 4. Principal Component Analysis of five colas from descriptive analysis using 11 attribute terms describing cola aftertaste. Attribute loadings are shown in italicized font whereas cola scores are shown as solid circles.

Whereas the aftertaste of colas containing sucrose or high fructose corn syrup were described primarily by the attributes sweet, sugary, malty and citrus, the aftertaste of colas containing artificial sweeteners were described quite differently. The aftertaste of sucralose was described as being long-lasting and having licorice, medicinal, and tongue drying attributes. On the first two principal components, aspartame was described as lacking a lingering aftertaste and lacking the sugary, malty character of the natural sweeteners; on the third principal component, aspartame was described as being bitter, astringent and licorice. The aftertaste of the cola containing the blend of aspartame (250 ppm) and acesulfame K (100 ppm) was described primarily as tongue drying, medicinal, astringent and bitter and to a lesser degree, licorice.

Temporal Dominance of Sensation

Results from the descriptive analysis suggest that multiple attributes contribute to the aftertaste differences perceived between and among natural and artificial sweeteners. However, descriptive analysis gives only a static representation of perceptual differences. The attributes are likely to have a dynamic temporal profile, with some attributes being perceived rather early and others being perceived at later time points. To study the dynamic nature of attribute intensity, the method of time intensity has often been employed (for review see *10*). Time intensity is a methodology in which the intensity of a particular attribute is scored over a period of time and allows the investigator to study such variables as attribute onset, decay and duration. However, time intensity is limited to the evaluation of a single attribute over time; assessing the temporal evolution of multiple attributes is not possible. As such, if an investigator has more than one attribute that needs to be characterized, time intensity methods become time consuming and labor intensive. Recently, a new methodology has been developed that addresses some of these limitations associated with time intensity.

Temporal Dominance of Sensation (TDS; *11*) is a method that borrows elements from both descriptive analysis and time intensity. Compared to time intensity, TDS is better suited for studying multivariate temporal changes. The method identifies and tracks the sensations that contribute most to the perception of a product at any given time and results in a map that illustrates the dynamic sequence of attribute dominance. Initially, attributes describing the aftertaste of artificially and naturally sweetened cola-flavored carbonated soft drinks were identified using descriptive analysis (see above). This list of descriptors served as the basis for subsequent TDS evaluations. Immediately after swallowing the cola, panelists were asked to identify the single attribute that dominated the perceived aftertaste. If, at any time during the ensuing 150 second evaluation period, a different attribute was perceived to dominate the aftertaste, panelists were asked to select that descriptor as the dominant sensation. Twelve panelists

completed 5 replications of this evaluation for each of the naturally- or artificially-sweetened colas. From these 60 evaluations, an index of dominance is calculated. The index is considered significant when a significant proportion of the evaluations identified a particular attribute as being dominant at a given time.

The TDS profile of cola containing high fructose corn syrup (HFCS) is shown in figure 5. The aftertaste of HFCS cola is characterized as being primarily sweet with citrus contributing significantly to the perception early (20-60 secs) and dryness later (50-90 sec). By 130 sec, the aftertaste of HFCS-sweetened cola had completely dissipated. This profile is consistent with anecdotal reports from consumers suggesting that the aftertaste of naturally sweetened beverages tends to be "clean" and devoid of the "surprise" tastes that define the aftertaste of artificial sweeteners. In contrast, the aftertaste of cola containing sucralose (figure 6) was initially (0-15 sec) characterized as sweet followed by a prolonged sensation that was dominated by a licorice note (15-130 sec) and finally a drying note (45-140 sec). Similarly, the aftertaste of cola sweetened with aspartame (figure 7) was initially characterized as sweet (0-25 sec) followed by a sensation dominated by licorice (20-110 sec) and drying notes (45-95 sec). Finally, the aftertaste of cola containing a blend of aspartame and acesulfame K (figure 8) was shown to be dominated initially by sweetness (0-40 sec) and bitterness (10-15 sec) followed later by licorice (15-120 sec) and drying notes (45-150 sec). In comparison to the information obtained from time intensity studies (see figure 9), the TDS experiments provide unique insight into the temporal dynamics of natural and artificial sweetener aftertaste. The single attribute that tends to most dominate the aftertaste of all three artificial sweeteners is licorice. Follow-up studies suggest that this attribute is derived from non-volatile components of the artificial sweetened colas as its intensity was not suppressed when retronasal olfaction was blocked using nose clips. Moreover, although the degree (dominance index) to which the aftertaste was dominated by the licorice and drying notes differed across the artificial sweeteners, it is interesting to note that the onset and duration of these attributes was remarkably consistent. The relevance of this finding is still unclear.

Aftertaste Masking Solutions

Results from our various studies suggest that a simple "magic-bullet" capable of making artificially sweetened products taste like naturally sweetened products is unlikely. Indeed, the descriptors that define different artificial sweeteners only partially overlap and the perceived intensities of these descriptors differ across artificial sweeteners. Moreover, the perceptual onset and duration of the various attributes is not the same for each artificial sweetener. Therefore, to alter sensory profiles of artificial sweeteners such that
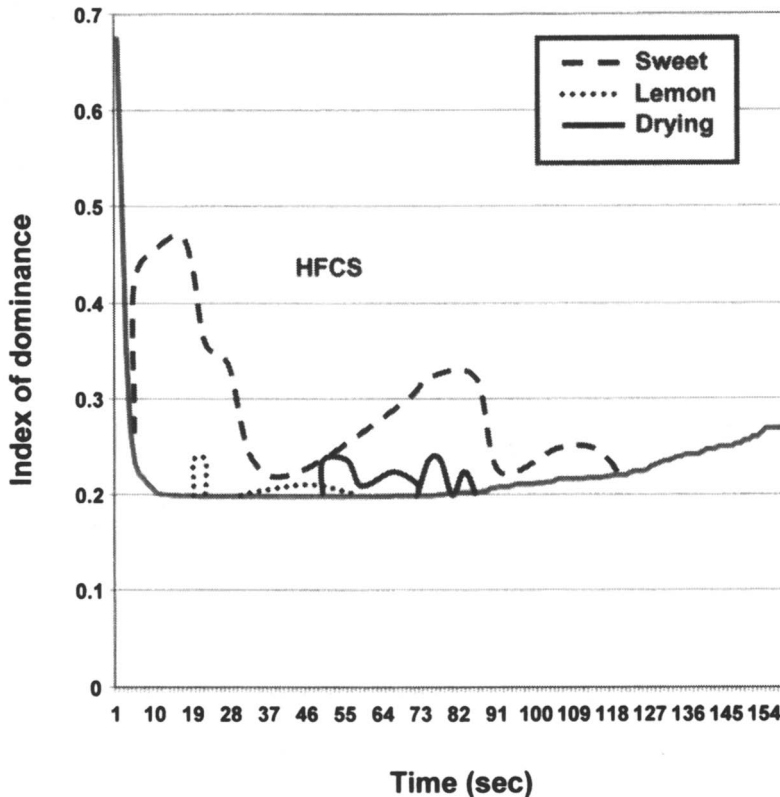
Figure 5. Temporal dominance profile of the aftertaste of cola containing HFCS. Graph shows the attributes at particular time points that were selected in a significant proportion of evaluations as dominating the perceived aftertaste. Light gray line shows the proportion of evaluations (from 60) needed at each time point to achieve statistical significance. Hatched line shows proportion of evaluations in which sweet was selected as being the dominant attribute. Dotted line shows proportion of evaluations in which lemon was perceived as the dominant attribute. Solid black line shows proportion of evaluations in which drying was perceived as the dominant attribute.
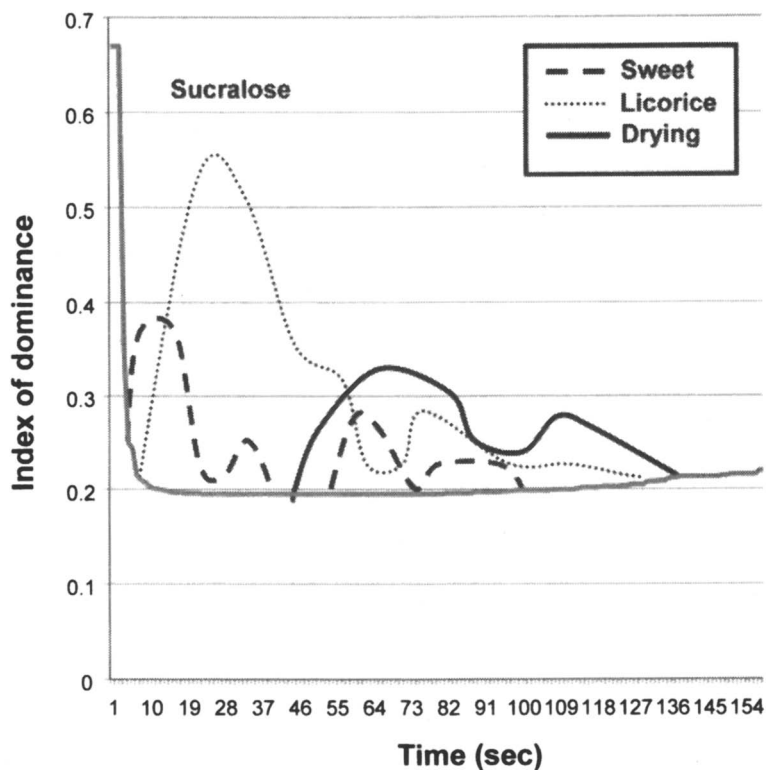
*Figure 6. Temporal dominance profile of the aftertaste of cola containing
sucralose. Graph as in figure 5. Light gray line shows the proportion of
evaluations needed at each time point to achieve statistical significance.
Hatched line shows proportion of evaluations in which sweet was selected
as being the dominant attribute. Dotted line shows proportion of
evaluations in which licorice was perceived as the dominant attribute.
Note difference in the dotted lines depicting lemon in figure 5 and licorice
in the current figure. Solid black line shows proportion of evaluations in
which drying was perceived as the dominant attribute.*
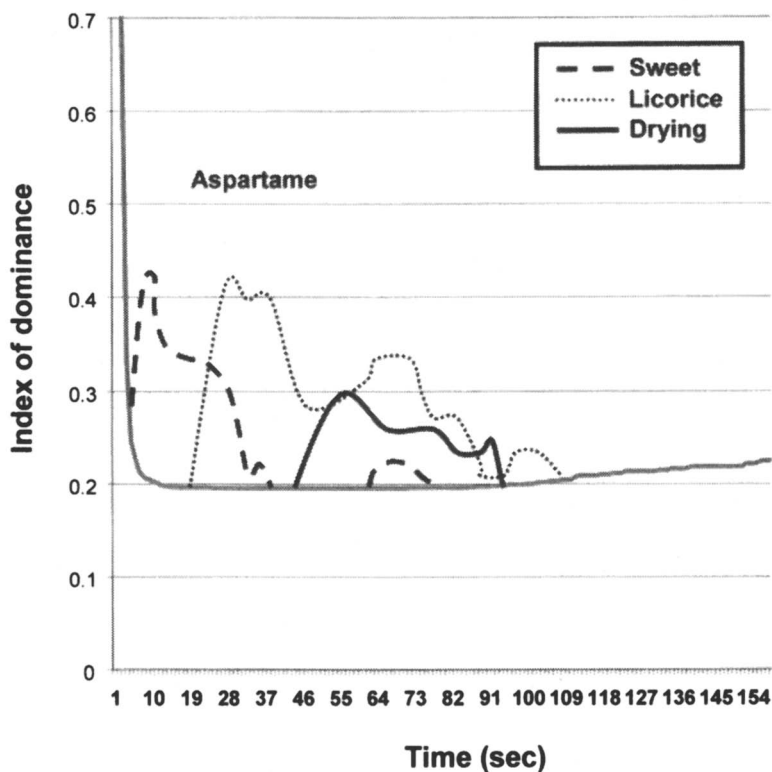
Figure 7. Temporal dominance profile of the aftertaste of cola containing aspartame. Light gray line shows the proportion of evaluations needed at each time point to achieve statistical significance. Hatched line shows proportion of evaluations in which sweet was selected as being the dominant attribute. Dotted line shows proportion of evaluations in which licorice was perceived as the dominant attribute. Solid black line shows proportion of evaluations in which drying was perceived as the dominant attribute.
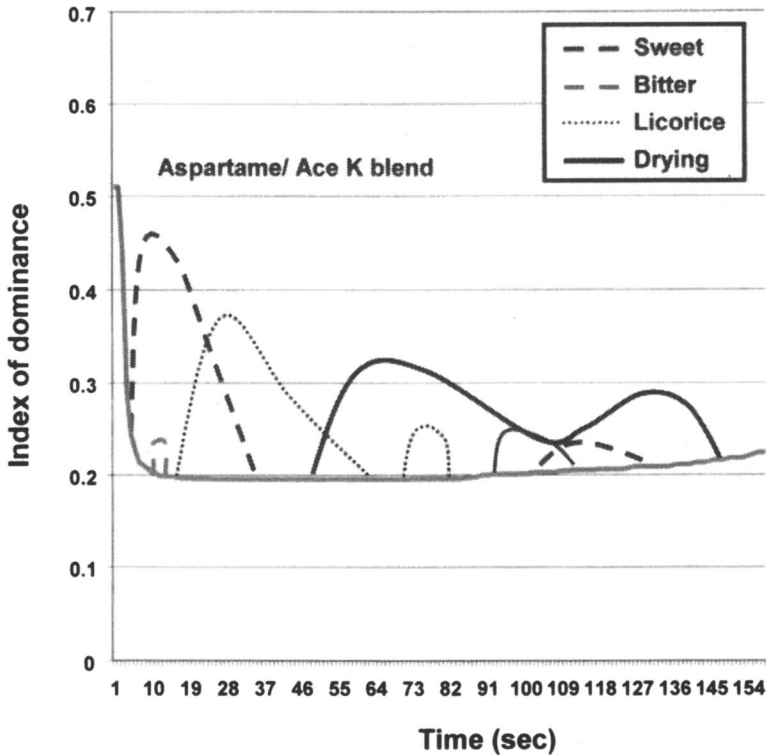
*Figure 8. Temporal dominance profile of cola containing the blend of
aspartame (250 ppm) and acesulfame K (100 ppm). Light gray line shows
the proportion of evaluations needed at each time point to achieve
statistical significance. Hatched line shows proportion of evaluations in
which sweet was selected as being the dominant attribute. Dotted line
shows proportion of evaluations in which licorice was perceived as the
dominant attribute. Solid black line shows proportion of evaluations in
which drying was perceived as the dominant attribute. Gray hatched line
depicts the proportion of evaluations in which bitter was selected as he
dominant attribute.*
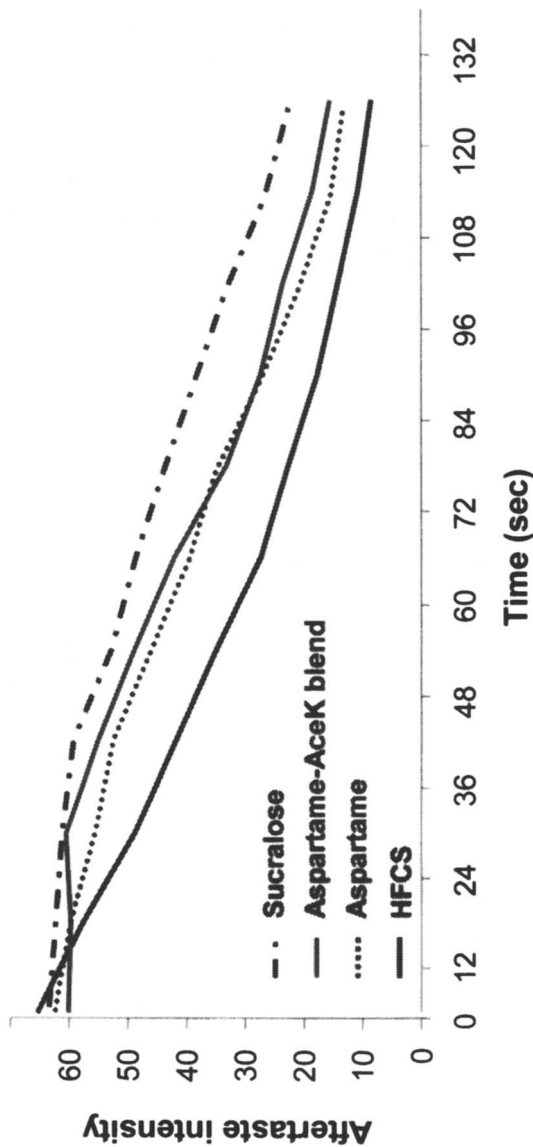
Figure 9. Aftertaste time intensity profiles of cola containing HFCS, sucralose, aspartame and the blend of aspartame and acesulfame K. Graph shows the perceived intensity of cola aftertaste over a 125 second period. Note that the aftertaste perceived in colas containing artificial sweeteners is more intense than that evoked by colas containing HFCS. However, the attributes contributing to this difference are unknown.

they are more similar to the profiles evoked by natural sweeteners, a multidimensional approach must be employed. To that end, we evaluated over 100 complex ingredients in an effort to identify those that are capable of making aspartame- or sucralose-sweetened colas indiscriminable from a cola containing HFCS. We employed a novel methodology using ten previously identified aspartame- or sucralose-sensitive panelists each of whom performed three replications of the test. Panelists were given four cola samples: a reference containing HFCS and three unknown samples containing HFCS (positive control), artificial sweetener (negative control) and artificial sweetener + ingredient. Panelists were asked to rank the three unknown samples from most similar to reference to least similar to reference (figure 10). If the complex ingredient works, the sample containing only the artificial sweetener will be ranked last, and the sample containing the artificial sweetener + ingredient will be confused with the HFCS control. Under such circumstances, an R-index analysis will show that the samples containing artificial sweetener and artificial sweetener + ingredient are significantly different whereas samples containing HFCS and artificial sweetener + ingredient are not (figure 10a). If, on the other hand, the complex ingredient does not work, HFCS will always be ranked first, and the ingredient will be confused with the artificial sweetener control. Under these circumstances, the R-index analysis will show that the samples containing artificial sweetener and artificial sweetener + ingredient are not significantly different whereas samples containing HFCS and artificial sweetener + ingredient are (figure 10b).

Aspartame and Sucralose

In the aspartame-sweetened cola base, we found it was possible to add complex flavor ingredients such that the perceptual profile evoked by the artificially sweetened beverage was not different from that evoked by HFCS containing cola. Indeed, the colas containing HFCS and aspartame + ingredient 1 were not significantly different whereas those containing aspartame alone and aspartame + ingredient 1 were (Table 2). Interestingly, multiple other ingredients, despite their ability to mask the bitter off-taste of aspartame, were not fully capable of making aspartame-sweetened cola indiscriminable from HFCS-sweetened cola. This finding underscores the need for identifying multiple key compounds that can be used to modify various aspects of the perceptual experience. Simply blocking the bitterness of artificial sweeteners, although crucial, is unlikely to deliver the same perceptual profile as that elicited by sucrose or HFCS. In the sucralose-sweetened cola base, a different complex ingredient (ingredient 2) was found to modify the profile such that it was not perceived as significantly different from cola containing HFCS (table 2). Interestingly, ingredient 2 had no effect on the perceptual profile evoked by an aspartame-containing beverage. Moreover, the ingredient that modified the
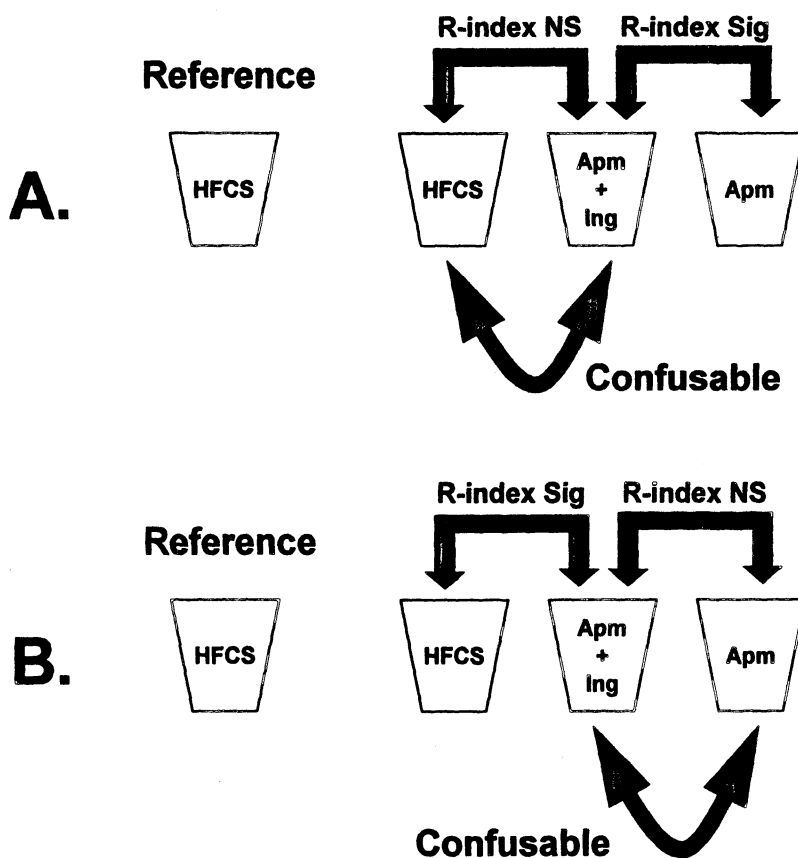
Figure 10. Testing paradigm to ascertain the possibility of making a carbonated soft drink containing aspartame or sucralose indistinguishable from one containing HFCS. A. Figure depicts the expected results of a similarity test when a complex ingredient is successful at making cola containing aspartame (apm) taste like cola containing HFCS. B. Figure depicts the expected results of a similarity test when a complex ingredient is unsuccessful at making cola containing aspartame taste like cola containing HFCS.

352

Table 2. Results from similarity ranking test. Cola containing aspartame (Apm) and ingredient 1 was not perceived as significantly different from cola containing HFCS. Similarly, cola containing sucralose and ingredient 2 was not perceived as significantly different from cola containing HFCS.

	Stimuli	R-index	Significantly different?
Aspartame	Apm + Ing 1 vs. HFCS	36%	No
	Apm + Ing 1 vs. Apm	79%	Yes
Sucralose	Sucralose + Ing 2 vs. HFCS	37%	No
	Sucralose + Ing 2 vs. Sucralose	68%	Yes

aspartame profile (ingredient 1) had no effect on the sucralose profile. These findings suggest that the combination of ingredients needed to mimic the HFCS profile is dependent on the artificial sweetener used in the base application.

Conclusions

A number of studies were undertaken in an effort to more fully understand the perceived negative aftertaste that is associated with many artificial sweeteners. From large scale screening studies, we determined that variability in sensitivity to the aftertaste of artificial sweeteners exists within the population. This variability most likely has genetic underpinnings that further impact consumer's behavioral choices as they relate to food and beverage consumption. By using panelists with documented sensitivity to the aftertaste of aspartame, acesulfame K or sucralose, we were able to identify the attributes that differentiated colas containing natural sweeteners from those with artificial sweeteners. Utilizing this information, we tracked the temporal profiles evoked from artificial sweeteners and showed how they differ from the temporal profiles elicited by HFCS. Whereas the aftertaste of cola containing HFCS is dominated by sweet perception alone, the aftertaste of cola containing sucralose, aspartame or a blend of aspartame and acesulfame K is dominated by a licorice note, bitterness and a drying effect. Finally, by attending to the specific attributes that differentiate natural sweeteners from a specific artificial sweetener, we showed that it is possible to minimize the aftertaste and make artificially sweetened beverages taste like naturally sweetened beverages. However, this is not a trivial task. The various attributes associated with the aftertaste of artificial sweeteners are likely to be mediated by separate mechanisms. Indeed, even for a single attribute like bitterness, it is likely that different receptors signal the presence of different artificial sweeteners. Thus, it is likely that complex solutions, involving multiple ingredients, will be needed to mitigate the negative aftertaste of artificially sweetened foods and make these products taste like their naturally sweetened counterparts.

References

1. Integrated Market Information System. Euromonitor, 2006.
2. Internal marketing research data. Givaudan Global Consumer Insights, 2006.
3. Kim, U. K.; Jorgenson, E.; Coon, H.; Leppert, M.; Risch, N.; Drayna, D. *Science* **2003**, *299*, 1221.
4. Bufe, B.; Breslin, P. A.; Kuhn, C.; Reed, D. R.; Tharp, C. D.; Slack, J. P.; Kim, U. K.; Drayna, D.; Meyerhof, W. *Current Biology* **2005**, *15*, 322.

5. Kuhn, C.; Bufe, B.; Winnig, M.; Hofmann, T.; Frank, O.; Behrens, M.; Lewtschenko, T.; Slack, J. P.; Ward, C. D.; Meyerhof, W. *Journal of Neuroscience* **2004**, *24*, 10260.
6. Kanazawa, H. *Archives of Histology and Cytology* **1993**, *56*, 533.
7. Witt, M.; Reutter, K. *Anatomical Records* **1996**, *246*, 507.
8. McBurney, D. H.; Smith, D. V.; Shick, T. R. *Perception and Psychophysics* **1972**, *11*, 228.
9. Frank, M. E.; Bouverat, B. P.; MacKinnon, B. I.; Hettinger, T. P. *Physiology and Behavior* **2004**, *80*, 421.
10. Piggott, J. R. *Food Research International* **2000**, *33*, 191.
11. Pineau, N.; Pessina, R.; Cordelle, S.; Imbert, A.; Rogeaux, M.; Schlich, P. *Presented at 7th Sensometrics Meeting, Davis, California,* **2004**.

Chapter 22

The Use of Rodent Models to Link Neurobiological Processes with the Psychophysics of Sweet Taste

A. C. Spector, S. Eylam, and C. D. Dotson

Department of Psychology and Center for Smell and Taste, University of Florida, Gainesville, FL 32611–2250

The gustatory effects of sweeteners begin with ligands binding with receptors in the oral cavity and ends with the behavioral and physiological responses of an animal. One of the primary goals of taste research is to fully understand this process. The psychophysical analysis of sweet taste in nonhuman animals, in which the gustatory system can be experimentally manipulated, is challenging, but essential in linking the underlying neurobiology to behavior. This chapter focuses on how the authors have used various behavioral techniques in rodents to help provide a context for interpreting molecular and neurophysiological findings regarding the taste effects of sweeteners.

Introduction

This decade has seen remarkable advances in understanding the molecular biology of sweet taste transduction in taste receptor cells. The discovery of the T1R family of taste receptors as well as the identification of intermediary enzymes and channels, such as PLCβ2 and TRPM5 respectively, critical in the transduction mechanisms triggered by sweet-tasting compounds, have been major breakthroughs (1-11). Transduction, however, represents only the first

stage of signal processing and there are many other events that intervene between contact of a ligand with its receptors and the behavior it ultimately generates. Any comprehensive understanding of the neurobiological mechanisms underlying sweet taste perception requires a rigorous assessment of the consequences of anatomical, pharmacological, or genetic manipulations of the gustatory system on taste-guided behavior in animal models (12). In this paper, we discuss some of the issues and complexities associated with such approaches with respect to the study of sweet taste in rodent models.

Benefits of Psychophysical Analysis of Sweet Taste in Animal Models

Why not just rely on human psychophysics to understand sweet taste perception? The answer, of course, is that there are several distinct benefits to conducting rigorous psychophysical analyses of taste function in rodent models. First, unlike with humans, experimenters can maintain control over exposure to chemical stimuli during development. Second, control can be maintained over the internal (physiological) and external environment. Third, rodents, especially mice, are optimal for testing genetic influences on taste function because of their high rate of breeding and the ability of the genome to be altered in embryonic stem cells. Finally, it is possible to invasively manipulate the gustatory system to test hypotheses on the neural mechanisms underlying taste function.

Methodological and Interpretive Issues

Although most researchers understand the necessity of conducting behavioral studies in rodent models, such efforts are laden with significant methodological and conceptual challenges (see (12,13). The most commonly used behavioral measure of taste responsiveness in animals is the two-bottle intake (preference) test. This procedure measures taste responsiveness in the context of ingestion and thus enjoys some connection to a functionally relevant natural behavior. Moreover, it is relatively easy to use and does not involve much training of animals or personnel. However, at its best, the two-bottle test only provides a rough approximation of an animal's responsiveness to taste stimuli. This is because non-gustatory factors such as postingestive events can influence choice and intake. Also, because it is the hedonic characteristics of a taste stimulus that drive the intake, if a taste compound is identifiable but neither preferred nor avoided, the two-bottle preference test will not provide much information. This can be problematic with compounds that have complex tastes or with taste stimuli presented at low concentrations that animals might not prefer or avoid, but might, nonetheless, be able to detect and identify.

Particular behavioral procedures have been developed to circumvent some of the interpretive constraints associated with preference and intake tests. These possess certain preferable methodological features. First, small volumes of taste stimuli are delivered in very brief-access trials. Second, immediate responses are measured. These two features increase the confidence that the animal's behavior is guided by orosensory cues. Third, taste stimuli are used as signals for other reinforcing events (such as reward or punishment in operant conditioning tasks). In this way, the animal's responses are driven by the motivation to obtain the reinforcer or avoid punishment, not by the hedonic properties of the taste stimulus.

Taste Detection Thresholds for Sweeteners in "Taster" and "Non-Taster" Mouse Strains

In the spirit of the methodological issues just noted, Eylam and Spector (*14*) employed an operant two-response taste detection procedure to examine whether inbred strains of mice possessing the "taster" allele of the *Tas1r3* gene are more sensitive at detecting "sweet-tasting" compounds compared with "non-taster" strains. The *Tas1r3* gene is one of a three-member gene family that also includes the *Tas1r1* and the *Tas1r2* genes (*1-11*). The proteins encoded by these genes (T1R1, T1R2, and T1R3) are thought to dimerize to form functional receptors such that the T1R1+T1R3 binds with L-amino acids and the T1R2+T1R3 binds with sugars, a subset of D-amino acids, and some synthetic sweeteners (and sweet-tasting proteins in some species). In laboratory mice, the *Tas1r3* gene, first identified through quantitative trait loci analysis as the *Sac* locus, is polymorphic. Strains that express alternative alleles of *Tas1r3* differ in their preference for low concentrations of sweeteners in two-bottle tests (*1,5,15-21*). The terms "taster" and "non-taster" are a bit of a misnomer because the phenotype merely reflects a shift in sensitivity and all of these strains are able to respond to these stimuli at midrange to high concentrations. Eylam and Spector examined whether this phenotype would also be expressed in a signal detection paradigm that incorporated some of the methodological features detailed above. Moreover, because glycine appears to bind with both the T1R1+T1R3 as well as the T1R2+T1R3 heterodimer (*5*), and the *Tas1r3* polymorphism appears to only affect the ligand binding affinity of the latter receptor complex, they included this amino acid in the test stimulus array along with the two prototypical sweet-tasting sugars sucrose and glucose.

The results of the Eylam and Spector study confirmed the prediction that the "taster" strains (C57BL/6 (B6) and SWR/J (SWR)) could detect both sucrose and glucose, on average, at significantly lower concentrations than "non-taster" strains (129P3/J (129) and DBA/2J (D2)). Detection thresholds to glycine, however, were not easily explained by the "taster" status of the strains (*Figure 1*).

358

Accordingly, when the thresholds for all of the mice were collapsed across strain, sucrose thresholds correlated with glucose thresholds very highly ($r=0.81$), but the correlation between each sugar and glycine was much more modest ($r=0.40 - 0.43$) (*Figure 2*). Eylam and Spector speculated that the reason there was much less correspondence in sensitivity between glycine and the sugars than between sucrose and glucose in mice is because glycine also binds with the T1R1+T1R3 heteromer which is unaffected by the polymorphism (*5*). It is also worth noting that when definition of threshold is adjusted by determining the lowest concentration at which the hit rate is significantly different than the false alarm rate, sucrose thresholds measured in this operant signal detection paradigm generally match those measured with two-bottle preference tests (although it is not always the case that the two procedures lead to similar conclusions; see (*22-24*)). This suggests that the hedonic value of sucrose begins to rise once the compound is detected.
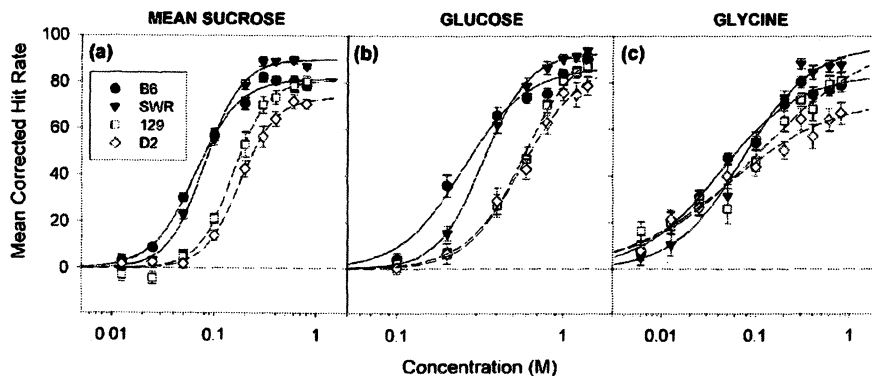
Figure 1. Taste detectability functions for sucrose, glucose and glycine in 4 inbred strains of mice. Reproduced with permission from reference 14. Copyright 2004.

Suprathreshold Hedonic Responsiveness to Sucrose, Glycine, and L-Serine in "Taster" and "Non-Taster" Mice

Even non-taster mice display robust preferences once the concentration of the sweetener reaches a certain level. Dotson and Spector examined whether the *Sac*-related phenotype would be observable in the suprathreshold concentration range (*25*). They used a brief-access test in which mice were trained to lick a drinking spout in a special gustometer, commonly referred to as a "Davis Rig" (DiLog Instruments, Tallahassee, FL), that delivered different taste solutions for very brief-access trials (5 s) (see (*26,27*)).
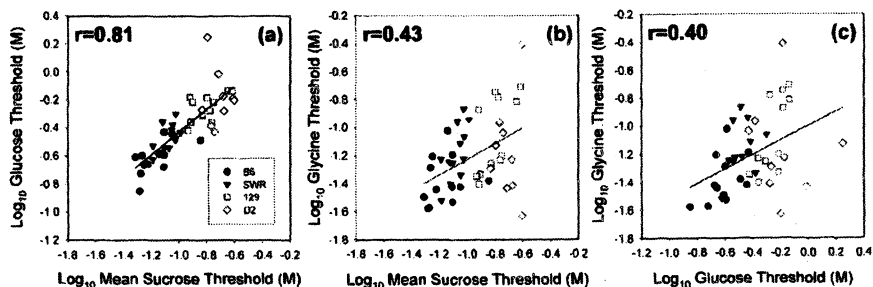
Figure 2. Scatter plot and correlations between sugar taste thresholds.
Reproduced with permission from 14. Copyright 2004.

Interestingly, the nondeprived non-taster mice displayed concentration-dependent licking of sucrose and responded to mid-range and high concentrations at levels similar to or even higher than taster mice (*see Figure 3, left panel*). What was somewhat surprising was that L-serine and glycine, two putatively sweet-tasting amino acids, did not generate much licking behavior at any of the concentrations with perhaps the exception of 1.0 M and 1.5 M glycine for the 129 strain. Clearly, the responses to these amino acids paled in comparison to sucrose (*Figure 3, middle and right panel*). The basis for this difference remains to be explained, but might be due to sideband tastes associated with these amino acids. Dotson and Spector concluded that hedonic responsiveness to suprathreshold concentrations of sucrose, as assessed in a brief-access test, is unrelated to *Sac* taster status suggesting that genes other than *Tas1r3* contribute to this taste-related behavior. Glendinning et al. (*28*) as well as Sclafani (*29*) have reached similar conclusions. It is also clear from these results that measures of taste threshold detectability do not necessarily
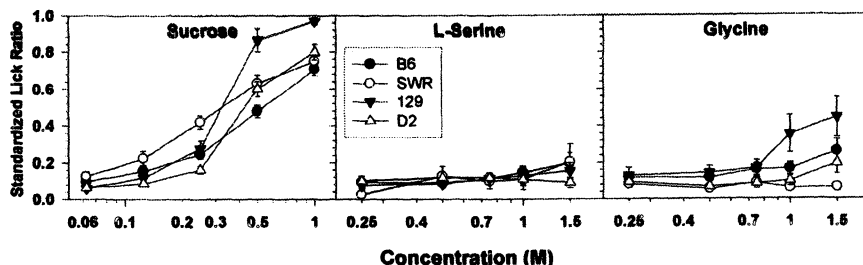
Figure 3. Licking responses in non-deprived mice from 4 inbred strains in a brief-access taste test. Reproduced with permission from 25. Copyright 2004.

correspond with suprathreshold measures of taste responsiveness. It is important to stress here, that one should not view one measure as good and the other as bad. The disparity simply means that the behaviors assessed in the two procedures are not under the control of identical factors.

Taste Discrimination between Maltose and Sucrose by Rats

Up to this point, we have discussed the methodological and interpretive ramifications of signal detection procedures and brief-access tests with regard to "sweet-tasting" stimuli. The former provides information on the absolute limens of sensitivity and the latter offers insight into motivational properties (i.e., hedonic) of stimuli in the suprathreshold range. We now turn our attention to taste discrimination paradigms. In these procedures, animals are trained to respond one way to one taste stimulus and another way to a different taste stimulus. If the animals can learn the discrimination, and intensity and extraneous cues can be ruled out, one can conclude that the animals can distinguish the qualitative nature of the taste compounds. Such approaches have been used in a limited fashion to investigate sweeteners in rodent models, but we would like to provide one example from work conducted in this laboratory testing whether rats can discriminate sucrose from maltose on the basis of taste (*30*).
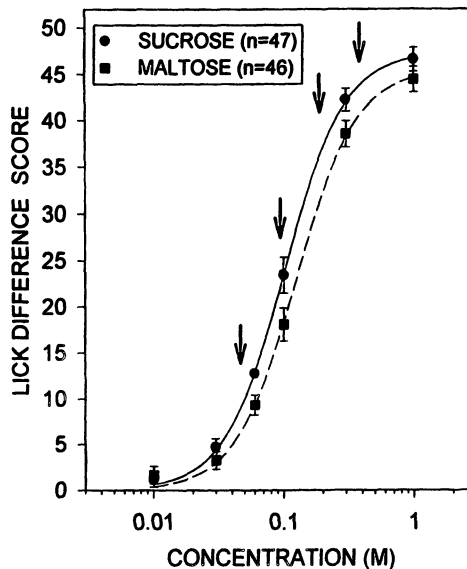
Figure 4. Licking responses to sucrose and maltose in rats relative to water. Adapted with permission from 30. Copyright 1997.

In this experiment, a range of concentrations were chosen based on concentration-response functions derived in prior work (*Figure 4*). This was done to render intensity a relatively irrelevant cue. In other words, provided that the range of concentrations overlap in intensity it would be difficult for the animal to learn a strong vs. weak intensity discrimination. Having chosen the concentrations, the animals were then trained in a conditioned shock avoidance paradigm. In this procedure thirsty rats were trained in a gustometer to lick a dry drinking spout for an average of 20 times in order to receive a fluid presentation. If the fluid was the S+ stimulus [1] (say maltose) the animal was required to entirely suppress licking within the first 2 s of the 5 s trial or else it received a brief and annoying shock to the paws at the end of the trial. If the fluid was the S- stimulus (say sucrose) the animal was free to lick the stimulus for the entire 5 s. If the animal suppressed licking during the latter 3 s of an S- trial then it received a 30 s time-out further delaying the opportunity to receive fluid.
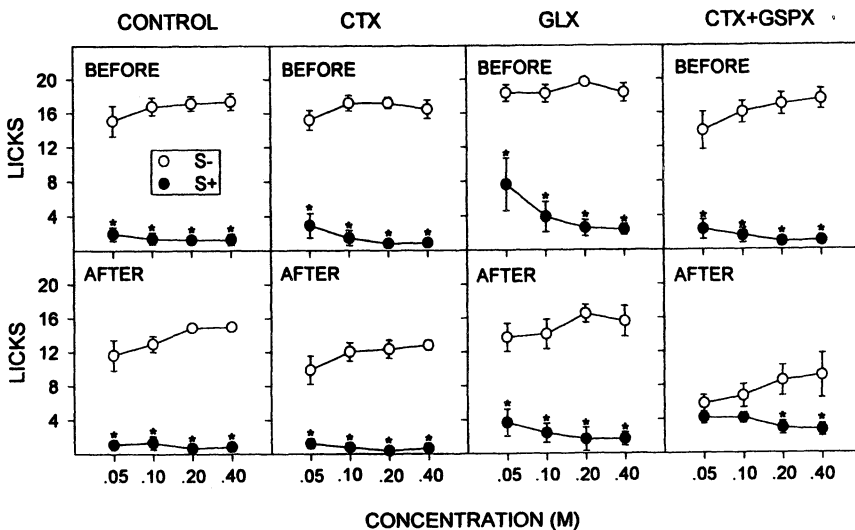
Figure 5. Licks to the sugar stimulus signaling shock (S+) and the sugar stimulus signaling no shock (S-) in a conditioned taste avoidance task used to assess the rat's ability to discriminate sucrose from maltose. Reproduced with permission from reference 30. Copyright 1997.

---

[1] In this experiment the S+ signified the stimulus associated with shock and the S- signified the stimulus associated with no shock. Some investigators use the term S+ to designate the stimulus signaling a "positive" event (i.e., no shock) and the S- to designate the stimulus signaling shock.

The rats learned the task well and displayed very high levels of performance to all of the concentrations as illustrated in the top panels of *Figure 5*. The animals were then assigned to surgical groups. Some animals received transection of the chorda tympani nerve (CT), which innervates taste buds on the anterior two-thirds of the tongue. Some animals received combined transection of both the CT and the greater superficial petrosal nerve (GSP), which innervates taste buds on the palate. Another group of rats had the glossopharyngeal nerve (GL), which innervates taste buds on the back of the tongue, cut. Finally a group of rats that received sham surgery was included. The rats that received combined transaction of the CT and GSP displayed severe impairments in performance suggesting that the taste signals carried by the fibers of the 7th cranial nerve are necessary to maintain normal discrimination between these two sugars.

What is the basis of the rat's ability to discriminate between sucrose and maltose? One possibility is that the two sugars generate similar but distinguishable taste qualities. Perhaps there are different types of "sweetness". Alternatively, perhaps one of the sugars, say maltose, binds with other, non-T1R receptors, leading to the generation of sideband tastes. These possibilities have conceptual ramifications regarding the organization of the gustatory system and are therefore quite appealing from a theoretical standpoint. However, there are other more pedestrian possibilities that must be considered.

First, it is possible that the animals are using a non-taste cue to make the discrimination. In taste discrimination experiments, the most suspicious of these possible cues is smell. Although the gustometer used in the experiment described above is designed to minimize olfactory stimulation (*31*), it is simply impossible to entirely eliminate the contribution of olfaction. However, there are times when the natures of the experimental results are not consistent with notion that olfactory cues were guiding performance. Such is the case in the sucrose vs. maltose experiment. The fact that CT+GSP nerve transection severely impaired performance in the task implies that smell was not sufficient to maintain normal discrimination between the two sugars.

Second, it is possible that there was a contaminant in one of the solutions that helped guide performance. This, of course, is a vexing possibility for all chemical senses researchers because regardless of how careful one can be in the laboratory preparing solutions, there is little practical control over the quality of the chemicals purchased from manufacturers other than the grade of purity ordered. Thus, stimulus contamination is an ever present caveat in taste discrimination experiments.

Third, another possibility is that the two compounds possess the same quality, but are distinguishable based on oral locus of stimulation because of differential distribution of their respective receptors. If, for example, sucrose stimulated Receptor A strongly and Receptor B weakly, and maltose did the converse, and if Receptor A was more densely distributed in the anterior tongue relative to the posterior tongue, and Receptor B was distributed in the opposite

fashion, then oral locus of stimulation could provide a discriminable cue. This could be the case even if the qualitative sensation produced were identical. Fourth, it is also possible that the two compounds possess the same quality, but produce differential rise and decay times of sensation due to respective ligand-receptor interactions[2].

It should be clear, therefore, that there are a number of possibilities underlying the ability of any subject to discriminate between two taste compounds that must be considered in interpretation. It is for this reason that conceptually, the demonstration of a failure to discriminate between taste compounds is more compelling than success provided that learning and intensity effects can be ruled out. Failure suggests that a perceptual identity relation exists between the two compounds in the pair. This sets the stage for searching for neurobiological processes that fail to distinguish between the stimuli.

Final Remarks

There are some general conclusions regarding the study of taste perception in animal models that transcend the issue of sweetness per se. First, manipulations of the gustatory system may not lead to concordant outcomes in different behavioral assays. Second, every behavioral method used to study taste function has its strengths and limitations. Therefore, a comprehensive depiction of taste function in animal models requires the complementary application of different types of behavioral assays.

In closing, from our perspective, there are several conceptual issues regarding sweetener taste in rodent models that would be meaningful to address in the future. First and foremost, are there pairs of sweeteners that rodents *cannot* discriminate? Second, in cases in which rodents can discriminate between sweeteners, what is the basis of the discrimination? Third, are T1R2 and T1R3, in heteromeric or homomeric form, the only receptors that bind with sweet-tasting ligands. Fourth, what taste functions rely on the presence of both T1R2 and T1R3? Fifth, are the motivational characteristics of sweeteners neurally dissociable from their perceptual quality? Finally, how is taste quality represented in the mammalian nervous system (labeled-line, across-neuron pattern, temporal codes, etc.)? Answers to these questions should bring us closer to understanding the neural basis of one of nature's greatest gifts – the experience of sweetness.

---

[2] It is worth noting that it is arguable that rise and decay could be considered part of taste quality, but we are taking a much more restrictive perspective here.

References

1. Bachmanov, A. A.; Li, X.; Reed, D. R.; Ohmen, J. D.; Li, S.; Chen, Z.; Tordoff, M. G.; de Jong, P. J.; Wu, C.; West, D. B.; Chatterjee, A.; Ross, D. A.; Beauchamp, G. K. Positional cloning of the mouse saccharin preference (Sac) locus. *Chem. Senses* **2001**, *26*, 925-933.
2. Kitagawa, M.; Kusakabe, Y.; Miura, H.; Ninomiya, Y.; Hino, A. Molecular genetic identification of a candidate receptor gene for sweet taste. *Biochem. Biophys. Res. Commun.* **2001**, *283*, 236-242.
3. Max, M.; Shanker, Y. G.; Huang, L. Q.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac. *Nat. Genet.* **2001**, *28*, 58-63.
4. Montmayeur, J. P.; Liberles, S. D.; Matsunami, H.; Buck, L. B. A candidate taste receptor gene near a sweet taste locus. *Nat. Neurosci.* **2001**, *4*, 492-498.
5. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y. F.; Ryba, N. J. P.; Zuker, C. S. Mammalian sweet taste receptors. *Cell* **2001**, *106*, 381-390.
6. Sainz, E.; Korley, J. N.; Battey, J. F.; Sullivan, S. L. Identification of a novel member of the T1R family of putative taste receptors. *J. Neurochem.* **2001**, *77*, 896-903.
7. Hoon, M. A.; Adler, E.; Lindemeier, J.; Battey, J. F.; Ryba, N. J.; Zuker, C. S. Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. *Cell* **1999**, *96*, 541-551.
8. Li, X. D.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. Human receptors for sweet and umami taste. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696.
9. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* **2003**, *112*, 293-301.
10. Nie, Y.; Vigues, S.; Hobbs, J. R.; Conn, G. L.; Munger, S. D. Distinct contributions of T1R2 and T1R3 taste receptor subunits to the detection of sweet stimuli. *Curr. Biol.* **2005**, *15*, 1948-1952.
11. Nie, Y.; Hobbs, J. R.; Vigues, S.; Olson, W. J.; Conn, G. L.; Munger, S. D. Expression and purification of functional ligand-binding domains of T1R3 taste receptors. *Chem. Senses* **2006**, *31*, 505-513.
12. Spector, A. C. Linking gustatory neurobiology to behavior in vertebrates. *Neurosci. Biobehav. Rev.* **2000**, *24*, 391-416.
13. Spector, A. C.; Travers, S. P. The representation of taste quality in the mammalian nervous system. *Behav. Cogn. Neurosci. Rev.* **2005**, *4*, 143-191.
14. Eylam, S.; Spector, A. C. Stimulus processing of glycine is dissociable from that of sucrose and glucose based on behaviorally measured taste signal detection in *Sac* 'taster' and 'non-taster' mice. *Chem. Senses* **2004**, *29*, 639-649.

15. Capeless, C. G.; Whitney, G. The genetic basis of preference for sweet substances among inbred strains of mice: Preference ratio phenotypes and the alleles of the Sac and dpa loci. *Chem. Senses* **1995**, *20*, 291-298.

16. Bedingfield, J. B.; King, D. A.; Holloway, F. A. Peripheral opioid receptors may mediate a portion of the aversive and depressant effect of EtOH: CPP and locomotor activity. *Alcohol* **1999**, *18*, 93-101.

17. Lush, I. E. The genetics of tasting in mice. VI. Saccharin, acesulfame, dulcin and sucrose. *Genet. Res.* **1989**, *53*, 95-99.

18. Blizard, D. A.; Kotlus, B.; Frank, M. E. Quantitative trait loci associated with short-term intake of sucrose, saccharin and quinine solutions in laboratory mice. *Chem. Senses* **1999**, *24*, 373-385.

19. Bachmanov, A. A.; Tordoff, M. G.; Beauchamp, G. K. Sweetener preference of C57BL/6ByJ and 129P3/J mice. *Chem. Senses* **2001**, *26*, 905-913.

20. Reed, D. R.; Li, S.; Li, X.; Huang, L.; Tordoff, M. G.; Starling-Roney, R.; Taniguchi, K.; West, D. B.; Ohmen, J. D.; Beauchamp, G. K.; Bachmanov, A. A. Polymorphisms in the taste receptor gene (Tas1r3) region are associated with saccharin preference in 30 mouse strains. *J. Neurosci.* **2004**, *24*, 938-946.

21. Inoue, M.; Reed, D. R.; Li, X.; Tordoff, M. G.; Beauchamp, G. K.; Bachmanov, A. A. Allelic variation of the Tas1r3 taste receptor gene selectively affects behavioral and neural taste responses to sweeteners in the F2 hybrids between C57BL/6ByJ and 129P3/J mice. *J. Neurosci.* **2004**, *24*, 2296-2303.

22. Eylam, S.; Spector, A. C. The effect of amiloride on operantly conditioned performance in an NaCl taste detection task and NaCl preference in C57BL/6J mice. *Behav. Neurosci.* **2002**, *116*, 149-159.

23. Spector, A. C. Psychophysical evaluation of taste function in non-human mammals. In *Handbook of Olfaction and Gustation*, 2nd ed.; Doty, R. L., Ed.; Marcel Dekker: New York, 2003; pp 861-879.

24. Spector, A. C. The functional organization of the peripheral gustatory system: lessons from behavior. In *Progress in Psychobiology and Physiological Psychology*, Fluharty, S. J., Grill, H. J., Eds.; Academic Press: San Diego, 2003; pp 101-161.

25. Dotson, C. D.; Spector, A. C. The relative affective potency of glycine, L-serine and sucrose as assessed by a brief-access taste test in inbred strains of mice. *Chem. Senses* **2004**, *29*, 489-498.

26. Glendinning, J. I.; Gresack, J.; Spector, A. C. A high-throughput screening procedure for identifying mice with aberrant taste and oromotor function. *Chem. Senses* **2002**, *27*, 461-474.

27. Smith, J. C. The history of the "Davis Rig". *Appetite* **2001**, *36*, 93-98.

28. Glendinning, J. I.; Chyou, S.; Lin, I.; Onishi, M.; Patel, P.; Zheng, K. H. Initial licking responses of mice to sweeteners: effects of tas1r3 polymorphisms. *Chem. Senses* **2005**, *30*, 601-614.

29. Sclafani, A. Sucrose motivation in sweet "sensitive" (C57BL/6J) and "subsensitive" (129P3/J) mice measured by progressive ratio licking. *Physiol. Behav.* **2006**, *87*, 734-744.
30. Spector, A. C.; Markison, S.; St.John, S. J.; Garcea, M. Sucrose vs. maltose taste discrimination by rats depends on the input of the seventh cranial nerve. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **1997**, *272*, R1210-R1218.
31. Spector, A. C.; Andrews-Labenski, J.; Letterio, F. C. A new gustometer for psychophysical taste testing in the rat. *Physiol. Behav.* **1990**, *47*, 795-803.

Chapter 23

Functional Characterization of the Human Sweet Taste Receptor: High-Throughput Screening Assay Development and Structural Function Relation

Xiaodong Li and Guy Servant

Senomyx, Inc., 4767 Nexus Center Drive, San Diego, CA 92121

The human sweet taste receptor is a heteromeric complex of T1R2 and T1R3. We have developed a high throughput screening (HTS) assay for the receptor and demonstrated a tight correlation between the *in vitro* receptor activity and *in vivo* taste behavior. Furthermore, we developed a human-rat chimeric receptor system, and discovered different functional domains on both subunits of the receptor.

Introduction

T1Rs, a family of class C G protein-coupled receptors (GPCRs), are selectively expressed in the taste buds (1-6). Functional expression of T1Rs in HEK293 cells revealed that different combinations of T1Rs respond to sweet and umami taste stimuli (6, 7). T1R2 and T1R3, when co-expressed in HEK293 cells, recognize a diverse set of natural and synthetic sweeteners. Similarly, T1R1 and T1R3, when coexpressed in HEK293 cells, respond to umami taste stimulus L-glutamate. This response is enhanced by 5'-ribonucleotides, a hallmark of umami taste. Recent experiments with knockout mice confirmed that T1Rs indeed mediate mouse sweet and umami tastes (8, 9).

The class C GPCRs possess a large N-terminal extracellular domain, often referred to as the Venus flytrap domain (VFD) (10), and are known to function as either homodimers, in the cases of metabotropic glutamate receptors (mGluRs) and the calcium-sensing receptor (CaR), or heterodimers, in the case of the γ-aminobutyric acid type B receptor (GABA$_B$R) (10). The functional expression data suggest a heterodimer mechanism for T1Rs: both T1R1 and T1R2 need to be coexpressed with T1R3 to be functional, which is supported by the overlapping expression patterns of T1Rs in rodent tongue. Nonetheless, there has been no direct evidence that T1Rs function as a heteromeric complex. It is possible that T1R3 is not a functional component of sweet and umami taste receptors, but merely a chaperone protein, which facilitates the proper folding or intracellular translocation of T1R1 and T1R2. The distinct ligand specificities of T1R1/T1R3 and T1R2/T1R3 receptors suggest that T1R1 and T1R2 play more important roles in ligand binding in sweet and umami taste receptors than T1R3. Support for this hypothesis was provided recently by results from mouse genetics where human T1R2 transgenic mice, generated on the T1R2 knockout background, displayed sweetener taste preferences similar to those of humans (9). However, the functional role of T1R3 and the overall structure/function relationship of T1R taste receptors remain largely unknown.

Another intriguing observation of the T1R2/T1R3 receptor is the structural diversity of its ligands. This receptor is able to recognize every sweetener tested, including carbohydrates, sweet amino acids and derivatives, sweet proteins, and synthetic sweeteners (7). Additionally, the receptor exhibits stereo-selectivity for certain molecules. For example, it responds to D-tryptophan but not L-tryptophan (7), which is in correlation with the sensory data. It is still a puzzle as to how this single receptor can recognize such a large collection of diverse chemical structures.

In this study, we report the development of an HTS assay using the human sweet taste receptor. The *in vitro* receptor activity is highly correlated with human taste behavior. Furthermore, we utilized the species differences in T1R ligand specificity to demonstrate that the sweet taste receptor indeed functions as a heteromeric complex, and that there are likely more than one ligand binding sites on the receptor (11).

A High Throughput Screening Assay for the Human Sweet Receptor T1R2/T1R3

We have developed a very sensitive cell-based assay platform for the detection of human sweet receptor modulators. Our assay was developed using the phospholipase C (PLC) effector pathway, where the human sweet receptor dimmer T1R2/R3 couples to Gα15 and PLC activation (7) causing a net increase

in calcium mobilization inside the cells. The sweetener-induced calcium mobilization can readily be monitored using calcium sensitive dyes such as Fluo3 and a Fluorometric Imaging Plate Reader (FLIPR) (7) and Figure 1.
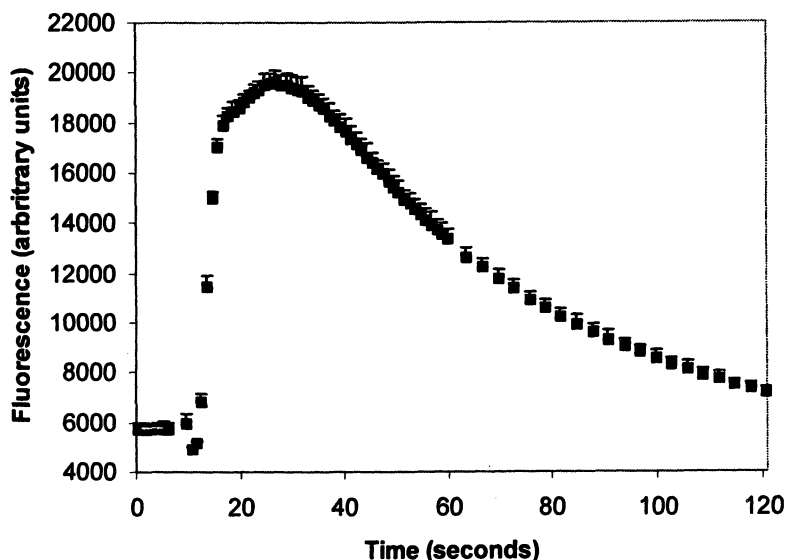
Figure 1. Aspartame induces an increase in intracellular calcium concentration within HEK293 cells stably expressing the human sweet receptor (T1R2/R3) and Gα15 on FLIPR^{TM}.

In Vitro Receptor Activity

We undertook the characterization of 44 different molecules reported to taste sweet or modulate sweet taste in humans. We performed dose-response analysis of each individual molecule in our Gα15 cell based FLIPR assay. A summary of the result can be found in Table I. Sweeteners fall into different classes based on potency and efficacy. The more potent sweetener class (with EC50s < 1 mM) contains the commonly known artificial sweeteners such as neotame, aspartame, alitame, saccharin, cyclamate, the sweet proteins as well as the terpenoids, terpenoid glycosides and dihydrochalcone (NHDC) (representative dose-response curves of some of these members are shown in Figure 2). Only one of these molecules, 2, 4-dihydroxybenzoic acid (DHB), is a partial agonist on the sweet receptor, inducing 45% of maximal receptor activity. The remaining molecules are as efficacious as D-Fructose at activating the sweet receptor. The less potent sweeteners class (EC50s > 1 mM) contains natural

carbohydrate sweeteners as well as D- and L- amino acids known to taste sweet to humans (Table I). D- Alanine and Glycine behave as partial agonists on the sweet receptor (Figure 2, Table I). EC50s for maltose, D-glucose, D-sorbitol, D-(+)-galactose, α-lactose and L-glucose could not be determined due to the low potency of these sweeteners in the assay. However, these molecules tested at a concentration of 100mM consistently induced activation levels corresponding to ~20%-50% of maximal receptor activity.

Table I. Summary of potencies and efficacies of sweet receptor agonists

Sweeteners	EC50 (μM)	Emax	N
Guanidinoacetic acid	0.11 ± 0.06	134 ± 7.00	28
Neotame	0.40 ± 0.21	106 ± 7.94	4
P-4000	0.83 ± 0.40	139 ± 4.00	3
Perillartine	1.45 ± 1.03	104 ± 3.10	4
SC-45647	1.62 ± 0.20	106 ± 1.53	10
Super Aspartame	2.16 ± 0.26	106 ± 3.06	10
Monellin	3.37 ± 0.76	95.5 ± 1.64	11
Pine tree Rosin sweetener	3.62 ± 1.41	125 ± 7.14	4
NC-002740-01	3.94 ± 0.59	95.6 ± 1.52	12
Thaumatin	6.11 ± 3.06	101 ± 8.96	19
NHDC	10.2 ± 4.01	128 ± 3.61	20
CC-00100	12.7 ± 1.49	97.7 ± 3.79	10
Mogroside V	13.4 ± 2.97	117 ± 6.93	5
NC-00420	13.6 ± 1.65	99.7 ± 1.53	10
Dulcin	13.8 ± 2.43	97.7 ± 3.79	5
Alitame	13.8 ± 0.92	99.7 ± 1.64	10
Rebaudioside A	14.4 ± 2.53	100 ± 3.49	14
NC-00576	20.2 ± 1.48	103 ± 1.00	10
Cyanosuosan	21.7 ± 0.58	102 ± 2.08	3
Steviocide	23.1 ± 4.29	105 ± 12.3	21
Sucralose	26.0 ± 4.00	106 ± 3.46	3
Glycyrrhizic Acid	29.3 ± 7.15	97.7 ± 2.52	30
CMB[a]	29.3 ± 12.9	87.7 ± 4.73	3
5-(3-hydroxyphenoxy)tetrazole	37.0 ± 3.00	128 ± 8.50	3
Saccharin	43.3 ± 11.2	105 ± 4.08	28
Aspartame	123 ± 33.7	117 ± 5.23	24
AcesulfameK	125 ± 21.4	102 ± 2.00	16
Cyclamate	490 ± 133	118 ± 5.12	13
D-Tryptophan	523 ± 115	112 ± 8.96	11
DHB[b]	957 ± 208	45.0 ± 8.55	12

Table I. *Continued*

Sweeteners	EC50 (mM)	Emax	N
L-Hydroxyproline	9.60 ± 1.14	77.4 ± 15.7	5
Sucrose	22.4 ± 5.36	93.4 ± 3.46	24
D-Alanine	37.6 ± 15.2	53.2 ± 8.30	7
D-fructose	44.6 ± 10.8	100	68
Glycine	59.9 ± 30.4	33.0 ± 9.38	9
D(-)tagatose	73.4 ± 33.3	80.6 ± 21.6	9
Xylitol	81.4 ± 14.6	111 ± 4.41	5

Sweeteners	% activity	Emax	N
Maltose	54.0 ± 1.73	--	5
α-D-glucose	40.4 ± 2.88	--	5
α-lactose	34.0 ± 2.35	--	5
D(+)galactose	27.3 ± 3.10	--	4
D-sorbitol	26.8 ± 2.95	--	5
L-glucose	20.4 ± 2.70	--	5

[a] 2-carboxy-4'-methoxybenzophenone
[b] 2, 4-dihydroxybenzoic acid
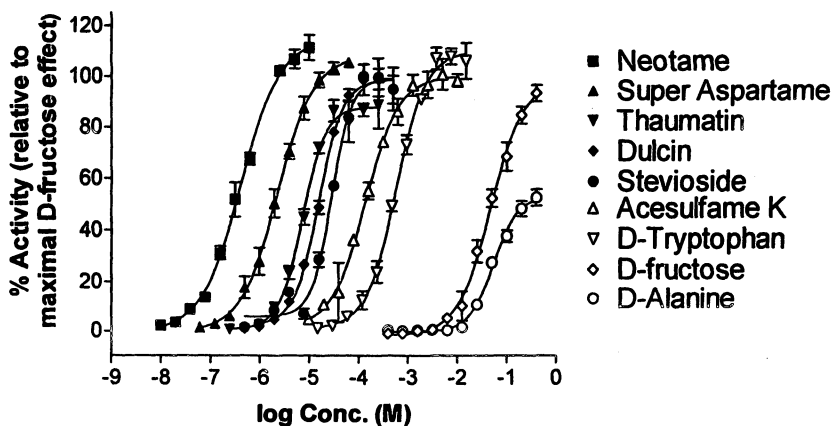
Figure 2. Dose-response analysis of different sweeteners in HEK293 cells stably expressing the human sweet receptor (T1R2/R3) and Gα15 on FLIPR^TM (Manuscript in preparation).

Correlation with taste data

A key question regarding the assay is whether the relative potencies of the different sweeteners characterized in the assay system correlates with their relative sweetness intensities in taste tests. The sweetness intensity of different sweeteners is often reported as sweetness relative to sucrose on a weight-by-weight basis (Pw) (12). This method, although still popular in the sensory field, is flawed by a lack of consideration for the significant variation of molecular weight that can be observed between different sweeteners. To correct for this limitation, Morini et al. (12) transformed Pw values of several sweeteners into molar relative sweetness (MRS) values. Table II shows the MRS values for 10 sweeteners and their corresponding relative potency to sucrose in our assay. The order of potency is almost identical between the two sets of data, with the only difference being that saccharin and aspartame have roughly the same MRS values while saccharin is about three fold more potent than aspartame in our assay. The most noticeable difference between the MRS and relative potency values were observed with neotame, P-4000 and perillartine. Although the order of potency for these sweeteners is the same in the two 2 sets of data, their absolute relative potency to sucrose is 5 to 15 fold greater than their corresponding MRS values. For the remaining sweeteners (guanidinoacetic acid, alitame, sucralose, saccharin, aspartame, cyclamate and D-tryptophan) the MRS and the relative potency values in the assay vary only by ~three fold at the most. The calculated correlation coefficient between the two data sets is 0.9625 (Figure 3).

Mapping of Functional Domains

Having demonstrated that a number of agonists exhibit differential activities between the human and rodent sweet taste receptors, we initiated experiments to begin to map where these agonists bind. The human and rat sweet receptors expressed in HEK293 cells functionally couple to a variety of G proteins including $G_{\alpha15/i1}$, a chimera of $G_{\alpha15}$ with the C-terminal tail of $G_{\alpha i1}$ (7). The human but not rat T1R2/T1R3 are selectively activated by a group of sweeteners, including aspartame, neotame, and cyclamate (7). These data are consistent to behavioral/sensory data with each of these sweeteners. To map the key domains, we generated chimeric T1Rs between human and rat receptors. Each T1R chimera consists of two halves, an N-terminal extracellular domain and the C-terminal transmembrane and intracellular domain from different species. For example, a chimeric T1R2, termed T1R2H-R, is composed of the N-terminus of human T1R2 linked to the rat T1R2 C-terminal sequence. Combinations of

Table II. Calculated Relative Potency of Different Sweeteners to Sucrose in the Assay and Their Relative Sweetness to Sucrose in Human Taste Tests

Sweetener	Potency in the assay relative to sucrose (A)	MRS values (B)	Log A	Log B
Guanidinoacetic acid	199815	168000	5.301	5.225
Neotame	55602	11057	4.745	4.044
P-4000	26856	2293	4.429	3.360
Perillartine	15434	996	4.188	2.998
Alitame	1622	1937	3.210	3.287
Sucralose	861	755	2.935	2.878
Saccharin	517	161	2.713	2.207
Aspartame	182	172	2.259	2.236
Cyclamate	46	26	1.659	1.415
D-Tryptophan	43	21	1.631	1.322
Sucrose	1	1	0.000	0.000

receptors were expressed in HEK293 cells with $G_{\alpha15/i1}$ and the cells were examined for their responses to aspartame, neotame and cyclamate (Fig. 4).

Aspartame and Neotame

The chimera in which the N-terminal domain of human T1R2 was replaced by the homologous rat domain failed to respond to aspartame or neotame, when co-expressed with human T1R3. This result was consistent with the model in which these sweeteners required the extracellular domain of human T1R2. The reverse chimera, in which the rat N-terminal domain of T1R2 was replaced with the human and co-expressed with rat T1R3, exhibited a gain of function and now responded effectively to aspartame and neotame (Fig. 4B). These data suggests that the same domain of human T1R2 is also sufficient (in the context of sweet taste receptors) to enable activation by those two sweeteners. Further support for this model comes from experiments in which a mouse strain engineered to expressed human T1R2 responded to aspartame (9).
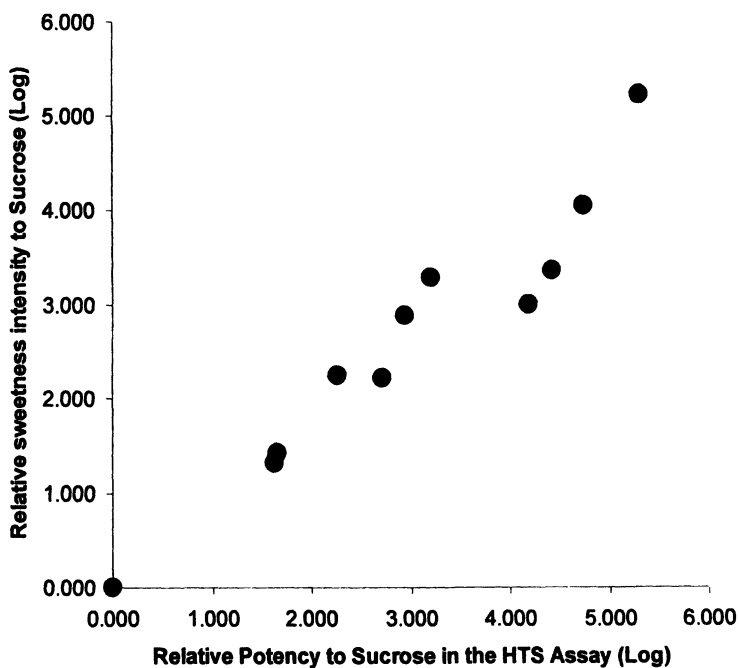
Figure 3. Correlation analysis between the relative potency of different sweeteners to sucrose in the HTS assay and their relative sweetness to sucrose in human taste tests (Manuscript in preparation).

Cyclamate

Cyclamate is another sweetener that is sweet to humans but not to rodents. Consistent with these findings, only the human sweet receptor responds to cyclamate in vitro. In contrast to the aspartame results, chimeric receptors composed of either the N- or C-terminal portion of human T1R2 and co-expressed with rat T1R3 failed to respond to cyclamate. Surprisingly, a chimeric receptor composed of the N-terminal domain of rat T1R3 and the C-terminal domain of human T1R3 when co-expressed with rat T1R2 was activated by cyclamate (Fig. 4C). It has been previously known that binding sites for certain positive and negative allosteric modulators of other family C GPCRs has been mapped to their transmembrane domains (13). Although the response to cyclamate was not tested in the mice engineered by Zhao et al, based on our data hT1R3 but not hT1R2 would be expected to convey cyclamate preference to mouse taste.
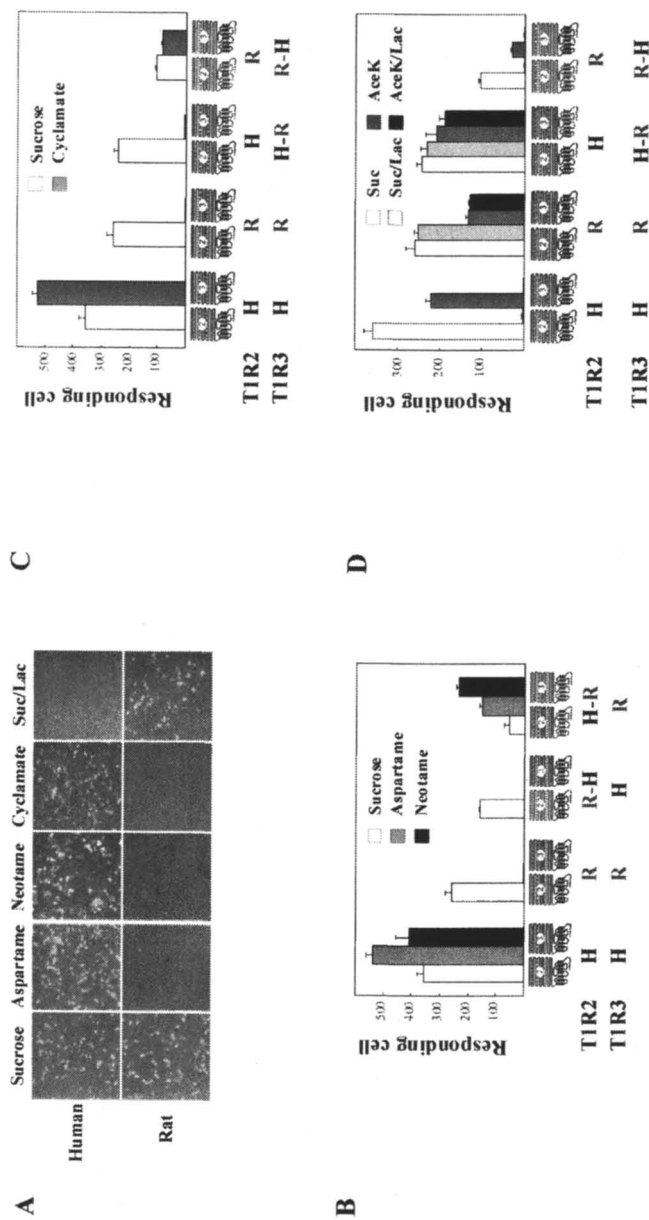
Figure 4. Sweeteners map to different domains/subunits of the human sweet taste receptor. (A) Differences between the activities of human and rat sweet taste receptors. (B) Aspartame and neotame were mapped to N-terminal extracellular domain of human T1R2. (C) Cyclamate was mapped to the C-terminal transmembrane domain of human T1R3. (D) Lactisole was mapped to the transmembrane domain of human T1R3. (Reproduced from reference 11. Copyright 2004 The National Academy of Sciences of the USA.)

Lactisole

Lactisole, an aralkyl carboyxlic acid, specifically inhibits the activation of the human taste receptor by sweeteners in vitro and blocks sweet taste in humans. It has no effect on the rodent sweet receptor or sweet taste response. The effects of lactisole on the human and rat receptors in our assay system are shown in Fig. 4A.

Using T1R chimeras we conducted mapping experiments and demonstrated that lactisole acts similar to cyclamate through the human T1R3 C-terminal domain to inhibit the receptor's response to sucrose and acesulfame K (Fig. 4D). This result further demonstrates the importance of T1R3 C-terminal domain in the sweet taste receptor function. To completely probe this response, we examined all 16 possible chimera combinations with lactisole. The results from are consistent with our model. Our results were later confirmed by other research groups (14-16).

Mutants with Ligand-selective Effect

To more precisely identify the amino acids essential for recognition of aspartame, neotame and cyclamate we generated receptor variants containing site-specific amino acid substitutions. Our hypothesis was that substitutions in the N-terminal domain of T1R2 that affected aspartame and neotame would not affect activation by cyclamate and substitutions in the T1R3 transmembrane domain that eliminated activation by cyclamate would not affect aspartame or neotame. Critical amino acid residues in the T1R2 N-terminal domain were selected by aligning the sequences of T1R2 with mGluR1 (Fig. 5A). Among the eight residues that are crucial in ligand binding in mGluR1 (17), three are conserved in human T1R2 (S144, Y218, and E302). Each of the three residues was changed to alanine and the resulting receptors were tested for their response to different sweeteners in HEK293 cells. Substitution of Y218 to A abolished the responses to all sweeteners tested including cyclamate (data not shown). Y218 might be important for the overall conformation of the sweet taste receptor. However, it is also possible that that Y218A failed to express or target to the cell surface, considering that equivalent substitutions in mGluR1 (18) and mGluR8 (19) led to partially functional receptors. In contrast, the variants containing S144A and E302A selectively reduced the response to aspartame and neotame but were still activated by cyclamate. Cell lines stably expressing the S144A and E302A hT1R2 variants and coexpressed with wild type hT1R3 and $G_{\alpha15}$ did not respond to aspartame or neotame at the physiologically relevant concentrations, but did respond to cyclamate (Fig. 5B). Cells stably expressing receptors are generally more sensitive than those produce by transient expression. However, in this case, the stable cells still failed to respond to aspartame or neotame.

The cyclamate-binding site was further dissected by creating substitutions within the three extracellular loops of the T1R3 C-terminal domain. Alignment of human and rodent T1R3s revealed multiple amino acid differences in the three extracellular loops. Replacing the human T1R3 second (loop-2) or third (loop-3) extracellular loops with the homologous rat sequences abolished the cyclamate response without affecting the sucrose or aspartame responses. In contrast, replacing the first extracellular (loop 1) had no obvious effect on the response to cyclamate, suggesting important roles for extracellular loop-2 and loop-3 in recognizing cyclamate. Interestingly, none of these loop replacements affected ability of lactisole to inhibit the receptor, suggesting a different binding mechanism. In summary, amino acid substitutions in T1R2 or T1R3 result in selective interference of activities induced by different sweeteners, consistent with the chimeric receptor results.

Taken together, the above results demonstrate that human sweet taste receptor functions as a heteromeric complex of T1R2 and T1R3. Both subunits are required for recognizing different sweeteners, and our data indicate the existence of multiple binding pockets on the receptor for different classes of agonists. The presence of multiple ligand-binding sites provides a possible explanation for the structural diversity of sweeteners.

G protein Interaction Site

Studies in HEK293 cells identified a different in G protein-coupling efficiency between the human and rat sweet taste receptors. For example, both human and rat receptors respond to sweeteners when co-expressed with $G_{\alpha15/i1}$ but only the human receptor efficiently responds when co-expressed with $G_{\alpha15}$ (7) (Fig. 6A). This differential response allowed us to map the receptor-G protein interactions using the chimeric receptors described above.

Replacing the C-terminus of human T1R2 with the corresponding rat sequence abolished coupling. In contrast, substitution of the rat T1R2 C-terminal half with human sequence enabled coupling to $G_{\alpha15}$ and respond to sucrose and acesulfame K (Fig. 6). These results suggest residues in T1R2 but not T1R3 are critical for $G_{\alpha15}$-coupling. Substituting the T1R3 C-terminal domain had no effect on $G_{\alpha15}$-mediated coupling (Fig. 6B). This observation demonstrates the important role of T1R2 in G protein-coupling in our functional expression system.

Gustducin (20) has been proposed to be an endogenous G protein for the sweet taste receptor, and we speculate that T1R2 should be the subunit responsible for coupling in taste cells. $GABA_BR$ is the other example of heteromeric family C GPCR, whereas one subunit ($GABA_BR1$) is responsible for ligand-binding, and the other ($GABA_BR2$) for G protein coupling (21-24). The sweet taste receptor is different from $GABA_BR$ in that the same subunit is required for both ligand recognition and G-protein coupling.
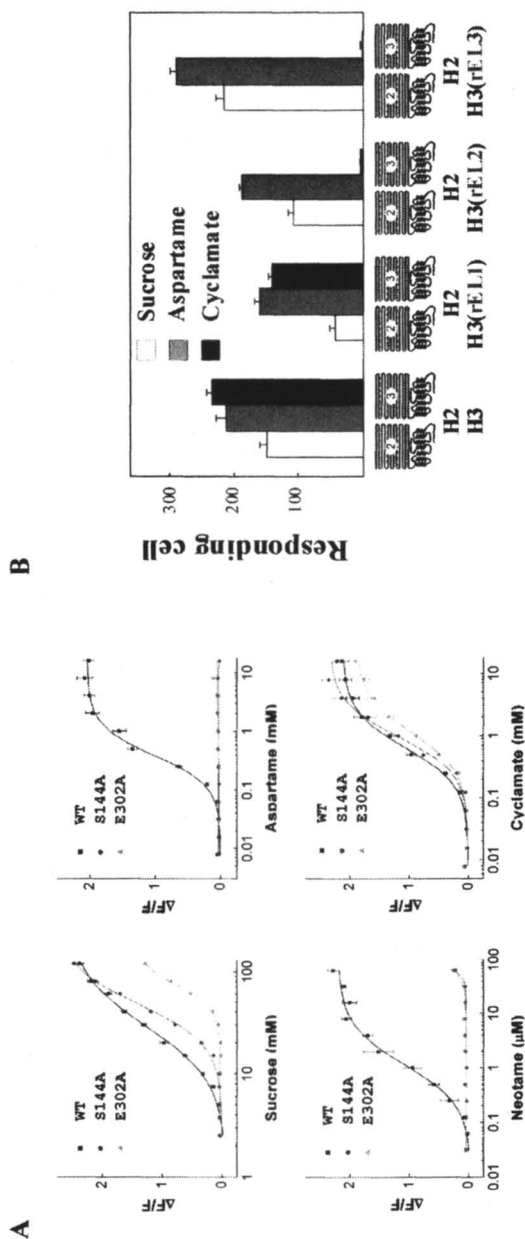
Figure 5. Mutants with sweetener-selective effect. (A) Two point mutations in the human T1R2 N-terminal extracellular domain abolish response to aspartame and neotame without affecting cyclamate. (B) Mutations in the extracellular loop of hT1R3 abolish response to cyclamate without affecting aspartame. (Reproduced from reference 11. Copyright 2004 The National Academy of Sciences of the USA.)
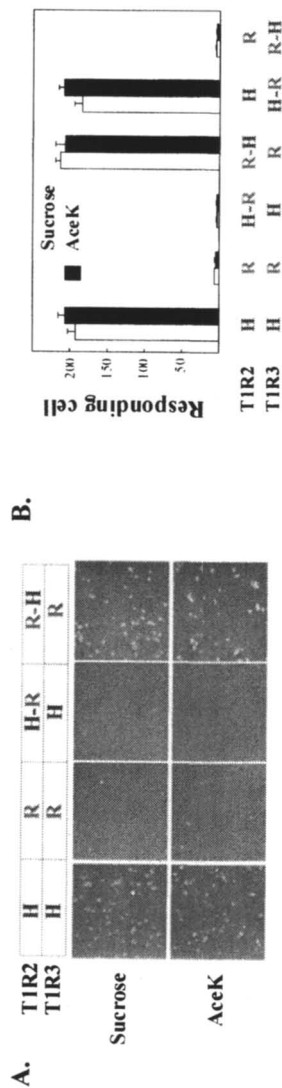
Figure 6. G protein is coupled to sweet receptor through the T1R2 TM domain. (A) Responses of human, rat and chimeric sweet taste receptors to sucrose and AceK. (B) G protein coupling is mapped to human T1R2. (Reproduced from reference 11. Copyright 2004 The National Academy of Sciences of the USA.)

Cyclamate Effect on Umami Receptor

Umami is characterized as the savory taste of monosodium glutamate (MSG). The umami receptor was shown to be composed of T1R1 and T1R3, thus sharing a subunit in common with the sweet receptor (7, 25). Following our finding that cyclamate likely binds to T1R3, we predicted that cyclamate would also modulate the activity of the human umami receptor. Initial experiments showed that cyclamate was not an agonist of the human umami receptor. However, cyclamate enhanced the response of the umami receptor to L-glutamate (Fig. 7A). This was demonstrated by a left shift in the dose response to glutamate (Fig. 7B). The effect of cyclamate was shown to require the human umami receptor since cyclamate had no effect on the carbachol response of the endogenous muscarinic acetylcholine receptor (Fig. 7A). It is noteworthy that cyclamate has comparable EC_{50}s for the sweet taste receptor (Fig. 7A) and umami taste receptor. Cyclamate shifted the dose-response curves for L-glutamate by ~2 fold either in the presence or absence of the glutamate enhancer inosine monophosphate (IMP). These results suggest IMP and cyclamate are acting via different binding sites. We speculate that IMP binds to T1R1, since it has no effect on the sweet taste receptor (7). Other sweeteners, including sucrose, aspartame, saccharin, and D-tryptophan, had no effect on the human T1R1/T1R3 activities (not shown). Due to the intense sweet taste of cyclamate, the effect of cyclamate on MSG taste is difficult to determine.

Conclusion

In summary, we developed a sweet receptor-based HTS assay and a series of human-rat chimeric receptors to probe the response of the sweet receptor to a variety of sweet taste modulators. Our work showed that the response of the receptor in the in vitro assay correlates with human sweet taste. Through functional mapping, we showed that 1) both T1R2 and T1R3 are required for function, 2) aspartame and neotame require the N-terminal extracellular domain of T1R2, 3) G protein coupling requires the C-terminal half of T1R2, and 4) cyclamate and lactisole require the transmembrane domain of T1R3. These findings demonstrated for the first time the different functional roles of T1R subunits in a heteromeric complex and the presence of multiple sweetener interaction sites on the sweet taste receptor. Because T1R3 is the common subunit in the sweet and the umami taste receptors, we predicted and confirmed the effect of cyclamate and lactisole on the umami taste receptor.

Based on these results, we proposed a model (Fig. 8) for the interaction of ligands with the sweet and umami taste receptors. Although the rat and human receptors both respond to carbohydrate sweeteners, we speculated that carbohydrate sweeteners, including sucrose and fructose, bind to the N-terminal
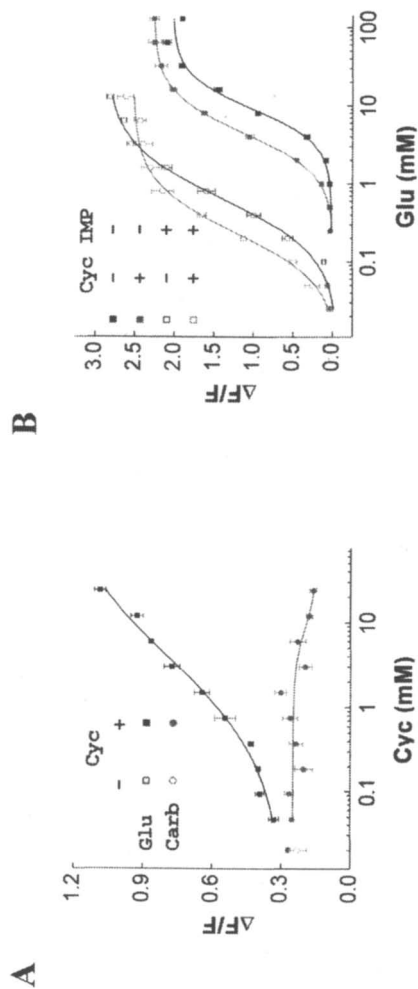
Figure 7. Effect of cyclamate on umami receptor. (A) The responses of human T1R1/T1R3 stable cell line to threshold level of L-glutamate and endogenous M2 receptor agonist carbachol in the absence and presence of various concentrations of cyclamate. (B) Dose-responses of the human T1R1/T1R3 stable cell line with or without 0.2 mM IMP in the absence and presence of cyclamate. (Reproduced from reference 11. Copyright 2004 The National Academy of Sciences of the USA.)
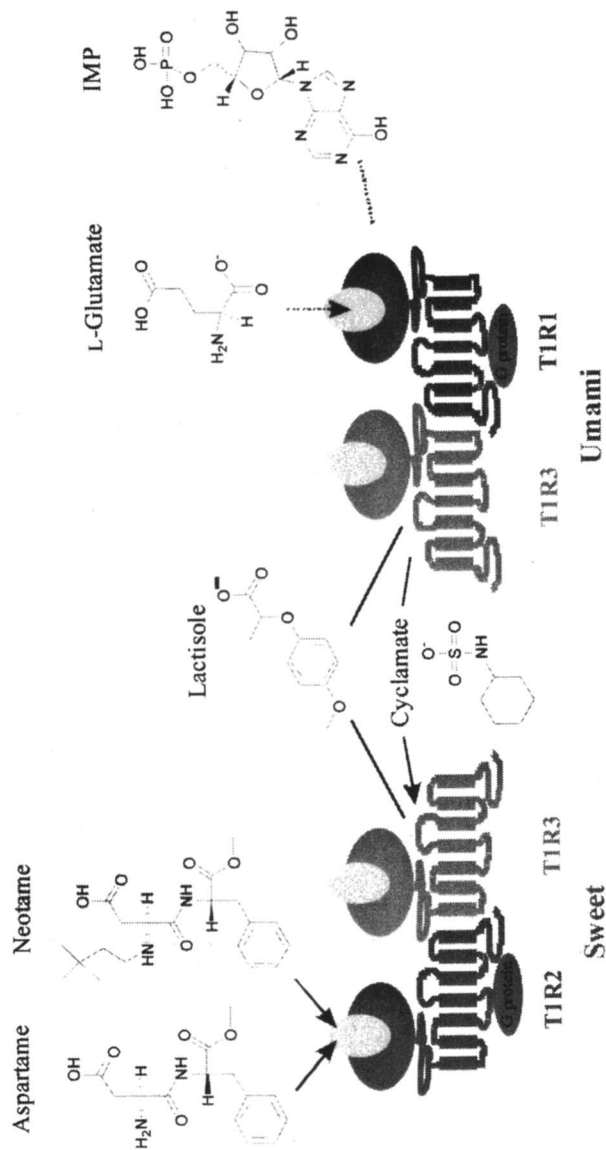
Figure 8. Different functional domains of the sweet taste receptor. (Reproduced from reference 11. Copyright 2004 The National Academy of Sciences of the USA.)

384

domain of T1R2. In addition to the N-terminal domain of T1R2 and the transmembrane domain of T1R3, other regions of the receptor, such as the transmembrane domain of T1R2, may also participate in ligand binding. The umami taste receptor is most likely to function in a similar fashion. Since neither glutamate nor IMP affects the sweet taste receptor we speculate that these umami modulators bind to T1R1. Furthermore, the coupling of the umami receptor with the G protein is mediated via the transmembrane domain of T1R1. A more detailed understanding of these receptors will emerge via additional mutagenesis, homology modeling, and x-ray crystallography experiments.

References

1. Hoon, M. A., Adler, E., Lindemeier, J., Battey, J. F., Ryba, N. J., & Zuker, C. S. (1999) *Cell* **96**, 541-551.
2. Bachmanov, A. A., Li, X., Reed, D. R., Ohmen, J. D., Li, S., Chen, Z., Tordoff, M. G., de Jong, P. J., Wu, C., West, D. B., *et al.* (2001) *Chem Senses.* **26**, 925-933.
3. Montmayeur, J. P., Liberles, S. D., Matsunami, H., & Buck, L. B. (2001) *Nat Neurosci* **4**, 492-498.
4. Max, M., Shanker, Y. G., Huang, L., Rong, M., Liu, Z., Campagne, F., Weinstein, H., Damak, S., & Margolskee, R. F. (2001) *Nat Genet* **28**, 58-63.
5. Kitagawa, M., Kusakabe, Y., Miura, H., Ninomiya, Y., & Hino, A. (2001) *Biochem Biophys Res Commun.* **283**, 236-242.
6. Nelson, G., Hoon, M. A., Chandrashekar, J., Zhang, Y., Ryba, N. J., & Zuker, C. S. (2001) *Cell* **106**, 381-390.
7. Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M., & Adler, E. (2002) *Proc Natl Acad Sci U S A* **99**, 4692-4696.
8. Damak, S., Rong, M., Yasumatsu, K., Kokrashvili, Z., Varadarajan, V., Zou, S., Jiang, P., Ninomiya, Y., & Margolskee, R. F. (2003) *Science.* **301**, 850-853. Epub 2003 Jul 2017.
9. Zhao, G. Q., Zhang, Y., Hoon, M. A., Chandrashekar, J., Erlenbach, I., Ryba, N. J., & Zuker, C. S. (2003) *Cell* **115**, 255-266.
10. Pin, J. P., Galvez, T., & Prezeau, L. (2003) *Pharmacol Ther.* **98**, 325-354.
11. Xu, H., Staszewski, L., Tang, H., Adler, E., Zoller, M., & Li, X. (2004) *Proc Natl Acad Sci U S A.* **101**, 14258-14263. Epub 12004 Sep 14257.
12. Bassoli, A., Drew, M. G., Merlini, L., & Morini, G. (2002) *J Med Chem* **45**, 4402-4409.
13. Gasparini, F., Kuhn, R., & Pin, J. P. (2002) *Curr Opin Pharmacol.* **2**, 43-49.
14. Jiang P, Cui M, Zhao B, Snyder LA, Benard LM, Osman R, Max M, Margolskee RF. (2005) *J Biol Chem.* **280**, 34296-34305.
15. Jiang P, Cui M, Zhao B, Liu Z, Snyder LA, Benard LM, Osman R, Margolskee RF, Max M. (2005) *J Biol Chem.* **280**, 15238-15246.

16. Winnig M, Bufe B, Meyerhof W. (2005) *BMC Neurosci.* **6**, 22.
17. Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., & Morikawa, K. (2000) *Nature* **407**, 971-977.
18. Sato, T., Shimada, Y., Nagasawa, N., Nakanishi, S., & Jingami, H. (2003) *J Biol Chem.* **278**, 4314-4321. Epub 2002 Nov 4319.
19. Bessis, A. S., Rondard, P., Gaven, F., Brabet, I., Triballeau, N., Prezeau, L., Acher, F., & Pin, J. P. (2002) *Proc Natl Acad Sci U S A.* **99**, 11097-11102. Epub 12002 Jul 11031.
20. Wong, G. T., Gannon, K. S., & Margolskee, R. F. (1996) *Nature* **381**, 796-800.
21. Kniazeff, J., Galvez, T., Labesse, G., & Pin, J. P. *J Neurosci 2002 Sep 1*; *22(17)*, 7352-7361.
22. Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prezeau, L., & Pin, J. P. *EMBO J 2001 May 1;20(9):2152-9*.
23. Margeta-Mitrovic, M., Jan, Y. N., & Jan, L. Y. (2001) *Proc Natl Acad Sci U S A* **98**, 14643-14648.
24. Margeta-Mitrovic, M., Jan, Y. N., & Jan, L. Y. (2001) *Proc Natl Acad Sci U S A* **98**, 14649-14654.
25. Nelson, G., Chandrashekar, J., Hoon, M. A., Feng, L., Zhao, G., Ryba, N. J., & Zuker, C. S. (2002) *Nature* **416**, 199-202.

Chapter 24

Development of Transient Receptor Potential Melanostatin 5 Modulators for Sweetness Enhancement

R. W. Bryant[1], K. S. Atwal[1], I. Bakaj[1], M. T. Buber[1], S. Carlucci[1], R. Cerne[1], R. Cortés[1], H. R. Devantier[1], C. J. Hendrix[1], S. P. Lee[1], R. K. Palmer[1], C. Wilson[1], Q. Yang[2], and F. R. Salemme[1]

[1]Redpoint Bio Corporation (formerly Linguagen Corporation), 2005 Eastpark Boulevard, Cranbury, NJ 08512–3515
[2]Current address: Merck & Company, Inc., 126 East Lincoln Avenue, Rahway, NJ 07065

The discovery of a family of G protein-coupled receptors (GPCRs) that can bind sweet, bitter, or umami tastants has established that these taste sensations are mediated by classical signal transduction cascades. Proteins downstream from the tastant binding GPCRs, such as the G_α protein gustducin, phospholipase $C_{\beta 2}$ ($PLC_{\beta 2}$) and the Transient Receptor Potential Melanostatin 5 (TRPM5) ion channel, have been identified as critical components in the transduction of these taste sensations. Using modern pharmaceutical discovery technology, we have discovered prototype compounds that specifically enhance TRPM5 activity in the presence of low levels of surrogate tastants. Enhancers operating through this novel mechanism could potentially allow for full taste sensations to be experienced from reduced concentrations of nutritive sweeteners.

Excess sugar in food has been identified as a key contributor to important public health problems facing developed countries worldwide. These include the rising trend in obesity (1) and its associated increased incidence of diabetes and other diseases (2), as well as oral health concerns (3). As a result, there is a growing interest, both from the general public and from the government, to develop cost-effective methods of reducing the amount of caloric sweetener that is added to processed foods and beverages while retaining their palatability and nutritional value. The food industry has made a large research and development investment that has led to the production of acceptable low-calorie sweeteners. Indeed, a large number of synthetic sweeteners have been identified and several have been successfully commercialized (4). Although many sugar substitutes are currently available, no one sweetener is thus far ideal. In particular, many have off-tastes and do not accurately replicate the temporal sensory experience of real sugar (5-8).

An alternative to artificial sweeteners is the identification of non-nutritive "sweetness enhancers" that can be used in concert with reduced quantities of nutritive sweeteners to enhance the perception of sweet taste. The ideal sweetness enhancer would have no taste of its own, but would increase the natural sweetness of sugar while preserving the temporal characteristics of the sugar taste. Although an enhancer providing a 1.5x reduction in the amount of sugar required for a specific sweetening application could be commercially useful, a larger reduction factor of 2-5x is more desirable from the public health perspective.

Research is currently in progress to identify molecules that could function as sweetness enhancers. One strategy involves identification of positive allosteric modulators of the G-protein coupled receptors (GPCRs) that bind sweet tastants (9). We describe an alternative approach involving identification of positive modulators of taste signaling components acting downstream of taste GPCR receptors. We focused initially on finding positive modulators of the Transient Receptor Potential Melanostatin 5 (TRPM5) ion channel, and have identified prototype compounds which selectively and strongly enhance the response of TRPM5 to low levels of surrogate tastants.

Signal transduction pathways involved in TRPM5 activation during sweet taste reception

The signaling cascade downstream of GPCR activation provides several targets for discovery of taste modulation, namely GPCRs, signal transduction proteins, and ion channels. The first step in the proposed pathway that ultimately leads to the perception of taste is the binding of a tastant to the extracellular domain of a GPCR of the T1R family (10, 11) (Figure 1). This

388
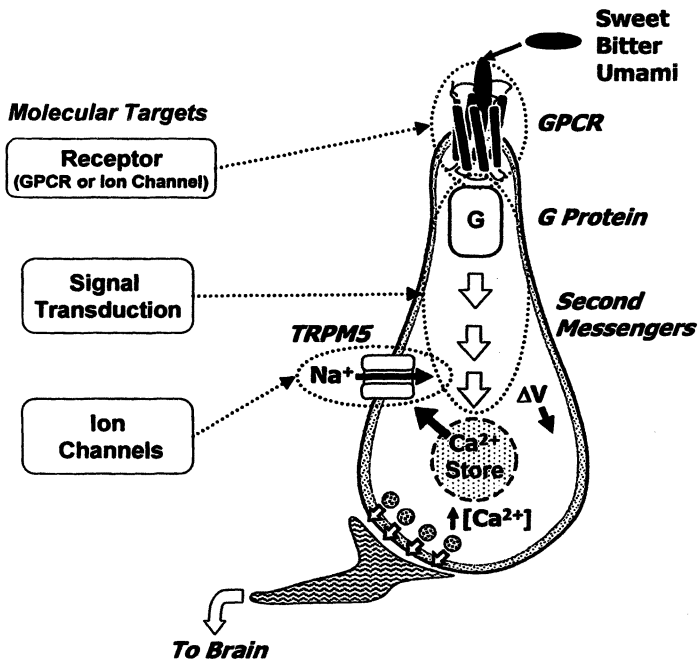
Figure 1. Potential targets for small molecule discovery in taste cell sensing.

family includes different subunits that form heteromeric receptors which mediate individual taste modalities. For example, sweet taste in mice and humans is transduced by the T1R2:T1R3 receptor (12, 13), while the umami taste is transduced by the T1R1:T1R3 receptor (13, 14). Approximately 25 cognate bitter receptors of the T2Rn class also exist (15-17). Sweet tastant binding to the T1R2:T1R3 receptor leads to the dissociation of heterotrimeric G proteins into their α and $\beta\gamma$ subunits (reviewed in (11)), although it is not completely clear yet which specific α and $\beta\gamma$ subunits are involved (18-23). Following a classical signal transduction cascade, the $\beta\gamma$ subunit activates $PLC_{\beta2}$ (24). This leads to inositol triphosphate (IP$_3$) generation, which then results in Ca^{2+} release from intracellular stores. This Ca^{2+} release is presumably mediated by the type III IP$_3$ receptor (IP$_3$R$_3$) (25). The resultant increase in intracellular calcium concentration ([Ca^{2+}]$_i$) causes the TRPM5 channel to open (26). TRPM5 activation depolarizes the taste cell which contributes to neurotransmitter release, thus transmitting tastant-mediated taste bud activation to the gustatory nerves. While GPCRs have been very successful targets for pharmaceutical development (27, 28), discovery programs for direct modulators of signal transduction targets such as G-proteins and phospholipases have not been as productive. Ion channels, however, have been successful targets for discovery of modulators in many areas of pharmaceutical research (29-32).

TRPM5 is a monovalent cation channel gated by Ca^{2+} (26, 33, 34), temperature (35), voltage (34), pH (36), and PIP_2 (33). Expression patterns suggest, and knockout animal data validates, the importance of the TRPM5 protein in taste transduction. Northern blot experiments show that TRPM5 mRNA is selectively expressed in taste tissue compared to non-taste tissue (37), and immunohistological staining of circumvallate papilla tissue localizes the protein to taste buds (38). In addition, the TRPM5 protein co-localizes with $G_{\gamma13}$, α-gustducin, and $PLC_{\beta2}$ (38), strongly implicating it as a component of the taste signaling pathway in which these three signaling molecules are involved. Consistent with these observations, TRPM5 knockout mice have greatly reduced responses to sweet, bitter, and umami tastants (39, 40). For example, there is a six-fold reduction in the chorda tympani nerve response to 400 mM sucrose and a seven-fold reduction in the glossopharyngeal nerve response to 10 mM quinine in *Trpm5* null mice, compared to wild-type control animals (39). The identification of small molecule modulators of the TRPM5 ion channel could therefore result in compounds that amplify the signaling cascade downstream of the sweet GPCRs. Of course, a modulator of TRPM5 could in theory also alter bitter and umami tastes, based on the mechanism described in Figure 1. The effect of TRPM5 enhancers would therefore be context sensitive. We describe here the results of our discovery program, which has led to the identification of several TRPM5 enhancers that could represent a novel approach to sweetness enhancement particularly for beverages where sugar is the predominant taste.

Results

A Pharmaceutical-Based Discovery Process for Identification of Taste Modulators

We have implemented a process for the identification of novel taste modulators using technology originally developed for pharmaceutical discovery. The discovery process starts with data from knockout animals validating TRPM5 as a component of the sweet signal transduction pathway, as was previously discussed. To isolate TRPM5 as a target and develop an assay for the rapid screening of small molecules that would enhance TRPM5 activity, stably transfected cell lines were developed. The full-length hTRPM5 cDNA sequence was cloned from an intestinal cDNA library and subcloned into the pcDNA 3.2/v5-DEST vector (Invitrogen). This construct was transfected into human embryonic kidney-293 (HEK293) and Chinese hamster ovary (CHO) cell lines in order to generate stable cell lines expressing hTRPM5. Stably transfected clones were selected using Geneticin (Invitrogen). Control cell lines containing the empty vector and counter-screening cell lines expressing other TRP family receptors were developed following the same methods.

An engineered cell assay, using stably transfected TRPM5-expressing cell lines, was established. This assay incorporated an automated high-throughput screening (HTS) process involving 384-well format liquid handling and a 384-well fluorescence imaging plate reader (FLIPR-Tetra®, Molecular Devices). Data were processed using an Excel-based data analysis package and Access-based data management system. Hits generated from the initial library screen were validated through chemical and pharmacologic means, incorporating LC/MS structure confirmation and specific assays to rule out blockade of calcium mobilization or artifactual membrane potential responses.

The specificity of validated hits were examined with a range of counter-screens using cell lines expressing other ion channels having potential pharmacological relevance or potentially acting as modulators of taste sensations. Compounds were additionally evaluated using whole-cell electrophysiological recordings of the TRPM5-expressing cells together with the engineered counter-screen cell lines. Validated hits with appropriate specificity and potency that emerged from this process define the starting point for a chemical optimization process aimed at the eventual identification of development compounds with all of the properties (e.g. potency, safety, process stability, manufacturing feasibility) required for a commercially viable product.

High-Throughput Screening Assay Development for TRPM5 Modulator Identification

The FLIPR screening assay took advantage of the native expression of purinergic metabotropic P2Y receptors in the parental HEK293 (41) or CHO (42) cell lines used for stable recombinant expression of TRPM5. In the assay, 10 µM adenosine 5'- triphosphate (ATP) acted as a surrogate tastant molecule to activate the GPCR-initiated signaling cascade that causes cytoplasmic $[Ca^{2+}]_i$ to rise, thus employing an endogenous mechanism to activate the TRPM5 ion channel and depolarize the cell. The TRPM5-expressing cell lines were plated in 384-well format plates one day prior to the experiment. The cells were loaded with membrane potential or calcium-sensitive dyes one hour before the assay. Changes in fluorescence intensity resulting from the addition of ATP were then recorded by the FLIPR-Tetra™ instrument (Figure 2A). ATP stimulation caused an equivalent transient increase in $[Ca^{2+}]_i$ in TRPM5- and vector-transfected HEK293 cells, as shown in Figure 2B, indicating that both cell lines are responsive to ATP. ATP stimulation, however, resulted in significant membrane depolarization only in cells expressing TRPM5. The ratio of maximum amplitude response for the TRPM5 transfectant was generally four-fold greater than the vector-transfected cells (Figure 2C). This assay provided very reliable data, reporting with Z'>0.5 for over 90% of plates.

A

B   **C**

★ 10 µM ATP addition
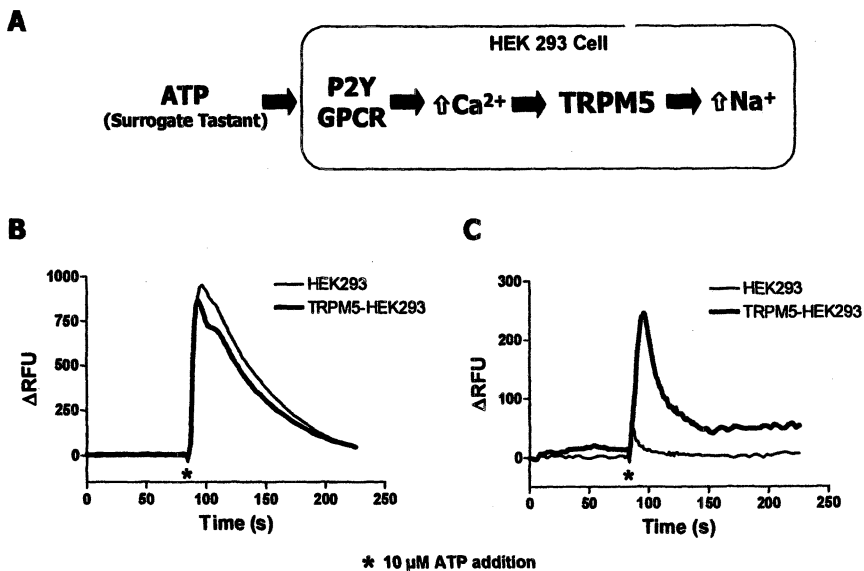
Figure 2. Basis of TRPM5 HTS membrane potential dye assay.
A. Activation strategy for TRPM5-expressing HEK293 cells utilizing
endogenous GPCRs. B. [Ca²⁺]ᵢ increase as reported by calcium dye in FLIPR
assay. C. Membrane potential change as reported by membrane potential dye
in FLIPR assay.

Following this development and optimization process, the FLIPR assay was used to screen a chemical library comprised of 83,580 diverse synthetic compounds. In this two-addition FLIPR assay, library compounds were applied at approximately 10 µM, followed by addition of 10 µM ATP three minutes later. Figure 3 shows the results of a portion of the compounds (59,238) from the initial screen as a frequency distribution of the percentage of inhibition of the TRPM5 response. A response of less than ±25% inhibition indicated an inactive compound. Potential TRPM5 blockers showed greater than 50% inhibition of TRPM5 activation, while enhancers showed -50% inhibition or better; i.e. they increased the activation of TRPM5 in the assay. At a compound concentration of approximately 10 µM, most compounds were shown to be inactive. The screen identified 601 compounds, or 0.7% of the compounds in the library, as potential TRPM5 blockers (<50% inhibition). In addition, 631 compounds that demonstrated greater than 69% enhancement (or -69% inhibition) of TRPM5 activity were identified and further evaluated as potential sweetness enhancers.
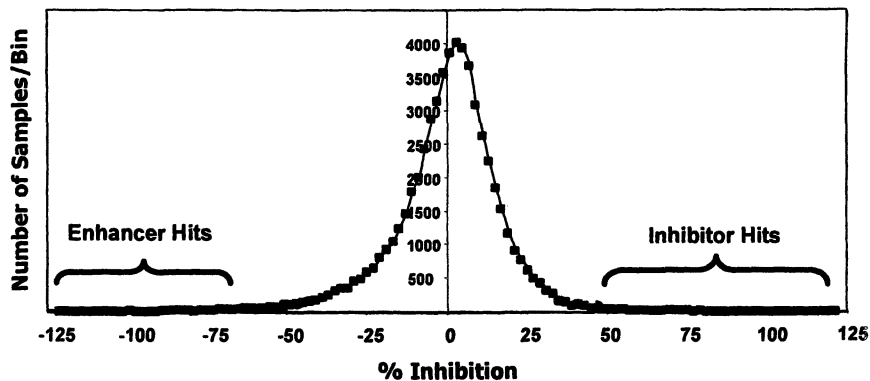
Figure 3. Frequency distribution of percentage of inhibition for 59,238 diverse synthetic compounds in an HTS assay for identification of TRPM5 modulators.

From these 631 potential enhancers, twenty compounds were identified in a secondary screen as having enhancement activity in TRPM5 channels expressed in both HEK and CHO parental lines. Of these twenty, a subset of compounds showed strong and selective enhancement at low surrogate tastant (ATP) levels, and were studied further.

Validation of Potential Hits Identified by the HTS Library Screen That Meet Sweetness Enhancer Criteria

Figure 4 illustrates the validation process for one potential TRPM5 enhancer, LGE20. In Figure 4A, increasing compound concentration resulted in an increase in the TRPM5 membrane potential response to 10 μM ATP in TRPM5-expressing HEK cells. Note that LGE20 did not produce a membrane potential response on its own prior to ATP application, indicating that the channel must be activated for the compound to have any effect. A similar dose-responsive potentiation of the ATP response by LGE20 was observed in CHO cells that were also engineered to express TRPM5 (Figure 4B), confirming the enhancement of the TRPM5 response. We also demonstrated that the compounds do not affect the purinergic P2Y receptor's ability to induce a rise in $[Ca^{2+}]_i$, as the calcium increase triggered by ATP was not altered by application of increasing concentration of LGE20 (Figure 4C). There was also no dose-responsive effect when HEK cells are depolarized with KCl, indicating the absence of a non-specific membrane potential dye effect (Figure 4D). To confirm that the compounds had no indirect metabolic effects on the cells, the cells were incubated overnight in the presence of 100 μM enhancer. This
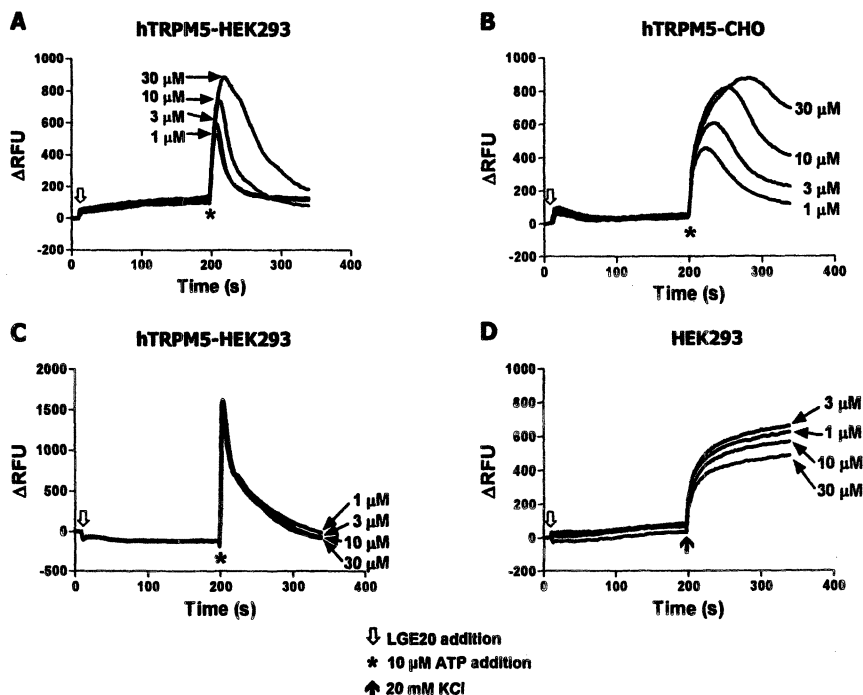
Figure 4. Validation of LG20, a specific TRPM5 enhancer.
Dose-responsive effect of LGE20 on:
A. ATP-stimulated membrane potential response in TRPM5-HEK293 cells;
B. ATP-stimulated membrane potential response in TRPM5-CHO cells;
C. ATP-stimulated intracellular calcium changes in TRPM5-HEK293 cells;
D. membrane potential response following KCl-induced depolarization
of naïve HEK cells.

extended treatment with compound did not result in any morphological or viability changes (data not shown).

A number of counterscreens were carried out to evaluate specificity of the initially identified compounds. Most notably, the prototype LG enhancers did not have any effect on TRPM4b, the most closely related channel to TRPM5 (43) of the several channels that were assessed.

Many of the prototype compounds identified in our screen display properties suggesting that TRPM5 enhancers could function as potent sweet enhancers. As discussed earlier, it would be most desirable to have compounds able to provide at least 2-3x enhancement, to allow a reduced amount of sweetener (e.g. 4% sucrose) taste like the full level of sweetener (approximating

A

B
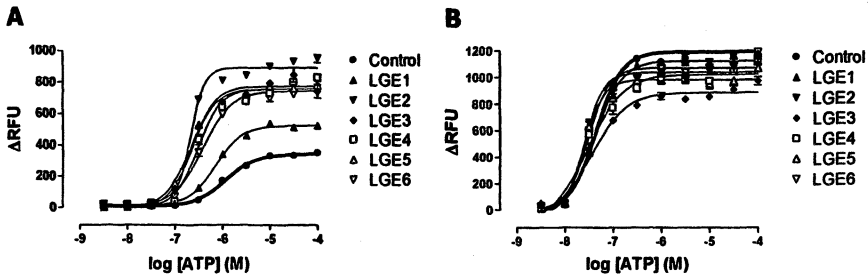
Figure 5. Effect of six TRPM5 enhancers on the ATP concentration-effect function in TRPM5-expressing HEK293 cells. The concentration of the enhancers was 30 μM. A. Membrane potential measurements in FLIPR assay. B. Intracellular calcium measurements in FLIPR assay.

12% sucrose) typically found, for example, in a full-sugar soft drink. The prototype TRPM5 enhancers that were identified increased the response of sub-maximal applications of ATP in the FLIPR assay by 5- to 10- fold, an effect particularly evident at very low concentrations of ATP (Figure 5A). This leftward and upward shift of the agonist dose response curves was very pronounced for 5 of the 6 enhancers tested at a concentration of 30 μM. These compounds did not significantly affect the $[Ca^{2+}]_i$ response to ATP (Figure 5B), indicating this enhancement was a direct TRPM5 effect and not a consequence of altering the triggering increase in $[Ca^{2+}]_i$.

The enhancement of TRPM5 activation by LG enhancer compounds was further validated by direct measurement of cell depolarization via whole-cell electrophysiological recordings. For these experiments, TRPM5-expressing HEK or CHO cells were acutely trypsinized, plated in bath solution on a coverslip in the recording chamber, and recorded in whole-cell voltage clamp mode. During the experiment, the cell being recorded was continuously perfused with bath solution or enhancer diluted in bath solution via a valve-controlled multi-barrel applicator. Solution exchange occurred within less than one second, allowing for the immediate application of the compound solution as well as for its immediate washout. The voltage protocol during the recording incorporated a ramp from a holding potential of -80mV to +80 mV (Figure 6, inset). The TRPM5 ion channel was activated in this assay by dialysis of Ca^{2+} from the pipette filling solution. This concentration of Ca^{2+} was optimized to produce a sub-threshold TRPM5 activation (Figure 6, 300 nM Ca^{2+}) that could be enhanced by LG compound application. No enhancement was observed when untransfected HEK cells were recorded during 10 μM LGE2 application (data not shown). The effect of the enhancer was absent in the zero $[Ca^{2+}]_i$ condition (Figure 6, left panel) and was most prominent at submaximal $[Ca^{2+}]_i$ threshold

Zero Ca⁺⁺   **300 nM Ca⁺⁺**   **30 μM Ca⁺⁺**
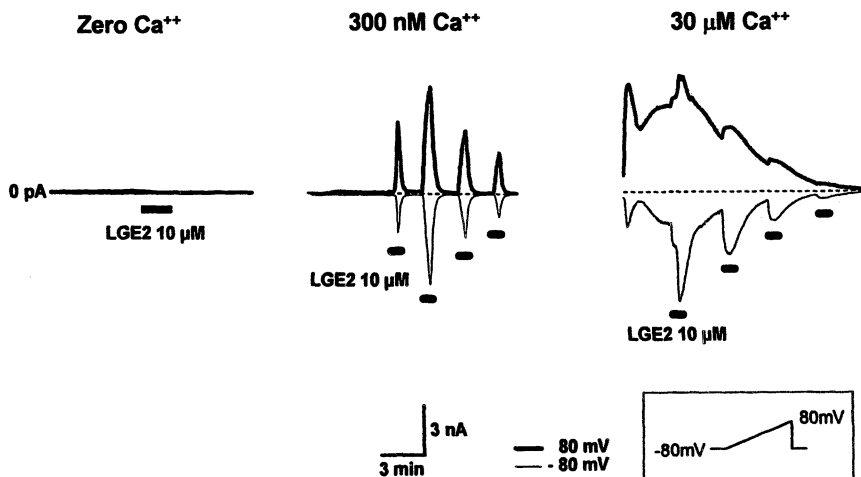
Figure 6. Whole-cell voltage clamp electrophysiological recordings demonstrate reversible TRPM5 current enhancement by LGE2.

levels (Figure 6, middle panel). This requirement of some TRPM5 activation for the compound to be active was also observed in FLIPR assay (Figure 4). The enhancer effect was still substantial at high $[Ca^{2+}]_i$ levels (Figure 6, right panel), but was not as dramatic in comparison because the channel was already at nearly maximal activation. This also recapitulates previous observations from the membrane potential FLIPR assay (Figure 5) and suggests that the enhancer may be increasing the sensitivity of TRPM5 to $[Ca^{2+}]_i$. Taken together, these results suggest that our compounds will not have a taste on their own, an important quality for an ideal sweetness enhancer, and that they will be most effective when combined with low quantities of caloric sweetener.

The potential enhancers we have identified may also possess the temporal characteristics required of sweetness enhancers. The action of the compounds appeared quickly and requires no pre-application (Figure 6). In additional studies involving a membrane potential FLIPR assay, there was no difference in TRPM5 enhancement between co-application and a 3- or 6-minute preincubation with LGE20 (data not shown). In addition, these compounds resulted in no persistent activation of the channel. 10 μM LGE2 augmented TRPM5 activation only during compound application, and its effects were eliminated immediately when the compound was washed out with bath solution (Figure 6). This property of reversible enhancement of TRPM5 currents is desirable to ensure the compound will not contribute to prolonged sweetness in taste modification applications.
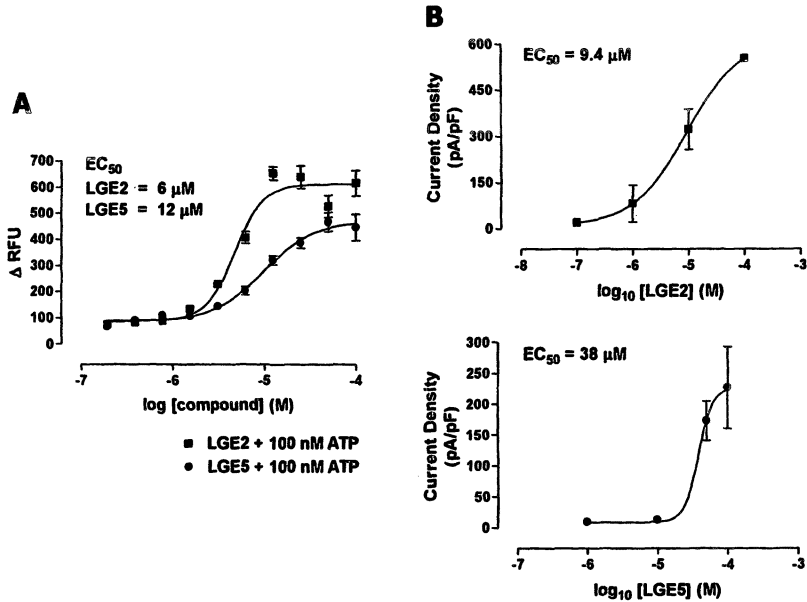
Figure 7. Potency measurements for two TRPM5 activators show comparable potencies for enhancers. A. Membrane potential assay and B. whole-cell electrophysiological recordings of TRPM5.

Whole-cell electrophysiological recordings were also used to confirm the EC_{50} values generated from the membrane potential FLIPR. To calculate EC_{50} values from whole cell recordings, cell capacitance was measured in every experiment and peak currents at each compound concentration were expressed as current density (pA/pF) for each cell. Enhancement dose response curves for two compounds, LGE2 and LGE5, generated by both FLIPR assay (Figure 7A, LGE2 EC_{50}=6 μM and LGE5 EC_{50}=9 μM) and whole-cell voltage clamp recording yielded comparable EC_{50} values (Figure 7B, LGE2 EC_{50}=9 μM, and LGE5 EC_{50}=38 μM), validating the potency of these compounds.

Discussion

There is a substantial interest in novel ways to reduce the sugar content of processed foods and beverages to maintain sweetness while reducing the negative health impact of excess sugar consumption. The strategy outlined here focuses on the development of sweetness enhancers that amplify the signaling step modulated through the TRPM5 ion channel. Using a pharmaceutically

inspired discovery process, we have identified prototype compounds that are potent, specific, and rapidly reversible enhancers. Our objective is to develop these prototype compounds so that they will produce 2-5x amplification of sweet taste and be effective in food or beverage products when present at a few parts per million level. Because TRPM5 functions as a downstream component of the sweet receptor signaling cascade, a TRPM5 enhancer would be effective with, and generate similar amplification effects for, both pure and mixed nutritive and non-nutritive sweeteners, creating a new set of sweetening options for processed foods and beverages.

Acknowledgements

References

1. Malik, V. S.; Schulze, M. B.; Hu, F. B. *Am J Clin Nutr.* **2006**, *84*, 274-288.
2. Mokdad, A. H.; Ford, E. S.; Bowman, B. A.; Dietz, W. H.; Vinicor, F.; Bales, V. S.; Marks, J. S. *Jama.* **2003**, *289*, 76-79.
3. Falco, M. A. *Gen Dent.* **2001**, *49*, 591-595.
4. DuBois, G. E. *Proc Natl Acad Sci U S A.* **2004**, *101*, 13972-13973.
5. Schiffman, S. S.; Booth, B. J.; Losee, M. L.; Pecore, S. D.; Warwick, Z. S. *Brain Res Bull.* **1995**, *36*, 505-513.
6. Schiffman, S. S.; Reilly, D. A.; Clark, T. B., 3rd. *Physiol Behav.* **1979**, *23*, 1-9.
7. Green, B. G.; Frankmann, S. P. *Physiol Behav.* **1988**, *43*, 515-519.
8. Yamamoto, T.; Kato, T.; Matsuo, R.; Kawamura, Y.; Yoshida, M. *Physiol Behav.* **1985**, *35*, 411-415.
9. Adler, J. E.; Li, X.; Staszewski, L.; Xu, H.; Echeverri, F. U. S. Patent 6,955,887, 2005.
10. Sugita, M. *Cell Mol Life Sci.* **2006**, *63*, 2000-2015.
11. Margolskee, R. F. *J Biol Chem.* **2002**, *277*, 1-4.
12. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S. *Cell.* **2001**, *106*, 381-390.
13. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc Natl Acad Sci U S A.* **2002**, *99*, 4692-4696.

14. Nelson, G.; Chandrashekar, J.; Hoon, M. A.; Feng, L.; Zhao, G.; Ryba, N. J.; Zuker, C. S. *Nature.* **2002**, *416*, 199-202.
15. Adler, E.; Hoon, M. A.; Mueller, K. L.; Chandrashekar, J.; Ryba, N. J.; Zuker, C. S. *Cell.* **2000**, *100*, 693-702.
16. Chandrashekar, J.; Mueller, K. L.; Hoon, M. A.; Adler, E.; Feng, L.; Guo, W.; Zuker, C. S.; Ryba, N. J. *Cell.* **2000**, *100*, 703-711.
17. Bufe, B.; Hofmann, T.; Krautwurst, D.; Raguse, J. D.; Meyerhof, W. *Nat Genet.* **2002**, *32*, 397-401.
18. McLaughlin, S. K.; McKinnon, P. J.; Margolskee, R. F. *Nature.* **1992**, *357*, 563-569.
19. Wong, G. T.; Gannon, K. S.; Margolskee, R. F. *Nature.* **1996**, *381*, 796-800.
20. Danilova, V.; Damak, S.; Margolskee, R. F.; Hellekant, G. *Chem Senses.* **2006**, *31*, 573-580.
21. Kusakabe, Y.; Yasuoka, A.; Asano-Miyoshi, M.; Iwabuchi, K.; Matsumoto, I.; Arai, S.; Emori, Y.; Abe, K. *Chem Senses.* **2000**, *25*, 525-531.
22. Blake, B. L.; Wing, M. R.; Zhou, J. Y.; Lei, Q.; Hillmann, J. R.; Behe, C. I.; Morris, R. A.; Harden, T. K.; Bayliss, D. A.; Miller, R. J.; Siderovski, D. P. *J Biol Chem.* **2001**, *276*, 49267-49274.
23. Ozeck, M.; Brust, P.; Xu, H.; Servant, G. *Eur J Pharmacol.* **2004**, *489*, 139-149.
24. Huang, L.; Shanker, Y. G.; Dubauskaite, J.; Zheng, J. Z.; Yan, W.; Rosenzweig, S.; Spielman, A. I.; Max, M.; Margolskee, R. F. *Nat Neurosci.* **1999**, *2*, 1055-1062.
25. Clapp, T. R.; Stone, L. M.; Margolskee, R. F.; Kinnamon, S. C. *BMC Neurosci.* **2001**, *2*, 6.
26. Prawitt, D.; Monteilh-Zoller, M. K.; Brixel, L.; Spangenberg, C.; Zabel, B.; Fleig, A.; Penner, R. *Proc Natl Acad Sci U S A.* **2003**, *100*, 15166-15171.
27. Schlyer, S.; Horuk, R. *Drug Discov Today.* **2006**, *11*, 481-493.
28. Felder, C. C. *Expert Opin Investig Drugs.* **2004**, *13*, 1071-1074.
29. Despotovski, S.; Razvi, E. *Assay Drug Dev Technol.* **2004**, *2*, 575-578.
30. Ford, J. W.; Stevens, E. B.; Treherne, J. M.; Packer, J.; Bushfield, M. *Prog Drug Res.* **2002**, *58*, 133-168.
31. Taylor, C. P.; Narasimhan, L. S. *Adv Pharmacol.* **1997**, *39*, 47-98.
32. Zheng, W.; Spencer, R. H.; Kiss, L. *Assay Drug Dev Technol.* **2004**, *2*, 543-552.
33. Liu, D.; Liman, E. R. *Proc Natl Acad Sci U S A.* **2003**, *100*, 15160-15165.
34. Hofmann, T.; Chubanov, V.; Gudermann, T.; Montell, C. *Curr Biol.* **2003**, *13*, 1153-1158.
35. Talavera, K.; Yasumatsu, K.; Voets, T.; Droogmans, G.; Shigemura, N.; Ninomiya, Y.; Margolskee, R. F.; Nilius, B. *Nature.* **2005**, *438*, 1022-1025.
36. Liu, D.; Zhang, Z.; Liman, E. R. *J Biol Chem.* **2005**, *280*, 20691-20699.

37. Perez, C. A.; Huang, L.; Rong, M.; Kozak, J. A.; Preuss, A. K.; Zhang, H.; Max, M.; Margolskee, R. F. *Nat Neurosci.* **2002**, *5*, 1169-1176.
38. Perez, C. A.; Margolskee, R. F.; Kinnamon, S. C.; Ogura, T. *Cell Calcium.* **2003**, *33*, 541-549.
39. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili, Z.; Perez, C. A.; Shigemura, N.; Yoshida, R.; Mosinger, B., Jr.; Glendinning, J. I.; Ninomiya, Y.; Margolskee, R. F. *Chem Senses.* **2006**, *31*, 253-264.
40. Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Mueller, K. L.; Cook, B.; Wu, D.; Zuker, C. S.; Ryba, N. J. *Cell.* **2003**, *112*, 293-301.
41. Schachter, J. B.; Sromek, S. M.; Nicholas, R. A.; Harden, T. K. *Neuropharmacology.* **1997**, *36*, 1181-1187.
42. Iredale, P. A.; Hill, S. J. *Br J Pharmacol.* **1993**, *110*, 1305-1310.
43. Ullrich, N. D.; Voets, T.; Prenen, J.; Vennekens, R.; Talavera, K.; Droogmans, G.; Nilius, B. *Cell Calcium.* **2005**, *37*, 267-278.

Chapter 25

Enhancers for Sweet Taste from the World of Non-Volatiles: Polyphenols as Taste Modifiers

Jakob P. Ley[*], Maria Blings, Susanne Paetz, Günter Kindel,
Kathrin Freiherr, Gerhard E. Krammer,
and Heinz-Jürgen Bertram

Flavor Research and Innovation, Symrise GmbH & Company KG,
P.O. Box 1253, 37601 Holzminden, Germany
[*]Corresponding author: jakob.ley@symrise.com, fax +49 5531 9048883

Several polyhydroxylated deoxybenzoins, benzoic acid amides and gingerdiones were synthesized and screened for their ability to enhance sweetness of sucrose. The most active compounds tested were able to increase the sweetness of a 5 % sucrose model solution by 20 to 30 %. The compounds showed only a slight intrinsic sweetness of about 0.5 % sucrose equivalents. Simple addition of normalized sweet ratings of sucrose and test compounds resulted in ratings about 10 % lower than those determined by the panel. Some of the structures were also able to restore some of the sweetness in sugar reduced bases. As a result, the compounds can principially be used as flavor molecules to increase sweetness in selected applications and may be a good starting point to develop more active sweet taste enhancers.

Due to the increased incidence of obesity and related diseases such as diabetes type II especially in western populations, there is a high demand for food products with reduced levels of sweet and caloric carbohydrates such as sucrose and high fructose corn sirup (HFCS). On the other hand, sweetness is always a positive hedonic signal to the consumer and therefore a strong preference driver for food consumption. As a result, the products reduced in sucrose or HFCS levels should have the same sweetness impression as compared to the full-sweetened product. For modern food development, three options offer viable solutions: the use of artificial sweeteners, the use of classical "sweet" flavors and the application of more or less tasteless sweet taste enhancers. Whereas the first two approaches are more or less state-of-the-art technology, the latter one is only rarely described in the literature (e.g. alapyraidine (*1*)). On the other hand the molecular basics of sweet taste reception and binding of sweet molecules seems to be clear now and a general enhancement should be possible (*2, 3*).

Some sweeteners such as sodium cyclamate show synergistic potential in combination with sugars (*4*), but they have to follow the legislation for food additives and often show off-tastes (bitterness, metallic taste) and in some cases a lingering sweetness perception. In the literature, there are some hints regarding more or less tasteless sweetness modifiers: Hofmann *et al.* described alapyraidin, a Maillard reaction product, as a sweetness enhancer (*1*) (alapyraidin shows in addition enhancing effects for all taste qualities (*5*)); lactisole, which occurs naturally in coffee beans, is a general sweet inhibitor. The modulation effects of such compounds can now indeed be explained on the molecular level (*6*).

Alapyraidin and lactisole contain phenolic structures and therefore we decided to investigate this structural class of compounds. In addition some polyhydroxylated phenolics with isovanillic patterns show intrinsic sweetness (such as neohesperedin dihydrochalcone and analogues (*7*)) and with vanillic pattern bitter inhibition activities (such as homoeriodictyol (*8*)), which may be correlated to sweet modulating activities. Thus, we decided to screen non-volatile polyphenolic food compounds and their derivatives with a weak intrinsic aroma profile.

Experimental

Syntheses

Synthesis of polyhydroxylated deoxybenzoins (for structures see Figure 1) was performed via the procedures described in literature (*9*) starting from polyhydroxybenzene and hydroxylated phenyl acetic acid derivatives (Figure 2a).
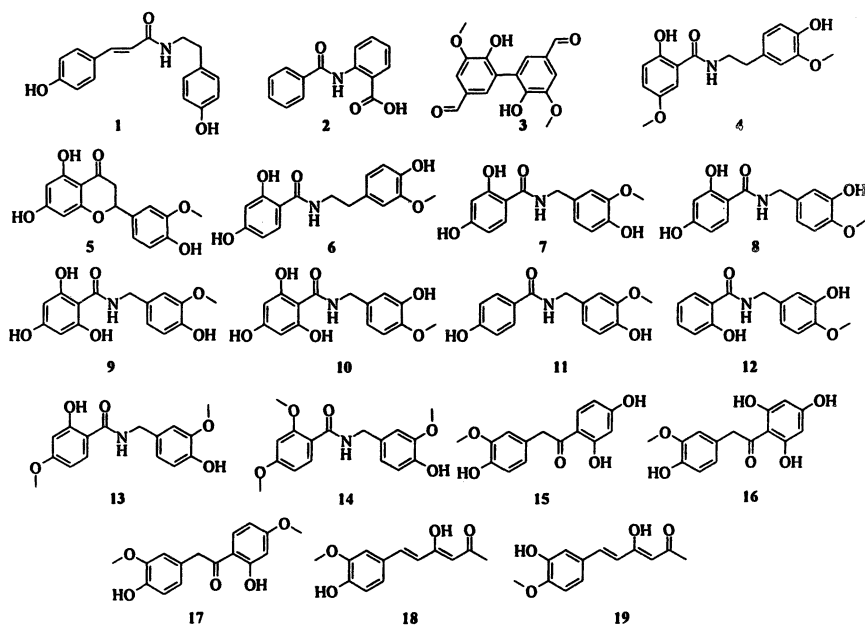
Figure 1. Molecules evaluated for sweet enhancing activity.
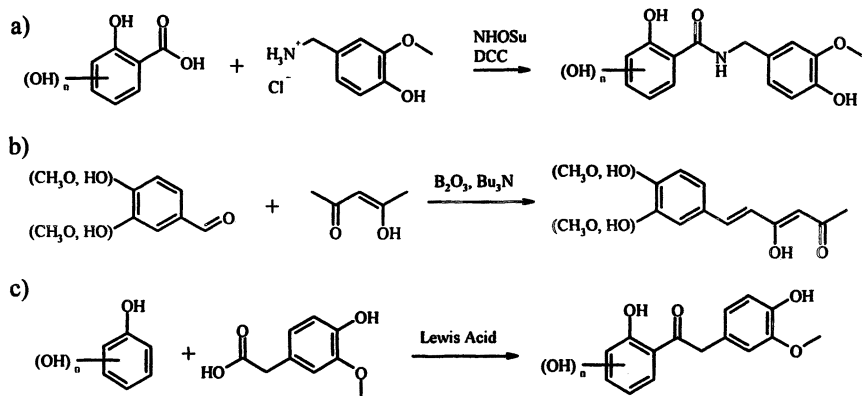
*Figure 2. Synthesis of polyhydroxylated deoxybenzoins (15, 16, 17),
short chain dehydrogingerdiones (18, 19), and polyhydroxylated benzamides
(4, 6 to 13).*

Short chain dehydrogingerdiones were synthesized according to published procedures (*10*) starting from vanillin or isovanillin and acetoacetone (Figure 2b). Polyhydroxylated benzamides of vanillylamine were prepared by the standard condensation procedure (*11*) using DCC/*N*-hydroxysuccinimide (Figure 1c). All new compounds were purified by crystallisation or chromatography to at least 95 % and characterized by spectroscopic methods (^{1}H- and ^{13}C-NMR, HRMS, LC-MS).

Sensory Evaluation

For screening of sweetness enhancing activity the test compounds were directly dissolved in an aqueous 5 % sucrose solution. Panelists (healthy adults, no tasting problems known) were trained to rate sweetness intensities of different sucrose concentrations between 0 and 15 % on a structured scale of 0 (no sweetness) to 10 (very strong sweetness). A minimum of 14 testers was used for duo comparision. Mean ratings of a 5 % sucrose solution ranged between 4 and 6. For all experiments the test solutions were coded and in the case of color or cloudiness they were covered. Panelists were advised to test randomly mixed samples in the given order by the sip and spit method. The raw sensory data were analysed using the standard functions of Microsoft Excel 97. For calculation of significance Student's matched pair test was used.

Results and Discussion

Sweetness of food and model applications can be influenced by volatile flavors or single flavor chemicals as well as by textural modifications (*13*). In such studies so called congruent flavors such as peach can increase and incongruent flavors such as lemon can decrease the perceived sweetness (*14*). In real applications the interactions between different taste qualities are much more important. Especially in the presence of acid a strong decrease in sweetness can be perceived. For example, the sweetness of a 8 % sucrose solution containing 0.2 % citric acid was rated 30 % lower compared to 8 % sucrose without acid; similiarly the sweetness of a 10 % sucrose/0.2 % citric acid solution against a 10 % sucrose soultion was decreased by about 20 % (own results).

In our study with typical "sweet" and volatile flavor molecules such as vanillin, damascenone and diacetyl, only non-significant enhancing effects lower than 15 % (based on a 5% sucrose solution) could be found. For example vanillin at 600 ppb showed a sweetness inhibiting activity of -3 % and diacetyl at 5 ppb was able to enhance the sweetness by 8 % (non-significant). Due to the strong flavor of aroma chemicals they can not be used in a broad range of applications. All tested volatile "sweet" flavor molecules showed no intrinsic

sweetness when tested at typical flavor concentrations in pure water with closed nose.

Therefore we performed a synthesis program mainly based on non-volatiles (e.g. polyhydroxylated benzoic acid benzylamides, dehydrogingerdiones, deoxybenzoins, see Figure 1) to evaluate the active structural elements responsible for the sweetness enhancing effects. In most cases the investigated molecules do not occur in nature but resembled known natural compounds. Some deoxybenzoins such as 2-(6-carboxy-2,4-dihydroxy-phenyl)-1-(4-hydroxyphenyl)-ethanone were isolated previously from white salsify (*15*) and short chain dehydrogingerdiones were found in ginger (*9*). Hydroxybenzoic acid amides are not so common in nature, but some prototypes such as anduncamide (**4**) were found in *Piper ssp.* (*16*) and cinnamamides such as *N*-coumaroyltyramine (**1**) were found in several plants (*17*).

Sweetness Enhancement

We have chosen 5 % sucrose solution as test medium because changes in sweetness could most easily be detected at this concentration. In Table I the screening results are summarized. The best sweetness enhancers were the artificial hydroxybenzamides **7**, **9** and **10** and the deoxybenzoins **15** and **16**. Interestingly small variations in the substitution pattern, especially by adding or "moving" methyl groups on the benzoic acid moiety or on the deoxybenzoin skeleton caused loss of activity. The exchange of vanillic patterns by isovanillic substitution (e.g. **18** compared to **19** and **7** versus **8**) decreased the activity dramatically. This was somewhat surprising because the isovanillic pattern occurs in many sweet structures.

Intrinsic Sweetness

To distinguish simple additive effects from synergistic activities, for the most active compounds the intrinsic sweetness was determined by comparision of the 100 ppm solution of test compound with reference solutions of sucrose (0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 %). The averaged sweetness (Table II) was calculated by

$$averaged\ sweetness\ (\%) = \frac{n_{0.5} \cdot 0.5 + n_1 \cdot 1 + n_2 \cdot 2 + n_4 \cdot 5 + n_5 \cdot 5}{n}$$

with: n = number of panelists and: n_x = number of panelists who rated x % sucrose equivalents.

Table I. Sweetness Enhancing Effects of Known and Newly Synthesized Compounds in 5 % Sucrose Solution

Compound (100 ppm)	Enhancing Activity (%)	Profile (100 ppm in Water)
N-coumaroyltyramine (1) (17)	6	sweet
dianthramide B (2)	11	soapy, sweet, bitter, mouthfeel
divanillin (3) (18)	9	fatty, vanillin-like, sweet, cream
aduncamide (4) (16)	-1	fresh, fruity, sweet, mouthfeel
homoeriodictyol (5) (8)	6	sweet, vanillin-like, phenolic, mouthfeel
6	2	vanillin-like, astringent, phenolic, dry-dusty
7	22	vanillin-like, licorice
8	0	herbal, bitter, phenolic
9	31^a	sweet, vanillin-like, licorice
10	20^a	fruity, ester
11	4	fruity, sweet, dry-dusty
12	12	milky, sweet, vanillin-like, balsamic
13	-8	fresh, cool, sweet
14	18	sweet, metallic
15	7	cream, sweet, mouthfeel
16	16	vanillin-like, spicy, woody, balsamic, clove
17	0	sweet, dry-dusty, balsamic
18	15	neutral, drying
19	-4	dry-dusty, balsamic

^{a} ($p < 0.05$)

Table II. Intrinsic Sweetness of Selected Compounds Compared to Enhancing Activity in 5% Sucrose solution

Compound (100 ppm)	Sweetness Comparable with Sucrose Solution of	Enhancing activity (%)
2	0.1%	11
16	0.3%	16
7	0.4%	22
9	0.6%	31

406

Indeed, a positive correlation exists for this small subset of compounds. In Figure 3 a comparision is shown between calculated sweetness by simple addition of normalized sucrose equivalents of a pure 5 % sucrose solution and a 100 ppm solution of the test compound, respectively, against the measured enhancing effect. In each case the measured effect is about 10 % higher than the calculated activity. The dataset is too small for final conclusions, but we conclude that according to Williams *et al.* (*19*) these data can be interpreted as synergistic and are not simply additive. For the future we will perform a broader dataset and tests on the molecular level on the sweet receptors in order to obtain more reliable information regarding the underlying mechanism.
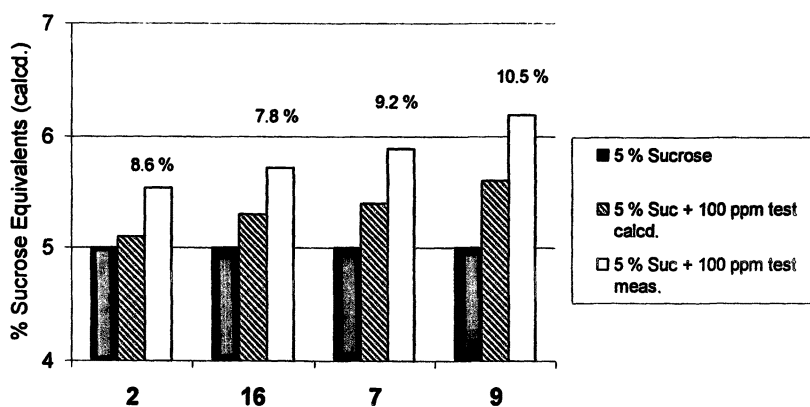
Figure 3. Comparision between calculated and measured normalized enhancing effects (values on top of the bars: relative synergism, Suc: sucrose).
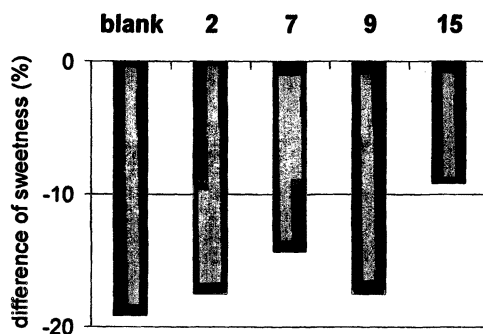
Figure 4. Comparision between modulation effects of 10 % sucrose solution vs. 8 % sucrose + sweet enhancer

Sucrose Replacement and Application

It is more realistic but much more difficult to obtain an isosweet solution or food product by reducing sucrose content and adding a sweetness enhancer. As test solutions we have chosen a 10 % sucrose solution as standard, an 8 % sucrose solution as a reduced version, and an 8 % sucrose solution containing the test compound. In Figure 4 the results show that only the deoxybenzoin **15** and to some extent the benzoic acid amide **7** were able to reduce the loss of sweetness caused by the lower sugar concentration. Therefore the activity of "sugar replacement" was not directly correlated to the intrinsic sweetness, otherwise the amide **9** should have been the best replacer.

As a more realistic model, a low fat yoghurt containing 5 % sucrose and some of the test compounds was the chosen medium. As shown in Figure 5, the amide **7** and the deoxybenzoin **15** were able to increase the sweetness by about 25 % (significance p < 0.05) and 18 % (non-signigicant), respectively.
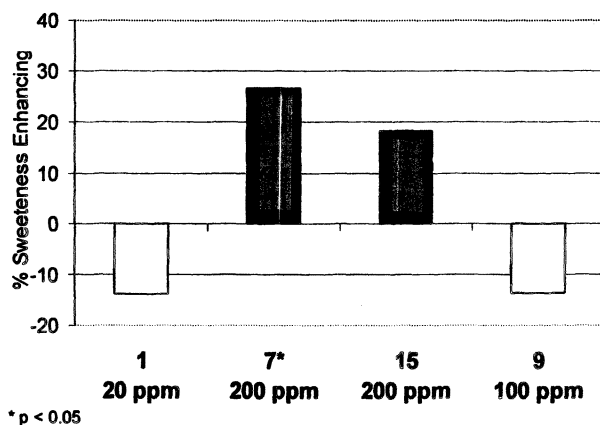
Figure 5. Modulation Effects in Low Fat Yoghurt (0.1 % fat), 5 % Sucrose Content.

Conclusions

We were able to detect more or less tasteless sweetness enhancing compounds in the area of polyhydroxylated benzoic acid vanillylamides and deoxybenzoins by a simple screening method in 5 % aqueous sucrose solution. It was very important to evaluate the intrinsic sweetness because there was a correlation between sweetness enhancing activity in 5 % sucrose model solution and intrinsic sweetness. But the enhancing effect could not be explained by a

simple additive activity. Some of the candidates were also able to recover the sweetness in sugar-reduced model solutions and in addition they were able to increase the sweetness of a fat-and sugar-reduced yoghurt.

Starting with these first promising results we intend to screen more natural molecules for their sweetness enhancing activities. Furthermore it is of high importance to obtain more structure-activity information and data from receptor studies in order to elucidate the mechanism of the sweetness enhancing effect.

Acknowledgements

References

1. Ottinger, H.; Soldo, T.; Hoffmann, T. *J. Agric. Food Chem.* **2003**, *51*, 1035-1041.
2. DuBois, G.E. *PNAS USA* **2004**, *101*, 13972-13973
3. McGregor, R. *Food Technol.* **2004**, *58*, 24-28.
4. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. *Proc. Nat. Acad. Sci. USA* **2004**, *101*, 14258-14263.
5. Soldo, T. ; Blank, I.; Hofmann, T. *Chem. Sens.* **2003**, *28*, 371-379
6. Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, Z.A.; Benard, L.M.J.; Osman, R.; Margolskee, R.F.; Max, M. *J. Bio. Chem.* **2005**, *280*, 15238-15246.
7. DuBois, G.E.; Crosby, G.A.; Stephenson, R.A. *J. Med. Chem.* **1981**, *24*, 408-428.
8. Ley, J.P.; Krammer, G.; Reinders, G.; Gatfield, I.L.; Bertram, H.-J. *J. Agric. Food Chem.* **2005**, *53* , 6061-6066.
9. Jolad, S.D.; Lantz, R.C.; Solyo, A.M.; Chen, G.J.; Bates, R.B.; Timmermann, B.N. *Phytochem.* **2004**, *65*, 1937-1954.
10. Masuda, T. ; Matsumura, H. ; Oyama, Y.; Takeda, Y. ; Jitoe, A.; Kida, A.; Hidaka, K. *J. Nat. Prod.* **1998**, *61*, 609-613.
11. Ley, J.P.; Kindel, G.; Bertram, H.-J.; Krammer, G. PCT patent application WO 2006 024,587, 2006.
12. Fokialakis, N.; Lambrinidis, G.; Mitsiou, D.J.; Aligiannis, N.; Mitakou, S.; Skaltsounis, A.L.; Pratsinis, H.; Mikros, E.; Alexis, M.N. *Chem. Biol.* **2004**, *11*, 397-406.
13. Lethuaut, L. ; Brossard, C. ; Meynier, A.; Rousseau, F.; Llamas, G.; Bousseau, B.; Genot, C. *Int. Dairy J.* **2005**, *15*, 485-493.

14. Djordjevic, J.; Zatorre, R.J.; Jones-Gotman, M. *Chem.l Senses* **2004**, *29*, 199-208.

15. Zidorn, C.; Lohwasser, U.; Pschorr, S.; Salvenmoser, S.; Ongania, K.H.; Ellmerer, E.P.; Börner A.; Stuppner, H. *Phytochem.* **2005**, *66*, 1691-1697.

16. Orjala, J.; Wright, A.D.; Rali, T.; Sticher, O. *Nat. Prod. Lett.* **1993**, *2*, 231.

17. Wu, P.-L.; Su, G.-C.; Wu, T.-S. *J. Nat. Prod.* **2003**, *66* (7), 996-998.

18. Reiss, I.; Gatfield, I.L.; Krammer, G.; Kindel, G.; Clerc, A. German patent application DE 10,310,204, **2004**.

19. Williams, J.G.; Bernhard, R.A. *Journal of Food Science* **1981**, *46*, 1245-1251.

Chapter 26

Evaluation of High-Intensity Sweetener Modulators

Cheryl R. Mitchell, Richard Ray, and Marian Schwartz

**Creative Research Management, 2029 East Harding Way,
Stockton, CA 95205**

Different compounds have been sought to function as modulators that stimulate sweetness perception, diminish lingering sweetness, or diminish off tastes that characterize most of the popular synthetic sweeteners. Sensory evaluation and determination of effectiveness of modulators is very difficult due to the cross-over or residual effects that characterize high intensity sweeteners. Using a three-way light switch as the model for the sweetness receptor circuit and sweetness perception, blocking and modulation, a protocol for testing the modulating effect of different compounds was developed. This sensory evaluation protocol enabled panelists to better evaluate and distinguish between and among different flavored beverage systems containing high intensity sweeteners that are not readily "cleared" and normally inhibit a panelist's ability to characterize and distinguish specific samples. The study provides an evaluation and summary of the effectiveness of Sucramask® (a natural flavoring) and acelsulfame-K as modulators for sucralose in beverage systems of neutral and high pH.

The symposium has provided research in the area of structure and modeling of taste receptors, taste transduction, and quantifying the sweetener response. In the area of current product formulation, manufacturing and packaging, the application of sweetener modulation is of primary concern in the current efforts to utilize existing high intensity sweeteners. Current manufacturers do not have the luxury of waiting for the proposed new and improved sweeteners. Food formulators must work with what they have and become aware of what modulators will improve the already existing high intensity sweeteners.

The food industry works with complex systems of foods and beverages having a multitude of taste responses of which sweetness or perceived sweetness is a key element. Perceived texture, flavor and sweetness will dictate the success or failure of a product. However, of these three, the intensity and quality of the sweetness, is the most significant.

As a sweetener, sucrose is the gold standard. Sucrose does not have any off tastes and is perceived as an example of "natural" sweetness. It is the primary or first ingredient in many foods and most beverages, providing both sweetness and texture or body. Significantly, sucrose *works in harmony* with many flavors and food systems including those of varying pH. Sucrose works in concert, like a classical string quartet, and does not stand out. It is a team player and provides homogenous and integrated sweetness along with texture and flavor.

Food compositions should produce a taste sensation that is balanced, rather than being segregated or producing "spikes". Sucrose works well to accomplish this task. However, since it is desirable to produce low calorie foods and beverages, sucrose must be replaced with a low calorie high intensity sweetener. Unfortunately, high intensity sweeteners for the most part do not integrate well with either the flavor or texture of the food product system. While work is being done to improve the quality of these high intensity sweeteners, because of the approval process, one can anticipate that the approval for use will not be in the immediate future. Since food formulators are producing products right now with the currently available high intensity sweeteners, the food formulator has no choice but to select and find appropriate modulators that will make the high intensity sweetener perform in the food system similar to the way sucrose would perform. To select appropriate modulators, it is important to understand the organoleptic issues associated with high intensity sweeteners.

High intensity sweeteners differ from sucrose in the quality and intensity of sweetness as well as the texture. Aftertastes such as bitter, cardboard, lingering sweetness, chemical, as well as textural sensations of coolness, powderiness, or a watery sensation may be associated with these non-sucrose sweeteners in food or beverage systems. Additionally, the sweet taste itself many times does not integrate well with the flavors at different pH of the system. Consequently, instead of getting an integrated sweet/sour for example, one is hit with flashes of spiking "sweet/sour/sweet/sour, etc." Sucrose on the other hand, is homogenous,

working together with other flavor and pH elements of a food system. The incongruous sweetness and flavor profiles produced by food systems containing high intensity sweeteners is a little like trying to get a rock and roll group to integrate homogenously with the classical string quartet. There are a lot of issues to resolve. However, it is the responsibility of the food scientist, to make the system work. Since at this point in time, there are limitations to the choice of high intensity sweeteners, and any food ingredients added as modulators must be an approved food additive or GRAS (generally recognized as safe), the challenge is to use food approved substances to modulate the currently accepted high intensity sweetener. The most popular and acceptable high intensity sweetener as of the writing of this paper is sucralose.

Sucralose is a high intensity sweetener that performs very well at neutral pH and in systems that undergo high heat processing such as beverage systems. Aspartame, is not as stable when subjected to the high temperatures and time necessary for product pasteurization or for aseptic commercial sterilization. Sucralose, while very heat stable, does not behave well in systems with low pH. In these systems, the sweetness and flavor profiles become erratic and it becomes very difficult to balance pH, sweetness level, and flavor. Consequently, it is necessary to perform numerous taste panels to determine the differences and perceived improvements of the formulations. However, these evaluations are flawed because of the lingering sweetness of sucralose which goes from one sample to another blinding the panelists taste from any perceived improvement in the formulation that may result from a modulator and the impact the modulator may have especially in its ability to diminish the lingering sweetness inherent to sucralose.

Panelists trained in sweeteners, flavors, and sourness, describe the incongruities of sucralose in beverage systems as: (a) Flavors: Intensity varies with pH (spiking); (b) Homogeneity: Lingering sweetness; and (c) Mouthfeel: Watery

Modulators

The food formulators' objective is to find the right modulator(s) that would impact the undesirable attributes of sucralose as well as a methodology that could correctly indicate the impact of the modulator when being evaluated from sample to sample. Specifically, in the case of sucralose, the food formulator is looking for modulator(s) that can impact the stabilization of the food/beverage system containing sucralose with regard to the incongruities of lingering sweetness, watery mouth feel, and the spiking or non integration of the acidity, sweetness and flavor.

To determine evaluation methodology, one might first want to deliberate on the causes of the incongruities of sucralose. The incongruities of spiking and

lingering may be associated with binding times of the sucralose to the receptor site. The incongruity of watery mouthfeel results from the differences in solids when using ppm of high intensity sweeteners versus grams of sucrose to achieve the same sweetness intensity. Regarding the latter, a thickening agent such as gums, would increase the viscosity and improve the mouthfeel. Unfortunately, it also reduces flavor impact. A gum that *does not* have viscosity but produces mouthfeel is the most desirable, and there are now modified pectins, that perform this action without increasing viscosity or reducing flavor impact. With regard to the binding and "release" of the sucralose, two modulators are actively considered with regard to the modulation in current food systems; Acelsulfame K (ASK) and Sucramask® (SM)(1). Both modulators appear to have an impact on sucralose, but it is difficult to understand exactly how and where they have their modulation effect as well as evaluating their effects using taste panels. First, a proposed binding/activation model will be provided to assist in an understanding of how these modulators may be impacting the sweetener receptor site, and then sensory evaluation methodologies and results using ASK and SM will be reviewed.

Binding/Activation Model

Sometimes it is much easier for the food formulator to work with easy conceptual models that are very familiar and help one to understand how systems are behaving and why they are having so much difficulty. In our laboratories, we have found the 3-way switch model to be very helpful and appropriate in understanding the binding of sucralose so that we could later target and look for modulators that target or impact that binding. To that end, Figure 1 represents the electrical schematic of a three-way switch and a possible representation of the sweetness receptors and their binding and impact on the sweetness signal.

Many people have three way switches in their homes and offices. They are designed such the light in a room for example, can be activated by either one of two different switches such that a person can enter or leave through two different doors and still turn on or off the same light. We believe that the binding of sucrose and sucralose as well as other molecules that impact the sweetness response can be simply considered in terms of this three-way mechanism. There are believed to be two different active receptor sites that control sweetness. The T1R2 and the T1R3. (2) Our model assumes that these represent the two switches in the room. The receptor site can be either filled or empty. When it is empty or filled, it is like flipping one of the switches. There is also a consideration as to the intensity with which these molecules bind or fill the switch.

In Figure 1, neither receptor site is filled and the light is off (no sweetness). In Figure 2 and Figure 3, when one or the other receptor site is filled, the light is activated (sweetness), in Figure 4, both receptor sites are filled, and the light is

off (no sweetness). The molecules that bind in the first receptor site may be considered to be sucrose, or analogous to sucrose such as sucralose. Interestingly, molecules like lactisole would have to then be able to bind to both sites, and apparently adhere to one site very tightly. Consequently, when lactisole is present alone or in combination with sucrose, (both receptor sites are filled), there is no apparent sweetness. (3) However, when the lactisole in the beverage is gone, sweetness is observed in water or liquids that do not contain other sugars or lactisole. The remaining binding lactisole keeps one switch filled and activates the sweetness response. In the case of sucralose, it apparently binds more tightly to the same receptor as sucrose (hence the lingering sweetness). Consequently, it is a matter of simply making sure one can remove the sucralose from the site. Modulators such as ASK and SM impact the binding of the sucrose site by either binding themselves or removing the sucrose or sucralose molecule from that site. They do not interfere with the second site
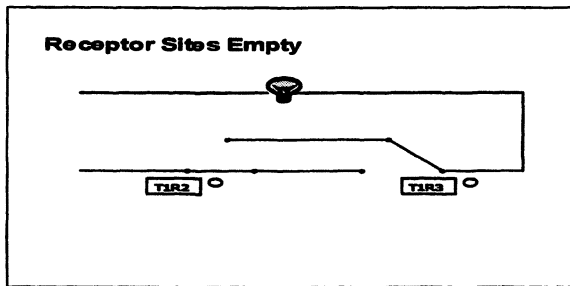
*Figure 1. Three way switch model. Both receptor sites empty
(no sweetness is perceived).*
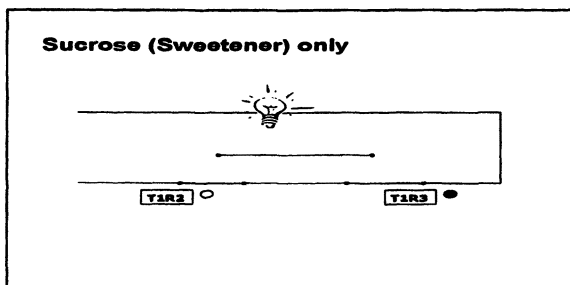
*Figure 2. Three way switch model. Sucrose only filling one receptor site
(perceived sweetness).*
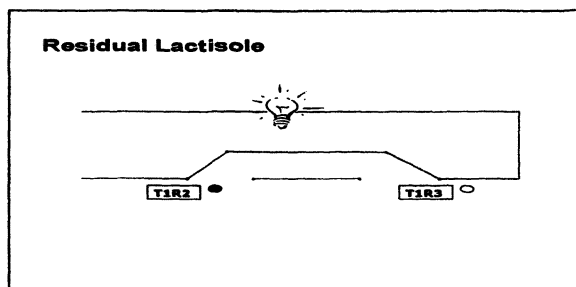
Figure 3. Three way switch model. Sucrose and lactisole filling both receptor sites (no sweetness is perceived).
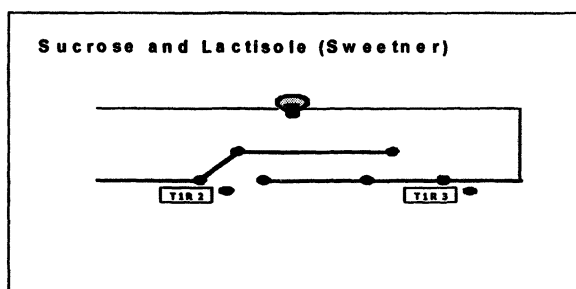
Figure 4. Three way switch model. Residual lactisole remaining in receptor site (perceived sweetness).

as the sweetness is not eliminated as is the case of lactisole. When developing test protocols to evaluate the impact of modulators on the sucralose system, keeping the above model in mind is helpful in understanding some of the test results.

Sensory Evaluations

Sensory evaluations were conducted with trained panelists from the National Food Laboratories.(4) These panelists have been extensively trained in the use of standardized vocabulary to describe the appearance, aroma, flavor and texture of a variety of products and are used on a regular basis to support contract research.

Lactisole Rinse Evaluation

The impact of both ASK and SM was evaluated in the lactisole system. That is the impact, of these modulators in a lactisole solution, that was perceived following a water rinse. For the lactisole rinse descriptive test, panelists rinsed with a 30 ml portion of a rinse, and then rated a sample of water which was coded with a three-digit random number. The panelists were unaware that the liquid they were rating was water. Four sets of rinses with water samples to rate (water rinse, lactisole rinse, lactisole/ASK and lactisole/SM rinse) were evaluated during each session. Two sessions were conducted with a 1-hour break between sessions. Two evaluations (replicates) were obtained from each panelist for each product; therefore, a total of 18 judgments were obtained for each product. Panelists placed a slash mark on 15-cm line scales to indicate the intensity of the sensory characteristics. Unsalted soda crackers were provided for cleansing the palate between samples. For data analysis, the slash marks on the line scales were converted to numbers ranging from 0 to 15 using a digitizer. The mean intensities were calculated for each sensory characteristic. Analysis of Variance and Duncan's Multiple Range Test, where appropriate, were used to determine significant differences among the samples for each attribute. When panelists-by-product interactions were significant, the mean square of the interaction term, instead of the mean square of the error term, was used in calculation of the product F values. Results of this test are presented in Table I.

Essentially, while there was significant difference between lactisole solution followed by water rinse versus a water followed by water rinse as anticipated, there was only a trending difference between lactisole solutions containing ASK or SM. The lactisole solutions containing the SM, followed by a water rinse, indicated a higher level of sweetness and sugar character. The latter would be expected if SM was dislodging the lactisole from the first site a bit more rapidly. ASK, produced a trending difference in the sugar character indicating that it was potentially in a competitive binding with the lactisole in the first site. More studies need to be performed to support these trending differences.

Triangle Test

The impact or binding of the sweetness receptor sites would also be a problem when one considers triangle tests which are very commonly used to determine the impact of modulators on high intensity sweetener systems. As indicated above, if the modulator clears the site or binds the site preferentially, the rate of release or binding is going to impact the system. The panelists were given three coded samples (two of the same product and one of the other product) and asked to indicate which sample was different from the other two.

Table I. Lactisole- Sweetener Rinses on Flavor Impact

Lactisole Rinses Quantitative Descriptive

QUANTITATIVE DESCRIPTIVE EVALUATIONS OF RINSES
(n=18, 9 Panelists, 2 Evaluations Each)

	Water after Water/ Rinse	Water After Water/ Lactisole Rinse	Water After Water/ Lactisole/ AceK Rinse	Water After Water/ Lactisole/ SucraMask™ Rinse	Conf. Level
FLAVOR:					
Total Flavor	2.62[b]	3.20[a]	3.27[a]	3.50[a]	95%
Sweet	0.94[b]	2.25[a]	2.26[a]	2.59[a]	95%
Sugar Character	0.13[b]	0.74[a]	0.59[a]	0.74[a]	95%
Chemical/Artificial	1.76	2.04	2.07	2.05	NSD
Bitter	1.28	1.07	1.15	1.16	NSD
Soft Water	0.93	1.21	1.07	1.17	NSD
Cardboard	0.55	0.89	0.64	0.68	NSD
Plastic	0.37	0.40	0.57	0.58	NSD
TEXTURE:					
Astringent	2.13[b]	2.59[a]	2.80[a]	2.57[a]	95%

NSD: Not significantly different at confidence levels of 90% or higher

The triangle test was conducted using a balanced reference (i.e. half of the time the two samples were the control product and half of the time the two samples were the test product). Each panelist repeated the test three times (i.e. replications) for a total of 30 evaluations within each set of samples. Serving order was balanced (i.e. each sample was seen approximately an equal number of times in each position). A ten minute break was given between trials. Ambient drinking water and unsalted soda crackers were provided for cleansing the palate between samples. The numbers of correct responses were tallied. A binomial test was used to determine if a significant difference was found between the samples in each set. Table II provides tallies of correct responses for the sucralose vs. sucraloseASK and sucralose vs. sucralose/SM triangle tests. In the sucralose vs. sucralose /ASK test, the panelists chose the correct sample 15 out of 30 times (p=0.043). The descriptive panel was able to perceive a difference between the two samples at the 95% confidence level. In the sucralose vs. sucralose/SM test, the panelists chose the correct sample 10 out of 30 times (p=0.568). The descriptive panel was not able to perceive a difference between the two samples at the 90% confidence level. However, in the triangle containing 2 sucralose vs. 1 sucralose/SM sets, the panelists chose the correct sample 5 out of 10 times (p=0.213). In the triangle tests utilizing 1 sucralose vs. 2 sucralose/SM sets, the panelists chose the correct sample 1 out of 10 times (p=0.983). Basically, the latter would indicate that there was a significant difference in the ability for panelists to distinguish during a triangle test with

sucralose and a modulator, as long as the modulator samples or the predominant samples. If the sucralose samples without modulator are the predominant samples, the lingering is so significant that they cannot distinguish the samples from each other.

Table II. Triangle Taste Evaluation Results
Sucralose vs. Sucralose with Modifier

Triangle Test – Number of Correct Responses

Panelist	Sucralose vs. Sucralose/ AceK (3/panelist)	Sucralose vs. Sucralose SucraMask™ (3/panelist)	2 Sucralose vs. 1 Sucralose/ SucraMask™ (1st viewing only) (1/panelist)	1 Sucralose vs. 2 Sucralose/ SucraMask™ (1st viewing only) (1/panelist)
1	3	2	1	0
2	2	0	0	0
3	1	0	0	0
4	1	0	0	0
5	0	1	1	0
6	1	2	1	0
7	1	0	0	0
8	2	1	0	0
9	3	1	1	0
10	1	3	1	1
Total Correct	15 of 30	10 of 30	6 of 10	1 of 10
p	0.043	0.568	0.213	0.983

Summary

Sucralose is a popular high intensity sweetener that is being used by food and beverage formulators. Certain incongruities resulting from the use of this sweetener such as wateriness, pH impact and lingering sweetness require that the food formulator use modulators to reduce or eliminate these incongruities. As such certain modulators such as gums, acelsulfame–K (ASK) and Sucramask® (SM) are used to modulate the texture and flavor of the food beverage system. The understanding of the binding of sucralose can be easily understood by relating to a 3 way switch model. The function of a modulator that impacts the binding of sucralose can be understood using this model. The potential of SM to remove lactisole is indicated but requires further evaluation. SM is a modulator that displaces the sucralose but at a relatively slow rate such that the intensity of sweetness is perceived but not the lingering. ASK apparently modulates by sharing the sucralose binding site providing a different type of sweetness

character. Care must be taken in selecting the order of triangle testing in that the modulator may be readily overcome by the sucralose only samples and hence the perceived difference with respect to lingering sweetness is very difficult to ascertain.

References

1. SucraMask® is a product of Creative Research Management, 2029 E. Harding Way, Stockton, California 95205 www.crmcorp.net.
2. Munger, Steven D.; Vigas, Stephan; Nie, Yling; Hobbs, Jeanette R.; Conn, Graeme L. *TIR2, TIR3 and the Detection of Sweet Stimuli*, presented at the 231st ACS National Meeting, Atlanta, GA, March 26-30, 2006.
3. Schiffman, Susan S.; Booth, Barbara J.; Sattely-Milller, Elizabeth A.; Graham, Brevick G.; Gibes, Kernon M. Selective Inhibition of Sweetness by the Sodium Salt of ±2-(4-Methoxyphenoxy)-propanoic acid. *Chem. Senses* **1999**, *24*, 439-447
4. The National Food Laboratories, 6363 Clark Avenue, Dublin, California 94568-3097.

Chapter 27

Improving the Taste of Artificial Sweeteners Using Flavors

Masashi Ishikawa[1], Akio Nakamura[1,*], Ayano Fujiki[1], Junichi Ide[1], and Kensaku Mori[2]

[1]Technical Research Center, T. Hasegawa Company, Ltd., 335 Kariyado, Nakahara-ku, Kawasaki 211–0022, Japan
[2]Department of Physiology, Graduate School of Medicine, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo, Japan

Aiming to create flavorings that would bring the taste of artificial sweeteners closer to that of preferred sugar, we used multi-channel near-infrared spectroscopy (NIRS) to measure cortical responses coupled with sensory evaluation. As a result, it was noted that a conditional sugar solution reduced the amplitude of the response to the test sugar or artificial sweetener solution. In other words, the cortical response to a test solution was found to show adaptation by the conditional sugar solution. Sugar-sugar self adaptation was significantly greater than sugar-artificial sweetener cross adaptation recorded at specific regions of the temporal and frontal cortex. The sugar-artificial sweetener difference in taste could thus be monitored by the difference in cortical responses. Furthermore, sugar-flavored artificial sweetener cross adaptation tended to come close to sugar-sugar self adaptation among the subjects who sensed improvement of the taste of an artificial sweetener by addition of a particular flavoring.

In response to recent, markedly growing consumer demand for low-calorie/sugar-free food products containing non-nutritive artificial sweeteners, such sweeteners have become increasingly used as sugar substitutes (*1*). However, it has been known for some time that despite improvement these sweeteners when compared to the preferred taste of sugar still differ in taste (*2,3*). This difference likely comes from complex multi-sensory modalities. For example, bitterness comes from gustatory modality (*4*). Astringency and the aftertaste might come from somatosensory modality. Flavor is also an important factor to differentiate the taste of artificial sweeteners from that of sugar. Commercial granulated sugar has its specific and preferred taste and odor. Artificial sweeteners do not possess such sugar-like flavor.

Here, our attention is focused on the importance of olfactory modality in sensing sugar flavor. We have sought to improve the taste of artificial sweeteners by applying sugar flavorings, because of the continuing preference of many consumers for the taste of sugar. We hypothesized that adding particular flavorings might reduce the sugar vs. artificial sweetener difference in taste and thereby improve the taste of artificial sweeteners.

In order to evaluate the improvement and the difference in taste, we have used two methods. The first method is by subjective sensory evaluation with the second method being by optical imaging of cortical responses to sweeteners using near-infrared spectroscopy (NIRS). NIRS is a non-invasive optical technique that continuously monitors cerebral hemodynamics (*5*) for the assessment of functional activity in the human brain (*6-11*). Although NIRS measurements are limited to the cortical surface, changes in the concentration of oxygenated and deoxygenated hemoglobin in the cerebral vessels can be measured and taken as indicators for cortical activation.

In the current study, using multi-channel NIRS, we sought to monitor cortical activity during the sensory evaluation. Our first objective was to detect the difference between cortical responses to sugar and artificial sweeteners using the optical imaging method. Our second objective was to create flavorings that would minimize the sugar vs. artificial sweetener difference in cortical responses and also minimize the difference found by the sensory evaluation.

Materials and Methods

Subjects

Twenty-four healthy volunteers (fifteen male and nine female, mean age 35.6 ± 8.3 years) participated in this study for three straight days. Written informed consent was obtained after a complete explanation of the study. To avoid any influence of environmental stress, each subject was seated comfortably

in a temperature, humidity, and brightness controlled room throughout the experiments.

Optical Imaging

Optical imaging was conducted with the ETG-4000 Optical Topography System (Hitachi Medical Co., Japan) using a 3 × 11 optode set (consisting of 16 photo-detectors and 17 light emitters) providing a total of 52-channels. Near-infrared laser diodes with two wavelengths (695 and 830 nm) were used as light emitters. Reflected lights were received by photo-detectors located 30 mm from the emitters. The optodes, which were mounted on a flexible cap, were carefully positioned on each subject's head so that the position was similar for all subjects. This configuration thus enabled us to detect signals simultaneously from the 52-channels which covered a 60 × 300 mm^2 frontal area of the cortex in both hemispheres. Signals reflecting the relative oxygenated hemoglobin concentration ([oxyHb]), deoxygenated hemoglobin concentration ([deoxyHb]) changes were recorded from a starting baseline.

Procedure

In this experiment, a high sweetness sugar substitute such as aspartame was used for the artificial sweetener solution. The degree of sweetness was converted to sugar equivalence. Ten mL granulated sugar (6%) for the sugar solution, aspartame (0.036%), or flavored aspartame solution was given to subjects as a test sample using a disposable cup with a straw. The flavorings contained volatile compounds from which subjects sensed granulated sugar-like sweet odor, based on analysis of raw cane sugar aroma constituents (12). Before measurement, subjects were trained to retain in mind taste characteristics of a sugar solution and that of an artificial sweetener solution. Test samples were given to the subjects one by one. After the optodes were placed on the subject's head, the subject was ready to start the sensory evaluation task. At time 0, we asked the resting subject to start the task. The subject would then pick up the cup and after a few seconds start to drink the given sample solution, then put back the cup on the desk. The subject would finish drinking by 5 seconds after the starting each, then concentrate on the sensory evaluation.

After each task, the subject filled out a sensory evaluation questionnaire, comparing test samples and the corresponding conditional sugar solution. Five descriptors were used, namely, sweetness, odor, bitterness/astringency, sweetness aftertaste, and bitterness aftertaste, with each being rate on a scale ranking from -3, indicating "much weaker" to +3, indicating "much stronger", with 0 as the same when compared to the conditioning.

Results and Discussion

Optical Imaging of Cortical Responses

When subjects tasted a sugar or an artificial sweetener solution, a distinctive increase in [oxyHb] and a decrease in [deoxyHb] were observed in specific regions of the frontal and temporal cortices. The intensity of the changes were maximum in the temporal portion of the measurable area in both left and right cortical regions with the middle area showing no clear increase and decrease. The left half of Figure 1 shows the time course of typical changes in [oxyHb] evoked by the sensory evaluation task. [OxyHb] increased to a maximum level with a peak latency at about 25 seconds, then gradually returned to the baseline. Among the twenty-four subjects examined, such clear and robust increases in [oxyHb] were observed in fourteen subjects (58%). However, the remaining ten subjects showed little or no responses. To further assess the difference in cortical responses to sweetener solutions, we concentrated on analysis of such clear changes in [oxyHb] as cortical responses obtained from the fourteen subjects.

The fourteen subjects showed such cortical responses to sugar and aspartame, regions evoked by the drinking of an aspartame solution being quite similar to those evoked by a sugar solution. As a first step, we addressed the question whether the amplitude of the cortical responses differ between a sugar solution and an artificial sweetener solution. Systematic analysis of the cortical responses in the fourteen subjects showed, however, that there are no statistically significant difference in the amplitude of the cortical responses between sugar and aspartame. Furthermore, a subject showed a greater response to aspartame in one experiment, whereas in another experiment the same subject showed a greater response to sugar.

In these experiments, we measured cortical responses to sugar, aspartame, and flavored aspartame in a sequential manner. Therefore, we thought that an amplitude of the cortical response to a sample was influenced by the previous response.

Self Adaptation and Cross Adaptation of Cortical Responses

When a subject drank a sugar solution, then after 60 seconds drank a second sugar solution, a significant reduction of the amplitude of the second response was noted. The amplitude of the response to the test sugar solution was apparently influenced by the previous response to the conditional sugar solution as shown in Figure 1. This is a self adaptation of cortical responses to sugar solutions. When the test solution was changed to aspartame, we noted that the conditional sugar solution also reduced the amplitude of the response to the test aspartame solution. This is a cross adaptation of cortical responses to sugar-

aspartame solutions. In other words, the cortical response to an aspartame solution showed cross adaptation by the conditional sugar solution. It can be noticed that the sugar-aspartame reduction was smaller than the sugar-sugar reduction as shown in Figure 1. This raised the possibility that sugar-sugar self adaptation might be greater than sugar-aspartame cross adaptation of cortical responses.
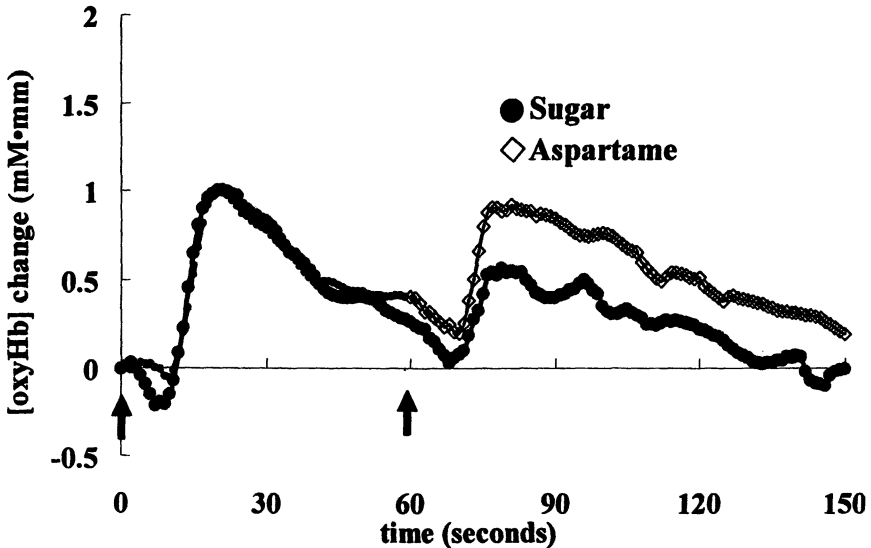
Figure 1. Typical cortical responses to conditional sugar–test sugar solutions and conditional sugar-test aspartame solutions.

If this is the case, the comparison between cross and self adaptation could be a useful tool to evaluate the difference between cortical responses to sugar and artificial sweeteners. We thus addressed the question whether sugar-sugar self adaptation was greater than sugar-aspartame cross adaptation of cortical responses. In order to quantify these adaptations, the sugar solution was always given to subjects as a conditioning before every test sample solution. We then compared the ratio of adaptations between test samples by calculating the ratio of the amplitudes of responses to test samples and those of the previous responses to the conditioning. In order to avoid order effect within one day, we compared the cortical responses to the first pair of conditioning and test solutions, although the measurement of the cortical responses to the conditioning and test solutions were repeated four times in one day.

Comparison between Self Adaptation and Cross Adaptation

Figure 2 shows a comparison between sugar-sugar self adaptation and sugar-aspartame cross adaptation recorded at a specific region (channel 41, CH 41). In the fourteen subjects, twelve subjects showed clear and robust responses in this region. Therefore, we compared the ratio of adaptations in these twelve subjects. Of the twelve, in ten subjects (83%), sugar-sugar self adaptation was greater than sugar-aspartame cross adaptation, and the opposite results were noted in the remaining two subjects. Statistical analysis indicated that sugar-sugar self adaptation was significantly greater than sugar-aspartame cross adaptation in CH 41 (P=0.012, paired t-test). These findings support the hypothesis that sugar-sugar self adaptation is greater than sugar-aspartame cross adaptation of cortical responses.

The solid circle in Figure 3 indicates the cortical region (CH 41), where we observed larger sugar-sugar self adaptation as compared to the sugar-aspartame cross adaptation. Also in the surrounding regions of the left side of the brain and in some regions of the right side (dotted areas in Figure 3), sugar-sugar self adaptation tended to be greater than sugar-aspartame cross adaptation. However the level of statistical significance was lower than that of CH 41 ($0.05 < P < 0.1$, paired t-test)

The difference between cortical responses to sugar and aspartame can thus be detected using the optical imaging method.

Sensory Evaluation

In the sensory evaluations provided by all the twenty-four subjects who participated in this research, subjects reported that the bitterness/astringency, the sweetness aftertaste and the bitterness aftertaste of aspartame were stronger than those of sugar. Subjects also reported that while a particular flavored aspartame had a profile which resembled aspartame, the bitterness/astringency and the sweetness aftertaste of aspartame were significantly reduced by this flavoring ($P < 0.05$, Wilcoxon's signed-ranked tests).

Figure 4 gives the summary of the sensory evaluations from the selected fourteen subjects. According to the results of the sensory evaluations of this particular flavored aspartame, the subjects were divided into two groups. Eight subjects reported that this flavoring effectively reduced the bitterness/astringency and the sweetness aftertaste of aspartame as shown in Figure 4A. These reductions were statistically significant ($P < 0.05$, Wilcoxon's signed-ranked tests). On the other hand, the remaining six subjects reported that the bitterness/astringency, the bitterness aftertaste, and the sweetness aftertaste of this flavored aspartame were stronger than that of aspartame as shown in Figure 4B.
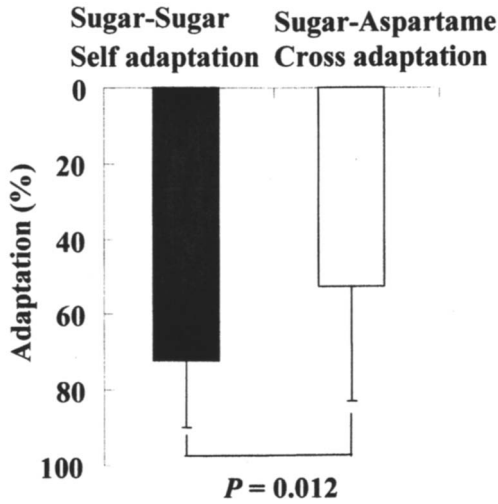
Figure 2. Comparison between sugar-sugar self adaptation and sugar-aspartame cross adaptation of cortical responses recorded at a specific region (CH 41).
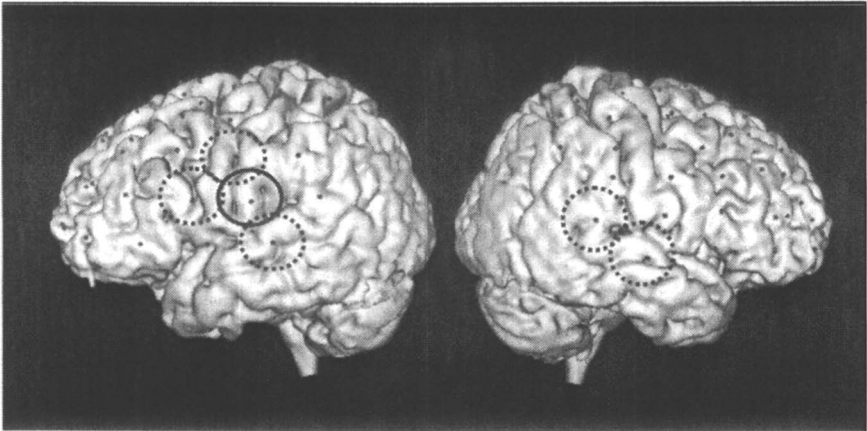
Figure 3. Comparison between sugar-sugar self adaptation and sugar-aspartame cross adaptation of cortical responses.

Screening of additional flavorings for effectiveness or combined usage of non-volatile components was consequently necessary, because almost half of the subjects reported that such added flavoring did not effectively improve the taste of aspartame. Our aim became that all subjects and by extension, all customers would perceive improvement by using a particular flavoring. However, in this research, eight subjects felt the taste of this flavored aspartame came close to that of sugar, whereas six subjects felt the taste was the same as that of aspartame.
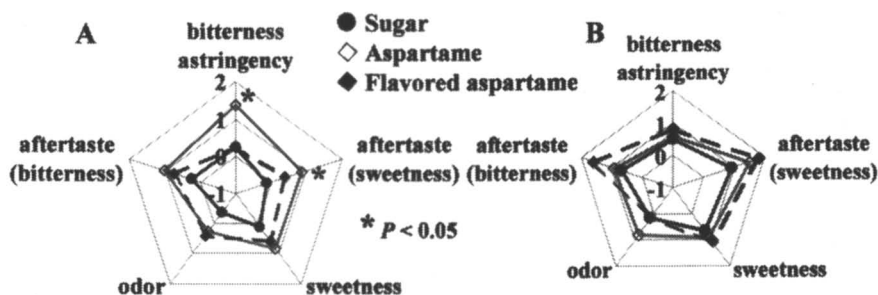
Figure 4. Spider-web-diagram resulting from tasting of sugar, aspartame, and flavored aspartame (A) in eight subjects who felt improvement in the taste of aspartame by the flavoring, (B) in six subjects who did not feel improvement.

Evaluation of the Effect of Added Flavorings

The difference between cortical responses to sugar and aspartame was detected, with likewise a different in sensory profile in aspartame versus sugar being also noted. These findings may indicate that the difference in taste correlates with the difference in cortical responses. This raised the possibility that if the better tasting sample were found to resemble a sugar solution, sugar-sample cross adaptation would come closer to sugar-sugar self adaptation. We thus compared sugar-flavored aspartame cross adaptation to sugar-aspartame cross adaptation in the eight subjects who sensed improvement by addition of the flavoring.

First, statistically significant larger sugar-sugar self adaptation as compared to sugar-aspartame cross adaptation was confirmed in the eight subjects ($P<0.05$, Dunnett's multiple comparison test) in regions including CH 41 (dotted areas in Figure 5). Second, sugar-flavored aspartame cross adaptation tended to be greater than sugar-aspartame cross adaptation ($0.1<P<0.11$, Dunnett's multiple comparison test) in the same dotted areas in Figure 5. Consequently, in the eight subjects who sensed that the taste of this flavored aspartame solution came close to that of a sugar solution by the flavoring, sugar-flavored aspartame cross

adaptation tended to come closer to sugar-sugar self adaptation. On the other hand, in the six subjects who did not sense improvement by addition of the flavoring, sugar-flavored aspartame cross adaptation differed further from sugar-sugar self adaptation.

According to these findings, the observed difference in adaptations was thought to reflect the difference in taste of sweetener solutions in the fourteen subjects. Therefore, the difference in adaptations could be an effective indicator for creating flavorings that minimized the difference found by sensory evaluation as well as the difference in cortical responses.
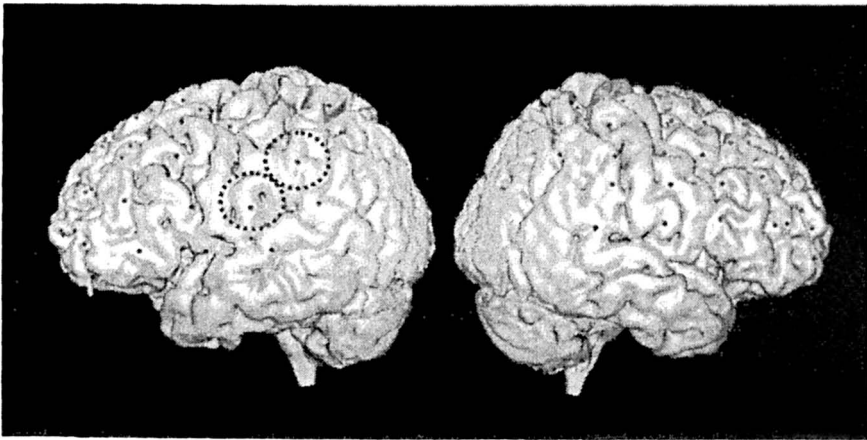
Figure 5. Comparison of adaptations between sugar, aspartame, and flavored aspartame in eight subjects who felt improvement of the taste of aspartame by the flavoring.

Conclusion

The sugar-artificial sweetener difference in taste can be monitored by the difference between sugar-sugar self adaptation and sugar-artificial sweetener cross adaptation of cortical responses. Multi-channel NIRS was sensitive enough to detect the difference. In addition, the sugar-flavored artificial sweetener similarity in taste might be estimated by the difference in adaptations of cortical responses.

It is still uncertain just how the brain functions in relation to the perception of sweetness. However, the method of recording cortical responses to various foods with flavors may help improving the perceptual quality of the foods.

References

1. Lindley, M. G. *World Rev. Nutr. Diet.* **1999**, *85*, 44-51.
2. Sprowl, D. J.; Ehrcke, L. A. *J. Am. Diet. Assoc.* **1984**, *84*, 1020-1022.
3. Horne, J.; Lawless, H. T.; Speirs, W.; Sposato, D. *Chem. Senses* **2002**, *27*, 31-38.
4. Kuhn, C.; Bufe, B.; Winnig, M.; Hofmann, T.; Frank, O.; Behrens, M.; Lewtschenko, T.; Slack, J. P.; Ward, C. D.; Meyerhof, W. *J. Neurosci.* **2004**, *24*, 10260-10265.
5. Jobsis, F. F. *Science* **1977**, *198*, 1264-1267.
6. Chance, B.; Zhuang, Z.; UnAh, C.; Alter, C.; Lipton, L. *Proc. Natl. Acad. Sci. USA.* **1993**, *90*, 3770-3774.
7. Villringer, A.; Planck, J.; Hock, C.; Schleinkofer, L.; Dirnagl, U. *Neurosci. Lett.* **1993**, *154*, 101-104.
8. Koizumi, H.; Yamashita, Y.; Maki, A.; Yamamoto, T.; Ito, Y.; Itagaki, H.; Kennan, R. *J. Biomed. Opt.* **1999**, *4*, 403-413.
9. Hoshi, Y. *Psychophysiology* **2003**, *40*, 511-520.
10. Obrig, H.; Villringer, A. *J. Cereb. Blood Flow Metab.* **2003**, *23*, 1-18.
11. Plichta, M. M.; Herrmann, M. J.; Baehne, C. G.; Ehlis, A. C.; Richter, M. M.; Pauli, P.; Fallgatter, A. J. *Neuroimage* **2006**, *31*, 116-124.
12. Abe, E.; Nakatani, Y.; Yamanishi, T.; Muraki, S. Proc. Japan Acad. Ser B **1978**, *54*, 542-547.

Chapter 28

Why Should an Organic Chemist Study Sweet Taste?

Angela Bassoli[1], Gigliola Borgonovo[1], Gilberto Busnelli[1], and Gabriella Morini[1,2]

[1]DISMA, Università degli Studi di Milano, Via Celoria 2,
I–20133 Milano, Italy
[2]Università di Scienze Gastronomiche, Piazza Vittorio Emanuele 9,
I–12060 Pollenzo (CN), Italy

The interest for sweeteners started well before the systematic study of sweet taste. Nevertheless, it was the hypothesis of the existence of a specific chemoreception mechanism that stimulated organic chemists to consider this area a fertile field for basic research and applications. Organic chemistry has given a large contribute to the elucidation of the sweet taste mechanism and to the discovery of new active compounds. The synthesis of new natural and artificial ligands with an appropriate control of conformational and stereochemical properties; the study of physico-chemical properties in order to optimise chemical stability and solubility; the calculations of molecular descriptors to be used in structure-activity relationships and to develop new models of ligand-receptor interactions: these are only some of the many aspects that an organic chemist could develop in studying sweet taste and sweeteners. The chapter will focus on the experience of our research group in various aspects of organic chemistry applied to the study of sweet taste.

Structure Complexity and Diversity

In the study of organic chemistry we often meet some basic (fundamental) concepts: structure complexity and diversity; intermolecular interactions; conformation; stereochemical features; structure-activity relationship are only some of these. All these aspects are easily found in the chemistry of sweet taste and therefore this research is a very good training for organic chemists.

One of the most fascinating aspects of organic chemistry, for people that for the first time approaches this matter, is the incredible diversity of organic compounds. While inorganic compounds seems to be formed by many atoms arranged in relatively few geometries, the organic material is formed by only few atoms that give rise to a plenty of different molecular architectures. Even more surprising is the fact that quite often compounds that "look" different are recognized as similar in a biological mechanism. In the chemistry of sweeteners this phenomenon is particularly evident, as shown schematically in Figure 1.
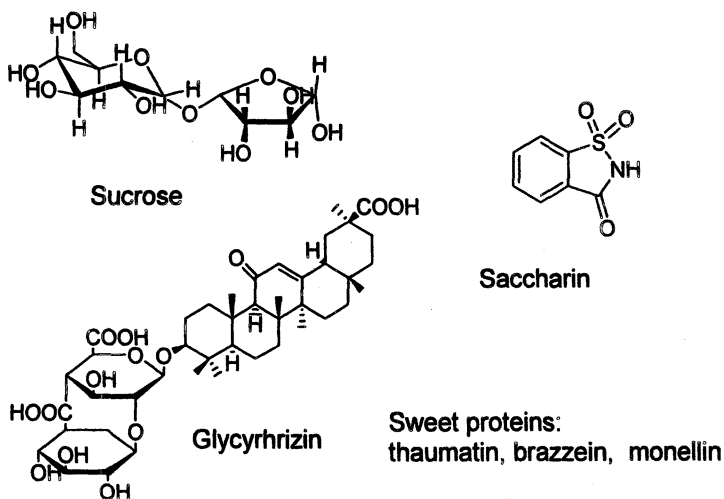
Figure 1. sweet compounds are a very good example of the concept of diversity in organic molecules.

Looking at the structures of sweet compounds, we found many classes of molecules of various molecular weight, from proteins as thaumatin, brazzein or monellin to small compounds as cyclamates or saccharine, passing through sugars, terpenes, flavonoids, amino acids and more. In fact, one of the puzzling

434

questions to which chemists who study taste have tried to answer for a long time is: how can a similar diversity account for a unique chemoreception mechanism? This question - from a theoretical point of view - can be formulated as: which are (if any) the molecular determinants that all these molecules have in common to explain their biological action? Even if diversity is commonly found in groups of compounds having similar functions, there is no doubt that the case of sweet compounds is extraordinary and probably is beyond comparison, since the activity is common to such different molecules.

This introduces another very well known topic in organic chemistry, that generally goes under the definition of "structure-activity relationship"; the action of simplify complex structures, discriminate groups with similar characteristics but different features, and compare them in a lot of compounds in order to make the common elements to come out. Similarly, the definition of "glucophore" clearly derives from the general concept of "pharmacophore" in the chemistry of biologically active substances. The definition of glucophores - the functional groups able to give the sweet taste to a molecule when put in a correct topological arrangement - came out from a linear and highly logic developement since the initial formulation of the theory by Shallenberger and Acree *(1)* up to now.

At the beginning and until few years ago, the existence of a receptor protein (or of several proteins) able to bind sweet ligand was already present but no information was available on its putative structure. Nevertheless as in many other cases, the starting point was to imagine which kind of credible binding could "hold together" the small molecule and the receptor. Obviously the attention was directed to the most common modes of intermolecular interactions found in biological and organic chemistry, as the system formed by two parallel hydrogen bonds holding together the sweet ligand and the taste receptor protein. Such intermolecular interactions are indeed very common and familiar to organic chemists: the double helyx of nucleic acids as well as the aminoacid strains in beta-sheet conformation are two well known examples of this. Similarly the functional groups that are effective in giving this kind of binding are very well known as alcohol, amine, carbonyl and carboxylic groups.

The initial postulated existence of the AH and B glucophores (hydrogen bond donor and acceptor) was therefore immediately understood and accepted by the organic chemists, and groups with consistent structure and positions to be identified as AH and B glucophores were quite easily found on most of the known sweet compounds. Similarly, the hypothesis of an hydrophobic area (the γ or X site) in the sweet molecules, able to establish positive hydrophobic interactions with hydrophobic residues in the receptor active site *(2)* sounded very attractive for organic chemists, who were used to find this kind of intermolecular interactions in biological systems. This first, famous three-sites topological model for the sweet taste receptor proved to be an excellent tool for organic chemists to a) compare known structures to the model in order to explain

experimental data and b) design new compounds based on this theoretical features. Many groups of synthetic organic chemists included us have extensively used the model to study different classes of compounds. Due to the simplicity of this model, initially even the simple help of framework molecular models was sufficient to verify the topology by constructing a new compound and measuring the distances among the putative glucophores, at least when the molecule was sufficently small and/or rigid, as in the case of saccharine derivatives. In the following years many evidences lead to the hypothesis of the existence of further glucophores; the well known multi point attachment model by Tinti and Nofre *(3, 4)* is the best example of this. This model, obtained by the systematic study of a series of hyperpotent guanidinic derivatives having rigid conformations, was extremely useful: the nine points of attachment were well described in their chemical features (hydrogen donor and acceptor, hydrophobic or pi- stacking interactions and so on), and their Cartesian coordinates in the space were given, allowing all researchers to compare other new and old compounds in order to identify the glucophores. Speacking with many colleagues at that time and in the following years, it was very funny to tell each other how we had found a way to reproduce the Tinti-Nofre model in our laboratories. I personally still have in my office a "solid" version made in the "pre-computer modelling era" by one of my students and her boyfriend with corks covered by playing paste as atoms, each one coloured in a different color, and wood skewer of appropriate leght as bonds, of course respecting the classic proportions of the Dreiding models. Figure 2 shows this model used to compare the likely glucophores in a new molecule and their relative distances, in order to predict its possible activity on the sweet taste.

The exercise of "docking" a molecule (i.e. a molecule madr by a framework molecular model) with the Tinti and Nofre model trying to make them fit to each other in every possible way was extraordinarily useful and stimulating, and I guess that many interesting intuitions and new hypotheses on the sweet taste mechanism and the role of glucophores came out from that kind of exercise made by organic chemists.

Of course not so easy is the case of compounds having a larger degree of conformational freedom. And again, this introduces another of the initially listed fundamental topics in organic chemistry, i.e. conformation analysis.

Conformation in Sweet Compounds: Active vs Minimun Energy Conformation

The concept of "minimum energy conformation" for an organic compound is introduced already in the basic chemistry courses. Molecules are flexible, due to the free rotation of atoms through sigma bonds and in the case of C-C bonds these rotations give rise to an infinite number of different possible conformers,
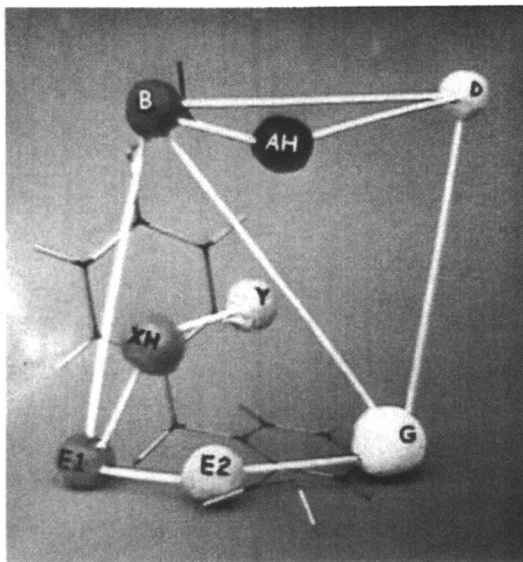
Figure 2. an "historic" example of the topological multi point attachment model by Tinti and Nofre.

each representing one of the "postures" that this flexible object can have in the three-dimensional space. The use of chemical formulas (that are "static" objects) sometimes induces the students (and even researchers) to forget the existence of many conformations, but organic chemists are well aware that this concept has not to be disregarded when dealing with biological phenomena as the interaction of a ligand with a receptor or an enzyme. In this case, it is fundamental to understand how the molecule arrange itself in order to give the best interaction, and such an arrangement is defined as the "active conformation", which is not necessarily identical or even similar to a minimum energy conformation. These aspects are easily studied with the instruments of molecular modelling, and even in the sweeteners area we have a lot of studies intended to understand which is the active conformation for known sweet compounds. The degree of complexity of this problem could be very different from the theoretical and practical point of view; small and very rigid compounds have little conformational freedom and high energetic barriers for rotations, so that the minimum energy conformation(s) is presumably similar to the active one and is easily obtained. This is for instance the case of compounds as saccharine and acesufame-K or polyciclic compounds such as for istance sweet naphthimidazolesulfonic acids *(5)* or haematoxylin derivatives *(6)* or for the very rigid guanidinic compounds as sucrononate *(3,4)* and analogues. These compounds were in fact very useful to "probe" the space available around the ligand in order to derive a 3D map of the cavity in the

receptor active site. This kind of approach was for istance very useful in defining this cavity as "flat" in a certain region, a description which very well accounted for the activity of different stereoisomers of simple aminoacids. Some conformational problems were much more difficult to be solved. Several studies have been made on the active conformation of aspartame and other sweet and bitter peptides *(7-9)* using both molecular modelling and NMR techniques in order to find the active conformation for this important class of tastants. The study of conformation was also particularly hard for sucrose itself -the most important sweet compound in nature. This compound is so complicated by the conformational point of view that many theoretical chemists have studied for a long time which it could be its active conformation and this point has been extensively reviewed (*10*).

In some cases analogues of known active compounds having blocked conformations have been expressly designed to give suggestions on the active conformation. We followed this approach in order to have some hints on the active conformation of isovanillic derivatives, which generally have the aromatic ring containing the AH and B system able to rotate freely respect to the heterocyclic fragment, corresponding to the hydrophobic G site. The problem was approached in two ways. First, we synthesised two groups of rigid derivatives representing the two "limit" situations, one having the two rings coplanar, and the other almost perpendicular. From point of view of synthetic organic chemistry the solutions were found by using the flat molecule of a steroid having all *cis* junctions as a synton in the first case, and by introducing a spiranic carbon in a strategic position in the other case *(11)*. Therefore, the search for a "ideal" 3D-structure was very stimulating to develop new synthetic approaches and solutions. Another feedback of this kind of problem, was the idea to develop structure – activity relationship based only on geometric descriptors of the molecules, such as torsion and dihedral angles defining the minimum energy conformation for each derivative of a series. Again this effort was surprisingly fruitful, since it permitted to obtain not only useful information on the active conformation, but also on the role of absolute stereochemistry on taste.

Stereochemistry and the Sweet Taste, an Ideal Combination

Stereochemistry is one of the main field of study for organic chemistry, especially in the field of synthesis and natural products. To have a synthetic pathway to access a natural-identical compound is important not only for confirming the chemical structure of a new active principle isolated from a natural matrix, but also for obtaining it in amounts advisable for performing several analyses such as spectroscopic investigations, toxicity tests, sensory

evaluation and any kind of measurement of physico-chemical properties as solubility, thermal stability and so on. This is particularly true for organic compounds that have a potential as food additives, since in this case the amount of experimental work to be done in order to verify a possible industrial application could be huge.

As the organic chemists well know, the existence of stereogenic centers on a molecule usually make much harder to find a convenient synthetic process, especially on large scale; it is not a case if some of the most important commercial sweeteners in the past (as saccharine or cyclamates) are very easyly obtained by synthesis, also due to the fact that they are achiral. Nevertheless, the challenge to manage appropriately the stereoselectivity of chemical transformations has been faced from a long time and today there are several methods available, either using chiral reagents and catalysts or enzymatic and microbiological transformations. In many cases, it is of fundamental importance the possibility to have a cheap precursor with the desired configuration: aspartame, wich is a modified peptide, has this advantage since aminoacids are - generally speaking - efficient and cheap chiral syntons. Aspartame is also a good example to introduce another familiar concept, i.e. the stereoselectivity of the recognition in biological system: only one of the four stereoisomers of aspartame is in fact sweet, the other being tasteless or bitter. However, sometimes things are not so easy, especially for complex structures as those of many natural sweet compounds such as the threeterpene osladin or glycirrizin.

Even molecules that could look quite simple can pose interesting problems of stereochemistry that are difficult to overcome. An example of this is that of phyllodulcin 1 and isovanillic derivatives, that our research group ave studied for a long time. These compounds , shown in Figure 3, have a single stereogenic center, but its position - at least in the most active terms of the series - is critical.
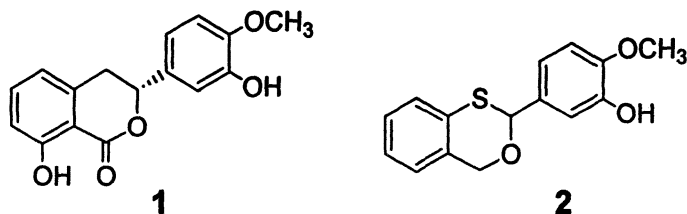
Figure 3. R-(+)-phyllodulcin and one of its synthetic analogues of the isovanillic family: two stereogenic centers not easy to manage.

The chiral carbon in R-(+)-phyllodulcin is on a benzylic lactone O-residue, and even if the compound is known from a long time *(12)* up to now only two

stereoselective syntheses *(13, 14)* are described and both have a little potential for industrial applications. Commercial phyllodulcin is in fact the natural compound obtained by extraction and it is therefore very expensive. The problem of stereochemistry is not easily overcome since the taste disappears if the sp^3 carbon is substituted by a planar sp^2 carbon atom; moreover, the role of absolute configuration is critical for taste, being the non-natural S-enantiomer completely tasteless. A similar situation is found also for isovanillic derivatives with different heterocyclic structures, in particular for the very sweet (relative sweetness = 9000) oxathiane derivative **2** shown in Figure 3 that has a thioacetalic arrangement for the stereogenic carbon. This discovery has stimulated our interest for the finding a suitable synthetic method to access these chiral compounds, and two alternatives of general use came out as a chemical feedback of this work, which are schematically shown in Figure 4.
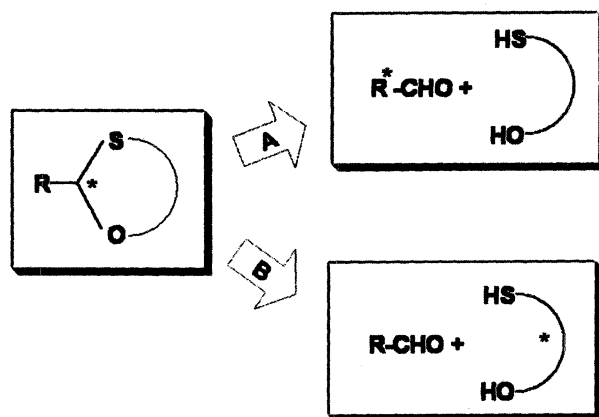
Figure 4. two different approaches to obtain stereoselectively the thioacetalic carbon.

The first approach (A in Figure 4) consists in the use of chiral chromium arene complexes to induce the correct configuration during the formation of the thioacetal *(15)*. The chromium moiety function as a classic "chiral auxiliary" that is removed after the reaction.

In the second approach (B in Figure 4) we used another methodology starting from natural chiral compouns such as canfosulfonic acid to induce diastereoselection in the acetalisation reaction *(16)*. By the way, none of these methods was compatible with all the functional groups and therefore they resulted unefficient in giving the sweet compound shown in figure with the

desired yields and enantiomeric excess. (I would definitely have used chiral lewis acids that put maybe the chirality closer to the reaction site)The single enantiomers in pure form were obtained later by using chiral chromatography, a very useful method that yet have the limitation of very high costs to be applied on industrial scale.

Again, these kind of studies are an excellent and stimulating training ground for organic chemists to apply all the techniques in order to obtain chiral compounds and determine the relative and absolute configuration: chiral separation methodologies, spectroscopic techniques as circular dicroism and anomalous diffraction of X-rays, NMR experiments, use of chiral auxiliaries and catalysts, use of enzymatic transformations.

Enzymes proved to be effective in solving the problem to obtain another very interesting sweet compound, the natural derivative monatin. Our research group has in fact developed a chemoenzymatic synthesis able to give access to all the four stereoisomers of monatin in a pure form (17) and some very efficient syntheses, also based on biotransformations, has been patented (18). Interestingly, these studies demonstrated that in the case of monatin the sweet taste is not a prerogative of only one enantiomer but three (or four, for other Authors) of them are sweet. This finding has a relapse on potential industrial applications, since the obtaining of racemates is much more easy than that of single enantiomers, and also some interesting theoric consequence. In fact, different diastereosiomers can also have different minimum energy conformations, and their comparative study could therefore to give some clues on the chemoreception mechanism (19).

Beyond the "Right Shape": Electronic Factors and Theoretical Organic Chemistry

Molecules are made by atoms, and there is no way to forget that our description of the "chemical objects" cannot leave aside the existence of electrons and of their interaction as the "core" of chemical binding phenomena. Actually, at the beginning of our story, we first stated that the electronic nature of the glucophores, beside their topology, is conclusive in establish their role. Also in this case, the study of sweeteners has been a fertile field of applications for all those theoretical methodologies that are intended to study the electronic distribution of a compound in relationship with its activity and there are many excellent examples of this (see for instance 20). The use of calculations to have information on molecular parameters as electrostatic potentials, charge distribution, HOMO and LUMO energy and much more are a prerogative of theoretical chemistry, but the barriers between this field and the "traditional"

organic chemistry is becoming thinner and thinner so that in every advanced laboratory these two professional skills work together in a synergistic mode. A number of big and small problems still wait to be solved with the help of this kind of calculations. Just to make an example, in our laboratory we synthesised the two oxathianes in Figure 5, having identical structures except for the positions of sulfur and oxygen atoms in the heterocyclic ring.

2
very sweet, RS 9000

3
low sweet, RS 500
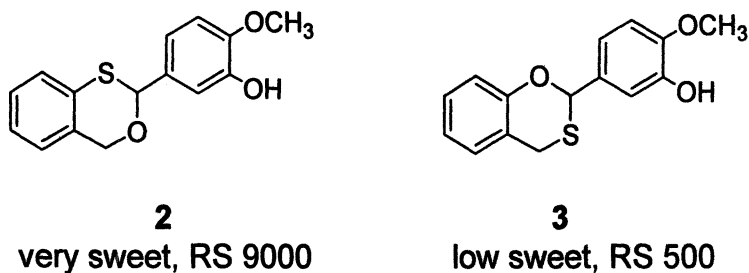
Figure 5. Electronic parameters are probably responsible of the difference in taste of these two oxathianes.

Up to now, and despite the advance models of chemoreception mechanism that are now available, we still do not have a reliable explanation of the very different taste intensity of these two compounds, which is presumably due to a "pure" electronic effect since the geometric and lipophilic features are almost identical.

In the last years the discovery of sweet taste receptor proteins has opened a new frontier in the structure-activity relationships of new active compounds. New models based on homology of taste receptor proteins have been proposed, and they are going to substitute the topological models based on the glucophore concept. This is an important improvement, since the topological models (such as Shallenberger or Tinti–Nofre) had the limitation that they suggested important features that a ligand should have to be active ("positive interactions") but did not give information on the regions that should not be occupied ("negative interactions") in order to fit correctly to the receptor. In other words, these models do not explain why several molecules do fit the model but indeed are not sweet at all ("false positive"). The new homology models allow to mimick the receptor active site(s), and in some cases even to estimate the binding energy among each ligand and the surrounding amino acid residues (*21*), therefore giving a much more reliable interpretation of the activity data and helping in eliminate the "false positive" cases. The new homology models also strengthen

the bridge between organic chemists and molecular biologists, since the study of molecular interactions together with the knowledge on receptor's sequence and structure help to give new hypotheses and explanations on the putative binding site(s), the role of allosteric modulators and inhibitors and the synergy phenomena. To sum up, several aspects of organic chemistry are found in the story of sweeteners developement. The contribution of organic chemistry is large and still growing, since the increasing knowledge of the chemoreception mechanism at molecular level give to organic chemists new suggestions, hints and hypotheses to work out. Most important, they have also important feedback from this research, in terms of developing new synthetic methods, stereoselective reactions, theoric calculations that can then be applied to a large number of different compounds and again contribute to the growing of scientific knowledge. Moreover, the sweeteners area is stimulating in addressing the interest of young chemists from the basic science towards the solution of practical problems with large impact on food industry, consumers science and also medicin, therefore being a very useful training ground for scientific and professional growth.

References

1. Shallenberger, R.S.; Acree, T.E. , *Nature*, **1967**, *216*, 480-482.
2. Kier, L.B., *J. Pharm. Sci.*, **1972**, *61*, 1394-1397.
3. Tinti, J.M.; Nofre, C. in *Sweeteners discovery, molecular design and chemoreception*, Eds. Walters, D.E.; Orthoefer, F.T.; DuBois, G.E. American Chemical Society, Washington, DC, **1991**, pp. 206-213.
4. Nofre, C.; Tinti, J.M. in *Sweet taste chemoreception*, Eds. Mathlouthi, M.., Kanters, J.A., Birch G.G., Elsevier, London, **1993**, pp. 205-236.
5. Castiglione Morelli, M.A.; Lelj, F.; Naider, F.; Tallon, M.; Tancredi, T.; Temussi, P.A., *J. Med. Chem.*, **1990**, *33*, 514-520.
6. Arnoldi A.; Bassoli A.; Borgonovo G.; Merlini L.; *J. Chem. Soc., Perkin Trans 1*, **1995**, 2447-2453.
7. Castiglione Morelli, M.A.; Lelj, F.; Naider, F.; Tallon, M.; Tancredi, T.; Temussi, P.A., *J. Med. Chem.*, **1990**, *33*, 514-520.
8. Kamphuis, J.; Lelj, F.; Tancredi, T.; Toniolo, C.; Temussi, P.A., *Quant. Struc.-Act. Relat.* **1992**, *11*, 486-491.
9. Yamazaki, T.; Benedetti, E.; Kent, D.; Goodman, M. *Angew. Chem., Int. Ed. Engl.*, **1994**, *33*, 1437-1451.
10. *Sweet taste chemoreception*, Eds. Mathlouthi, M.., Kanters, J.A., Birch G.G., Elsevier, London, **1993**.
11. Arnoldi A.; Bassoli A.; Borgonovo G.; Drew G. B.; Merlini L.; Morini G.;, *J. Agric. Food Chem.*, **1998**, *46*, 4002-4010.

12. Y. Asahima, Y.; Asano J.; *Ber. Dtsch. Chem. Ges.* **1931**, *64*, 1252-1256.
13. Zehnter R.; Gerlach H.; *Tetrah. Asymm.* **1995**, *6*, 2779-2786.
14. Ramacciotti A.; Fiaschi R.; Napolitano E.;, *J. Org. Chem.*, **1996**, *61*, 5371-5374.
15. Bassoli A.; Merlini L.; Baldoli C.; Maiorana S.; Drew M.G.B.; *Tetrahedron Asymmetry*, **1996**, *7*, 1903-1906.
16. Bassoli A.; Borgonovo G.; Merlini L.; Morini G.; *Tetrahedron: Asymmetry*, **2000**, *11*, 3177-3186.
17. Bassoli A.; Borgonovo G.; Busnelli G.; Morini G.; Drew M. G. B.; *Eur. J. Org. Chem.*, **2005**, 1652-1658.
18. Abushanab, E.; Arumugam, S.; Patent (1999): US 5994559 A, Nov 30. Kawahara S.; Amino Y.; Mori K.; Funakoshi N.; Takemoto T.; Patent (2003)b: WO2003059865. Abraham T. W.; Cameron D. C.; Dalluge J.; Hicks P.M.; Hobson R. J.; McFarlan S. C.; Millis J. Rosazza J.; Patent (2003c): WO2003091396. Sugiyama M.; Watanabe K.; Kashiwagi T.; Suzuki E.; Patent (2004): WO2004053125.
19. Bassoli A.; Borgonovo G.; Busnelli G.; Morini G.; Drew M. G. B.; *Eur. J. Org. Chem.*, **2005**, 2518-2525.
20. DuBois G.E.; Walters E.; Kellog M.S.; *Sweet taste chemoreception*, Eds. Mathlouthi, M., Kanters, J.A., Birch G.G., Elsevier, London, **1993**, 237-267.
21. Temussi P.A; Bassoli A.; Morini G.; *J.Med. Chem.* **2005**, *48*, 5520-5529.

Chapter 29

Sweeteners and Sweetness Modulators: Requirements for Commercial Viability

Grant E. DuBois

Strategic Research Department, The Coca-Cola Company, One Coca-Cola Plaza, Atlanta, GA 30313

Interest has never been greater in the development of new sweetness technologies which enable more cost-effective food and beverage product formulation, more sugar-like taste quality and reduction in caloric levels. Guidance, targeted to organizations considering development of new sweetness technologies, is given as to the specific requirements for successful commercialization of new sweeteners and sweetness modulators. And the argument is made that a successful new sweetness technology must deliver on nine metrics, including 1) high maximal sweetness response, 2) clean flavor profile, 3) sugar-like temporal profile, 4) sugar-like sweetness adaptation profile, 5) safety, 6) stability, 7) solubility, 8) cost-effectiveness and 9) patentability. Importantly, the argument is also made that a viable new sweetness technology must deliver on *all* nine of these metrics in order to realize commercial success.

Since the middle of the 19th century, chemists have synthesized and characterized from nature many, many sweet-tasting chemical compounds. And a source of fascination for chemists has been the highly diverse relationship between chemical structure and sweet taste. Numerous sweetener structure-activity-relationship (SAR) reviews are available.[1,2,3,4,5] At this time, sweet-tasting organic compounds can be grouped into at least 50 structural classes of compounds. As a result of this historical knowledge of sweetener SAR, the discovery of new and improved sweeteners within existing structural classes is straightforward. However, recent work by a collaborative team from the laboratories of Zuker (University of California, San Diego) and Ryba (National Institutes of Health) on the discovery of the rat sweetener receptor[6] and follow up work by Li and coworkers (Senomyx) on the discovery of the human sweetener receptor[7] should accelerate the discovery of new sweeteners and even new structural classes of sweeteners. An outcome of the work of these scientists is that a cell-based assay is now available which can be used for the high-throughput-screening (HTS) of large libraries of synthetic and naturally occurring compounds. Thus, there is no doubt that the number of new sweet-tasting organic compounds to be discovered will dramatically increase in the future.

In the work by the Zuker/Ryba collaborative team and by Li and coworkers, it was found that the sweetener receptor is a member of the G Protein-Coupled Receptor (GPCR) class of receptors. Further it was found that the sweetener receptor is a GPCR of Family C, a small sub-family of 12 GPCRs, out of the large family of nearly 1000 human GPCRs, which is unique in that its members are distinguished by large extracellular structures often referred to as Venus Flytrap Domains (VFDs). The VFD notation is used because Family C GPCRs bind their ligands in a manner resembling the closure of a Venus Flytrap plant on its prey. Other members of the Family C class of GPCRs include eight metabotrophic glutamate receptors (mGluRs), one γ-aminobutyric acid receptor (GABA$_B$R), one extracellular calcium receptor (ECR) and one glutamate taste receptor (umami receptor). Of considerable interest is the fact that positive allosteric modulators (PAMs) are known for the GABA$_B$R, the ECR, the umami taste receptor and nearly all of the mGluRs. And, while some of these PAMs were serendipitously discovered or identified by traditional methods of drug discovery, most of them have been identified by HTS methods employing cell-based assays.

In summary, a cell-based assay for studying sweetener receptor activation is now known and, while many sweet-tasting organic compounds have been discovered by traditional methods, it is expected that modern HTS methodologies with cell-based assays will enable the discovery of many novel sweeteners. And, importantly, HTS with cell-based assays should also lead to the discovery of sweetener receptor PAMs. However, in order for new sweeteners or sweetener receptor PAMs to be successfully commercialized, they must meet certain minimal criteria for viability. These criteria are as follows:

1. Taste Quality,
2. Taste Quality,

3. Taste Quality,
4. Taste Quality,
5. Safety,
6. Stability,
7. Solubility,
8. Cost Effectiveness and
9. Patentability

In this paper, these 9 criteria for commercial viability are discussed in some detail such that organizations considering commercial development of a sweetener or a sweetener receptor PAM may understand the requirements before embarking on a costly development program. The position of "Taste Quality" as first on the commercial viability list and its listing 4 times is noteworthy. The apparent redundancy is not an error. "Taste Quality" is absolutely critical. Without delivery on this criterion, it makes no difference if a sweetener is safe, stable, soluble, cost-effective and patent-protected. It will not be successful in the marketplace. "Taste Quality" is best quantified by four different metrics thus explaining the multiple listing. And clearly, in order to be used in foods or beverages, a sweetener, or sweetener receptor PAM, must be safe, sufficiently stable, sufficiently soluble, cost-effective and patentable.

1. Taste Quality Metric 1: High Maximal Response. Common sweet foods and beverages, dependent on sucrose for sweetness, contain sucrose in the 10-15% concentration range. Therefore, alternative sweetener systems must be able to deliver 10-15% sucrose equivalents of sweetness intensity. However, while carbohydrate and polyol sweeteners appear to exhibit equivalent high maximal responses, high-potency sweeteners uniformly exhibit lower and variable maximal response. Thus, high-potency sweeteners appear in Concentration/Response (C/R) function behavior as if they are partial agonists. These findings were made in work with an expert sensory panel trained in the scaling of attribute taste intensities (i.e., sweet, sour, salty, bitter, etc.) on a sweetness intensity scale calibrated in sucrose equivalents.[8] The reasons for the apparent partial agonism of high-potency sweeteners, relative to carbohydrate sweeteners is not known, although this observation suggests that carbohydrate sweeteners act through more than one receptor with high-potency sweeteners limited to a single receptor. In this work, the C/R functions for 18 sweeteners were determined. Included were C/R functions for saccharin and cyclamate as are illustrated in Figure 1. C/R function data for high-potency sweeteners are well modeled by the law of mass action $R = R_mC/(k_d + C)$, a model advantaged as it provides the predicted maximal response (R_m) as well as the apparent sweetener/receptor dissociation constant (k_d). Thus from Figure 1, it can be seen that saccharin and cyclamate are predicted to have R_m values equivalent in sweetness intensities to that of 10.1% and 15.2% sucrose, respectively. From the saccharin C/R function, it can be concluded that formulation of foods and beverages with sweetness equivalent to 10% sugar should not be attempted since this level of sweetness intensity can only be reached
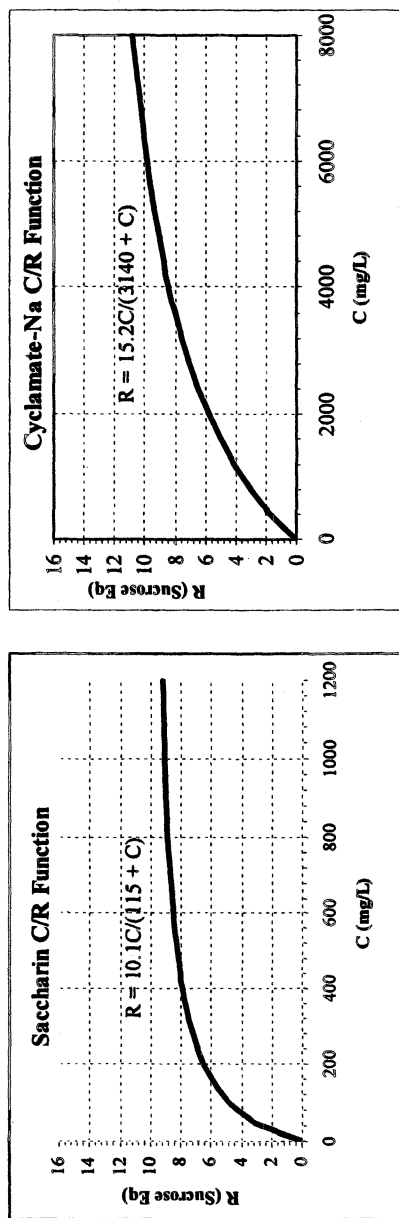
Figure 1. C/R Functions of sodium saccharin and sodium cyclamate in water.

at a very high concentration. At the same time, reaching 10% sucrose equivalency with cyclamate is possible since it exhibits a significantly higher R_m. In practice, however, saccharin is a successful product but, in order to realize this success, it is used in blends where it only contributes 3-4% sucrose equivalents to the sweetener system. As a bottom line, in order for a new sweetener or new sweetener system to be commercially viable, it must be able to deliver a sucrose equivalency of at least 10%. If it cannot, it must be used in blends with other sweeteners. R_m (Sucrose Equivalents) and k_d (mg/L) values for other common high-potency sweeteners, determined by the method described here for saccharin and cyclamate, are as follows: aspartame (16.0; 562), acesulfame-K (11.6; 472), sucralose (14.7; 142) and neotame (15.1; 9.2).

2.   Taste Quality Metric 2: Flavor Profile.   The number of food products sweetened with nonnutritive sweeteners has increased dramatically since the 1960s. The most successful of these products are beverages, especially carbonated soft drinks (CSDs).   Good tasting zero- or near zero-calorie CSDs are now available as alternatives to the sucrose and high fructose corn syrup sweetened products which carry approximately 150 kilocalories per 12-oz serving. This commercial success only occurred, however, following the discovery and commercialization of good-tasting non-caloric sweeteners. Consumers have never shown significant willingness to sacrifice taste quality in their choices of food products. Frederick Helgren of Abbott Laboratories reported a key discovery in 1957, which enabled the 1960s burst of growth in low-calorie foods.[9] He found that blending saccharin and cyclamate salts, in a ratio such that each sweetener contributed equally to the mixture, resulted in a sweetener formulation with improved taste quality relative to either sweetener taken separately. This blend is one of approximately 10/1 cyclamate to saccharin, since saccharin is approximately 10 times more potent than cyclamate.   Cyclamate salts, and especially saccharin salts, when tasted at levels high enough to provide 10% sucrose equivalents of sweetness intensity, exhibit bitter and other negative taste attributes.   Helgren found, however, that these negative taste attributes are substantially ameliorated in the 10/1 cyclamate/saccharin blend.   In fact, the improvement was so significant that, for the first time, zero- and low-calorie food and beverage product alternatives were possible without major compromise in taste.

The taste quality of a sweetener is really only meaningful in the context of a food or beverage product and the taste quality of such products is best assessed by consumer preference or acceptability studies. However, a less resource-intensive technique, often used to predict taste quality, is Flavor Profile Analysis (FPA).[10] In FPA, pioneered at the Arthur D. Little Company in the 1940s, expert sensory panels are used to break down complex and multiple-flavor-attribute systems and to rate attribute intensities. FPA is also used to assess sweetener taste attributes. Thus, for purpose of illustration, the flavor profiles of saccharin and cyclamate in water are provided in Figure 2. These data were obtained by methodology described by Carr and coworkers.[11] Aspartame, sucralose and neotame, when

evaluated in this way, exhibit sweet taste as the only perceptible taste attribute, while acesulfame-K is similar to saccharin with significant bitter and metallic attributes. It is noteworthy that saccharin and cyclamate exhibit bitter taste attributes in addition to sweetness. This finding is now understood for saccharin since saccharin has been demonstrated to activate specific bitterant receptors.[12] It is also of interest to note that a "metallic" taste attribute is reported for saccharin, perhaps similar in character to the metallic taste observed for iron salts. The source of this sensation is not understood. And finally, it is noteworthy that the high concentration of cyclamate evaluated in this study exhibits a salty taste attribute. The source of this note is obvious, however, since the evaluation was carried out with the sodium salt of cyclamate and, in general, sodium salts are salty. As a bottom line, in order to have commercial viability, a sweetener or a sweetener system must be able to deliver clean sweetness at intensity at least equivalent to that of 10% sucrose. Some sweeteners (e.g., saccharin and cyclamate) achieve this requirement only in blend systems.
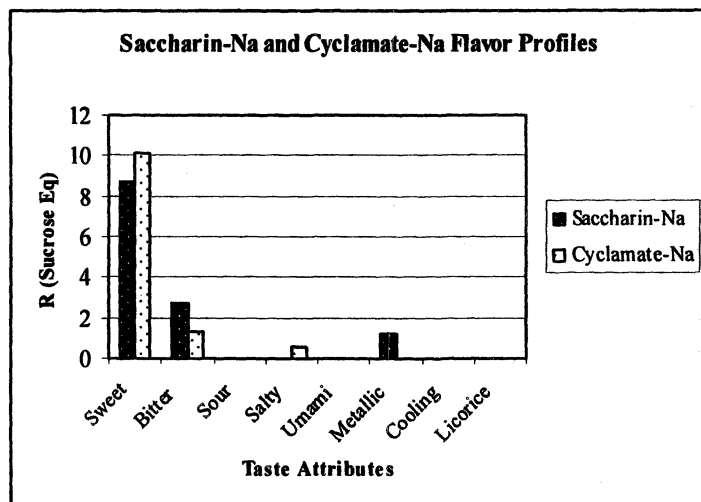
Figure 2. Flavor Profiles of saccharin-Na (384 mg/L) and cyclamate-Na (5930 mg/L) in water.

3. Taste Quality Metric 3: Temporal Profile. Some non-caloric sweeteners are quite similar to sucrose in flavor profile. One might expect that such sweeteners would enable zero- and reduced-calorie products equivalent in taste to sucrose-sweetened products. However, this is not the case. One factor contributing to the difference in taste between the high-potency-sweetener- and sucrose-sweetened products is a difference in sweetness perception over time. The author studied this effect in the flavonoid glycoside class of sweeteners[13] and developed a sensory

method termed Temporal Profile Analysis (TPA) for its quantification.[14] In this method, the time required for a sweetener to elicit maximal sweetness intensity is defined as the Appearance Time (AT) and the time for the perceived sweetness to decline to sweetness equivalent to that of 2% sucrose is defined as the Extinction Time (ET). It was found, in the development of this method, that AT and ET values are concentration dependent (i.e., dependent on sweetness maxima) and thus, it was found essential to normalize all AT and ET values determined, to a common maximal sweetness intensity level. In this work, this level was arbitrarily chosen to be that of 10% sucrose. Normalized temporal profiles for sucrose, aspartame and the licorice-root derived natural sweetener monoammonium glycyrrhizinate (MAG) are illustrated in Figure 3. It is noteworthy that aspartame is slightly delayed and MAG strongly delayed in AT from that of sucrose. And, it is also worthy of comment that aspartame and MAG exhibit ET values that parallel their delays in AT. Empirically, it is always observed that delayed ATs are accompanied with prolonged ETs. The biochemical rationale for delayed AT / prolonged ET behavior for high-potency sweeteners relative to those of carbohydrate sweeteners is not understood. Sweeteners evaluated in the study providing the data for Figure 3 include sucrose (AT = 4 sec; ET = 14 sec), sodium saccharin (AT = 4 sec; ET = 14 sec), sodium cyclamate (AT = 4 sec; ET = 14 sec), aspartame (AT = 5 sec; ET = 19 sec), neohesperidin dihydrochalcone (AT = 9 sec; ET = 40 sec) and MAG (AT = 16 sec; ET = 69 sec). As a bottom line, in order to have commercial viability, a sweetener or a sweetener system must be able to deliver clean sweetness at intensity, at least equivalent to that of 10% sucrose, and without noticeable delay in sweetness onset or prolonged sweet aftertaste. In beverage products today, the temporal problems described have been minimized by blending (e.g., aspartame/acesulfame-K, cyclamate).
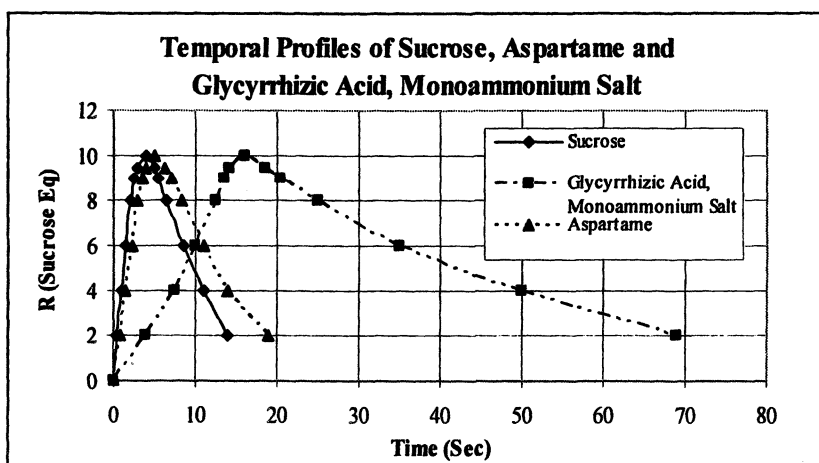
Figure 3. Temporal Profiles of 10% sucrose, aspartame (760 mg/L) and monoammonium glycyrrhizinate (5000 mg/L).

4. Taste Quality Metric 4: Adaptation Profile. In a new sweetener discovery program in the author's laboratory while at The NutraSweet Company, the promising aryl urea new sweetener candidate N-(4-Carboxamido-Phenyl)-N'-(1-(3-Pyridyl)-3-Carboxy-Propyl)-Urea (CPU) was identified.[15] CPU exhibited a high R_m, a clean sweet flavor profile and a temporal profile with acceptable AT and ET. This compound was advanced into preliminary safety assessment studies and into an extensive series of sensory assessments in multiple food and beverage prototypes. However, on evaluation in a cola carbonated soft drink (CSD) system, an unusual behavior was observed. The first sip of the beverage was very good in sweetness quality, quite similar to that of aspartame-sweetened beverages. Surprisingly, however, subsequent sips of the same beverage tasted noticeably less sweet. In order to quantify this effect, a sensory method was developed. In this method, trained panelists tasted cola samples at zero time, rating perceived sweetness intensity, and then tasted and rated again at 30 sec intervals out to an elapsed time of 90 sec. The results of this testing for commercial cola products sweetened with HFSS-55 (HFSS = High Fructose Starch Syrup) and aspartame as well as a cola product prototype sweetened with CPU are illustrated in Figure 4. Thus, it can be seen that the sweetness of the cola with the carbohydrate sweetener HFSS-55 does not significantly diminish over time while that for aspartame-sweetened cola does. However, the CPU beverage prototype sweetness almost drops to a threshold sweetness level at the 4th sip. Thus, CPU has an Adaptation Profile quite dissimilar from carbohydrate sweeteners as well as from aspartame. Subsequent to this finding, we incorporated Adaptation Profile Analysis (APA) into new sweetener candidate evaluation.

As discussed above, the sweetener receptor is a GPCR and it is well known that GPCRs undergo adaptation, often referred to as desensitization, following activation.[16] This process is a multi-step cascade of reactions and is initiated by specific kinases known as G Protein-Coupled Receptor Kinases (GRKs) which phosphorylate the receptor. This first step in the desensitization cascade is known to occur within seconds of agonist binding, thus blocking the receptor from further signaling via its cognate G protein. The second step in the desensitization cascade is known to occur over a period of minutes and proceeds by removal of the phosphorylated receptor from the cell surface and internal sequestration. The final steps in the cascade occur over a period of minutes to hours and involve recycling the internalized receptor back to the cell surface (re- sensitization) and/or degradation of the internalized receptor in lysosomes (down-regulation). Support for this pathway of sweetener receptor desensitization has been provided by Naim and coworkers with the identification of specific GRKs in taste bud cells.[17] It is surprising, however, that the degree of desensitization observed appears to be sweetener dependent. Thus, as illustrated in Figure 4, no desensitization is observed on stimulation with the carbohydrate sweetener, while modest desensitization is observed with aspartame and strong desensitization with CPU. Clearly, high-potency sweeteners, must have rate constants for release from the receptor that are lower than that of the weakly potent carbohydrate sweeteners and thus it seems reasonable that sweetener receptor desensitization may correlate with sweetness potency. Further work is needed to validate this speculation.

CPU
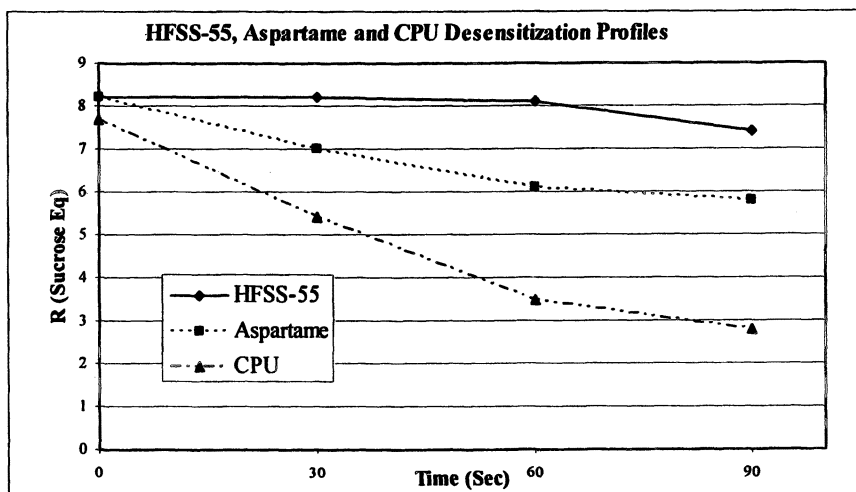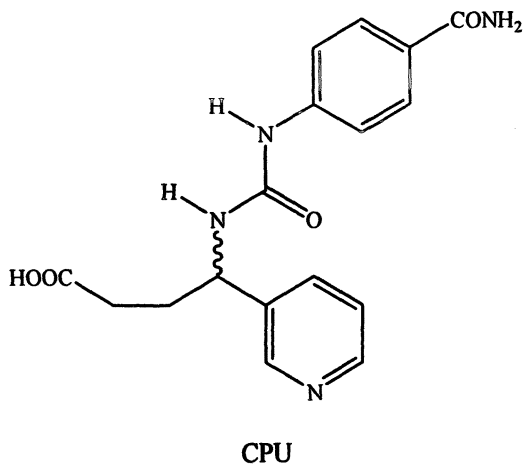
Figure 4. Adaptation Profiles of HFSS-55, aspartame and CPU in cola CSDs.

5. Safety. Requirements for the assurance of food ingredient safety differ somewhat from country to country and a comprehensive treatment of this topic is beyond the scope of this discussion. Here, a general overview is provided, limited in scope to procedures in place in the United States. In the US, the Food and Drug Administration (FDA) regulates the use of sweeteners, as well as other food additives, under the 1958 Food Additives Amendment to the Food, Drug and Cosmetic Act of 1938. This legislation and its effects on food additive regulation have been reviewed.[18,19] Initially, saccharin and cyclamate salts were exempted from this food additive regulation, as were many other commonly used food

ingredients. An ingredient category known as Generally Recognized As Safe (GRAS) food ingredients was established and saccharin and cyclamate salts were on the original GRAS list. Their GRAS statuses were later reversed and they are now regulated as food additives. Not all sweeteners were included on the GRAS list, however. Surprisingly, even sucrose was not. The omission of sucrose and many other obviously safe food ingredients prompted the following FDA comment:

> "It is impractical to list all substances that are generally recognized as safe for their intended use. However, by way of illustration, the Commissioner regards such common food ingredients as salt, pepper, sugar, vinegar, baking powder and monosodium glutamates as safe for their intended use." [20]

Since the original GRAS list, a few additional sweeteners have been determined to be GRAS. In addition, several others have been developed through the Food Additive Petition (FAP) process. Regulatory issues related to sweetener development by the FAP process have been reviewed by Broulik.[21] In addition, regulatory issues related to both GRAS and FAP processes have been reviewed by Noah and Merrill.[22] Thus, at the present time, two options exist for the commercial development of sweeteners, the GRAS and the FAP processes. Both require high degrees of rigor for assurance of safety. The principal difference between them is in the procedures employed in review of data supporting safety. A comparison of the GRAS and FAP options is presented here.

GRAS Option: A high-level overview on the GRAS determination process was recently provided by Heimbach.[23] In addition, comprehensive guidance has been provided by the FDA[24,25] and an informative history of the GRAS process is given in the Noah and Merrill review already referenced. It is noteworthy that an organization may determine an ingredient to be GRAS and market it without any input from FDA. However, this is a process fraught with peril in that FDA, at any time, may request the data upon which the GRAS determination is based and, if not in agreement, cause the marketing of the ingredient to be discontinued. Early on, organizations avoided this potential problem through a GRAS Affirmation Petition process in which documentation was filed with FDA containing all of the information upon which their GRAS determination was based. It was expected that this would be a rapid review process where FDA would either affirm GRAS status or rule that a substance was a food additive and therefore subject to the FAP process. In practice, however, the GRAS Affirmation Petition process was quite slow with an average of 7 years elapsing before FDA ruling. Then, in 1997, FDA replaced the GRAS Affirmation Petition process with the GRAS Notification process. By this new process, an ingredient sponsor would provide data upon which a GRAS determination was based and the FDA obliged itself to respond by letter within 90 days. The FDA correspondence would acknowledge receipt of the notification and, in the hope of the GRAS Notification sponsor, provide notice that

FDA has no objections with the GRAS determination. While such a No Objection Letter is the outcome of the GRAS Notification process desired by sponsors, FDA may also raise questions about the GRAS determination, thus delaying a no objection decision, or even rule that an ingredient is a food additive and not eligible for GRAS status.

In order to achieve GRAS status, a sweetener, or any food ingredient, must be generally recognized as safe among "experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food."[26] By this statement, in order for a sweetener to be GRAS, its safety must be evaluated by "experts". In practice this means a panel of experts competent to evaluate all aspects of an ingredient's safety and such a panel is commonly referred to as an Expert Panel. To further clarify, an Expert Panel may affirm a sweetener to be GRAS based on either of two considerations. The first consideration is "scientific procedures". The meaning of this is Expert Panel review of scientifically rigorous safety assessment studies of such breadth as to address all significant safety concerns under specific levels of human exposure. The second consideration is "in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food". The meaning of this phrase is Expert Panel comparison of historical human exposure levels and the exposure levels resulting from specific applications in foods and beverages. It is important to note that, upon Expert Panel GRAS determination, there is no legal requirement of FDA notification to precede ingredient marketing. In practice, however, FDA notification of a GRAS determination is the common route since food manufacturers generally will insist on FDA concurrence with a GRAS determination. It is also important to note that an ingredient is GRAS only for the uses for which the GRAS determination has been made. Thus an ingredient may be GRAS for limited applications but not GRAS for general usage.

One objective of the safety assessment studies carried out in support of either a GRAS Affirmation or a Food Additive Petition is the determination of the highest dose that may be given without effects. This dose is termed the No Observed Effect Level (NOEL). The NOEL, in the most sensitive animal species evaluated, is then used by the Expert Panel (GRAS) or FDA (FAP) to set a permissible human exposure level known as the Acceptable Daily Intake (ADI). And it is common practice to set the ADI at 1/100th of the NOEL in order to address the possibilities that 1) humans may be more sensitive than the animal species used in testing and 2) increased sensitivity may exist in human population subsets. NOEL and ADI exposure levels are given in milligrams per kilogram (mg/kg) body weight. Thus, as an example, if the NOEL of a sweetener is determined to be 500 mg/kg, an ADI of 5 mg/kg would likely be set. The question then naturally arises as to how an ADI (e.g., 5 mg/kg) is used to regulate usage. Simply put, the ADI is employed to determine the specific food categories, and levels of use in those categories, which will be permitted. This ensures that the ADI is not to be exceeded on a chronic basis. In order to do this, food and

beverage consumption data are employed. The sweetener may then be approved for use at specific levels in food and beverage categories where aggregate consumption does not exceed the ADI. Category and level approvals granted for many sweeteners in both the U.S. and abroad have been summarized by Marshall and Pollard.[27] And recently, Renwick reviewed published studies on non-caloric sweetener intakes in multiple geographical areas.[28]

Food Additive Petition Option: Sweetener development through the FAP process is somewhat more formal than the GRAS process in that 1) FDA guidelines for the scientific studies to be conducted for assurance of safety have been provided and 2) the review of the studies supporting the requested use are conducted by scientific experts at the FDA. The FDA first provided guidelines for assurance of safety in the 1982 publication *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food* which is generally referred to as the "Redbook".[29] The Redbook has been updated several times with the most recent edition being the *Office of Food Additive Safety Redbook 2000 Toxicological Principles for the Safety Assessment of Food Ingredients*.[30]

As noted at the beginning of this section, this discussion of food ingredient safety assessment procedures is limited to the methods in place in the U.S. Internationally, there has been an attempt at harmonization and these efforts have been reviewed by Vettorazzi.[31] In 1956 the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) established the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The fundamental objective of JECFA is the establishment of ADIs for food additives following the assembly and interpretation of all relevant biological and toxicological data. Presently, some countries accept JECFA recommendations while others maintain independent review processes. And yet other countries, substantially adopt the approval decisions of countries conducting independent reviews.

At the present time, 10 sweeteners have been commercially developed in the U.S. through either the GRAS or FAP processes.[32] The 5 polyol/carbohydrate type sweeteners maltitol, lactitol, isomalt, erythritol and D-tagatose have been developed through the GRAS process. Of these, only erythritol is a non-caloric sweetener, with maltitol, lactitol, isomalt and D-tagatose being partially caloric. All of these polyol type sweeteners are less potent than sucrose and thus their applications are limited to those in which properties other than sweetness are critical (e.g., freezing point suppression / frozen desserts, starch gelatinization point elevation / baked goods, bulking / confections, etc.). The 5 high-potency sweeteners saccharin, aspartame, acesulfame-K, sucralose and neotame have been approved by the FDA for food and beverage use. Of these, aspartame, acesulfame-K, sucralose and neotame have been developed through the FAP process and ADIs established for them are 50, 15, 5 and 0.3 mg/kg body weight, respectively. All of these sweeteners are effectively non-caloric under conditions of use. In addition, a petition for the approval of cyclamate is presently under FDA review. Cyclamate is currently approved for use in EU countries as well as

in many Latin American and Asian countries. The ADI established by JECFA for cyclamate is 11 mg/kg body weight.[33]

On reflection on the non-caloric sweetener ADIs given above, the high ADI for aspartame merits comment. Aspartame is unique due to its chemical composition. Aspartame is a methyl ester of the di-peptide aspartyl-phenylalanine and undergoes digestion in the gut by the same enzymes which break down common foods. Thus aspartame is digested into its three building blocks, the two amino acids aspartic acid (Asp) and phenylalanine (Phe), and methanol. The amino acids Asp and Phe are the same amino acids present in all dietary protein and it is noteworthy that Phe is an *essential* amino acid, thus being essential for life. The methanol formed on aspartame digestion is the same as the free methanol present in fruits and fruit juices and formed on digestion of pectin, a complex carbohydrate present in many fruits and vegetables. Thus all three products of aspartame digestion are already obtained in a normal diet. The uniqueness of aspartame leading to its digestion as a food is discussed in detail in a recent review by Butchko and others.[34] Ironically, in spite of aspartame's very high ADI, food and beverage products formulated with it are required to carry a warning label. The warning is provided for a very small subset of the population (ca. 1 in 15,000) with a genetic disorder known as phenylketonuria (PKU) which, today in developed countries, is diagnosed at birth. People with PKU, while requiring low levels of Phe in their diets, must carefully control levels ingested.

In view of the fact that digestion of aspartame leads only to Asp, Phe and methanol, all of which are obtained in a diet of common foods (e.g., meat, fish, dairy products, fruits and vegetables), it seems that safety assessment studies should not even be necessary. Nonetheless, aspartame has been extensively tested for safety in both animals and humans, under sound scientific protocols, thus leading to FDA establishment of an ADI of 50mg/kg body weight. As aspartame is metabolized to common dietary components, it prompts the obvious question.......What are the amounts of Asp, Phe and methanol provided by aspartame-containing foods and beverages relative to that in a normal diet? The data in Table I address this question. Suffice it to say, aspartame is a very unique food ingredient in which safety is inherent in its molecular structure.

Table I. Comparison of Asp and Phe[35] as well as methanol[36] provided by common foods and beverages with that provided by a diet beverage sweetened with aspartame (520mg/L)

Food	Aspartic Acid	Phenylalanine	Methanol
Roasted Chicken (100g)	36X	13X	
Skim Milk (12 oz)	13X	6.6X	
Orange Juice (12 oz)			1.3X
Apple Juice (12 oz)			1.6X
Tomato Juice (12 oz)			5.9X

6. Stability. To be commercially viable, a sweetener must be stable to the intended conditions of use. Stability is critically important for two reasons. First, the sweetener must not degrade such that the level of sweetness of the food product is substantially reduced during the food or beverage product lifetime. Also, the sweetener must not break down leading to any negative sensory attributes. Hydrolytic stability is of obvious importance in this regard. However, photochemical stability is also critical for some applications such as beverages. In work in the author's laboratory, analogues of the sweetener D-tryptophan were found to be particularly labile to light exposure, leading to malodorous degradation products.[37] This photochemical instability appeared to be a general characteristic of indole compounds. The second reason for the stability requirement is a safety issue. For either the GRAS Affirmation or FAP development process, degradation products must be shown to be safe. Currently, except for the case where a degradation product is a metabolite, if its exposure reaches or exceeds 0.0063 mg/kg body weight, then safety assessment studies equal to those mandated for the sweetener itself are required.[38] Thus, a sweetener's stability is a critical factor in assessment of commercial viability.

7. Solubility. Many sweeteners are insufficiently water soluble to be of general utility. Commonly, sweetness intensity levels at least equivalent to 10% sucrose are required in foods and beverages and, in some systems (e.g., frozen desserts), soluble sweetener levels which match the sweetness of 15-20% sucrose are needed. In general, to ensure that sweetener crystallization or precipitation does not occur in finished food or beverage products, the equilibrium solubility at the pH and temperature of interest should be at least 2-fold higher than the requisite concentration. For many foods and beverages, rapid dissolution may be important in addition to high equilibrium solubility. Carbonated soft drinks are a relevant example. In CSD manufacturing, sweetener/flavor concentrates are prepared where it is important that the sweetener be rapidly soluble at a substantially higher level than is present in finished beverages. A new sweetener that does not rapidly dissolve and fit into existing manufacturing processes is unlikely to be easily accepted by food and beverage manufacturers.

8. Cost Effectiveness. Non-caloric sweeteners are always compared to sucrose, the consumer's standard and new sweeteners must be cost-competitive with sucrose. Sucrose prices are highly variable from country to country and are dependent on many factors, including volumes of local production, price supports for domestic manufacturers and levels of usage for ethanol manufacture. At this writing, pure refined sucrose is priced on the London Futures Exchange at 37.0¢/kg (16.8¢/lb).[39] At the same time, however, for the reasons noted above, prices for sucrose in major markets can often be 2 to 4-fold higher. The effective cost or cost per sucrose equivalent (CSE) for a non-caloric sweetener is the quotient of its cost ($/kg or $/lb) and its sweetness potency (P) relative to sucrose. And P for a sweetener is defined as the quotient of a specific concentration of sucrose (e.g., 10.0% w/v) and the equi-sweet concentration of a sweetener of

interest (e.g., 0.050% w/v). Thus, if a concentration of 0.050% of a non-caloric sweetener is needed to match the sweetness of 10.0% sucrose, then the sweetness potency is 10.0/0.050 = 200. Sweetness potency is sometimes expressed on a molar basis, but most commonly, as in the preceding example, it is expressed on a weight basis (P_w). It is important to recognize that high-potency sweetener P_w values are not constant relative to sucrose reference concentration, while they are for carbohydrate sweeteners including polyols.[40] C/R functions demonstrating saccharin and cyclamate nonlinear dependencies of P_w on sucrose reference concentration are illustrated in Figure 1.

In addition to the sucrose reference concentration effect on sweetener potency, variation in sweetener potency is also observed in different food and beverage systems and on variation of temperature. As example of the system effect, aspartame has been reported to be 133 times more potent than sucrose in water, whereas in CSDs, it is 180, in both cases relative to a 10% sucrose reference.[41] As example of the temperature effect it is generally accepted that fructose is more potent than sucrose on a weight basis.[42] At elevated temperatures, however, sucrose is more potent than fructose. Thus calculation of sweetener CSE requires specification of the application to ensure that the relevant value of P_w is used.

Carbohydrate sweeteners (e.g., glucose, maltose, etc.), in general, are less potent than sucrose. As such, CSE values for carbohydrate sweeteners usually exceed that of sucrose. In the 20th century, many high-potency sweeteners have been discovered and some of them, including saccharin, cyclamate, aspartame, acesulfame-K, sucralose and neotame, commercially developed. And, because of their high sweetness potencies, quite low CSE values are often realized. For illustration, consider aspartame, now a commodity, which currently is available at prices < $20/kg. As noted above, aspartame exhibits a sweetness potency of 180 for CSD applications. The CSE of aspartame, used in a CSD application at a 10% sucrose sweetness equivalent level, would therefore be < $20/180 = < 11.1¢/kg (5.0¢/lb). This contrasts quite sharply with the cost of sucrose which, even at world market prices is > 3-fold higher. Clearly such sweetener system cost improvements attainable with high-potency sweeteners offer food manufacturers substantial improved economies in the low-calorie sector of product categories.

9. **Patentability.** Over the last 25 years, the four non-caloric sweeteners, aspartame, acesulfame-K, sucralose and neotame, have been commercially developed as new food additives in the US as well as in other countries. The aggregate cost for commercialization of a new sweetener [i.e., 1) preclinical and clinical safety assessment studies, 2) stability studies under projected food/beverage conditions of use, 3) safety assessment studies of degradation products which form in food/beverage applications, 4) manufacturing process development and 5) manufacturing facility construction] can be well in excess of $100,000,000. Given this very significant investment necessary for commercialization, it is critical for the organization making the investment to

ensure a period of exclusivity of sufficient duration to earn a reasonable return. And typically the means of ensuring exclusivity is through patent protection. In general, patent protection is obtained through the following four types of utility patents:

> Composition of Matter Patents
> Application or Use Patents
> Process Patents
> Formulation Patents

In order to obtain patent protection, a discovery must be novel and must have utility. The knowledge of this novelty and utility is often referred to as intellectual property (IP). In the sweetener field, it is typical, soon after a new chemical entity is discovered to have sweet taste activity of sufficient promise for use in foods and beverages, to file for patent protection. In the U.S., the patent application is filed with the U.S. Patent and Trademark Office and, within one year, equivalent foreign applications are filed to ensure patent protection in all other countries of business interest. This first patent application will claim the novel Composition of Matter based on utility as a sweetener for use in foods and beverages. Sometimes, however, a chemical compound found to be sweet may already be known in the scientific or patent literature, although the taste activity was unknown. In this case, an Application or Use patent application may be filed. This was the case with aspartame. The sweet taste of aspartame was discovered by Schlatter, a chemist working in the laboratory of Mazur at Searle Pharmaceutical Company, some years later than it was described in the literature by chemists at Imperial Chemical Industries.[43] Once it is clear that a new sweetener has commercial potential, it is common IP protection strategy to obtain broad patent protection on both Processes for its manufacture and on Formulations. In this manner, it is often possible to extend the period of exclusivity enabled by Composition of Matter and/or Use patents by blocking competitors from cost-effective manufacture and/or formulation of the sweetener in a manner providing good tasting foods and beverages.

In summary, given the very significant investment required for commercialization of a new sweetener, the assurance of a reasonable period of exclusivity for the commercializing organization is crucial. In the absence of such exclusivity enabled by patents, an investment in commercial development will be a money losing proposition. Over the last century, chemists determined the chemical structures of nearly 100 natural non-caloric sweeteners and, for the most promising of these, inadequate attention was given to patent protection. As a consequence, without assurance of exclusivity through patent protection, no natural non-caloric sweeteners have been commercially developed to the point of obtaining regulatory agency approvals in the U.S. or the European Union. Thus sweeteners which have been commercially developed to the point of regulatory approvals, are all synthetic sweeteners protected by Composition of Matter / Use patents.

Conclusion

In the discussion above, 9 metrics have been described for assessment of the commercial viability of new sweeteners or sweetener receptor PAMs. These metrics are provided here to provide guidance to organizations considering commercial development programs on specific product candidates. *The critically important point intended here is that, in order to realize commercial success and to therefore justify the substantial investment requisite for commercialization, the new sweetener or sweetener receptor PAM must perform well against all 9 metrics.* It is insufficient to deliver on 7 or 8. If organizations make the investment on sweeteners or sweetener receptor PAMs that do not deliver on all 9 metrics, they will likely be wasting their resources. Due to 1) the business opportunity afforded by the cost-savings possible in zero- and reduced-calorie products, 2) the business opportunity which may be realized upon delivery of sugar-like sweetness in zero- and reduced calorie products, and 3) increasing health and wellness issues associated with high calorie / high glycemic load diets and sedentary lifestyles, the need has never been greater for breakthrough innovations in sweetness technologies. *Thus it is essential that organizations initiating development programs on new sweeteners or sweetener receptor PAMs be certain that their product candidates are products that the food and beverage industry will use.* If resources are wasted on product candidates of insufficient commercial potential, the advancement of the much-needed breakthrough innovations in sweetness technology will be delayed.

References

1. Lee, C.-K. *Advances in Carbohydrate Chemistry and Biochemistry*; Volume 45; Academic Press, Inc.: New York, NY, 1987, pp 199-351.
2. Shallenberger, R. S. *Taste Chemistry*, Blackie Academic & Professional: London, UK, 1993.
3. *Alternative Sweeteners*, 3rd Edition; O'Brien Nabors, L., Ed.; Marcel Dekker: New York, NY, 2001.
4. *Handbook of Sweeteners*; Marie, S.; Piggott, J. R., Eds.; Blackie & Son, Ltd.: London, UK, 1991.
5. Kim, N.-C.; Kinghorn, A. D. *Studies in Natural Products Chemistry*; Vol. 27; Atta-ur-Rahman, Ed.; Elsevier Science B. V.: New York, NY, 2002, pp 3-57.
6. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J. P.; Zuker, C. S. *Cell* 2001, *106*, 381-390.
7. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M; Adler, E. *PNAS* 2002, *99*, 4692-4696.
8. DuBois, G. E.; Walters, D. E.; Schiffman, S. S.; Warwick, Z. S.; Booth, B. J.; Pecore, S. D.; Gibes, K.; Carr, B. T.; Brands, L. M. *Sweeteners: Discovery, Molecular Design and Chemoreception*; Walters, D. E.;

Orthoefer, F. T.; DuBois, G. E., Eds.; ACS Symposium Series 450, Chapter 13, American Chemical Society: Washington, D.C., 1991, pp 261-276.

9. Helgren, F. J. U.S. Patent 2,803,551 (August 20, 1957), to Abbott Laboratories.

10. Meilgaard, M.; Vance Civille, G.; Carr, B. T. *Sensory Evaluation Techniques,* Vol. II; CRC Press: Boca Raton, FL, 1987, pp 5-6.

11. Carr, B. T.; Pecore, S. D.; Gibes, K. M.; DuBois, G. E. *Flavor Measurement*; Ho, C.-T.; Manley, C. H., Eds., Marcel Dekker, Inc.: New York, NY, 1993, pp 219-237.

12. Pronin, A. N.; Tang, H.; Connor, J.; Keung, W. *Chemical Senses* **2004**, *29*, 583-593.

13. DuBois, G. E.; Crosby, G. A.; Stephenson, R. A. *Journal of Medicinal Chemistry* **1981**, *24*, 408-428.

14. DuBois, G. E.; Lee, J. F. *Chemical Senses* **1983**, *7*, 237-246.

15. DuBois, G. E. Unpublished Results; The NutraSweet Company; Mt. Prospect, IL, 1990.

16. Oakley, R. H.; Barak, L. S.; Caron, M. G. *G Protein-Coupled-Receptor Interactions*; George, S. R.; O'Dowd, B. F., Eds., Wiley-Liss: Hoboken, NJ, 2005.

17. Zubare-Samuelov, M.; Shaul, M. E.; Peri, I.; Aliluiko, A.; Tirosh, O.; Naim, M. *Am. J. Physiol. Cell Physiol.* **2005**, *289*, C483-C492.

18. Ronk, R. J. *Sweeteners and Dental Caries*; Shaw, J. H.; Roussos, G. G., Eds.; Information Retrieval, Inc.: Washington, D.C., 1978, pp 131-144.

19. Schultz, H. W. *Food Law* Handbook; Avi Publishing Company: Westport, CT., 1981.

20. *Code of Federal Regulations, Title 21 (Food and Drugs),* Section 182.1, April 1, 1988, pp 385-386.

21. Broulik, F. J. *Advances in Sweeteners*; Grenby, T. H., Ed., Blackie Academic & Professional: London, UK, 1996, pp 35-55.

22. Noah, L.; Merrill, R. A. *Boston University Law Review* **1998**, *78*, 329-443.

23. Heimbach, J. *Prepared Foods* **2004**, January, 123-129.

24. www.cfsan.fda.gov/~dms/opa-frgr.html.

25. *Federal Register* 62 FR 18937, April 17, 1997; www.cfsan.fda.gov/~lrd/fr970417.html.

26. *Code of Federal Regulations, Title 21 (Food and Drugs),* Section 570.30, April 1, 1989, p 611.

27. Marshall, J. P.; Pollard, J. A. *Food Legislation Surveys No. 1: Sweeteners-International Legislation,* 4th ed., Leatherhead Food, R. A., 1987.

28. Renwick, A. G. *Food Additives & Contaminants,* **2006**, *23*, 327-338.

29. *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food,* U.S. Food and Drug Administration, Bureau of Foods: Washington, D.C., 1982.

30. *Office of Food Additive Safety Redbook 2000 Toxicological Principles for the Safety Assessment of Food Ingredients,* July, 2000, Updated October

2001, November 2003, April 2004 and February 2006, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition; Washington, D.C.; www.cfsan.fda.gov.

31. Vettorazzi, G. *International Food Regulation Handbook*, Middlekauf, R. D.; Shubik, P. Eds., Marcel Dekker; New York, NY, 1989.

32. DuBois, G. E. *Encyclopedia of Food Science & Technology*; John Wiley & Sons, New York, NY, 2000, pp 2245-2265.

33. *Twenty-Sixth Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series, No. 683; Geneva, Switzerland, 1982.

34. Butchko, H. H.; Stargel, W. W.; Comer, C. P.; Mayhew, D. A.; Benninger, C.; Blackburn, G. L.; de Sonneville, L. M. J.; Geha, R. S.; Hertelendy, Z.; Koestner, A.; Leon, A. S.; Liepa, G. U.; McMartin, K. E.; Mendenhall, C. L.; Munro, I. C.; Novotny, E. J.; Renwick, A. G.; Schiffman, S. S.; Schomer, D. L.; Shaywitz, B. A.; Spiers, P. A.; Tephly, T. R.; Thomas, J. A.; Trefz, F. K. *Regulatory Toxicology and Pharmacology* **2002**, *35*, S1-S93.

35. USDA Nutrient Database for Standard Reference, Release 14, U.S. Department of Agriculture, Agricultural Research Service, 2001; http://www.nal.usda.gov.fnic.foodcomp.

36. Wucherpfennig, K.; Dietrich, H.; Bechtel, J. *Flussiges Obst.* **1983**, 348-354.

37. DuBois, G. E.; King, G. A., Unpublished Results, The Coca-Cola Company, Atlanta, GA, 1995.

38. *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food*, U.S. Food and Drug Administration, Bureau of Foods: Washington, D.C., 1982, pp 1-19.

39. www.sugartech.co.za/sugarprice/index.php.

40. Moskowitz, H. R. *Product Testing and Sensory Evaluation of Foods*, Food & Nutrition Press: Westport, CT, 1983, pp 110-120.

41. Homler, B. E. *Aspartame: Physiology and Biochemistry*, Stegink, L. D.; Filer, Jr., L. J., Eds., Marcel Dekker: New York, 1984.

42. Hyvonen, L.; Koivistoinen, P. *Nutritive Sweeteners*; Birch, G. G.; Parker, K. J, Eds., Applied Science Publishers: Englewood, NJ, 1982, pp 135-137.

43. Mazur, R. H. *Aspartame: Physiology and Biochemistry*, Chapter 1, Stegink, L. D.; Filer, Jr., L. J., Eds., Marcel Dekker: New York, NY, 1984.

Chapter 30

Development of New, Low Calorie Sweetener: New Aspartame Derivative

Yusuke Amino[1], Kenich Mori[1], Yasuyuki Tomiyama[2], Hiroyuki Sakata[2], and Takeshi Fujieda[1]

[1]AminoScience Laboratories and [2]Food Products Development Center, Ajinomoto Company, Inc., 1-1, Suzuki-Cho, Kawasaki-Ku, Kawasaki 210–8681, Japan

Since its launch in the market over 20 years ago, the amino acid based sweetener Aspartame has substantially contributed to the improved taste quality of uncountable number of reduced calorie food and beverage products around the globe. During this period, value added functionalities of Aspartame was developed for a wide spectrum of food products. In parallel, the Ajinomoto Company has explored a great number of molecules to develop a novel sweetener. By using advanced technology, in conformational analysis, molecular design, and receptor model building, Ajinomoto has developed the next generation sweetener. In this paper, we will describe Ajinomoto's research and development work on the new sweetener (Laboratory code name: ANS9801).

Introduction

A brief history of research on sweet molecules, especially aspartyl-based molecules can be summarized as follows. In the later 1960s, after Aspartame was accidentally discovered (1), almost at the same time, the Shallenerger and Acree's "AH-B" model of the chemical basis of sweetness was proposed. In the 1970s through the 1980s, other new peptide sweeteners such as Alitame were discovered by trial and error (2). In the later 1970s, Dr. Ariyoshi proposed a novel idea of structural modeling of Aspartame derivatives as sweeteners (3). Since then, some mode of interaction between sweet peptides and sweet taste receptor has been hypothesized. In the 1990s, computational chemistry was applied to the analysis of sweet molecules and construction of sweet molecular models. In this century, the sweet taste receptor was identified by several groups.

Our research on sweeteners started in the late 1960s together with the development of Aspartame as a sweetener. Through these experiences, we have continued to find and develop new sweet molecules, such as aspartyl-based sweeteners, applying advanced theory and technique.

Discovery

Strategy

The following strategy was utilized to explore new aspartyl-based sweet molecules.
- Select lead compounds
- Conduct lead optimization and SAR study
- Apply computer aided molecular modeling
- Synthesize compounds and screen potential new sweeteners

Lead compounds

Aspartame and compounds discovered by The Coca-Cola Company (4) and Dr. Nofre (5) were chosen as lead compounds for this study (Figure 1).

Lead optimization and SAR study

Lead optimization and structure-activity relationships studies were carried out based on the sweet taste recognition models of Dr. Ariyoshi or Dr. Goodman

(6, 7), (Figure 2 and 3). Computer aided molecular modeling was also applied to synthesize sweet compounds (8).

Aspartame

The Coca-Cola Company (1994)   **Dr. Nofre (1994)**
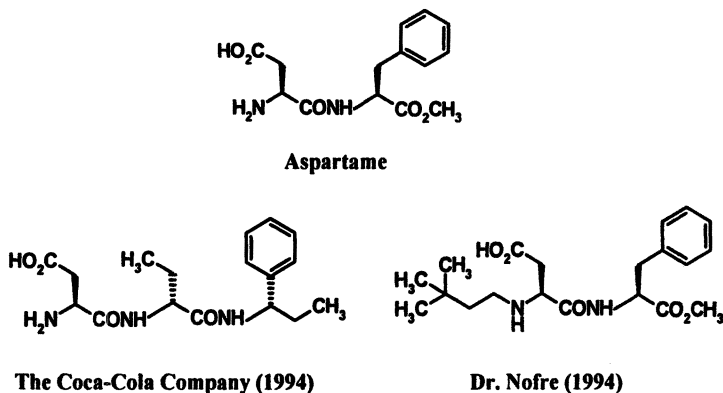
Figure 1. Lead compounds.

Fisher Projection

A: sweet   **B: not sweet**   **B: not sweet**

$R_2 > R_1$ $L > M > S$
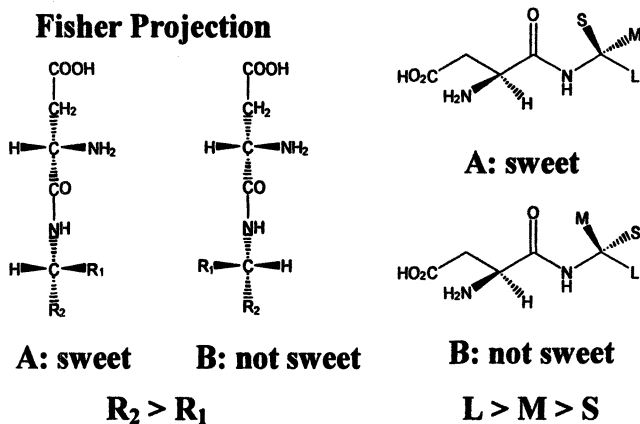
Figure 2. Ariyoshi model for aspartyl dipeptide sweeteners.

Applying the Ariyoshi model, we could predict whether the aspartyl dipeptide derivative is sweet or not sweet (Figure 2). Namely, in the Fisher projection formulae "A" and "B", the sweet dipeptide ester group is uniformly of "A" type while the inactive analogues are of the "B" type molecular topography.

Goodman model was proposed based on the conformational analysis of sweet-tasting, tasteless and bitter-tasting aspartyl-based peptides and peptidemimetics. The taste recognition model shown in Figure 3 describes the relationship between topochemical array and taste in aspartyl-based ligands. The zwitterionic glucophore (termed AH/B) of the Shallenberger-Kier model is oriented on the +y axis, and the hydrophobic group plays a decisive role in determining the taste class (I-VI Class). In the taste recognition model of Figure 3, of the two conformers that contribute to the sweet taste of the aspartyl-based ligands, the "L-shaped" hydrophobic glucophore occupies the +x axis region of space (Class I) and the extended glucophore lies along the –y axis (Class VI).

Crystal structure of Aspartame
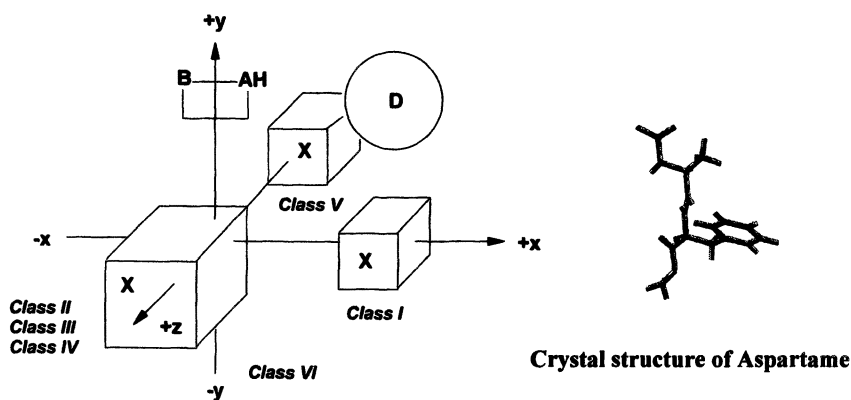
Figure 3. Goodman model.
Class-I : an L-shaped structure with the AH- and B-containing zwitterionic ring of the N-terminus forming the stem of the L in the y axis, and the hydrophobic X group projecting along the base of the L in the +x axis. Sweet:Classes I,VI; Bitter:Class V; Tasteless:Classes II, III, IV; D zone:key to the enhancement of sweet potency. (Reproduced with permission from reference 14. Copyright 2006 Wiley.)

Structure-Activity Relationships

We have found hundreds of high-potency Aspartame derivatives from the structure-activity relationships study (9~18). Conformational analysis of aspartyl dipeptide amide (the compound of the Coca-cola company and its analogue), N-alkylated aspartame (the compound of Dr. Nofre and its analogue) and N-alkylated aspartyl dipeptide amide was carried out and the detailed descriptions given in the published papers (11, 12, 13). When examining the preferred conformations in solution as determined by NMR and molecular modeling, these

compounds can adopt "L-shaped" conformation. In addition, it was found that a hydrophobic substituent of N-terminal residue which is positioned above the base of the "L" in the +x, +y quadrant of space might be important for enhancement of sweet potency. The Tinti-Nofre model for sweet taste ligands assigns special regions to a number of pharmacophoric groups considered to be essential for sweet taste (15). When the Tinti-Nofre model is compared with the Goodman model, the G domain of this model can be viewed as equivalent to the X domain in the Goodman model since it accommodates the hydrophobic group. The D zone of the Tinti-Nofre model remains unexplored in term of molecular arrangement. We assumed that the hydrophobic substituent (the second hydrophobic binding domain) above the base of the "L" in the Goodman model oriented to the D zone of the Tinti-Nofre model. As a result, we sought to design and synthesize sweet ligands to probe D zone (the second hydrophobic substituent) and to determine its role in sweet taste.
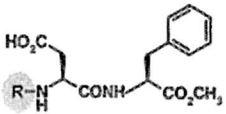
An example of SAR study on the N-substituted aspartyl-based sweet molecules is shown in Figure 4. By introducing an "arylpropyl" substitution on the N-atom of aspartic acid moiety of Aspartame, the sweetening potency of Aspartame was increased. Moreover, comparison of the structure of hydrophobic substitution and sweetening potency of each compounds clearly showed that the replacement of aromatic hydrogen atoms at 3,4-positions by hydroxyl, alkoxy or alkyl group and substitution of hydrogen atom(s) at 3-position of propyl group by alkyl group(s) can lead to dramatically high sweetness potencies. Among them, the sweetest compound has the sweetening potency 50,000 times more than that of sucrose.

The extraordinary potency of the N-arylalkylated compounds can be explained by the effect of a second hydrophobic binding domain in addition to interactions arising from the "L-shaped" structure of the original ligand Aspartame. The "arylpropyl" substitution of this series of compounds is arrayed above the base of "L" (D zone) in the +x, +y plane and might be responsible for the increased sweetness potency of the original molecule (Figure 3). We believe that the "arylpropyl" substitution play a fundamental role in enhancing the sweet potency because of introduction of orientational constrains on the whole molecule by hydrophobic (aromatic)-hydrophobic (aromatic) interactions (7, 14).

Screening for Development

At the first screening, about 10 compounds were chosen from hundreds of compounds based on ;

- sweet potency
- taste
- availability of synthesis
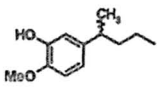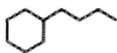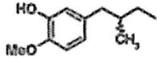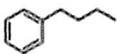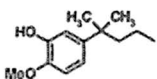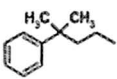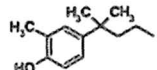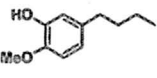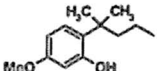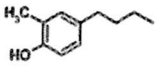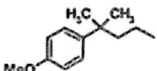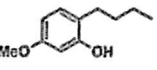
468

R	4%SE	R	4%SE
(phenylpropyl)	faintly sweet	(3-hydroxy-4-methoxy, CH₃)	40,000
(cyclohexyl)	0	(3-hydroxy-4-methoxy, CH₃)	5,000
(phenylbutyl)	1,000	(3-hydroxy-4-methoxy, H₃C CH₃)	50,000
(H₃C CH₃ phenyl)	4,000	(H₃C CH₃ dihydroxy)	50,000
(HO MeO)	20,000	(MeO OH)	20,000
(H₃C HO)	35,000	(H₃C CH₃ MeO)	20,000
(MeO OH)	15,000		

Figure 4. The SAR study on the hydrophobic binding domain of aspartyl-based sweet molecules.

At the secondary screening, the candidate was decided based on the following viewpoints;

- sweet potency
- detail of taste profile
- physico-chemical property
- feasibility of industrial production
- estimation of metabolic dynamics in human body by *in vitro* assay

New Sweetener

Through these processes, a novel non-nutritive sweetener, N-[N-[3-(3-hydroxy-4-methoxyphenyl)propyl-α-aspartyl]-L-Phenylalanine 1-methyl ester, monohydrate (hereafter referred to as ANS9801) was discovered (Figure 5).

ANS9801 has a structural similarity to natural sweeteners. The functional group 3-hydroxy-4-methoxyphenyl exists as part of a component of Phyllodulcin, a Japanese traditional sweetener obtained from Saxifragaceae amateur's leaf and Neohesperidin dihydrochalcone, a precursor obtained from citrus fruits (Figure 6).

Lab. Code name: ANS9801

Chemical names (IUPAC):

    N-[N-[3-(3-hydroxy-4-methoxyphenyl) propyl] -α-aspartyl]-
L-phenylalanine 1-methyl ester

CAS No.:   714229-20-6

Molecular formula: $C_{24}H_{30}N_2O_7 \cdot H_2O$

Relative molecular mass: 476.52

Structural formula:
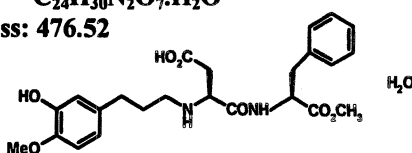
Figure 5. Characteristics of ANS9801.
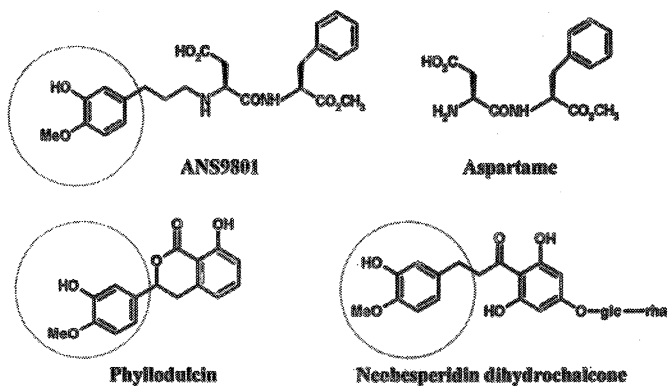
Figure 6. Structural formulas of ANS9801 and other sweeteners

Synthesis

ANS9801 is synthesized from Aspartame and (3-hydroxy-4-methoxy-phenyl)propylaldehyde in a one step process by reductive N-alkylation, which is carried out by treating APM and the aldehyde with hydrogen in the presence of a platinum (Pt/C) in a methanolic solution. The important intermediate, (3-hydroxy-4-methoxyphenyl)propylaldehyde, is derived from vanillin via four steps (Figure 7).

Vanillin

Aspartame

reductive N-alkylation
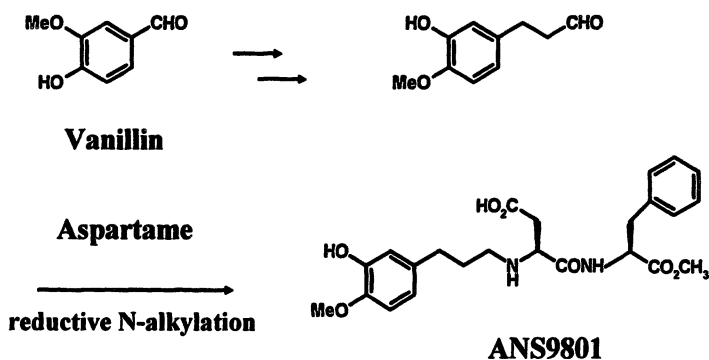
ANS9801

Figure 7. Preparation of ANS9801.

Properties

ANS9801 is an odorless white crystalline compound and obtained as a hydrate (empirical formula $C_{24}H_{30}N_2O_7 \cdot H_2O$; formula weight 476.52). The melting point of the ANS9801 hydrate is 101.5°C. The advanced analysis results to set the specification for Good Manufacturing Practice are summarized in Table I. Tests like heavy metals, catalysts, residual solvents, microbial limit will be added on the current specification.

The solubility of ANS9801 in water is 0.099g/dl at 25°C for 30 minutes. This is far greater than the necessary solubility required to obtain a sweetness level matching a 10% sucrose solution. A co-crystal of ANS9801 and Aspartame was prepared to improve the dissolution rate. As shown in Figure 8, in the case of the mixture initial dissolution rate of ANS9801 is slower than that of Aspartame, however, in mixed crystal (ANS9801:Aspartame = 0.022:1 mol/mol), the dissolution rate of ANS9801 is the same as that of Aspartame.

Table I. The advanced analysis results of ANS9801

Specification Parameter	Specification Value
Identification	
Description	White to yellow powder
IR absorption spectrum	Same as Reference standard
Purity	
Assay	Not less than 97.0% and not more than 102.0% on anhydrous basis
N-[N-[3-(3-hydroxy-4-methoxyphenyl)propyl-α-aspartyl]-L-phenylalanine	Not more than 1.0%
Total other related substances	Not more than 1.5%
Water	Between 2.5% and 5.0%
Residue on ignition	Not more than 0.2%
Lead	Not more than 1 ppm
Specific rotation [α]20D	Between −45° and −38°
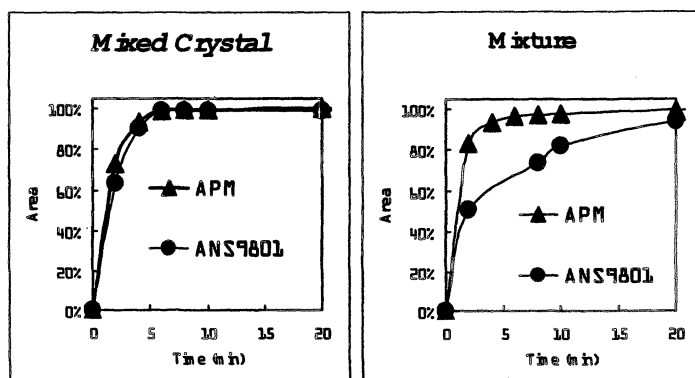
Figure 8. Dissolution rate of co-crystal of ANS9801 and Aspartame(APM)
(Temp.:25 degree, Concentration:PSE=10).

Functionality

Functionality as a Flavor Enhancer

Functionality of ANS9801 in various foods was evaluated. The ANS9801 has flavor enhancement effects in foods such as lemon tea, orange juice and strawberry yogurt at very low concentrations. According to the definition of the U.S. FDA, a flavor enhancers is a substances added to supplement, enhance, or modify the original taste and/or aroma of food, without imparting a characteristic taste or aroma of its own. For example, in the case of strawberry yogurt, the difference threshold value, or minimum concentration to be able to detect the change in specific taste intensity under 10 wt% sugar solutions, is 0.005mg/dl. Other examples of evaluation of vanilla, lemon, orange and strawberry flavor are summarized in Table II. In strawberry yogurt, the aroma becomes fresher with more characteristic strawberry taste and flavor.

Table II. Flavor enhancer effects of ANS9801

Item	PSE (%)	Addition at below difference threshold value ():equivalent conc. to sugar		Effects of ANS9801
Vanilla flavored water	10	0.005mg/100ml (0.10%)	Aroma	enhancing the overall flavor, improving the palatability
			Taste	enhancing the mouthfulness, full-body
Lemon flavored water	10	0.005mg/100ml (0.10%)	Aroma	impressing the top flavor, enhancing the overall flavor
			Taste	adjusting the balance between sweetness and flavor, improving the palatability
Lemon tea	10	0.009mg/100ml (0.18%)	Aroma	more natural lemon flavor, real lemon flavor
			Taste	adjusting the balance between sweetness and sourness, mouthfulness, no astringency of tea, clear after sweetness
100% orange juice	10	0.005mg/100ml (0.10%)	Aroma	more fresh flavor, suitable time-intensity balance of sweetness and flavor extension
			Taste	enhancing the mouthfullness, improving the balance of overall taste
Strawberry yogurt	10	0.005mg/100ml (0.10%)	Aroma	more fresh flavor, more characteristic and pleasant flavor
			Taste	more pleasant, more characteristic taste like strawberry

Functionality as a Sweetener

The taste profile and the effectiveness of ANS9801 as a sweetener was examined through a series of sensory evaluations at various concentrations in water and compared to sucrose or aspartame-sweetened solutions.

Sweetness Potency

The sweetness potency of ANS9801 was compared to Aspartame in water using a qualified sensory panelists, where the panelists measured the sweetness profile of ANS9801 using the sucrose equivalency scale. The panelists were screened and specially trained to correctly identify the level of sweetness (i.e., strong, slightly strong, moderate, slightly weak, weak and trace level sweetness) and familiarized with the sucrose equivalency line scale (31 points). The perceived sweetness intensity of ANS9801 and Aspartame increased with increasing concentrations of each as shown in table 3. The relative sweetness potency of ANS9801 compared to Aspartame (i.e., ratio of equi-sweet concentrations) varied from approximately 119 to 70 times the sweetness potency of Aspartame over a wide range of sweet intensities (4% to 14% SE).

Table III. Sweetness potency of ANS9801

Sweet Intensity (% Sucrose Equivalency)	ANS9801 Relative Potencies	
	Relative to Aspartame (Aspartame/ANS9801)	Relative to Sucrose (Sucrose/ANS9801)
3	120	47778
4	119	44074
5	118	40370
6	116	36637
7	114	32963
8	112	29259
9	109	25556
10	105	21852
11	100	18148
12	94	10741
13	85	10741
14	70	7037

Taste Profile

Descriptive analysis of taste profile of ANS9801 in water was carried out using the QDA® methodology (19). To identify the flavor profile of ANS9801 in water, it was compared to Aspartame at different concentrations. Flavor profile was evaluated at 500 ppm and 1600 ppm for Aspartame and 5 ppm and 16ppm for ANS9801. Results are summarized in figure 9. ANS9801 has

dominant sweet flavor, while the perceived intensities for bitter flavor and sour flavor are very weak. The sensory profile of ANS9801 is similar to that of Aspartame.

Aftertaste attributes of ANS9801 is shown in Figure 10. At lower concentration, ANS9801 has a little bit higher after taste compare with the same sweetness Aspartame solution. However at higher concentration, these differences are negligible.

ANS9801 has very clean sweet taste similar to Aspartame and as previously stated its sweetness potency is twenty thousands times sweeter than sucrose and 100 times than that of Aspartame. Due to its high potency, very small amount of ANS9801 will be effective to add sweetness with various foods. Moreover, ANS9801 can be used in combination with sugar or other high intensity sweeteners. The amount of ANS9801 used for some foods in case all sweetness comes solely from it is shown in Table IV.
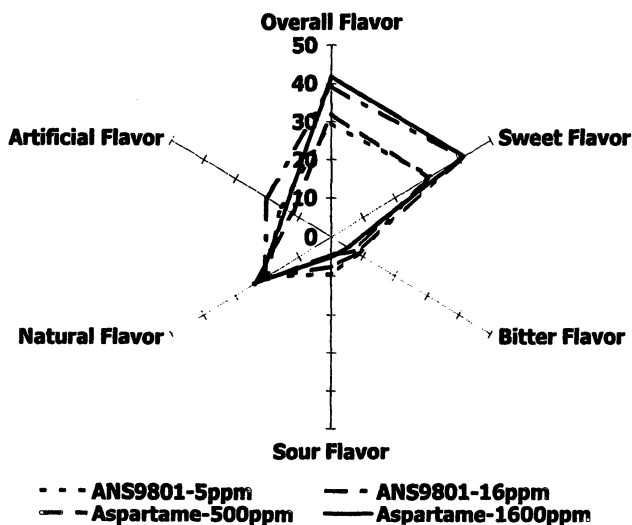
Figure 9. Flavor profile of ANS9801 at higher concentrations

Stability

ANS9801 in dry form such as table top sweetener or powdered soft drink mix is very stable and keeps its functionality under the usual storage conditions (25°C /60%RH, Figure 11).
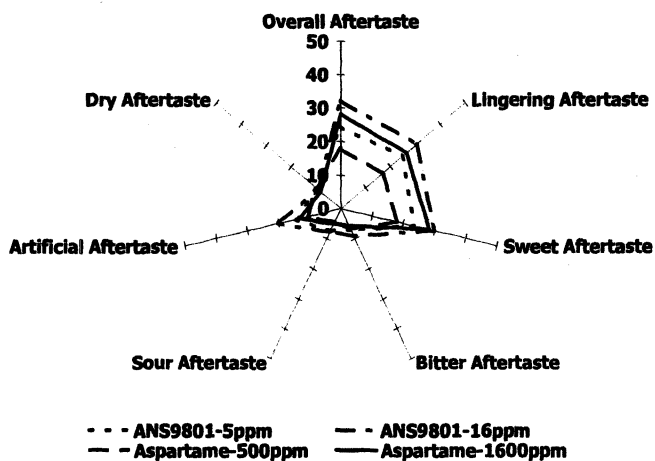
Figure 10. Aftertaste attributes of ANS9801.

Table IV. Calculated use level of ANS9801 in various foods

Item	concentration of ANS9801
Table top sweetener	500ppm in 1.3g sachet (in drink:2~4ppm)
Powder soft drink	110~600ppm (in drink:2~7ppm)
Carbonated soft drink	4~7ppm
Hot packed beverage	2.5~3.5ppm
Chewing gum	35~50ppm
Fruit yogurt	4~7ppm
Yellow cake	10~14ppm

Figure 12 shows the stability of ANS9801 in model carbonated soft drink. The stability was measured using a typical ingredient, pH=3.2 that means typical pH of CSD under storage conditions of 25°C /60% relative humidity. After 20 weeks, ANS9801 remained about 60%. However, the satisfactory sweetness level remained throughout the experiment. At 0, 8, 15 and 20 weeks, sensory evaluation was carried out to check the sweetness acceptability. About 50% of panelist accepted the sweetness of test sample as just about right even at week 20. This result means physico-chemical stability and functional stability is different. High intensity sweeteners have relatively higher sweetness potency in a lower concentration. This phenomenon can be explained by the concentration-response relationships of HIS, the equation of which asymptotically approaching maximal response and fits to the Beidler equation (20).

Usage Example

A usage example of ANS9801 in combination with other sweeteners is shown in Figure 13. In lime flavored water sweetened with high fructose corn syrup, 30% of HFCS can be replaced by ANS9801 without any change of taste character.

Conclusion

In the discussion above, the procedure to discover new sweet molecules and the approach taken to develop a new sweetener has been described.

A novel non-nutritive sweetener, N-[N-[3-(3-hydroxy-4-methoxyphenyl) propyl-α-aspartyl]-L-Phenylalanine 1-methyl ester, monohydrate (Laboratory code; ANS9801) was discovered by applying the lead optimization and structure-activity relationships studies based on the sweet taste recognition models of Dr. Ariyoshi or Dr. Goodman. After evaluations of the potentiality as a sweetener, ANS9801 was chosen as a candidate for the development.

ANS9801 is made from vanillin and Aspartame. It has very clean sweet taste similar to Aspartame and its sweetness potency is twenty thousands times of sucrose and 100 times of Aspartame. Also ANS9801 has flavor enhancement effects in foods such as lemon tea, orange juice and strawberry yogurt at very low concentrations. Since its high intensity, reduction of sweeteners cost by combination with other nutritive or non-nutritive sweetener is a possible utilization of ANS9801.

In the sweetener market, nutritive sweetener has a 90% share as sweetness equivalent. Currently, the population with obesity and diabetics are increasing in the world including developing countries, and they are major risk factor for other health problems. In this situation, high intensity sweeteners can contribute toward calorie management and open opportunities for expanded usage.
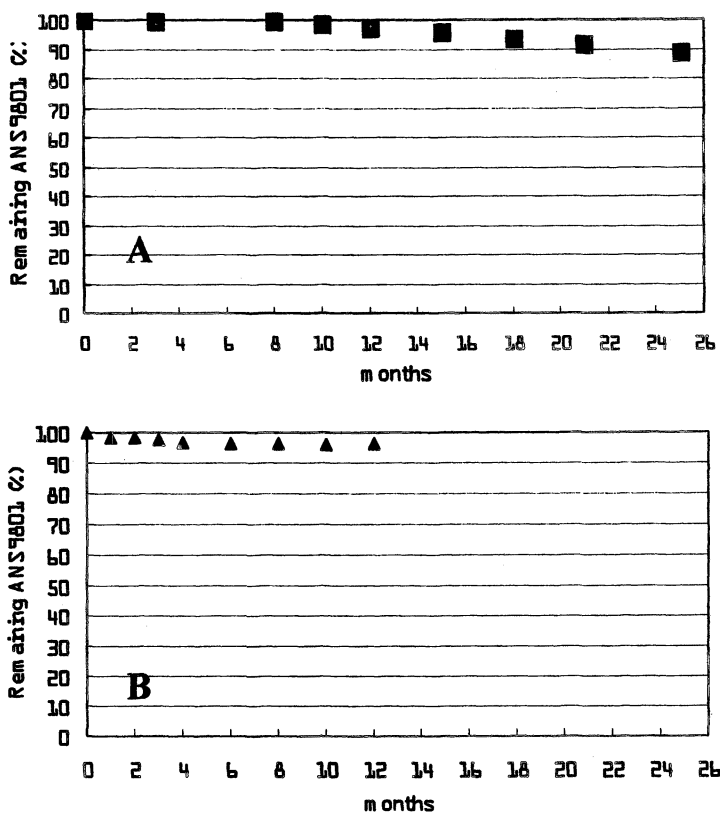
Figure 11. Stability of ANS9801 in dry form.
A; Tabletop form B; Powdered soft drink mix stored at 25°C/60%RH

478

Stability
- using a typical ingredient
- pH3.2, typical pH of CSD
- Storage conditions
    25°C/60%RH - 20 weeks
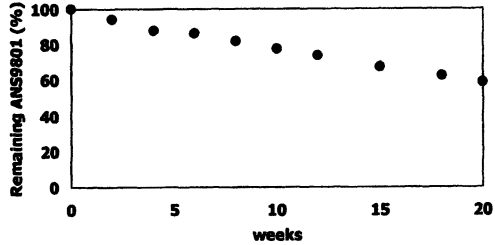
Sweetness Acceptability
- Sensory evaluation at each stability time point

☐ Not Quite Enough
☐ Just About Right
☐ Slightly Too Sweet
☑ Much Too Sweet
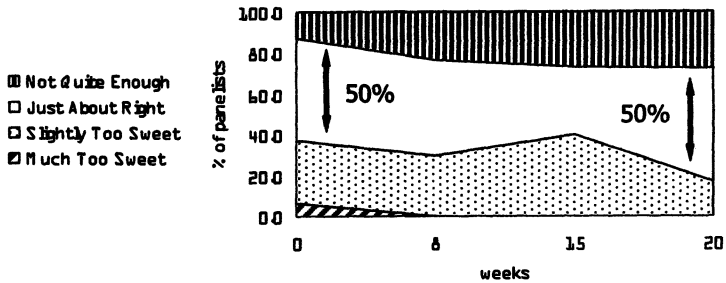
Figure 12. Stability in carbonated soft drink and sweetness acceptability of ANS9801.
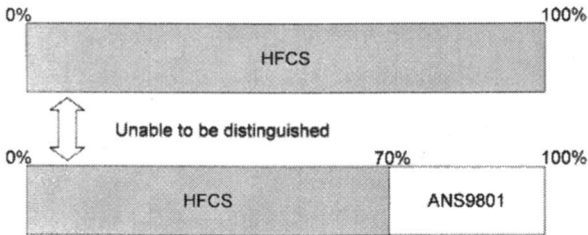
Figure 13. High Fructose Corn Syrup (HFCS) substitution by ANS9801 in lime flavored water at PSE 5 and 8%, pH3.2, tested by triangle method with 15 expert panels.

Acknowledgments

Y.A. wishes to thank late Dr. Goodman for many helpful discussions during the course of SAR study.

References

1. Mazur, R. H.; Schlatter, J. M.; Goldkamp, A. H. J. Am. Chem. Soc. **1969**, *91*, 2684-2691.
2. Glowaky, R. C.; Hendrick, M. E.; Smiles, R. E.; Terres, A. In: Sweeteners: Discovery, Molecular Design, and Chemoreception; Walters, D. E., Orthoefer, F. T.; DuBois, G. E, Eds; ACS Symposium Series 450, American Chemical Society: Washington, DC, 1991; p57.
3. Ariyoshi, Y.; Agric. Biol. Chem. **1976**, *40*, 983-992.
4. D'Angelo, L. L.; Sweeny, J. G.; US 5,286,509 (Feb. 15, 1994).
5. Nofre, C.; Tinti, J-M.; FR 2697844 (May. 13, 1994).
6. Yamazaki, T.; Benedetti, E.; Kent, D.; Goodman, M. Angew. Chem. Int. Ed. Engl. **1994**, *33*, 1437-1451.
7. Goodman, M.; Del Valle, J. R.; Amino, Y.; Benedetti, E.; Pure Appl. Chem. **2002**, *74*, 1109-1116.
8. Nakamura, R.; Takahashi, M.; Amino, Y.; Sakamoto, K.; Abstract of Papers, 2nd IUPAC International Symposium on sweeteners, Hiroshima; IUPAC, 2001; P-02, p117.
9. Ariyoshi, Y.; Kohmura, M.; Hasegawa, Y.; Ota, M.; Nio, N. In: Sweeteners: Discovery, Molecular Design, and Chemoreception; Walters, D. E., Orthoefer, F. T.; DuBois, G. E, Eds; ACS Symposium Series 450, American Chemical Society: Washington, DC, 1991; p41.
10. Ando, T.; Ota, M.; Kashiwagi, T.; Nagashima, N.; Ariyoshi, Y.; Chadha, R. K.; Yamazaki, T.; Goodman, M. J. Am. Chem. Soc. **1993**, *115*, 397-402.
11. Goodman, M; Zhu, Q.; Kent, D. R.; Amino, Y.; Iacovino, R.; Benedetti, E.; Santini, A. J. Peptide Science, **1997**, *3*, 231-241.
12. Mattern, R-H.; Amino, Y.; Benedetti, E.; Goodman, M. J. Peptide Res. **1997**, *50*, 289-299.
13. Mattern, R-H.; Amino, Y.; Benedetti, E.; Goodman, M. Biopolymers, **1999**, *49*, 525-539.
14. De Coupa, A.; Goodman, M.; Amino, Y.; Saviano, M.; Benedetti, E. ChemBioChem, **2006**, *7*, 377-387.
15. Tinti, J.-M.; Nofre, C. In: Sweeteners: Discovery, Molecular Design, and Chemoreception; Walters, D. E., Orthoefer, F. T.; DuBois, G. E, Eds; ACS Symposium Series 450, American Chemical Society: Washington, DC, 1991; p206.

16. Amino, Y.; Yuzawa, K.; Takemoto, T.; Nakamura, R. WO 9952937 A1 (Oct. 21, 1999).
17. Amino, Y.; Yuzawa, K.; Takemoto, T.; Nakamura, R. WO 2000000508 A1 (Jan. 6, 2000).
18. Amino, Y.; Yuzawa, K.; Takemoto, T.; Nakamura, R. WO 2000017230 A1 (Mar. 30, 2000).
19. H. Stone and J. L. Sidel; Food Technology, **1998**, *52(8)*, 48-52.
20. DuBois, G, E.; Walters, D. E.; Schiffman, S. S.; Warwick, Z. S.; Booth, B. J.; Pecore, S. D.; Gibes, K.; Carr, B. T.; Brands, L. M. In: Sweeteners: Discovery, Molecular Design, and Chemoreception; Walters, D. E., Orthoefer, F. T.; DuBois, G. E, Eds; ACS Symposium Series 450, American Chemical Society: Washington, DC, 1991; p261.

Chapter 31

Challenges to Reducing Sugar in Foods

D. Kilcast, C. den Ridder, and C. Narain

Leatherhead Food International, Randalls Road, Leatherhead, Surrey KT22 7RY, United Kingdom

Sweetness is an important characteristic of food and drink products that at the appropriate levels receives universally positive consumer responses. In most products sweetness has traditionally been delivered by sucrose, but health and nutritional concerns have for many years obliged the food and drinks industry to find ways of reducing the sugar content of products. This has resulted in a wide range of sugar-free or reduced-sugar products on the market, but the pressure to take this process further is unrelenting. In taking this process further companies need to take a systematic approach to sugar reduction, and recognise that in addition to achieving the required sweetness level, they must also build in a range of other related characteristics. Model systems in aqueous solution can give valuable information, but do not necessarily predict responses in a typical product matrix. In addition, manufacturers should take steps to understand possible consumer segmentation patterns in their target market, and formulate modified products with this in mind. Finally, interactive effects in reducing sugar, fat and salt, either in pairs or together, should be investigated.

Introduction

Human beings are born with the capacity to enjoy the sweetness of foods, and this liking continues to be reinforced throughout life. This importance has provided the driving force to understand more fully the mechanism of the sweetness response, not only to sucrose, which remains the dominant sweetener of choice, but also to other sweet molecules. In addition to this positive force, however, there have emerged several important negative influences. Most important of these have been health concerns associated with high levels of sucrose consumption, and which currently focus on the issue of obesity. These have resulted in efforts to find alternative sweeteners that will help achieve a reduction in energy consumption. One consequence has been the intensive efforts to find suitable intense sweeteners that are non-cariogenic and non-caloric, but concerns are frequently expressed that high intake of such sweeteners might also be accompanied by negative effects on health. There is, then, a need to deliver to consumers the required level and quality of perceived sweetness, but using lower quantities of sweeteners.

Problems in sucrose replacement

Sucrose remains the standard sweetener in most sweet foods, which is generally regarded as a consequence of its clean sweet taste. That sucrose has a clean sweet taste is increasingly being questioned, and an alternative view that has been proposed is that sucrose has other non-sweet characteristics (such as a caramel note) that we have adapted to and recognise as the ideal profile. Sucrose also possesses other important functional properties, one of the most important of which is its bulking properties, and the ideal sucrose substitute would retain the characteristic sweet taste and functional properties, but would have none of the unhealthy connotations. However, introduction of new sucrose replacers can involve long and expensive approval procedures that are aimed primarily at ensuring consumer safety, but even sweeteners that have successfully overcome this hurdle show defects in terms of sensory characteristics when compared with sucrose.

Substitution of sucrose in different food products is generally achieved via the addition of bulk sweeteners, intense sweeteners or a mixture of both. Bulk sweeteners, as the name suggests, exhibit similar bulking properties to sucrose. Their sweetness response characteristics can usually be modelled by a linear concentration-response function, but most are less sweet than sucrose, and polyols such as sorbitol and xylitol give an additional cooling effect that can limit applications. In contrast, intense sweeteners exhibit non-linear concentration-response behaviour. An important practical consequence of this is that the equivalent concentrations of intense sweeteners to be used as sucrose

substitutes depend on the level of sweetness to be achieved. The range of permitted intense sweeteners is growing slowly, but the use of intense sweeteners alone cannot deliver the bulking properties of sucrose or other bulk sweeteners, and specifically in soft drinks sector, the required mouthfeel. As a consequence, there are increasing uses of combinations of bulk sweeteners and intense sweeteners: the bulk sweeteners provide the bulk but usually insufficient sweetness, with the intense sweetener providing the required sweetness.

In recent years there has been increasing interest in the possible use of combinations of sweeteners in order to maximise the potential of individual sweeteners, and often simultaneously minimising the unwanted characteristics, both sensory and physicochemical, of some sweeteners. This can be most commonly seen in the use of binary or ternary mixtures of intense sweeteners in soft drinks. A further advantage of using intense sweetener combinations lies in the potential exploitation of synergistic combinations of certain sweeteners. Such combinations exhibit sweetness levels that are greater than the sum of their components, and opens up the possibility of substantial cost savings to users. Extending the principle of combinations to bulk and intense sweeteners, however, carries the potential of realizing the high sweetening power of intense sweeteners with the mouthfeel delivery properties of the bulk sweeteners, in addition to potential quality improvements and cost-savings through synergy.

Designing sweetness quality

The first step in any programme in substituting for sucrose is usually to interrogate the literature for information on the sensory and other functional characteristics of alternative sweeteners. In practice, most of the information found in the literature describes properties of sweeteners in aqueous solution. This is to be expected, both from a logical point of view – water is the most common component of food matrices – but also from a practical viewpoint, as soft drinks form one of the most common classes of sweetened products. The importance of soft drinks is emphasised even further if the usage of intense sweeteners is considered; for example, it has estimated (1) that soft drinks represent 65% of the market for intense sweeteners. This can be seen in the reduced sugar food and drink market data for 2004 (Figure 1).

One direct consequence of the dominance of the soft drink market is that the bulk of the published literature on sweeteners focuses on the sensory properties in aqueous solution and, with few exceptions (the major one being the confectionery market) pays less attention to the effect of the product matrix on the sweetener properties. Consequently, in addition to researching the basic sweetener characteristics, the product developer must also consider the effect of the product matrix. Small changes in the product matrix (equivalent to the
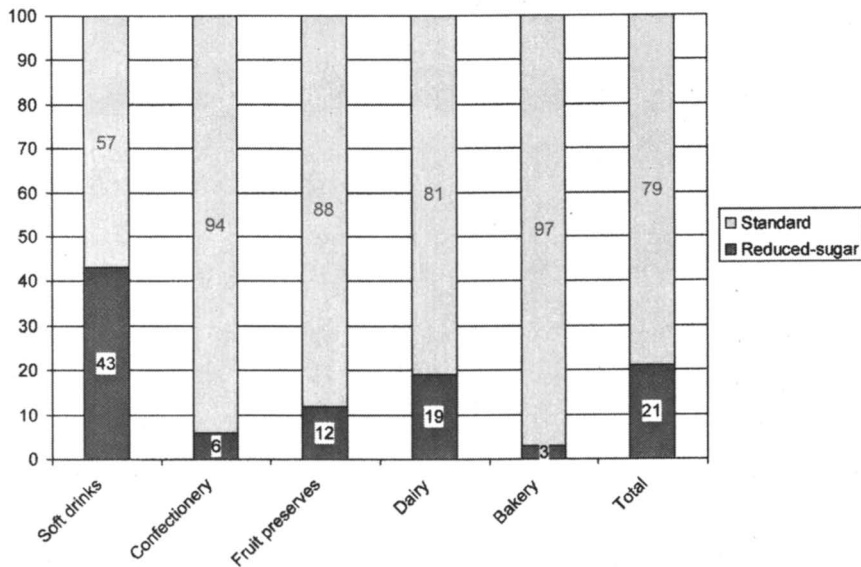
Figure 1. Market for reduced-sugar food and drink products in the UK, 2004 (LFI estimates)

external environment of the sweetener) can have a major influence on the characteristics of the sweetener. An example of this is shown in Figure 2, which summarizes the effect of a single environmental change, that of pH, on synergistic effects in combinations of selected bulk and intense sweeteners *(2)*. In the sucrose system, lowering the pH to 3.1 (typical of a soft drink) changes the suppressive combination of sucrose/aspartame found in neutral solution to synergistic, and the additive combination of sucrose/alitame to suppressive. Fewer changes are seen in the maltitol system, however.

Most intense sweeteners (and some bulk sweeteners) exhibit a range of non-sweet flavours that can reduce their appeal in certain product applications. For example, acesulfam-K and cyclamate show bitter and burnt flavours, whilst NHDC has a strong liquorice flavour. The detailed sensory characteristics can be identified and quantified using trained sensory profile panels. The sensory characteristics can be presented and visualised in a number of different ways, but one useful method by which the quality attributes of a number of samples can be compared and examined visually is through the use of various multivariate statistical methods, the most common being principal component analysis. Mapping of sensory profile data using principal component analysis can illustrate dramatically the sensory relationships between the sweeteners. In such

	Neutral solution		Acidic solution (pH 3.1)
Sucrose - aspartame	suppression	→	synergy
Sucrose - acesulfam-K	additivity		(additivity)
Sucrose - cyclamate	synergy		synergy
Sucrose - alitame	additivity	→	suppression
Maltitol - aspartame	additivity/suppression	→	synergy
Maltitol - acesulfam-K	synergy		synergy
Maltitol - cyclamate	synergy		synergy
Maltitol - alitame	suppression		suppression

Figure 2. Effect of pH on synergy in bulk and intense sweetener combinations.

a map, sweeteners that are closely positioned are perceived as more similar than those positioned far apart. The superimposed attribute plot shows the basis on which the sweeteners differ.

An additional problem is that the sweeteners can also show temporal responses in the mouth that are different from that of sucrose. The importance of such responses lies in the fact that chemical stimuli are not released from the food matrix, and nor are they perceived instantly once they arrive at the sweetness receptors (Figure 3). If the time-intensity profile curves of two perceptible chemical stimuli behave as shown in the figure, then an imbalance may occur. This could be an imbalance between different sweeteners, between a sweetener and another tastant (e.g. an acidulant), or between a sweetener and a volatile. In the case of sweeteners such as thaumatin and NHDC, this is manifested in an extremely long persistence time, which can limit their applications in food. However, these same properties can be of value in other applications, for example in masking bitterness and other flavours in pharmaceutical preparations and nutritional supplements. Other sweeteners might show responses different from that of sucrose, and which can give a poor harmonisation with the responses from other tastants and flavours.

486
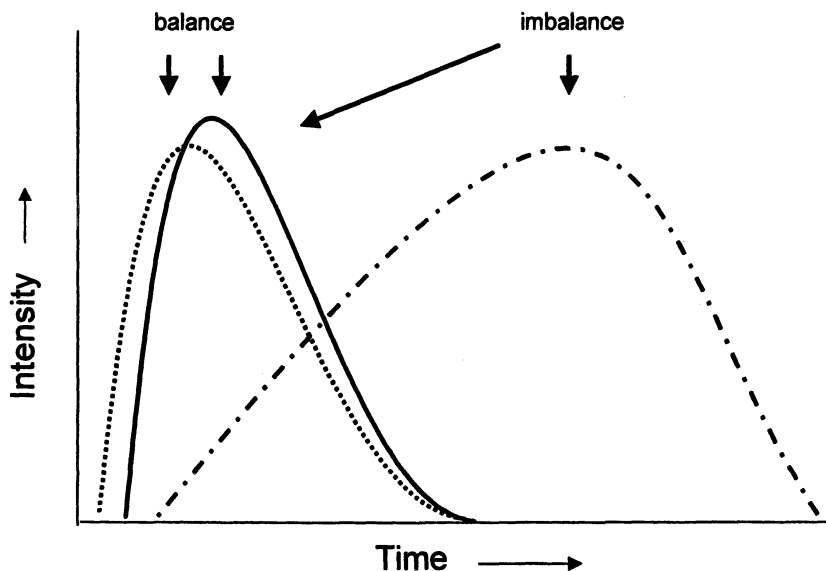
Figure 3. Schematic tine-intensity curves showing balanced and imbalanced flavour release

Quality benefits of synergistic mixtures

Synergy between bulk and intense sweeteners is less well-documented than that between intense sweeteners, but in principle can combine the desired bulk properties with the desired high sweetness levels in a cost-effective way. The summarized data in Figure 2 shows that synergistic combinations of bulk and intense sweeteners can be identified in aqueous solution. Such combinations can benefit in a number of ways. The most obvious benefit lies in cost reductions, and this has been realised for many years in the use of synergistic combinations of two or more intense sweeteners. This practice has also highlighted another major benefit, that of improved sensory quality of the combinations in comparison with the individual components. Such quality benefits can also be seen in combinations of bulk and intense sweeteners, and this effect is shown in Figure 4. This shows how the high levels of bitterness and liquorice flavour associated with cyclamate in solution can be reduced by using equisweet mixtures with either sucrose or with maltitol. The same combinations have also been shown to give improved temporal characteristics.
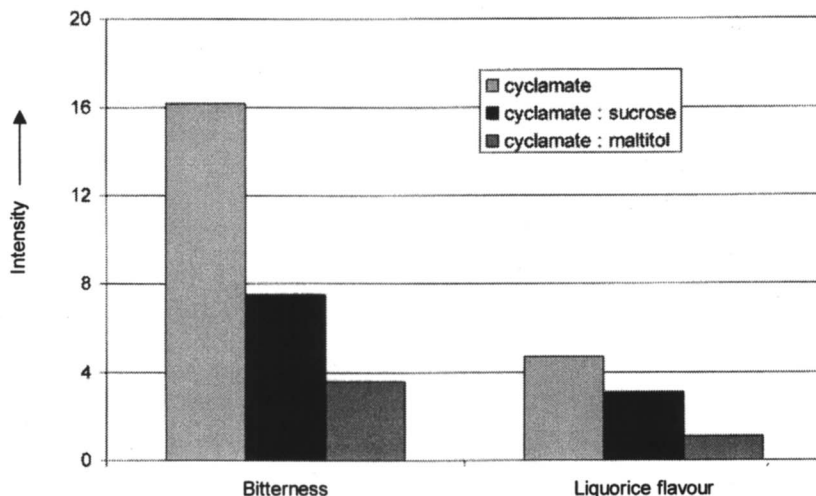
Figure 4. Effect of bulk and intense sweetener combinations on improving sweetness quality characteristics

Sweeteners and mouthfeel

In soft drinks and other beverages, bulk sugars contribute to a mouthfeel characteristic that has been ascribed to both the viscosity contribution and also to the density of the sweetener solution. The most likely contribution to the mouthfeel is thought to lie through the viscosity contribution, and in some markets considerable efforts are made to compensate for this effect when sucrose is replaced by intense sweeteners. This effect is illustrated in Figure 5, which shows how the sensory characteristics of a sucrose solution change when the sucrose is substituted by an aspartame/acesulfam-K combination, giving a less smooth and thinner mouthfeel, and how the characteristics can be retrieved, at least partially, when hydrocolloids are incorporated that mimic the mouthfeel properties of the sucrose. The hydrocolloid type and concentration needs to be chosen carefully in order to avoid unwanted mouthfeel characteristics, however.

Consumer segmentation

Any product developer team formulating products for a diverse market must take into account probable segmentation in consumer requirements. Whilst this is commonly recognised on an international basis, such segmentation is common even on a more local scale in most markets. Optimising formulations must accept
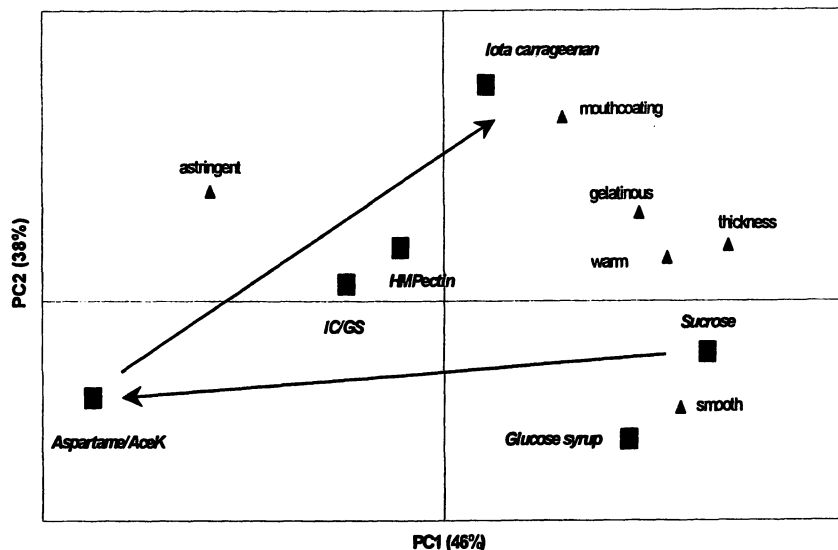
Figure 5. Principal component plot showing changes in the sensory characteristics of sucrose solutions on substitution by intense sweeteners, and partial compensation by hydrocolloid additions.

the possibility of segmentation, and even if development of different formulations for these segments is not commercially feasible, then this segmentation must be understood if the major market is to be satisfied. Statistical tools are available for identifying such segmentation, and are often used in combination with experimental design techniques. As an example of such techniques, a recent study carried out at Leatherhead Food International investigated the optimisation of a fruit juice formulation using colour, sweetness, pulpiness and thickness as formulation variables. Each of these variables was manipulated at 3 levels (coded Low, Medium, High) using a d-optimal design that reduced the 81 sample treatments that would have been generated by a full 3^4 factorial design down to 16 sample treatments. The samples were evaluated by consumer testing and also sensory profiling by a trained panel, and the results analysed by a number of techniques such as cluster analysis, internal preference mapping, partial least squares regression and response surface modelling. the cluster analysis revealed 3 distinct clusters based on consumer liking, as shown in Table I.

Whilst 76% of the respondents wanted high levels of sweetness, 24% preferred low levels. Whether this size of the market is significant involves commercial judgements, any product developer would have to accept that formulating to satisfy the majority of the market is likely to alienate a significant

Table I. Cluster Analysis of Consumer Preference Data

	Cluster group		
	1	2	3
Number of respondents	94	29	40
	58%	18%	24%
Sweetness	High	High	Low
Colour	Low	High	Low
Pulpiness	Medium	(Low)	Low
Thickness	Low	(Low)	Low

minority. This situation is likely to become more complex if sweetness quality differences are introduced in sucrose substitution programmes.

Sugar reduction and reduction in other components

In addition to pressures on manufacturers to reduce sugar, there are corresponding pressures to reduce both salt and fat as measures to improve healthy eating in the more affluent societies. Each of these other types of reduction carries issues that are not dissimilar to those associated with sugar reduction. Like sugar, fat has both structural and sensory functions that must be simulated. Salt has fewer structural functions, but has both sensory and microbiological functions. Many foods contain at least two of these components, and some all three, and there is little systematic information available on how reduction of two or three components simultaneously can be achieved without destroying the basic structure of the product or its desired sensory characteristics. As an example of possible interactions, it has been known for some time that additions of sodium chloride to sucrose solutions increase the intensity of sweetness (3). Investigations into the interaction of different salts with a range of bulk sweeteners shows a much more complex pattern however. As shown in Figure 6, sweetness enhancement of other bulk sweeteners is greater than with sucrose, and the pattern is highly unpredictable when potassium chloride and magnesium chloride (both potential sodium chloride replacers) are used (4).

Conclusions

There is a continuing drive to extend the range of sweeteners available to food and drink manufacturers, but attaining the unique physical and sensory
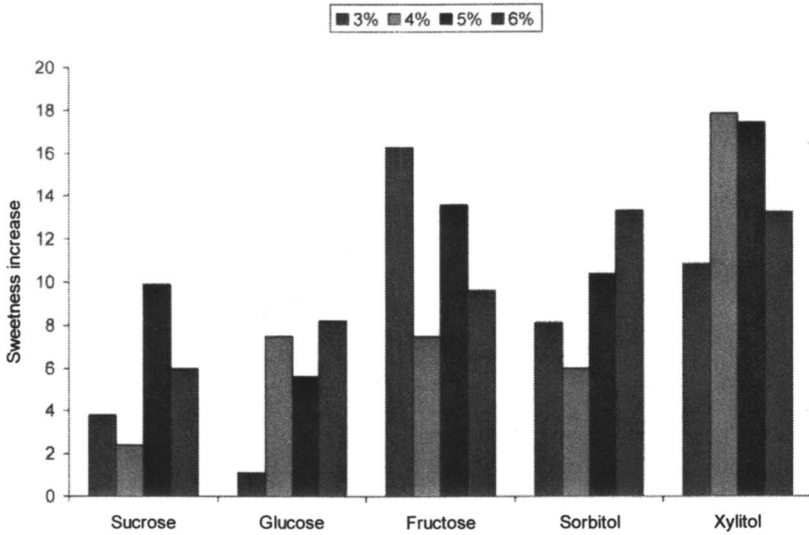
*Figure 6. The effect of 2% sodium chloride addition on the sweetness of bulk
sweeteners at increasing concentrations. (Reproduced with permission from
reference 4. Copyright 2000 Elsevier.)*

properties delivered by sucrose remains elusive. Consumer demands for quality are increasing in parallel with pressure to reduce sugar consumption. A small number of new sweeteners, such as sucralose, alitame and neotame are now being approved in various countries, but the costs of developing new intensive sweeteners is likely to limit any extensive future activities. The industry needs to examine more closely the potential benefits in using combinations of existing sweeteners, and the research being carried out at various centres has demonstrated the possibilities. Additionally, we need to improve the understanding of the interactive nature of flavour and texture in order to compensate for the mouthfeel changes that can occur when replacing bulk sweeteners by intense sweeteners. Increasing pressures on the food industry to contribute to healthier diets also requires a better understanding of how to achieve reductions of sugar in conjunction with other major food components successfully.

Acknowledgements

References

1. Fry, J. C. *Food Science & Technology Today* **2005**, *19(2)*, 26+28-30.
2. Hutteau F., Mathlouthi M., Portmann M.O. and Kilcast D. *Food Chemistry* **1998**, *63(1)*, 9-16.
3. Pangborn, R. M. *J. Food Sci.* **1962**, *26*, 648-55.
4. Kilcast D., Portmann M.-O. and Byrne, B. *Food Chemistry*, **2000**, *70(1)*, 1-8.

Chapter 32

Development of a New, No Calorie Commercial Sweetener Neotame

Indra Prakash[1] and Ihab E. Bishay[2]

[1]The Coca-Cola Company, One Coca-Cola Plaza, Atlanta, GA 30313
[2]The NutraSweet Company, 222 Merchandise Mart Plaza, Suite 936, Chicago, IL 60654

Neotame, a new high potency sweetener and flavor enhancer, is derived from aspartame and is 8000 times sweeter than sucrose. It provides zero calories and has clean, sweet sugar-like taste with no undesirable taste characteristics. It is functional in a wide array of beverages and foods and can be used with other high potency or carbohydrate sweeteners. It is stable under dry conditions, and has comparable stability to aspartame in aqueous food systems. It is safe for use by general population and has been approved by U. S. FDA and several other countries around the world. Development of neotame i.e. discovery, manufacture, physical and chemical characteristics, taste profile, blends, stability and applications will be reviewed.

Neotame is a new high-intensity sweetener and flavor enhancer developed by The NutraSweet Company. It is a derivative of the dipeptide composed of the amino acids aspartic acid and phenylalanine and is approximately 7,000 – 13,000 times sweeter than sugar (30 – 60 times sweeter than aspartame). Neotame provides zero calories and has a clean sweet sugar-like taste with no undesirable taste characteristics. It is functional in a wide array of beverages and foods and is well suited for blending with other high-intensity or carbohydrate sweeteners. Neotame is stable under dry conditions and in aqueous food systems its stability is similar to aspartame with greater stability in neutral pH conditions and/or at higher temperatures (e. g., heat processing, baking and yogurt).

The results of numerous safety studies confirm that neotame is safe for use by the general population, including children, pregnant women, and people with diabetes. In addition, no special labeling for phenylketonuric (PKU) individuals is required.

Neotame has been approved for general use as a sweetener and flavor enhancer in the United States, Mexico, China, Australia and New Zealand and in many other countries. Its unique properties will provide the food technologist with another tool to produce innovative new foods and beverages to meet the demand of consumers to have available great tasting foods without all of the calories of sugar.

Discovery of Neotame

Neotame was the result of a long-term research program to discover new high-intensity sweeteners with desirable taste characteristics. French scientists Claude Nofre and Jean-Marie Tinti invented neotame by substitution of the N-terminus of aspartame. The chemical structures of aspartame and neotame are compared in Figure 1.

Nofre and Tinti prepared a series of N-substituted aspartame derivatives that are listed in Table 2 along with their corresponding sweetness potencies
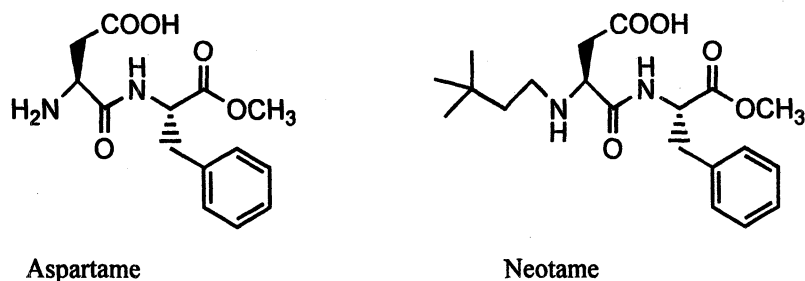
Figure 1. Comparison of the chemical structures of aspartame and neotame

Table 1. Approximate sweetener indices of some of the common high-intensity sweeteners

Sweetener	Approximate sweetness index
Sucrose[a]	1.0
Acesulfame-K	200
Aspartame	200
Neotame	8000
Saccharin	300
Sucralose	600

[a] Included for reference.
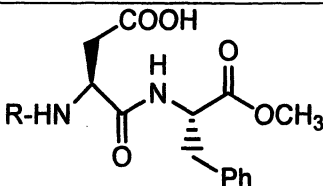
relative to a 2% solution of sucrose. As noted in Table 2, aspartame substituted with a 3,3-dimethylbutyl group results in the sweetest of the compounds tested.[1,2]

Sweetness Potency of Neotame

In sweetener research sucrose is the standard against which other compounds are compared. "Sucrose equivalence" or "% SE" is the standardized sweetness intensity scale established for comparing sweet compounds. An x% SE is equivalent in sweetness to an x% sucrose in water solution.

Neotame is 7,000 to 13,000 times sweeter than sucrose and is more potent than the high-intensity sweeteners currently marketed in the U.S. (Table 1). Though it is a derivative of aspartame, neotame is 30 to 60 times sweeter than aspartame. The actual sweetness potency is dependent on the neotame concentration required in various food or beverage products. Because of its remarkable sweetness potency, neotame is used in food and beverage products at considerably lower concentrations than other high-intensity sweeteners. In fact, consumer exposure to neotame will be much lower than that from flavoring ingredients such as vanillin, cinnamon, and menthol that are commonly used in food and beverages. The concentration-response curve for neotame was established using a trained sensory panel to evaluate the sweetness intensity of five solutions of neotame at increasing concentrations. The results are presented in Figure 2. Based on these data, neotame can reach an extrapolated maximum sweetness intensity (plateau) of 15.1% SE in water. Sweeteners such as aspartame, acesulfame-K, sodium cyclamate, and sodium saccharin attain their maximum sweetness intensity in water at approximately 16.0% SE, 11.6% SE, 11.3% SE, and 9.0% SE, respectively.[3]

Table 2. Hydrophobic groups substituted on the terminal nitrogen of aspartame and the corresponding sweetening potencies of the resulting compounds

No.	R = (hydrophobic group)	Relative sweetener potency[a]
1	$CH_3CH_2CH_2CH_2$	400
2	$(CH_3)_2CHCH_2$	500
3	$(CH_3)_2CHCH_2CH_2$	1300
4	$(R,S)-CH_3CH_2CH(CH_3)CH_2$	900
5	$(CH_3CH_2)_2CHCH_2$	2000
6	$(CH_3)_3CCH_2CH_2$ = (neotame)	10,000
7	Cyclohexyl	800
8	Cycloheptyl	900
9	Cyclooctyl	1000
10	Cyclopentylmethyl	1500
11	Cyclohexylmethyl	800
12	$C_6H_5CH_2CH_2CH_2$	1500
13	$(R,S)-C_6H_5CH(CH_3)CH_2CH_2$	1200
14	3,3-Dimethylcyclopentyl	150
15	(R,S)-3-Methylcyclohexyl	1000
16	3,3,5,5-Tetramethylcyclohexyl	1000
17	(R,S)-2-Hydroxycyclohexyl	800
18	$(3-OCH_3,4-OH)C_6H_3CH_2CH_2CH_2$	2,500
19	$(3-OCH_3,4-OH)C_6H_3CH=CHCH_2$	2,000
20	$(R,S)-(3-OCH_3,4-OH)C_6H_3CH_2CH_2CH(CH_3)$	500
21	$(R,S)-(3-OCH_3,4-OH)C_6H_3CH=CHCH(CH_3)$	500

[a] Sweetener potency is given on a molar basis relative to a 2% sucrose solution.

— Predicted

• Observed
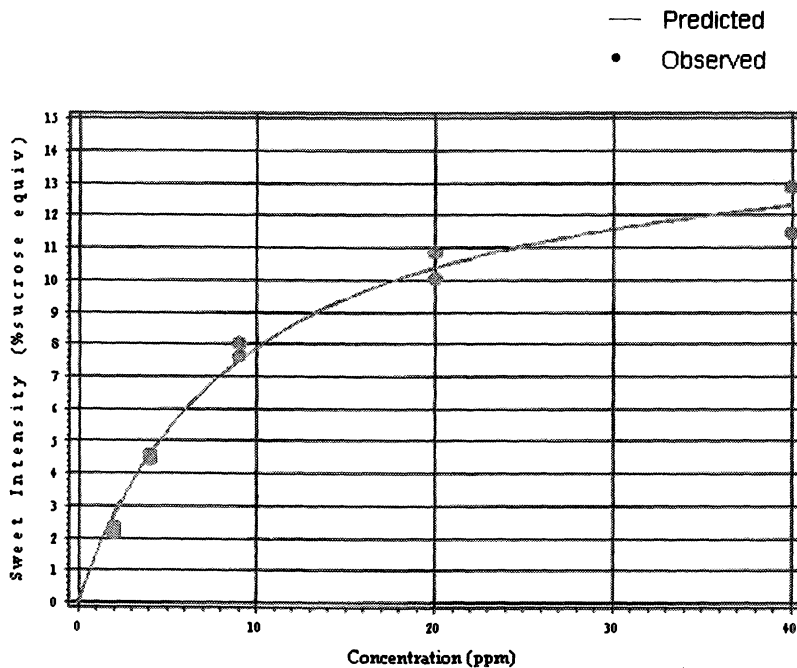
Figure 2. Neotame concentration response curve in water

Manufacture of Neotame

Neotame can be made in one step by the reaction of aspartame with 3,3-dimethylbutyraldehyde in methanol using hydrogen and a catalyst (palladium or platinum) under mild conditions (Figure 3).[4,5] Other possible methods of preparation are from aspartame precursors, aspartic acid derivatives via anhydride or other peptide coupling methods, and by the aminolysis of substituted oxazolidinone derivatives.[6,7,8]
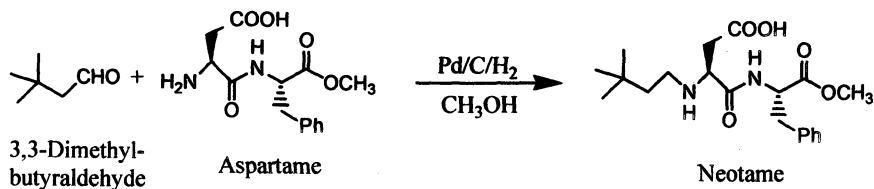
Figure 3. Manufacture of neotame

Properties of Neotame

Chemical

Neotame is N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1- methyl ester (CAS registry number 165450-17-9, INS number 961). It is a derivative of a dipeptide composed of the amino acids aspartic acid and phenylalanine. Neotame contains both a carboxylic acid and a secondary amino group, with pK_a values of 3.03 and 8.08, respectively. It is capable of forming both acidic and basic salts, as well as complexes with various metals, thus affording unique delivery forms having improved solubility and other characteristics.

The two amino acids in neotame, aspartic acid and phenylalanine, are the natural L-configuration. The other three possible isomers, L,D-, D,D-, and D,L-, lack the sweet taste of neotame.[9]

Physical

Neotame is a fairly low-melting hydrate (m.p. 80.9 – 83.4 °C). It is a white to off-white crystalline powder with 4.5% water of hydration, the empirical formula $C_{20}H_{30}N_2O_5 \cdot H_2O$, and a molecular weight of 396.48. Neotame has similar solubility in water as aspartame (12.6 g/L vs 10 g/L at 25 °C) but is more readily soluble compared to aspartame in some solvents that are typically used in food systems and pharmaceuticals. For example, neotame is more readily soluble in ethanol compared to aspartame (Table 3).

The solubility of neotame in water and ethyl acetate increases with increasing temperature (Table 4). Using neotame in a salt form (e.g., as a phosphate salt) significantly increases the rate of dissolution.

Table 3. Comparison of the solubility of neotame and aspartame in water and some typical solvents at 25 °C

Solvent	Solubility of neotame (g/100g of solvent)	Solubility of aspartame (g/100g of solvent)
Soybean oil	0.03	0.01
Water	1.26	1.02
Ethyl acetate	7.7	< 0.1
Glycerin	10.29	0.06
Propylene glycol	21.20	0.16
Ethanol	> 100	0.372

Table 4. Comparison of the solubility of neotame in water
and ethyl acetate at increasing temperatures

Temperature (°C)	Solubility of neotame in water (g/100g of solvent)	Solubility of neotame in ethyl acetate (g/100g of solvent)
15	1.06	4.36
25	1.26	7.70
40	1.80	23.8
50	2.52	87.2
60	4.75	> 100

Stability of neotame

The stability of neotame is dependent upon pH, moisture, and temperature. Dry powder is stable for at least five years under proper storage conditions (Figure 4). In aqueous systems pH stability follows a bell-shaped curve at a given temperature. The optimum pH for neotame's maximum stability is about 4.5. As expected, stability decreases with increasing temperature.

In aqueous systems (pH 2 - 8) the major decomposition pathway of neotame is through the hydrolysis of the methyl ester forming de-esterified neotame (Figure 5), also the major metabolite of neotame in humans.
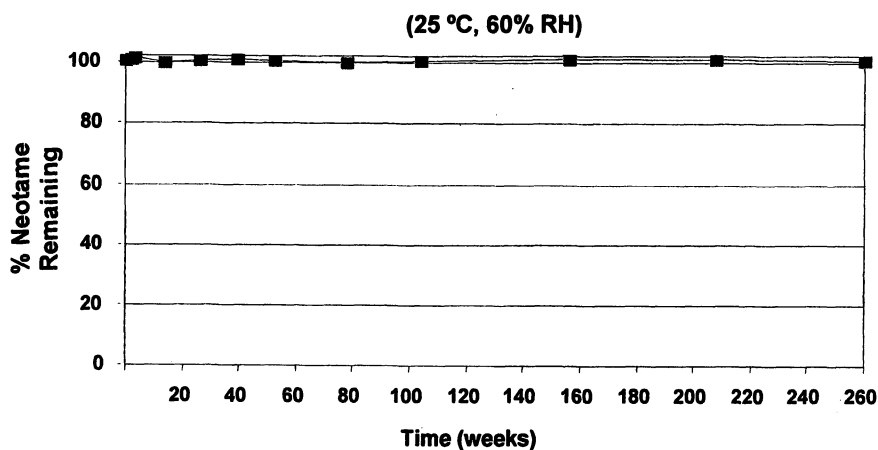
Figure 4. Stability of Neotame in the Dry Form (Reproduced with permission from Food Technol., July 2002, 56(7), 36. Copyright 2002.)
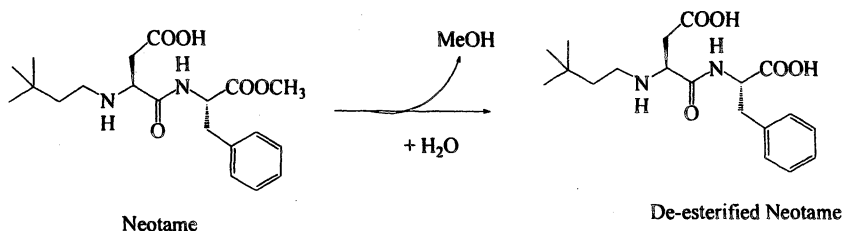
*Figure 5. Major pathway of degradation of neotame under hydrolytic condition
(Reproduced with permission from Food Technol., July 2002, 56(7), 36.
Copyright 2002.)*

De-esterified neotame is not sweet. Under conditions of use neotame does not degrade to phenylalanine. Unlike aspartame, neotame does not form a diketopiperazine (DKP) derivative (Figure 6). Neotame is compatible with reducing sugars and will not participate in Maillard browning reactions and is compatible with many aldehyde or ketone-based flavoring agents.

Neotame has similar stability to aspartame in many applications at low pH and temperature, stability improves at higher pH's & temperatures. For example in cola beverage neotame shows similar stability at the lower temperatures, but shows slightly better stability at the higher temperatures (Figure 7).
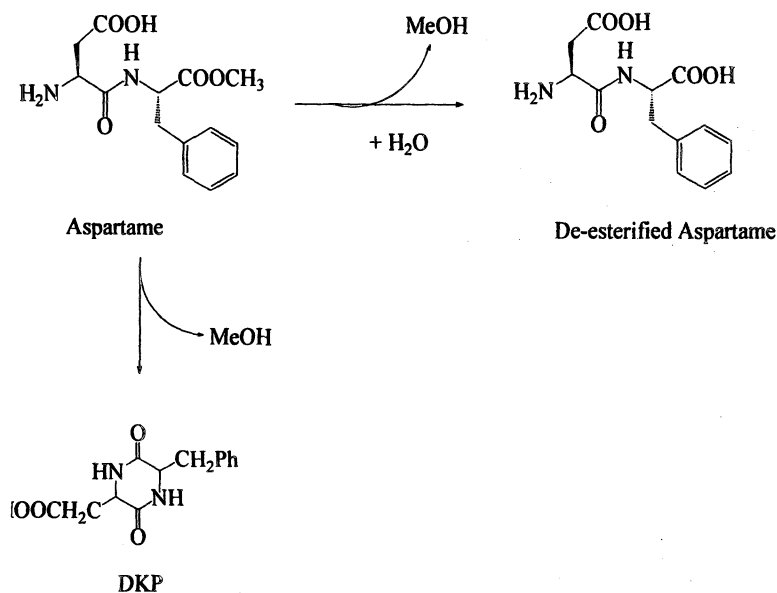
*Figure 6. Major pathways of degradation of aspartame under hydrolytic
conditions (Reproduced with permission from Food Technol., July 2002,
56(7), 36. Copyright 2002.)*
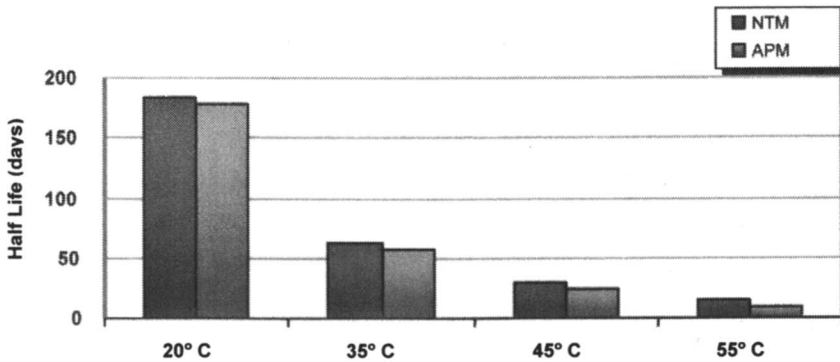
Figure 7. Stability of Neotame and Aspartame in Cola (pH 3.1) (Reproduced with permission from Food Technol., July 2002, 56(7), 36. Copyright 2002.) (See color insert in this chapter.)

In heat-processed beverages, such as lemon tea, both neotame and aspartame show good stability after High Temperature-Short Time (HTST) heat processing and during the beverage's shelf life (Figure 8). Unlike aspartame, neotame is not digested by the cultures found in yogurt, therefore neotame can be added before the incubation stage without loosing a significant amount of the sweetness (Figure 9). Since neotame is more heat stable than aspartame, it may not need to be encapsulated in application where aspartame requires encapsulation, such as in baking goods (Figure 10).
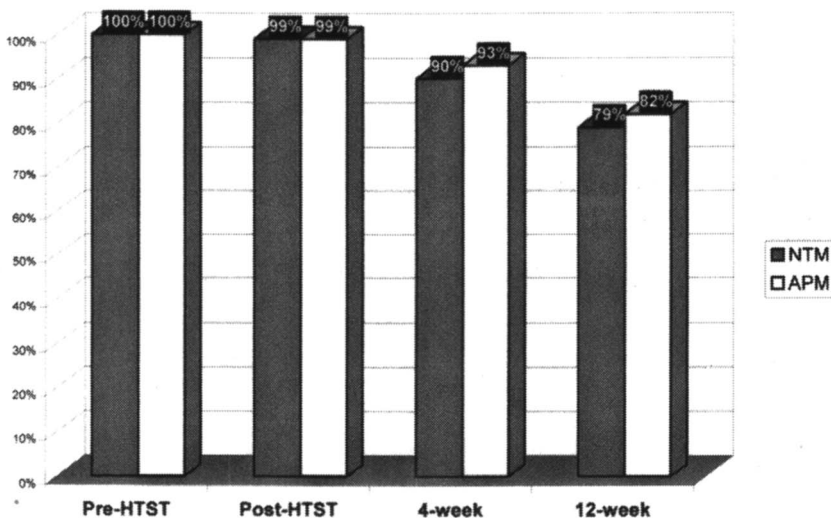
Figure 8. Stability of Neotame and Aspartame in Lemon Tea (Reproduced with permission from Food Technol., July 2002, 56(7), 36. Copyright 2002.)
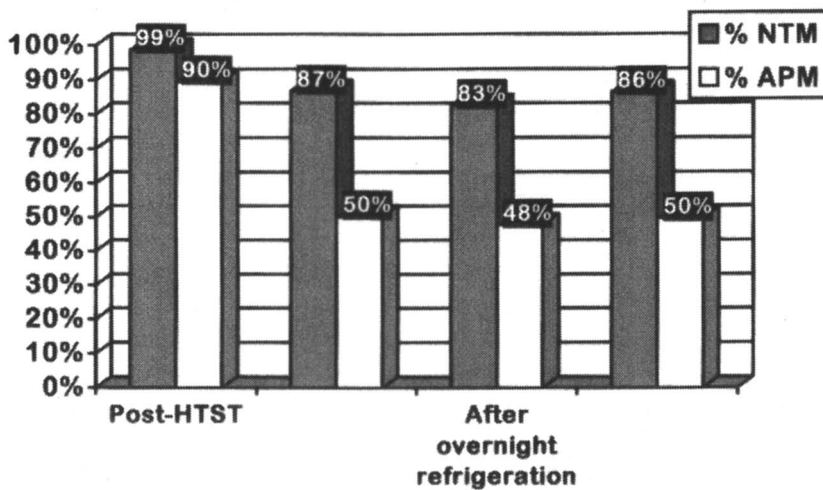
Figure 9. Stability of Neotame and Aspartame in Yogurt
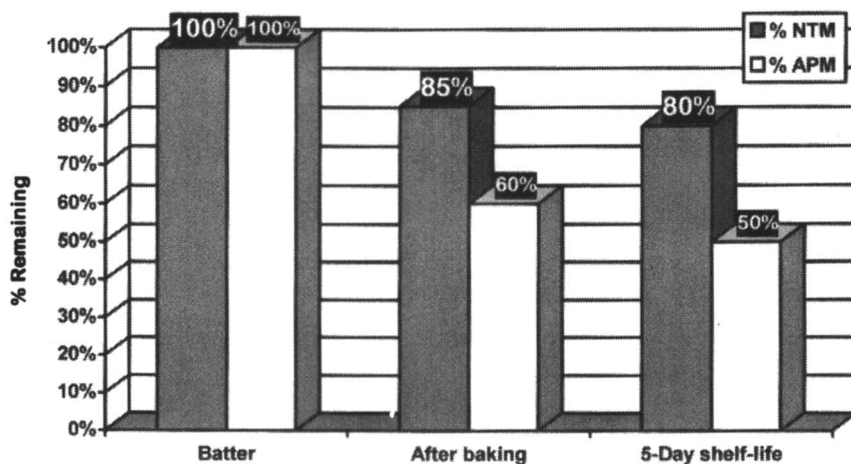
Figure 10. Stability of Neotame and Aspartame in Yellow Cake

Sensory properties of Neotame

Taste profile

Neotame exhibits a clean sweet taste profile at use levels in product applications. A trained descriptive panel evaluated neotame and sucrose at comparable sweetness levels in water. The taste profile of neotame is very similar to that of sucrose, with the predominant sensory characteristic of neotame being a very clean sweet taste. The sweetness of neotame increases as the concentration in water increases but taste attributes such as bitterness, sourness, and metallic taste are undetectable (Figure 11). In a similar study with neotame in a cola drink, increasing the sweetener concentration from 9 to 46 ppm improved the desirable flavor attributes (cola flavor, sweet taste, and mouthfeel) but not the undesirable notes (Figure 12).
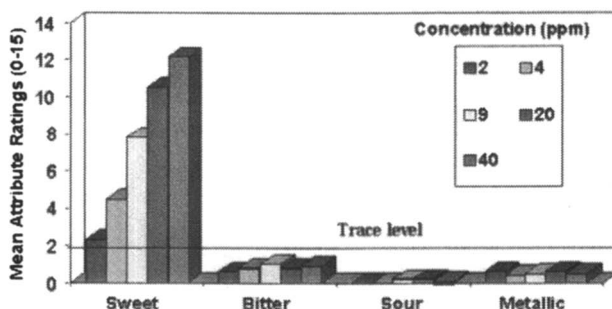
Figure 11. Descriptive taste profile of neotame at various concentrations in water (See color insert in this chapter.)

Sweetness temporal profile

The temporal profile of sweeteners demonstrates the changes in the perception of sweetness over time. This property is a key to the functionality of a sweetener and is complementary to its taste profile. Every sweetener exhibits a characteristic onset or response time and an extinction time. Most high-intensity sweeteners, in contrast to sugar, display a prolonged extinction time. As shown in Figure 13, the sweetness temporal profile of neotame in water is close to that of aspartame, with a slightly slower onset and slightly longer extinction time. A longer extinction time can be beneficial in some products such as chewing gum where prolonged sweetness is a desirable quality.

The sweetness temporal profile of neotame may also be modified by the addition of hydrophobic organic acids (e.g., cinnamic acid) and certain amino
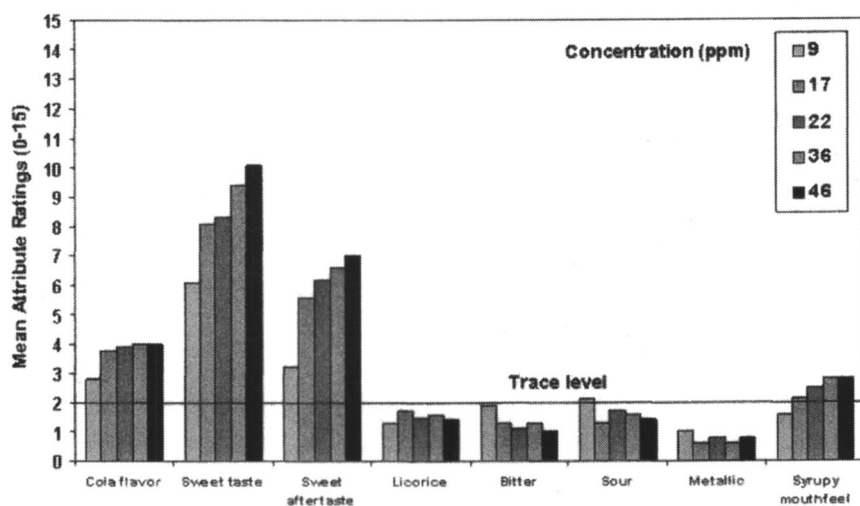
*Figure 12. Taste profile of neotame at various concentrations in cola
(See color insert in this chapter.)*
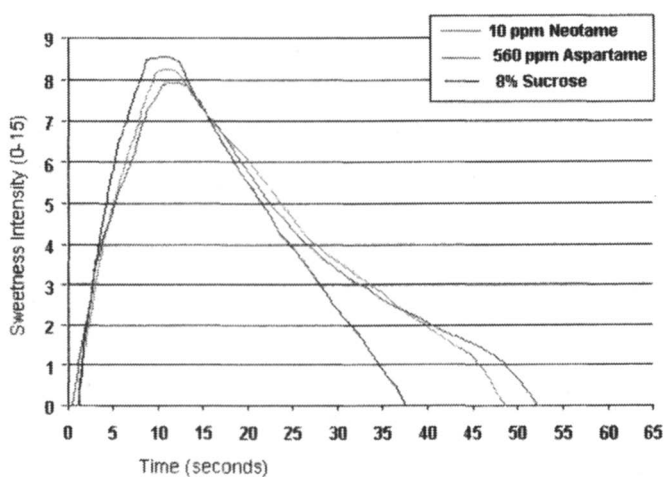
*Figure 13. Comparative temporal profile of neotame vs sucrose and aspartame
at isosweet concentrations in water (See color insert in this chapter.)*

acids (e.g., serine and tyrosine).[10,11,12] Taste modifiers may be used in concentrations necessary to achieve the desired taste profile of a product for a desired application.

Blending

Blending of sweeteners (nutritive as well as non-nutritive) is well known in the literature[13,14,15,16,17,18] to improve the taste characteristics, stability, and synergy. A blend of neotame and saccharin shows sweetness synergy and provides 14 to 24% greater sweetness than would be predicted by adding together the sweetness intensities of the individual sweeteners.[19] Such synergistic blends offer cost savings by decreasing the total amount of sweetener needed. Neotame can be blended with nutritive sweeteners as well as other high-intensity sweeteners such as aspartame, acesulfame salts, cyclamate, sucralose, saccharin, etc.[1] Furthermore, the clean sweetness of neotame permits its substitution for substantial amounts of carbohydrate sweeteners without altering the flavor of the product.

Because time-intensity and taste profiles of each sweetener is different and are also different from sucrose, the resulting profiles in blends combine properties of the different sweeteners and reduce the negative characteristics such as bitterness, metallic, etc. For example, the sweetness of acesulfame-K is generally perceived fairly quickly, it therefore, provides a quick impact sweetness, but often fades fairly quickly. Acesulfame-K also has a bitter-metallic taste that limits it use in many applications. Therefore, acesulfame-K combines particularly well with sweeteners having a more delayed onset of sweetness and a longer lasting sweetness, such as aspartame or neotame. So for example a blend that has neotame or aspartame contributing 70% of the sweetness and acesulfame-K contributing 30% of the sweetness, has a sweetness profile much closer to sugar with a quick onset, significantly reduced lingering sweetness, and no bitterness or metallic off-taste.

Neotame has also been shown to be very effective at replacing up to 50% of the sucralose in a beverage sweetened with 100% sucralose or a sucralose/acesulfame-K blend. Triangle taste tests have shown that consumers cannot differentiate between the sucralose sweetened beverage and beverage containing sucralose and neotame.[20] Because of neotame's attractive cost position, replacing sucralose with neotame can yield significant economic benefits.

Sugar substitution

Neotame's clean sweet taste is well suited for substitution of a portion of a carbohydrate sweetener while maintaining a taste that is indistinguishable from

the 100% carbohydrate product. For example, studies have shown that 20% of the carbohydrate sweetener such as high fructose corn syrup (HFCS) can be replaced with 2.1 ppm of neotame in a cola carbonated soft drink and the taste is indistinguishable from the 100% HFCS-sweetened cola beverage (Figure 14). Because neotame is $1/5^{th}$ the cost of HFCS and $1/10^{th}$ the cost of sugar, it can offer a significant economic benefit and, because neotame has no calories, offers a positive caloric benefit.
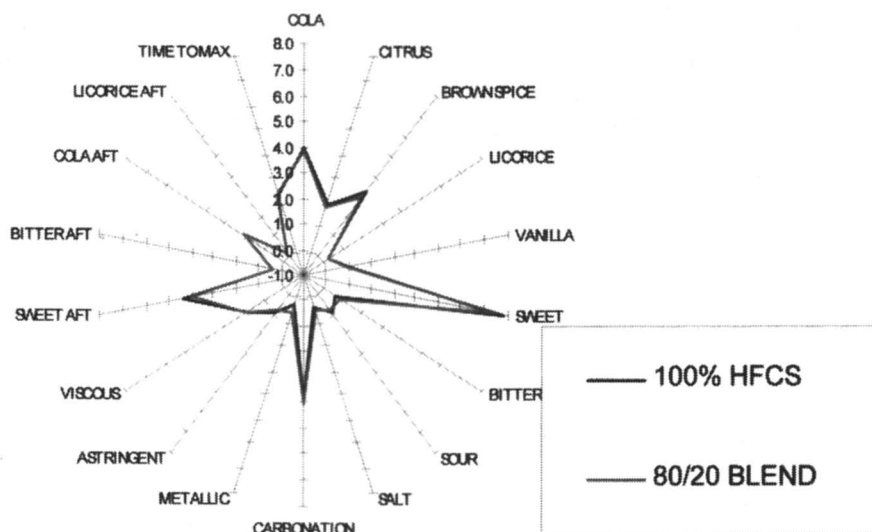
Figure 14. Descriptive test results of cola beverages – 100% High Fructose Corn Syrup (See color insert in this chapter.)

Flavor Modification and Enhancement

Flavor modifiers are substances that are added to enhance or modify a product's flavor, which includes the combined perception of taste, smell, and aroma. Products containing vitamins, nutraceuticals, pharmaceuticals, salt substitutes, and soy in various applications are often either bitter or harsh in flavor. The addition of neotame at a subsweetening level modifies or masks undesirable notes/qualities such as bitterness, astringency, and burning or cooling sensations. Undesirable attributes such as the potential bitterness of caffeine, cocoa, and potassium chloride and the harsh notes of medicinals and plant extracts can be modified or masked.

Neotame reduces the bitter taste of potassium chloride in salt substitutes, thereby providing a cleaner salty taste. In soy products neotame reduces or

eliminates "beany" flavor notes. Neotame modifies or enhances the attributes of many flavoring chemicals, including essential oils, oleoresins, plant extracts, reaction flavors, and mixtures thereof.[21]

Food Applications and Functionality

Historically, the stability and functionality of a new sweetener or an ingredient was determined for each food product before the sweetener was approved. This process generated redundant data. This redundancy could be avoided if products with similar ingredients and processing conditions could be reduced to representative test products for evaluation.

The functionality of neotame was demonstrated with a three-dimensional food matrix model representing the intended conditions of use in foods.[22] Based on experience with aspartame and the structural similarities of neotame and aspartame, product moisture, process temperature, and product pH were considered to be the key factors responsible for neotame stability and were selected to represent the three dimensions of the matrix. These products were prepared according to standard formulas, then packaged appropriately, stored at room temperature conditions (25 °C and 60% relative humidity)[23], and evaluated for stability at appropriate intervals. Neotame concentrations were determined using validated HPLC methods.

Functionality (sweetness) of the test products was determined using panels consisting of 35 to 50 persons. Samples were appropriately prepared, served, and evaluated using a five-point scale of categories ranging from 5 (much too sweet) to 1 (not at all sweet). The samples were considered functional if no more than 75% of the panelists rated the sweetness in categories 2 (not quite sweet enough) and 1. The evaluations of the test products are presented below:

1) Carbonated soft drink: Neotame remained functional as a sweetener in cola flavored carbonated soft drink through at least 16 weeks, consistent with currently marketed low-calorie carbonated soft drinks.[24]

2) Hot pack lemon tea: Neotame remained functional as a sweetener in hot pack lemon tea for approximately 25 weeks.

3) Powdered soft drink: At each evaluation the sweetness of the reconstituted drink received a rating of just about right, indicating that the product was stable and functional as a sweetener during 52-weeks of storage at 25 °C and 60% relative humidity.

4) Tabletop product: Neotame was considered stable and functional in tabletop products for 156 weeks when stored at 25 °C and 60% relative humidity.[25,26]

5) Chewing gum: Using neotame that was double encapsulated with modified starch and hydroxypropyl methyl cellulose (HPMC) resulted in a 52 weeks shelf-life.[27] Subsequent work has shown that in some formulations, encapsulation is not needed for neotame stability in chewing gum.

6) Dairy products/ strawberry yogurt: At the end of a 6-week period, the typical shelf life of these products, about 98% of the initial neotame remained. Sensory results showed that neotame had excellent functionality as a sweetener in yogurt.[25,28]

7) Bakery products/ yellow cake: Neotame was functional as a sweetener in yellow cake with 82% remaining after baking at 350 °F. After storage for five days, which is longer than cakes are commercially held for optimum freshness, there was only a 4% loss of neotame. The combined losses of less than 20% did not affect sweetener functionality.[25,29]

8) Other products: Functionality of neotame has been demonstrated in cereals and cereal-based foods[30], nutraceuticals[31], pharmaceuticals[32], edible gels[33], and confectionery products[34].

Benefits of Neotame

Neotame provides several benefits as a sweetener and/or flavor enhancer in food and beverage systems. Some of these benefits are reviewed below.

- 8,000 times more potent than sugar
- Because of its high potency, the quantity of neotame required to sweeten a product is about 1/30 to 1/60 of the amount of aspartame required and 1/12 to 1/15 of the amount of sucralose required.
- Has a clean, sweet taste, like that of sugar
- Neotame is non-caloric.
- Neotame requires no PKU labeling.
- Based on its chemistry, neotame is not likely to react with aldehydes and, consequently, may be compatible with flavors containing aldehydes.
- Neotame enhances the flavor of some ingredients such as mint, citrus, and fruit flavors in various food and beverage systems.
- Neotame is very cost effective

Regulatory Status of Neotame

Safety of neotame

The results of extensive research done in animals and humans using amounts of neotame that far exceed expected consumption levels clearly confirm the safety of neotame for the general population including children, pregnant women, and people with diabetes. Neotame is not mutagenic, teratogenic, carcinogenic and has no effect on reproduction. In addition, no special labeling for phenylketonuric individuals is required. The major route of metabolism of neotame is de-esterification. Both neotame and de-esterified neotame have short plasma half-lives with rapid and complete elimination.[25,35]

508

United States regulatory status

The United States Food and Drug Administration has approved of neotame for general use in food as a sweetener and flavor enhancer under GMP.

International regulatory status

The NutraSweet Company has filed for regulatory approval in a number of foreign countries. Approval has already been received from Mexico, China, Philippines, Indonesia, Australia and New Zealand and in over 20 other countries for use of neotame as a sweetener and flavor enhancer.

References

1. Nofre, C. and Tinti, J.-M. *N-Substituted derivatives of aspartame useful as sweetening agents.* U.S. patent 5,480,668. 1996.
2. Nofre, C. and Tinti, J.-M. Neotame: discovery, properties, utility. *Food Chem.* 2000, *69*, 245-297.
3. DuBois, G., Walters, D, Schiffman, S., Warwick, Z., Booth, B., Pecore, S., Gibes, K., Carr, B., and Brands, L. Concentration-response relationships of sweeteners. in *Sweeteners Discovery, Molecular Design, and Chemoreception*, D.E. Walters, F.T. Orthoefer, and G.E. DuBois eds., American Chemical Society, Washington, DC. 1991, Chapter 20, pp 261-276.
4. Nofre, C. and Tinti, J.-M. *Method of preparing a compound derived from aspartame, useful as a sweetening agent.* U.S. patent 5,510,508. 1996.
5. Prakash, I., *Method for preparing and purifying an N-alkylated aspartame derivative.* U.S. patent 5,728,862. 1998.
6. Prakash, I., *Synthesis of N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester using oxazolidinone derivatives.* U.S. patent 6,852,875. 2005.
7. Prakash, I. and Chapeau, M.-C. *N-3,3-Dimethylbutyl-L-aspartic acid and esters thereof, the process of preparing the same, and the process for preparing N-(N-(3,3-dimethylbutyl)-α L-aspartyl)-L-phenylalanine 1-methyl ester therefrom.* U.S. patent 6,077,962. 2000.
8. Orlovski, V., Prakash, I., Scaros, M. and Moore, C. *A method for the preparation of N-neohexyl-α-aspartyl-L-phenylalanine methyl ester from imidazolidin-4-one intermediates.* U.S. patent 6,465,677. 2002.
9. Prakash, I., Bishay, I., and Schroeder, S. Neotame: synthesis, stereochemistry and sweetness. *Synthetic Communications*, 1999, *29(24)*, 4461-4467.

10. Bishay, I., Prakash, I., Desai, N., and Gelman, Y. *Modification of the taste and physicochemical properties of neotame using hydrophobic additives.* PCT International application WO 00/69282. 2000.

11. Gerlat, P., Hatchwell, L., Walters, G., Miragilo, A., and Sawyer, H. *Use of N-neohexyl-α-aspartyl-L-phenylalanine methyl ester as flavor modifier.* U.S. patent application No. 09/465,837. 2000.

12. Prakash, I., Bishay, I., Desai, N., and Walters, D. Modifying the temporal profile of the high-potency sweetener neotame. J. Agric. Food Chem. **2001**, *49(2)*, 786-778.

13. Lavia, A. and Hill, J. *Sweeteners with masked saccharin aftertaste.* French patent 2,087,843. 1972.

14. Paul, T. *Chem. Zeit.* **1921**, *45*, 38-39.

15. Schiffman, S., Booth, B., Carr, B., Losee, M., Sattely-Miller, E., and Graham, B. Investigation of synergism in binary mixtures of sweeteners. *Brain Research Bulletin*, **1995**, *38(2)*, 105-120.

16. Scott, D. Saccharin-dipeptide sweetening compositions. British patent 1,256,995. 1971.

17. Verdi, R. and Hood, L. *Food Technol.* **1993**, *47*, 94-102.

18. Walters, E. High intensity sweetener blends. *Food Product Design.* Weeks Publishing Co. Northbrook, IL. 1993.

19. Pajor, L. and Gibes, K. N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester synergistic sweetener blends. U.S. patent 6,048,999. 2000.

20. Northland Laboratories Report. *NutraSweet Triangle Discrimination Study, Mixed Berry Flavored Beverage.* Northland Laboratories, Northbrook, IL July 11, 2005.

21. Gerlat, P., Walters, G., Bishay, I., Prakash, I., Jarrett, T., Desai, N., Sawyer, H., and Bechert, C.-L. *Use of additives to modify the taste characteristics of N-neohexyl-α-aspartyl-L-phenylalanine methyl ester.* U.S. patent 6,368,651. 2002.

22. Pariza, M., Ponakala, S., Gerlat, P., and Andress, S. Predicting the functionality of direct food additives. *Food Technol.* **1998**, *52(11)*, 56-60.

23. FDA. International conference on harmonization; guidelines for room temperature storage. *Fed. Reg.* **1994**, *59(183)*, 48754-48759.

24. Gerlat, P., Milovanovic, S., Ponakala, S., Ziegler, J., Sawyer, H., and Walters, G. *Neotame-based sweeteners for beverages.* PCT International application WO 99/30576. 1999.

25. FAP. Monsanto Co.: Filing a food additive petition. FAP 8A4580. Food and Drug Admin., *Fed. Reg.* **1998**, *63(27)*, 6762.

26. Towb, A., Dron, A., and Walters, G. *Drying of neotame with co-agents.* PCT International application WO 02/05660. 2002. Ponakala, S. V., Gerlat, P. A., Ziegler, J. G., Jarrett, T. N. and Cheng, J. *Tabletop sweetener compositions comprising sweetener with extremely high potency.* PCT International application WO 99/30577. 1999.

27. Roefer, W. Unpublished data. Dept. of Analytical Chemistry, The NutraSweet Company, Mt. Prospect, Ill. 2002.

28. Gaughan, W., Gerlat, P., Ziegler, J., Walters, G., Logli, L., Corliss, G., and Finley, J. *Neotame sweetener for dairy products and dairy product substitutes.* PCT International application WO 99/30578. 1999.

29. Chinn, B., Solter, S., Ponakala, S., Ziegler, J., Bakhoum, M., Jarrett, T., Paeschke, T., and Corliss, G. *Use of N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester in baked goods, frostings and bakery fillings.* PCT International application WO 99/30566. 1999.

30. Ponakala, S. and Corliss, G. *Cereals and cereal-based food sweetened with neotame.* PCT International application WO 00/056175. 2000.

31. Ponakala, S., Walters, G., Gerlat, P., and Hatchwell, L. *Nutraceuticals having N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester.* PCT International application WO 00/057726. 2000.

32. Ponakala, S. *Pharmaceutical compositions containing neotame.* PCT International application WO 01/028590. 2001.

33. Ponakala, S., Walters, G., and Schroeder, S. *Manufacture of edible gels sweetened with neotame.* PCT International application WO 00/056176. 2000.

34. Jarrett, T. *Confectionery food products sweetened with N-[N-(3,3-dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester.* PCT International application WO 01/010236. 2001.

35. FAP. Monsanto Co.: Filing a food additive petition. FAP 9A4643. Food and Drug Admin., *Fed. Reg.* 1999, *64(25)*, 6100.

Chapter 33

Sensory Properties of Neotame: Comparison with Other Sweeteners

Susan S. Schiffman[1], Elizabeth A. Sattely-Miller[1], and Ihab E. Bishay[2]

[1]Department of Psychiatry, Duke University Medical Center, Durham, NC 27710–3259
[2]The NutraSweet Company, 200 World Trade Center, The Merchandise Mart, Suite 936, Chicago, IL 60654

Neotame is a high potency sweetener with a molecular formula of $C_{20}H_{30}N_2O_5$. It is over 11,000 times sweeter by weight than sucrose at a sweetness equivalent to 5% sucrose (in water). Neotame reaches a maximum sweetness intensity equivalent to 15.1% sucrose. The temporal properties of neotame, like all other high potency sweeteners, differ somewhat from sucrose. The time of onset of sweetness of neotame is later than sucrose and it lingers longer. Cross-adaptation studies support ligand-receptor binding studies that indicate neotame along with aspartame and sucralose preferentially interact with the T1R2 subunit of the sweetener receptor. Neotame can substitute for 20-30% of the sweetness of soft drinks with no perceived difference in taste.

Neotame (N-[N-(3,3-dimethybutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester, CAS registry No. 165450-17-9) is a high potency sweetener that was designed by the French scientists Claude Nofre and Jean-Marie Tinti in 1994 (1,2). Its molecular formula is $C_{20}H_{30}N_2O_5$, and its molecular weight is 378.47 grams/mole (2). The chemical structure is given in Figure 1. Neotame is manufactured from the dipeptide sweetener aspartame and 3,3-dimethyl-butyraldehdye in a one-step chemical synthesis that includes a reductive alkylation, followed by purification, drying, and milling. Neotame was approved for general use as a sweetener and flavor enhancer in foods and beverages in 2001 in Australia and New Zealand and in 2002 in the United States of America. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) determined the safety of neotame as a food additive in 2003 with an Acceptable Daily Intake (ADI) of 2.0 mg/kg/body weight/day. For a 60 kg person, this ADI translates to the sweetness equivalence of over two pounds of sugar per day. Neotame is currently used as a sweetening agent in many countries throughout the globe.
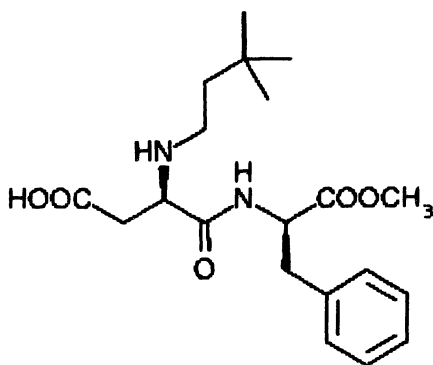
Figure 1. Chemical structure of neotame

The purpose of this chapter is to provide an overview of the sensory properties of neotame including similarities and differences with other sweet compounds. The topics covered in the chapter include: a) taste detection and recognition thresholds, b) concentration-response characteristics and maximum sweetness, c) potency, d) sensory profile including temporal properties, e) cross-adaptation studies, f) mixture studies, and g) sweetener substitution with neotame in carbonated media. The results of these studies provide insight into how sweeteners including neotame interact with the sweetener receptors.

Taste Thresholds of Neotame and Other Sweeteners

The taste detection and recognition thresholds for neotame (and sucralose) were determined using a forced choice procedure and compared with threshold values for other sweeteners previously published by Schiffman et al. (3). The results given in Table I indicate neotame along with the large protein sweeteners monellin and thaumatin have the lowest thresholds while carbohydrate sweeteners such as fructose have the highest thresholds. The magnitude of the sweetness recognition thresholds in Table I varies over 6 orders of magnitude depending on the chemical structure of the sweetener. Detection and recognition thresholds for sucrose (not given in Table I) are similar to those for fructose (4). The mean thresholds given in Table I are based on data for individuals under the age of 50 years. Mean thresholds for older individuals tend to be slightly higher (approximately twice as high for most sweeteners including neotame). Overall, the sweetness recognition thresholds for neotame are four orders of magnitude lower than those for sucrose and fructose.

**Table I. Mean Sweetener Thresholds in Ascending Order
[Modified from (3)]**

Sweetener	Detection threshold	Recognition threshold
Monellin	0.0195μM	0.0676μM
Thaumatin	0.0716μM	0.210μM
Neotame	0.621μM	1.14μM
Neohesperidin dihydrochalcone	2.20μM	5.28μM
Rebaudioside	4.61μM	13.6μM
Stevioside	5.31μM	23.7μM
Sucralose	8.77μM	13.0μM
Sodium saccharin	0.0147mM	0.0497mM
Aspartame	0.0224mM	0.0449mM
Acesulfame-K	0.0444mM	0.161mM
D-tryptophan	0.109mM	0.546mM
Calcium cyclamate	0.266mM	1.33mM
Fructose	4.39mM	16.6mM

Concentration-Response (C-R) Curve and Maximum Sweetness

Figure 2 shows the concentration-response (C-R) curve for neotame in water established by a trained sensory panel [see (5) for procedural details]. The

sweetness intensity is expressed in sucrose equivalence (%SE), i.e. the concentration of neotame (ppm) that is equivalent in sweetness to an x% sucrose solution in water. If the C-R curve for neotame were extended to an infinite neotame concentration, the maximum sweetness intensity (plateau) attainable would be 15.1% in water. The equation in Figure 2 is a standard equation used in physiology [see (5)] to relate concentration and response (here sweetness intensity).
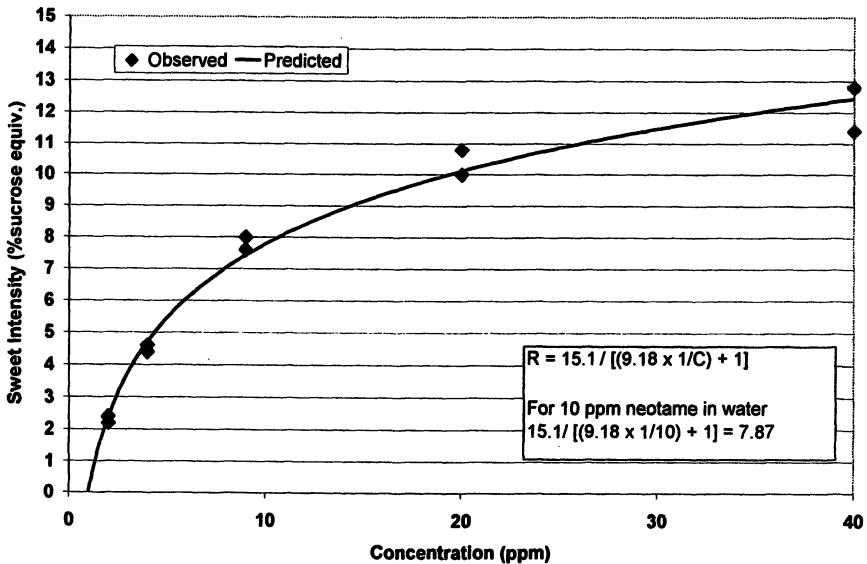
Figure 2. The concentration-response curve (C-R) curve for neotame in water (Reproduced with permission from reference 1. Copyright 2002.)

Other high potency sweeteners such as acesulfame-K, aspartame, calcium cyclamate, sodium saccharin, and sucralose have C-R curves with shapes similar to neotame such that the gradient decreases with increasing concentration. However, each individual sweetener varies uniquely with regard to the maximum sweetness reached (see Table II for maximum sweetness reached). Sucrose, unlike high potency sweeteners, continues to increase in perceived intensity at concentrations higher than 16% in water. The maximum sweetness intensity reached for all sweeteners is reduced when flavoring and other ingredients are included in the mixture. For neotame, the maximum sweetness reached in a cola drink is 13.4% compared with 15.1% in water.

Potency

The potency of a sweetener relative to sucrose by weight is calculated from its concentration-response (C-R) curve (5). The potency of neotame in water at sweetness levels equivalent to 2.5% (Pw2.5), 5% (Pw5), 7.5% (Pw7.5), and 10% (Pw10) sucrose are given in Table III along with potencies of other sweeteners. For example, the potency of neotame at a sweetness equivalent to 5% sucrose (i.e. 50,000 ppm sucrose) is calculated as follows: 50,000ppm/4.54ppm equals 11,013. Thus neotame is over 11,000 times sweeter by weight than sucrose at a sweetness equivalent to 5% sucrose. In Table III it can be seen that neotame is the most potent sweetener at sweetness intensities equivalent to 7.5 and 10% sucrose. Potency, like maximum sweetness, is generally reduced in mixtures with food and beverage ingredients compared to potency in water.

Table II. Maximum Sweetness in Sucrose Equivalence (%SE) Reached for High Potency Sweeteners in Water

Sweetener	%SE
Aspartame	16.0
Neotame	15.1
Sucralose	13.0
Acesulfame-K	11.6
Na Cyclamate	11.3
Thaumatin	10.1
Rebaudioside	10.0
Stevioside	9.9
Neohesperidin dihydrochalcone	9.8
Na Saccharin	9.0
Monoammonium glycyrrhizinate	7.3

Temporal Properties and Sensory Profile of Neotame

The temporal properties of neotame, like other high potency sweeteners, differ somewhat from sucrose (see Figure 3). The time of onset of sweetness is later than sucrose and it lingers longer. When trained panelists were asked to rate the time of onset of sweetness for a broad range of sweet compounds at moderate sweetness intensity comparable to 5-7% SE (using a scale labeled early, early to middle, middle, middle to late, and late), sweeteners tended to fall into three categories. Those with "early" and "early to middle" onset included fructose, glucose, sorbitol, sucrose, sodium cyclamate, sodium saccharin, acesulfame-K, and aspartame. Those with intermediate or "middle" onset included sucralose, neotame, stevioside and rebaudioside. Thaumatin always

had the latest onset in sweetness (6). Ratings of lingering at concentrations equivalent in sweetness intensity to 5-7% sucrose (i.e. intensity of sweetness at 30 and 45 seconds after tasting) by the trained Duke University panel gave a similar grouping. That is, sweeteners with early (and early to middle) onset had the least lingering; those with intermediate or "middle" onset had intermediate lingering; sweeteners with late onset displayed the greatest lingering. Overall, the comparison of temporal properties of neotame with the temporal properties of a broad range of sweeteners indicates that the onset of sweetness as well as the lingering properties for neotame are in the middle of the range.

The sensory profile of neotame determined by a trained panel indicates that neotame is predominantly sweet with minimal off-tastes compared with other high potency sweeteners. Figure 4 shows a plot of the perceived sweetness versus bitterness for several sweeteners including neotame [see (7) for the relationship of bitterness to sweetness as a function of concentration for a broader range of sweeteners]. It can be seen that neotame has little bitterness relative to stevioside which becomes quite bitter as concentration is increased.

Table III. Potencies of Sweeteners in Water Relative to 2.5%, 5%, 7.5% and 10% Sucrose by Weight

	Pw2.5	Pw5	Pw7.5	Pw10
Thaumatin	21,111	14,167	7,222	278
Neotame	13,736	11,013	8,278	5,556
Neohesperidin dihydrochalcone	1377	906	434	
Sucralose	633	636	546	385
Na Saccharin	515	444	247	
Rebaudioside	375	250	125	
Aspartame	241	196	152	107
Acesulfame-K	194	140	87	34
Stevioside	180	120	59	
Monoammonium glycyrrhizinate	229	110		
Na Cyclamate	28	32	29	18
Fructose	1.3	1.3	1.3	1.3
Glucose	0.6	0.6	0.6	0.6

Cross-Adaptation Studies: Implications for Receptor Binding

The psychophysical method of cross-adaptation has been used to investigate whether two sweeteners are occupying the same sites on the sweetener receptor
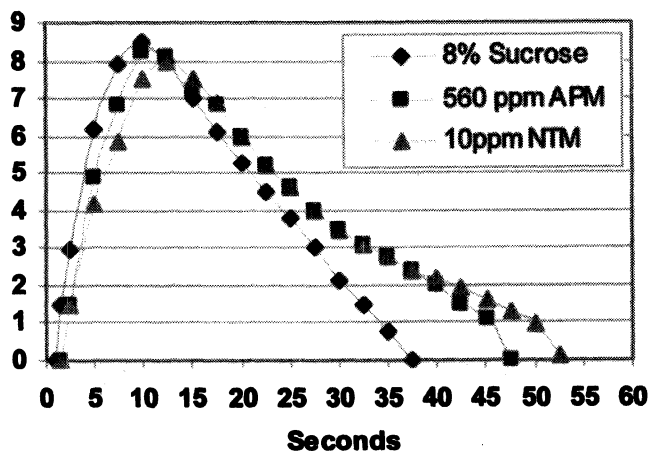
Figure 3. Temporal properties of neotame (NTM) compared with aspartame (APM) and sucrose (Reproduced with permission from reference 1. Copyright 2002.) (See color insert in this chapter.)

(8). If adaptation to one sweetener results in a decreased sweet response to another sweetener, this suggests that the two stimuli are binding to a similar site on the sweetener receptor. However, if adaptation to one sweetener does not decrease the sensation of another sweetener, a possible implication is that different areas of the sweetener receptor code these stimuli. In the study by Schiffman et al. (8), fourteen sweeteners (seven high potency sweeteners and seven sugars) were cross-adapted with one another. When a sugar was utilized as the adapting stimulus, there was a consistent reduction in the intensity of all other sweeteners. However, when the adapting stimulus was a high potency sweetener, the effect was unpredictable and could be a reduction or an enhancement depending on the pair of stimuli tested. This indicated that multiple receptor sites must mediate sweet taste, a conclusion that has now been confirmed in ligand-binding studies (9,10).

The cross-adaptation paradigm was extended here to pairs of neotame combined with 8 other sweeteners at four intensity levels (equivalent to 3%, 6%, 9%, and 12% sucrose, see Table IV). Cross-adaptation was performed with all pairs of sweeteners with neotame at the same sucrose equivalence using a trained taste panel of 16 individuals. Each pair of sweetener combinations was presented in an ABA experimental design. The panelist first swirled Sample A around his/her mouth for 5 seconds and then expectorated. Immediately after expectoration of Sample A, the panelist rated the sweetness intensity (i.e. preadaptation sweetness intensity). The panelists then waited for 1-minute during which period they rinsed their mouths thoroughly with deionized water. After the 1-minute interval had elapsed, the experimenter instructed the panelists
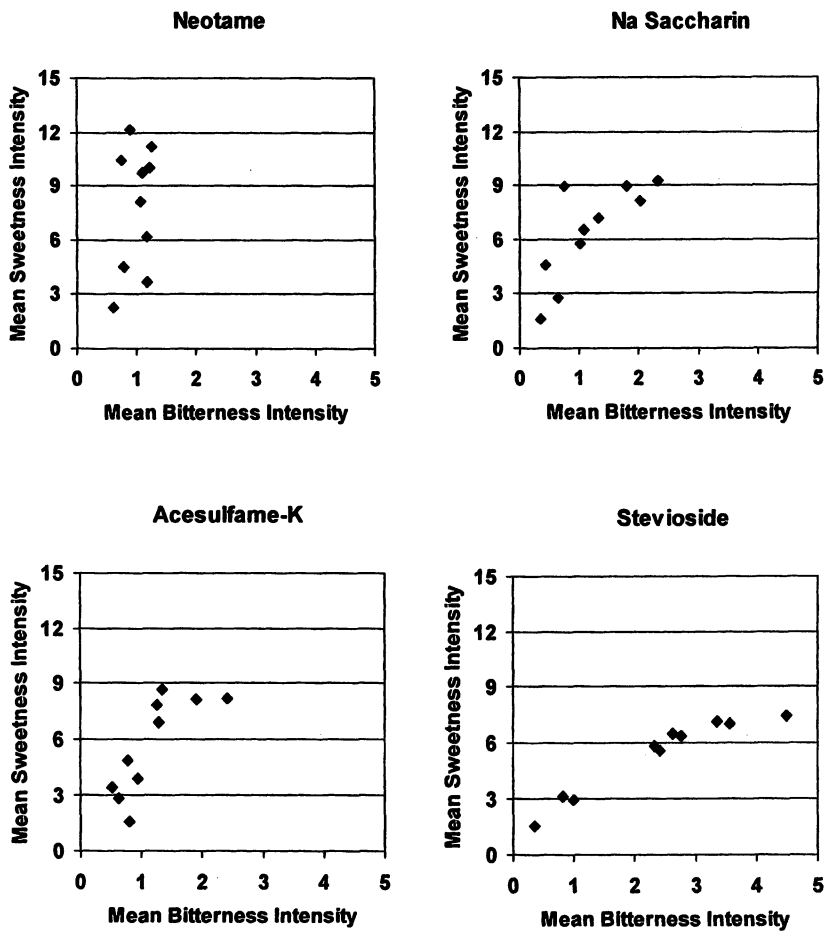
Figure 4. The relationship between mean sweetness and bitterness ratings for neotame and three other sweeteners (Na saccharin, acesulfame-K, and stevioside)

to sip Sample B (i.e. the adapting stimulus), holding it in their mouths for one full minute. The experimenter indicated when to expectorate Sample B. Immediately following expectoration of the adapting stimulus (Sample B), the experimenter instructed panelists to sip the 3rd sample which was another cup of Sample A, hold it in their mouths for 5 seconds, and then expectorate. Panelists then rated the sweetness intensity of Sample A (i.e. postadaptation sweetness intensity). See Figure 5 for a diagram of the methodology. The results of this cross-adaptation experiment will be shown to give strong insight into the binding sites for various sweeteners.

Before describing the results, it is first helpful to briefly describe our current understanding of the recognition of sweet compounds at the receptor level. We know that there is a sweet receptor that is a dimer composed of two G-protein-coupled receptors (GPCRs), each of which is comprised of 7 transmembrane helices with the N- and C-termini being extra- and intracellular, respectively (9,10). These two GPCRs are termed T1R2 and T1R3 (see Figure 6). The large extracellular N-terminal domain (NTD) on each sweet taste receptor subunit is thought to be involved in ligand recognition although some sweeteners such as cyclamate salts appear to bind to other sites in the transmembrane domain (9). Physiological studies indicate that there are multiple binding sites on the NTD with which diverse chemical classes of sweet compounds can interact. The N-terminal domain consists of an extracellular Venus flytrap domain (VFTD) which is connected to the transmembrane helices via a cysteine rich domain (CRD).

Table IV. Concentrations of Sweeteners Tested in the Neotame Cross-Adaptation Study

Sweetener	3 sweet	6 sweet	9 sweet	12 sweet
Acesulfame-K	164ppm	504ppm	1627ppm	n/a
Aspartame	129ppm	336ppm	720ppm	1680ppm
Fructose	2.3%	4.7%	7.1%	9.4%
Glucose	5%	10%	15%	20%
Na saccharin	58.5ppm	157.5ppm	n/a	n/a
Neotame	2.28ppm	6ppm	13.5ppm	35.3ppm
Sorbitol	0.34M	0.54M	0.78M	1.1M
Sucralose	46.5ppm	78.6ppm	196.3ppm	649ppm
Sucrose	3%	6%	9%	12%

Results of the cross-adaptation studies confirm physiological reports about activation sites for specific sweeteners. Figures 7a-d show the results of the cross-adaptation of neotame paired with aspartame, sucralose, sucrose, and glucose respectively. An asterisk indicates a significant change after cross-
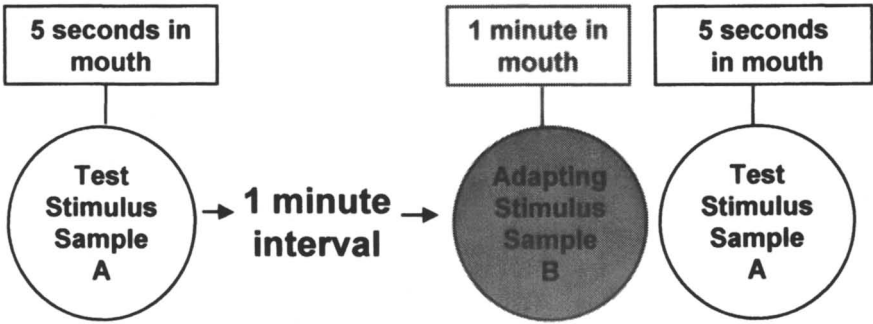
Figure 5. Diagram of the cross-adaptation paradigm
(See color insert in this chapter.)
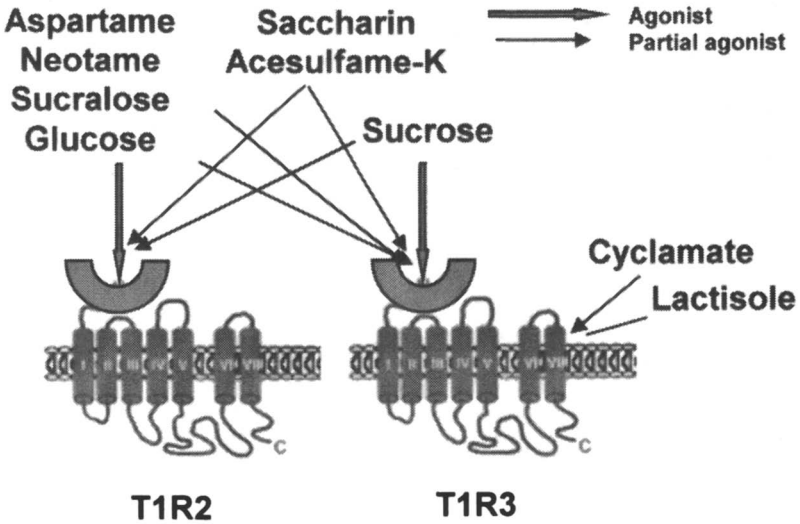
Figure 6. The sweet receptor dimer (T1R2 and T1R3) along with
probable binding sites for various sweeteners and modifiers
(See color insert in this chapter.)

adaptation. The mutual cross-adaptation between pairs of neotame with aspartame and sucralose supports ligand-receptor binding studies that indicate these three sweeteners preferentially interact with the VFTD of the T1R2 subunit (9,10). That is, sweeteners that have a greater affinity for the T1R2 subunit tend to cross-adapt with one another (i.e. their binding sites are overlapping). For sugars, the NTD of T1R3 binds sucrose with 5 times higher affinity than the NTD of T1R2, and the relationship is reversed for glucose which is bound at 2.8 times higher affinity by T1R2 than T1R3. Interestingly, there is only minor cross-adaptation between sucrose and neotame at low to moderate concentrations and none at the highest concentration. This lack of significant cross-adaptation is probably due to the fact that sucrose binds preferentially to T1R3 [based on the Kd values, see Nie et al. (10)], while neotame preferentially binds to T1R2 (9,10). When neotame is the adapting stimulus, there are higher than expected responses to some carbohydrate sweeteners (glucose, fructose, and sorbitol)—but not sucrose. The enhancement of the taste of glucose (see Figure 7d) after adaptation with neotame is probably due to its higher affinity for T1R2 than other natural sugars. Overall, this elevated sweetness of glucose after neotame adaptation suggests the sweet receptor is an allosterically modulated receptor that supports cooperative binding. That is, neotame is a positive allosteric modulator of the T1R2 receptor subunit, i.e. neotame enhances the binding of monosaccharides and a polyhydric alcohol that is hydrogenated from a monosaccharide. Another finding was that adaptation to neotame blunts the response to acesulfame-K but the reverse is minimal. This is possibly due to the fact that acesulfame-K, like saccharin, binds to both T1R2 and T1R3 (11-14); also acesulfame-K, like saccharin, activates bitter receptors T2R43 and T2R44 (15).

Neotame Mixed with Other Compounds

The purpose of this experiment was to determine the effect on the sweetness of neotame, if any, of other compounds blended in a mixture with neotame. The concentrations of neotame and blend compounds tested are given in Table V and include lactisole [the sodium salt of ±2-(4-methoxyphenoxy)propanoic acid) which is used as a sweetness blocker, see (16,17)], chloride salts, ascorbate salts, zinc salts, gluconate salts, bitter compounds, and amino acids. Sweetness intensity was evaluated by a trained panel over time (30 seconds). The results showed only minor effects of blending neotame with other compounds, and only two significant findings emerged. First, lactisole (at both 250 and 500 ppm) partially suppressed the sweetness at 2.5, 5, 7.5 and 10 sweetness equivalence. This pattern of partial suppression is similar to the partial suppression of other sweeteners with an intermediate onset (stevioside and rebaudioside) (see 16). Schiffman et al. (16) showed stronger sweetness suppression by lactisole for

APM-Adapting Stimulus

Neotame-Adapting Stimulus
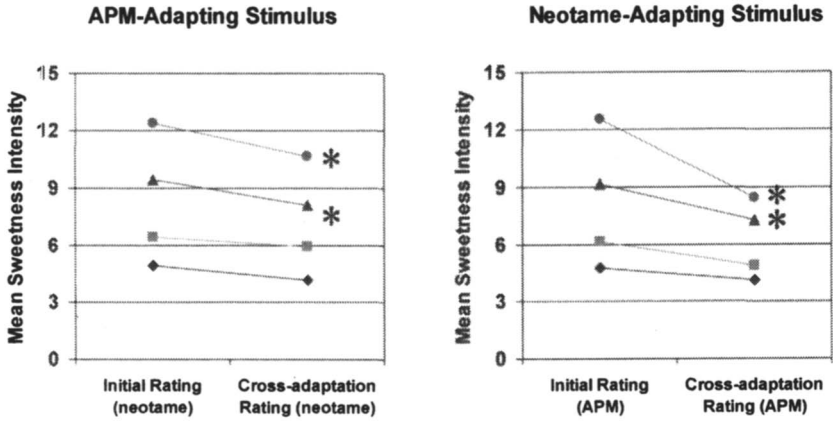
Figure 7a. Cross-adaptation of neotame paired with aspartame (APM)
(See color insert in this chapter.)

Sucralose-Adapting Stimulus

Neotame-Adapting Stimulus
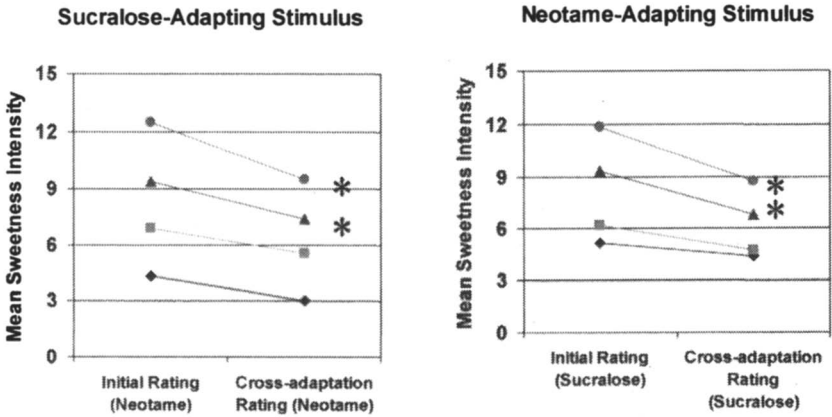
Figure 7b. Cross-adaptation of neotame paired with sucralose
(See color insert in this chapter.)
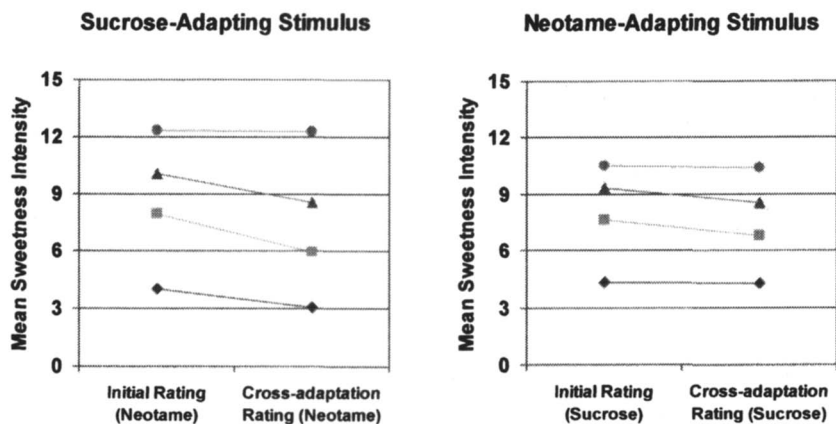
Figure 7c. Cross-adaptation of neotame paired with sucrose
(See color insert in this chapter.)
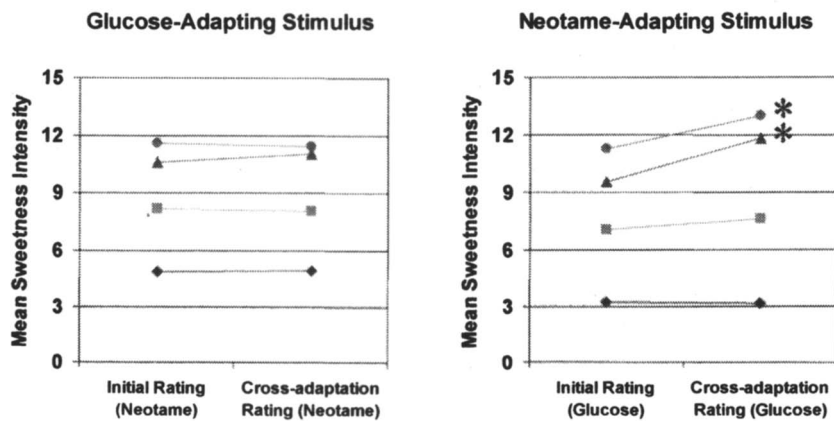
Figure 7d. Cross-adaptation of neotame paired with glucose
(See color insert in this chapter.)

524

sweeteners with an early onset (acesulfame-K, aspartame, fructose, glucose, sodium cyclamate, sorbitol, and sucrose) and a lack of suppression was found for sweeteners with a later onset and more complex tastes (monoammonium glycyrrhizinate, neohesperidin dihydrochalcone, and thaumatin). Thus the degree of lactisole suppression appears to involve a temporal factor in that it is most effective with quick-onset sweeteners, least effective with late-onset sweeteners, and moderately effective with sweeteners having intermediate onset such as neotame.

Second, for the sweetness intensity ratings at multiple time-lapsed intervals for mixtures of neotame with salts, an ANOVA found significant differences between neotame/salt blends at the 10-second, 20-second, and 30-second intervals. Further analysis indicated that at the 10-second time interval, neotame with $CaCl_2$ and neotame with NH_4Cl had small but significantly higher sweetness intensity ratings than neotame alone and neotame with NaCl. At the 20-second time interval, neotame with $CaCl_2$ and neotame with NH_4Cl had significantly higher sweetness intensity ratings than neotame with NaCl. No other significant effects were found. These data suggest that neotame sweetness is not substantially changed by these ingredients that could be present in neotame-sweetened product.

Sweetener Substitution with Neotame in Carbonated Media

Because neotame (like all other high potency sweeteners) differs somewhat in temporal properties from carbohydrate sweeteners such as sucrose or high fructose corn syrup (HFCS), neotame is often used as part of a blend. When 20-30% of sweetness of a carbohydrate-sweetened product is replaced by neotame, consumers are unable to distinguish a change. In one study, test stimuli consisted of bottled cola-flavored carbonated soft drinks (CSDs). Samples contained either 100% HFCS-55 which contains 55% fructose or were blends of HFCS-55 with neotame in which 20% of the sweetness of the syrup came from 2.9 ppm neotame. All carbonated soft drinks (CSD's) used in the study were composed of 80% carbonated water and 20% syrup by weight. The sensory profile for the 100% HFCS55 sample and the 80/20 HFCS55/NTM blend were statistically identical as shown in Figure 8. If a person typically consumed 2 HFCS sodas per day at 150 calories per soda, they would consume 109,500 calories from soda per year (300 x 365). Use of an 80/20 HFCS55/NTM blend would result in a 20% reduction of caloric intake (i.e. 21,900 less calories). If all other energy intake and expenditure remained the same, this could result in a weight loss of 6.25 pounds in a year (assuming 3500 excess calories leads to a weight gain of 1 pound).

Table V. Concentrations Used for Mixture Studies in which Neotame Was Blended with Other Compounds

Compounds blended with neotame	Neotame Levels Tested	Blend Compound Levels
Lactisole	1.82 ppm (2.5% SE) 4.54 ppm (5% SE) 9.09 ppm (7.5% SE) 18 ppm (10% SE)	250 ppm, 500 ppm
Chloride salts	10.34 ppm (8% SE)	0.0024M NaCl, 0.0024M KCl, 0.00155M $MgCl_2$, 0.00336M $CaCl_2$, 0.0015M NH_4Cl
Ascorbate salts	10.34 ppm (8% SE)	Detection threshold levels: 0.00589M Ca ascorbate 0.00404M Na ascorbate Suprathreshold levels: 0.02356M Ca ascorbate 0.01616M Na ascorbate
Zinc salts	10.34 ppm (8% SE)	Detection threshold levels: 0.387mM ZnCl, 0.456mM $ZnSO_4$ Suprathreshold levels: 1.548mM ZnCl, 1.824mM $ZnSO_4$
Gluconate salts	10.34 ppm (8% SE)	Detection threshold levels: 0.002M each of calcium, magnesium, sodium, and potassium gluconate Suprathreshold levels: 0.008M each of calcium, magnesium, sodium, and potassium gluconate
Bitter compounds	10.34 ppm (8% SE)	Detection Threshold Levels: 4.795µM quinine HCl 0.02% caffeine 4.164nM denatonium benzoate 0.101M urea Suprathreshold levels: 9.59µM quinine HCl 0.08% caffeine 9nM denatonium benzoate 0.404M urea

Table 5. *Continued.*

Compounds blended with neotame	Neotame Levels Tested	Blend Compound Levels
Amino acids	10.34 ppm (8% SE)	Detection threshold (DT) levels: 0.182mM L-aspartic acid 0.0630mM L-glutamic acid 6.61mM L-phenylalanine 4 x DT level: 0.728mM L-aspartic acid 0.252mM L-glutamic acid 26.4mM L-phenylalanine 8 x DT levels: 1.46mM L-aspartic acid 0.504mM L-glutamic acid 52.9mM L-phenylalanine
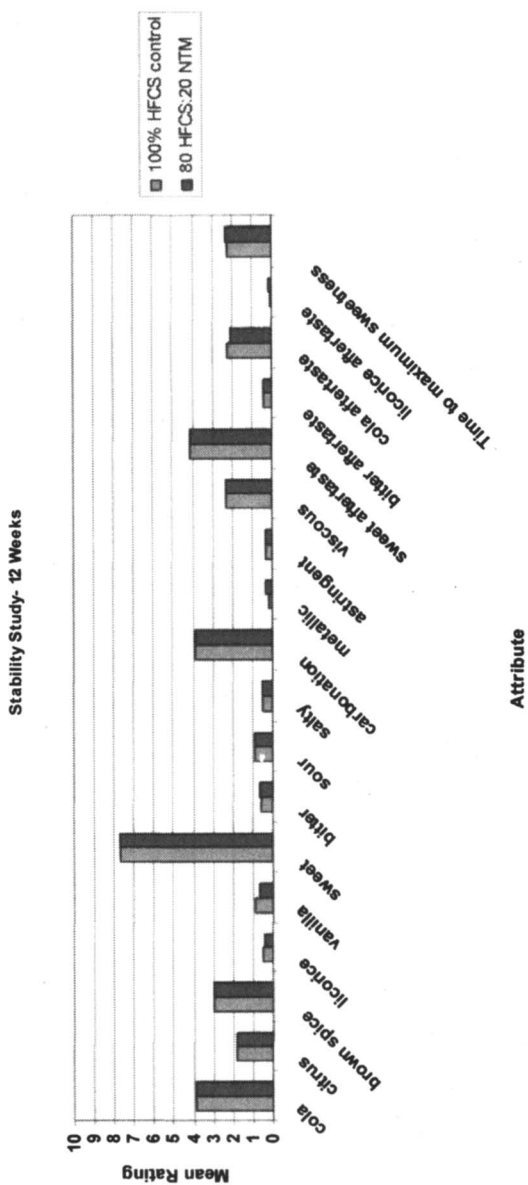
*Figure 8. Comparison of sensory profile of 100% HFCS and 80/20 HFCS55/NTM blend.
(See color insert in this chapter.)*

528

Summary

Neotame has a taste detection threshold of 0.621 µM and a sweetness recognition threshold of 1.14 µM. The recognition threshold is four orders of magnitude lower than that of sucrose or fructose. Neotame is predominantly sweet with minimal bitterness, sourness, metallic or other off-tastes It is over 11,000 times sweeter by weight than sucrose at a sweetness equivalent to 5% sucrose (in water). At sweetness intensities equivalent to 7.5 and 10% sucrose, neotame is more potent than any other sweetener utilized in foods internationally. The concentration-response curve reveals that neotame reaches a maximum sweetness intensity equivalent to 15.1% sucrose. The temporal properties of neotame, like all other high potency sweeteners, differs somewhat from sucrose. The time of onset of sweetness of neotame is later than sucrose and it lingers longer. Cross-adaptation studies support ligand-receptor binding studies that indicate neotame along with aspartame and sucralose preferentially interact with the T1R2 subunit of the sweet receptor. Neotame can substitute 20-30% of the sweetness of soft drinks with no perceived difference in taste.

References

1. Prakash, I.; Corliss, G.; Ponakala, R.; Ishikawa, G. Neotame: The next-generation sweetener. *Food Technol.* **2002**, *56(7)*, 36-45.
2. Nofre, C.; Tinti, J.-M. Neotame: discovery, properties, utility. *Food Chem.* **2000**, *69*, 245-257.
3. Schiffman, S. S.; Lindley, M. G.; Clark, T. B.; Makino, C. Molecular mechanism of sweet taste: Relationship of hydrogen bonding to taste sensitivity for both young and elderly. *Neurobiol. Aging* **1981**, *2*, 173-185.
4. Pfaffmann, C. The sense of taste. In *Handbook of Physiology, Vol. I*. Field, J. (Ed.) American Physiological Society: Washington, DC., 1959;: pp. 507-533.
5. DuBois, G. E.; Walters, D. E.; Schiffman, S. S.; Warwick, Z. S.; Booth, B. J.; Pecore, S. D.; Gibes, K.; Carr, B. T.; Brands, L. M. Concentration-response relationships of sweeteners. A systematic study. In *Sweeteners. Discovery, Molecular Design, and Chemoreception*. Walters, D. E.; Orthoefer, F. T.; DuBois, G. E. (Eds.) American Chemical Society: Washington, D.C., 1991; pp. 261-276.
6. Schiffman, S. S.; Sattely-Miller, E. A., Bishay, I. E. Time to maximum sweetness intensity of binary and ternary blends of sweeteners. *Food Qual. Pref.* **2007**, *18*, 405-415.
7. Schiffman, S. S.; Booth, B. J.; Losee, M. L.; Pecore, S. D.; Warwick, Z. S. Bitterness of sweeteners as a function of concentration. *Brain Res. Bull.* **1995**, *36*, 505-513.

8. Schiffman, S. S.; Cahn, H.; Lindley, M. G. Multiple receptor sites mediate sweetness: Evidence from cross adaptation. *Pharmacol. Biochem. Behav.* **1981**, *15*, 377-388.

9. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X. Different functional roles of T1R subunits in the heteromeric taste receptors. *Proc. Nat. Acad. Sci. USA.* **2004**, *101(39)*, 14258-14263.

10. Nie, Y.; Vigues, S.; Hobbs, J. R.; Conn, G. L.; Munger, S. D. Distinct contributions of T1R2 and T1R3 taste receptor subunits to the detection of sweet stimuli. *Curr. Biol.* **2005**, *15(21)*, 1948-1952.

11. Inoue, M., Reed, D. R., Li, X., Tordoff, M. G., Beauchamp, G. K.; Bachmanov, A. A. Allelic variation of the Tas1r3 taste receptor gene selectively affects behavioral and neural taste responses to sweeteners in the F2 hybrids between C57BL/6ByJ and 129P3/J mice. *J. Neurosci.* **2004**, *24*, 2296–2303.

12. Lu, K.; McDaniel, A. H.; Tordoff, M. G.; Li, X.; Beauchamp, G. K.; Bachmanov, A. A.; VanderWeele, D. A.; Chapman, C. D.; Dess, N. K.; Huang, L.; Wang, H.; Reed, D. R. No relationship between sequence variation in protein coding regions of the Tas1r3 gene and saccharin preference in rats. *Chem. Senses.* **2005**, *30(3)*, 231-240.

13. Zhao, G. Q.; Zhang, Y.; Hoon, M. A.; Chandrashekar, J.; Erlenbach, I.; Ryba, N. J; Zuker, C. S The receptors for mammalian sweet and umami taste. *Cell* **2003**, *115*, 255-266.

14. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili, Z.; Varadarajan, V.; Zou, S.; Jiang, P.; Ninomiya, Y.; Margolskee, R. F. Detection of sweet and umami taste in the absence of taste receptor T1r3. *Science* **2003**, *301(5634)*, 850-853.

15. Kuhn, C.; Bufe, B.; Winnig, M.; Hofmann, T.; Frank, O.; Behrens, M.; Lewtschenko, T.; Slack, J. P.; Ward, C. D.; Meyerhof, W. Bitter taste receptors for saccharin and acesulfame K. *J. Neurosci.* **2004**, *24(45)*, 10260-10265.

16. Schiffman, S. S.; Booth, B. J.; Sattely-Miller, E. A.; Graham, B. G.; Gibes, K. M. Selective inhibition of sweetness by the sodium salt of +/-2-(4-methoxyphenoxy)propanoic acid. *Chem. Senses* **1999**, *24(4)*, 439-447.

17. Lindley, M. G. Method for inhibiting sweetness. US Patent 4,567,053. **1986**, January 28.

Chapter 34

Recent Developments in Structure–Taste Studies of Sulfamates

William J. Spillane[*], Damien P. Kelly, Jean-Baptiste Malaubier, Gary G. Hanniffy, Brendan G. Feeney, Catherine C. Coyle, Lorraine M. Kelly, Emer F. Thompson, Danny G. Concagh, and Maryanne B. Sheahan

Department of Chemistry, National University of Ireland, University Road, Galway, Ireland

The effect of sulfamation on known tastants has been investigated using several series of compounds containing a primary amine function namely, nitroanilines, phenylureas and –thioureas and amino acids and peptides. Profound changes in taste took place on sulfamation. The effect of chirality on the taste portfolios of various sulfamates has also been examined by preparing sets of enantiomeric pairs from aliphatic, aliphatic/aromatic and alicyclic/aromatic precursor amines and aminoalcohols. Some interesting taste differences have emerged, though these are not as great as observed in the first study.

Introduction

For some years we have been synthesizing sodium sulfamates RNHSO$_3$Na, where R may be aliphatic (straight chain or branched), alicyclic, aromatic and hetero- (open chain and cyclic) and developing both quantitative and semi-quantitative structure-taste (SAR) relationships (*1-3*). The SARs developed have involved the use of various spatial, electronic and other parameters and the use of a number of techniques, such as linear and quadratic discriminant analysis (*4,5*) and more recently classification and regression (CART) analysis (*6,7*). The derived relationships generally have good predictive ability in the sense that if a large training set is used they can usually predict the predominant tastes of most of the members of a small test set correctly. Secondly they have faired quite well in correctly predicting the tastes of compounds that at the time had not been synthesized. Thirdly, because steric or volume parameters are always involved in the SARs constructed, a reasonable estimate can be made of the 'opening' dimensions of the receptor site.

Apart from seeking quantitative structure-taste relationships qualitative ones have also been sought by examining the effects of changes in the –NHSO$_3^-$ Na$^+$ portion of the sulfamate moiety. Thus, replacement of the amino hydrogen by an alkyl group destroys sweetness (*8*) but the replacement of the negative charge on the sulfamate anion does not and sulfamate esters RNHSO$_3$R' tend to be strongly sweet with concomitant bitterness (*9*).

However, we have not produced a new highly potent sulfamate (cyclamate) sweetener and the sweetest known sodium sulfamate, sodium *exo*-norbornylsulfamate with a relative sweetness (RS) ~ 76 (*3*) has been known for more than twenty years (*10*). Now in two fresh approaches (i) known (usually) sweet tastants have been sulfamated and the taste portfolios of the products examined and (ii) the effect of chirality on sulfamate tastes has been looked at.

Sulfamation of Known Tastants

One could list a variety of ways of trying to induce sweetness or prepare new sweet compounds. They would include:

(i)   use of templates such as suosan, aspartame etc. (*11*),
(ii)   use of the Nofre and Tinti Multicomponent Attachment Theory (MCA) (*12*) or other structure-taste relationships (*6,13,14*),
(iii)   the identification of atoms, groups or molecules known to be sweet taste potentiators and the introduction of these into other molecules (*15*).

An interesting early example of (iii) is shown in Figure 1 where an attempt was made to prepare a new sweet molecule(s) by combining two known sweeteners, namely, dulcin and saccharin (*16*).

Dulcin

Saccharin

"Saccharin-6-dulcin"
(Tasteless)

"Saccharin 2-*p*-ethoxyformanilide"
(Tasteless)
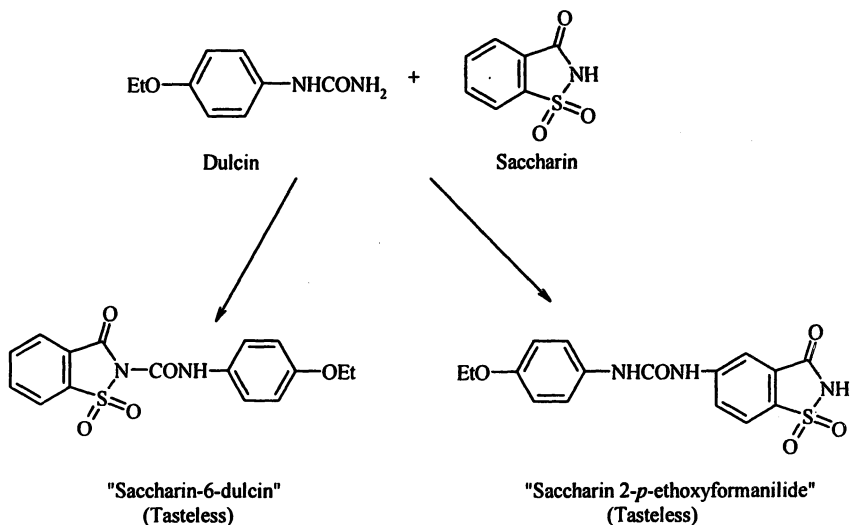
Figure 1. Combining the known sweeteners Dulcin and Saccharin

A more or less similar strategy has been tried with the sweet dihydrochalcone (DHC) shown in Figure 2 which was sulfamated to give a sulfamate with the same level of sweetness (*17*). This latter example shows one can try to induce additional sweetness by introducing a 'sweet-conferring entity' into a known sweet molecule. Probably the reason that the taste did not change is because the crucial 'receptor' sites of the DHC molecule were not interfered with as a result of sulfamation at the relatively distant $-NH_2$ position.

A dihydrochalcone (DHC)

A dihydrochalcone-sulfamate
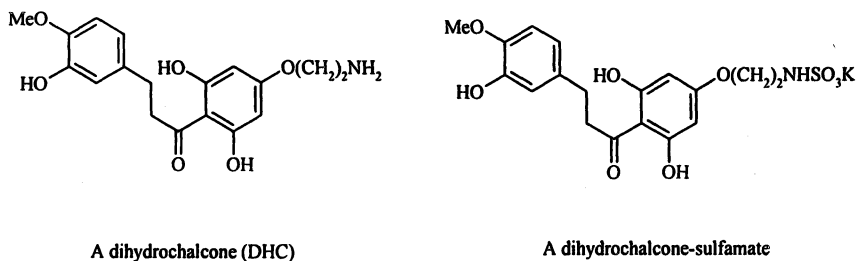
Figure 2. Effect of sulfamation on taste of a dihydochalcone (DHC)

This is the kernel of our approach in this part of our work but in order to proceed we have to (i) identify molecules with a primary amine function (suitable 'sulfamatable' site – the molecule must retain a hydrogen atom on the sulfamate nitrogen after sulfamation (*vide infra*) to have the possibility of being sweet and (ii) have available taste data in the literature for these molecules (see Figure 3).

Suitable

$$NH_2 \quad \xrightarrow{SO_3} \quad NHSO_3^-$$

Not suitable

$$R'NH \quad \xrightarrow{SO_3} \quad R'NSO_3^-$$

$$R'R''N \quad \xrightarrow{SO_3} \quad R'R''N.SO_3^-$$

Requirements:
1) Primary amine function
2) Taste data already available in the literature
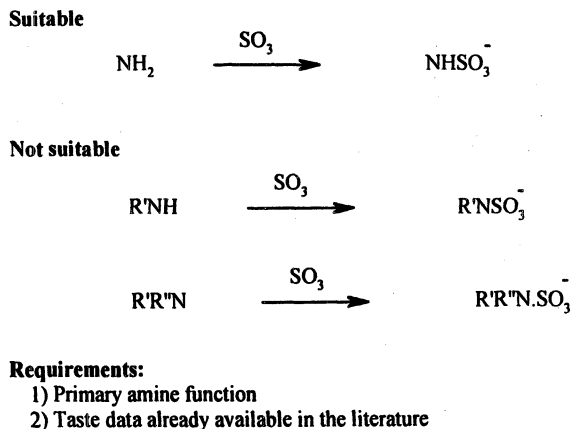
Figure 3. Criteria for suitable molecules for sulfamation

Fortunately the, mostly older, literature contains large amounts of taste data since there was a tendency then to include tasting of a new compound together with a plethora of other determinations such as solubility, mp/bp, Kjeldahl analysis for nitrogen (when appropriate) and various additional physical measurements. Several extensive compilations of systematic taste data are available (*18-21*).

The molecules that we have chosen for sulfamation are shown schematically by class in Figure 4. At the top left of the Figure anilines (mostly nitroanilines) are shown and moving anti-clockwise through the Figure are phenylureas, phenylthioureas, amino acids and dipeptides.

Anilines

Anilines were either available commercially or synthesized as shown in Figure 5 with separation by flash chromatography and/or recrystallization.

All sulfamates irrespective of precursor were synthesized by the procedures illustrated in Figure 6.

In the third vertical column in Table I the literature tastes of the parent anilines are reported and most of these can be obtained from two major listings
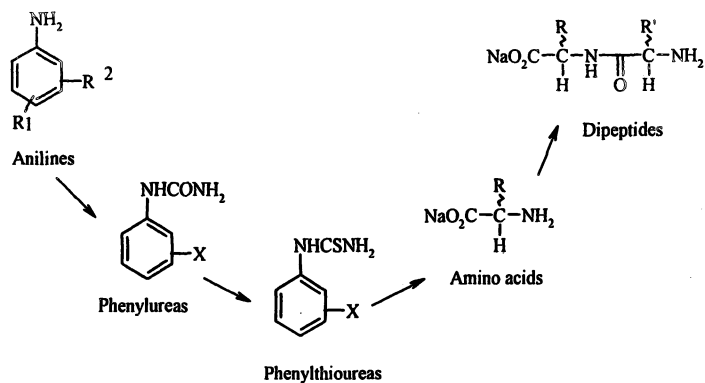
Figure 4. Molecules of known tastants which possess a "sulfamatable" site
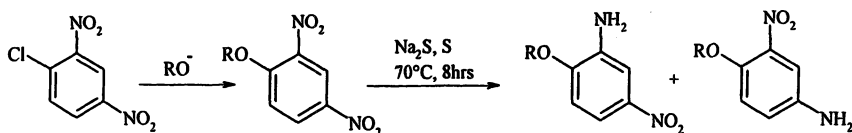
Figure 5. Synthesis of nitroanilines

Preparation of reagent *in situ*

Sulfamation

α-Pic =
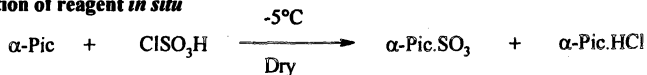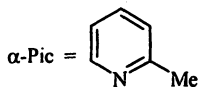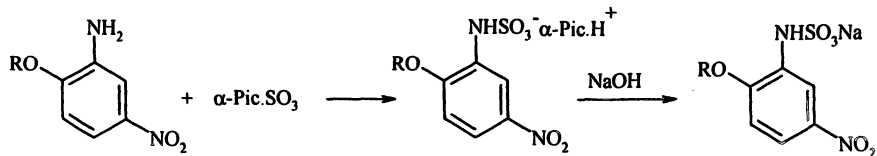
Recrystallization from aqueous EtOH
Tests for Cl^-, SO_4^{2-}, $-NHSO_3^-$
Characterization by H^1 and C^{13} NMR, IR and C, H and N analysis

Figure 6. Synthesis of sulfamates

Table I. Comparative taste studies of various mono- and disubstituted anilines and their sulfamates

Aniline $R^1R^2C_6H_3NH_2$		Lit. taste of Anilines	Predominant taste(s)[a] & lesser taste(s) of sulfamates $R^1R^2C_6H_3NHSO_3Na$
R^1	R^2		
2-NO$_2$	H	Tasteless	Very bitter
3-NO$_2$	H	Sweet	Bitter
4-NO$_2$	H	Almost Tasteless	Sour
3-CN	H	Sweet	Sweet
2-Cl	5-CF$_3$	Sweet	Bitter/Sweet a.[b]
2-Br	5-CF$_3$	Sweet	V. bitter
2-F	5-CF$_3$	Sweet	V. bitter
2-Pr^{n}O	5-NO$_2$[c]	Sweet	Bitter/Sweet
2-F	5-NO$_2$	Sweet	Sweet/Bitter
2-Cl	5-NO$_2$	Sweet	Sweet/Sour
2-Me	5-NO$_2$	Sweet	Sweet/Bitter
2-MeO	4-NO$_2$	Sweet	Sour

[a]Bold font indicates predominant taste. [b]Sweet a. means sweet aftertaste. [c]Synthesised in 34% yield.

of the tastes of organic compounds (*18,20*) and some tastes can be found in Verkade (*21*) and Blanksma and van der Weyden (*22*).

There are some interesting anilines listed in Table I. For example, 3-nitroaniline (R^1 = 3-NO$_2$, R^2 = H) is believed to be the earliest sweet compound reported in the literature in 1846 (*23*) and 2-n-propoxy-5-nitroaniline, known as P-4000, was the sweetest compound known (*24*) until the discovery of the hyperpotent sweeteners about 20 years ago. Comparsion of the literature taste data for the anilines in column 3 of Table I with that for the sulfamated anilines in column 4 shows that sweetness is frequently retained on sulfamation though it is often tempered with bitterness or sourness. The literature tastes for the anilines are qualitative but probably reliable and in a few cases different groups have reported the same taste. For example, 2-chloro-5-nitroaniline (R^1 = 2-Cl, R^2 = 5-NO$_2$) is reported to be sweet by Wheeler in 1895 (*25*) and by Blanksma in 1946 (*26*).

Phenylureas and –thioureas

About half of the phenylureas needed were available commercially and the others were synthesized by the methods (*27,28*) outlined in Figure 7.

$$ArNH_2 \quad + \quad HNCO \xrightarrow[35°C]{Dil.\ CH_3CO_2H} ArNHCONH_2$$

$$ArNH_2 \quad + \quad SCHNCOC_6H_5 \xrightarrow{\text{Heat}} ArNHCSNHCOC_6H_5$$

$$\downarrow \text{Debenzoylation}$$

$$ArNHCSNH_2$$
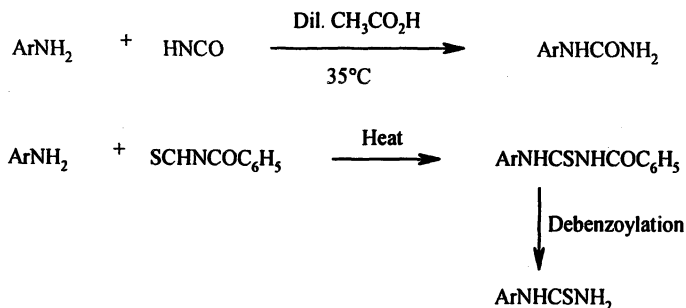
Figure 7. Synthesis of phenylureas and phenylthioureas

The % yields of those synthesized are given in Table II. The third and fourth columns in the Table record the literature tastes (*18-21*) and the tastes found by us for the parent ureas and generally there is good agreement where comparsion is possible. The sulfonation of the ureas was carried out as for the anilines above (see Figure 6). The last column contains the tastes recorded for the resulting phenylureasulfonates and there are a number of interesting changes. 2-Fluorophenylurea (X = 2-F) a bitter compound becomes predominantly sweet on sulfonation, 3-methylphenylurea (X = 3-Me) has some slight sweetness introduced and 4-methoxyphenylurea (X = 4-MeO) retains substantial sweetness. The former well known commercial sweetener Dulcin, 4-ethoxyphenylurea (X = 4-EtO) used in the US in the fifties for a time, retains a little sweetness after sulfonation. The final compound on the list is 2-methylphenylthiourea which retains its bitterness on sulfamation.

One issue which arises in the case of the ureas is the possibility of various products forming. These are shown in Figure 8. Disulfamation occurs readily with some ureas and thus (A) and (B) are possible products of the sulfamation reaction. The expected and desired monosubstituted product would be (C) but (D) could also form. Formation of (A) and (B) can be inhibited by using less pyridine sulfur trioxide adduct in the sulfamation. Proton NMR analysis of the product shows if (A) or (B) are present since some or all of the Hs on the two nitrogens N^1 and N^2 will be missing. Similarly if (C) forms the two Hs on N^1 and N^2 respectively can be seen and if (D) is present the N^1 H is missing and the N^2 Hs can be clearly discerned (*29*).

Amino acids and dipeptides

In Table III literature tastes for a series of amino acids and dipeptides are brought together. The first seven amino acid tastes in column 2 have been reported by Birch and Kemp (*30*) and the taste of L-phenylalanine is given by

Table II. Comparison of tastes of various phenylureas, thioureas and their sulfonates

Phenylurea/thiourea $XC_6H_4NHCYNH_2$[a]	% yield	Lit. taste	Found taste	Taste(s) of sulfonates $XC_6H_4NHCYNHSO_3Na$[a]
4-Br	85	Sweet	Sour/Sweet a.[c]	Bitter
H	-	Bitter	Bitter	Sour
2-Me	-	Tasteless	Tasteless	Bitter/Sour
3-Me	-	Bitter	Bitter	Bitter/Sour/Sweet a.
4-Me	-	Sweet	Sweet	Sour
2-MeO	38	Tasteless	Bitter	Tasteless/Bitter
4-MeO	-	Sweet	Sweet	Bitter/Sweet a.
2-EtO	53	-	Tasteless	Bitter
3-EtO	46	-	Tasteless	Bitter
4-EtO	83	Sweet	Sweet	Bitter/Sweet a.
2-F	-	-	Bitter	Sweet
4-F	-	-	Bitter	Sour
2-Me	17	-	Tasteless/Bitter a.[c]	Bitter

[a]Y = O for the first 12 compounds, Y = S for the last entry. [b]Bold font indicates predominant taste and normal font indicates lesser tastes. [c]a. means aftertaste.

A   B

C   D
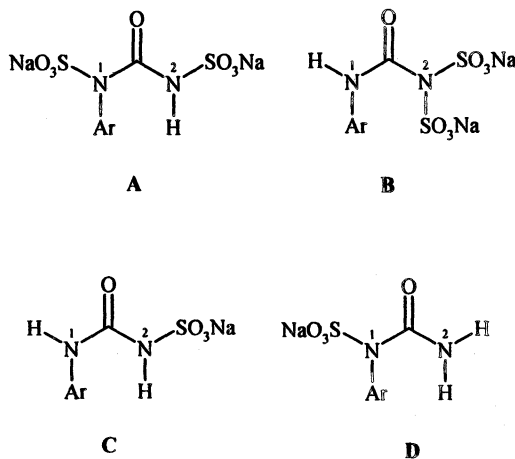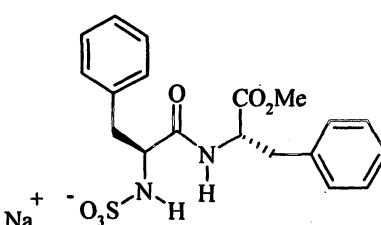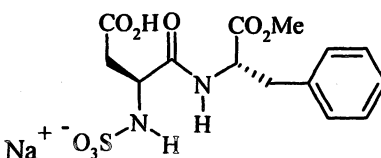
Figure 8. Possible phenylureasulfonate products

538

Table III. Tastes of amino acids and dipeptides and of their sulfamates

Amino acids	Lit. taste	Sulfamates RNHSO$_3$Na R=	Predominant taste(s)[a] & lesser taste(s)
Glycine	Sweet	NaO$_2$C—CH$_2$—	Salty
L-alanine	Sweet	NaO$_2$C—CH(CH$_3$)—	**Bitter**
L-methionine	Bitter	NaO$_2$C—CH—(CH$_2$)$_2$—S—CH$_3$	Sweet/Sour
L-valine	Sweet/Bitter	NaO$_2$C—CH—CH—(CH$_3$)$_2$	**Sweet**/Sour
L-glutamic acid	Sour	NaO$_2$C—CH—(CH$_2$)$_2$—CO$_2$Na	Salty/Sour
L-aspartic acid	Sour	HO$_2$C—CH$_2$—CH—CO$_2$H	**Sour**
L-aspartic acid	Sour	NaO$_2$C—CH$_2$—CH—CO$_2$Na	Salty
L-phenylalanine	Bitter	HO$_2$C—CH—CH$_2$—C$_6$H$_5$	Sour/Salty
L-phenyl-L-phenylalanine methyl ester	Bitter		**Bitter**
L-aspartyl-L-phenylalanine methyl ester (Aspartame)	Sweet		**Sour**

[a]Bold font indicates predominant taste.

Solms, Vuataz and Egli (*31*). The tastes of the two dipeptides are reported by Mazur, Schlatter and Goldkamp (*32*). There are some features of interest in Table III, which records the literature tastes in column 2 and then in the final column the taste assessments of the 'sulfamated amino acids and dipeptides'. Glycine and L-alanine lose sweetness on being sulfamated, however sweetness is induced in L-methionine and retained in L-valine. Less change may be noted in the other amino acids when sulfamated. L-aspartic acid can retain its sourness in the monosodium salt but becomes salty when it is isolated as the trisodium salt. Sulfamation of the first dipeptide produces no change but sulfamation of aspartame destroys sweetness producing a sour compound.

Effect of Stereoisomerism on Sulfamate Taste

There have been some studies on the tastes of structural isomers of sodium sulfamates (*1,6*) and some of these are illustrated in Figure 9, where it is clear that the position of a particular group or atom or of the sulfamate grouping can have a major effect on taste.
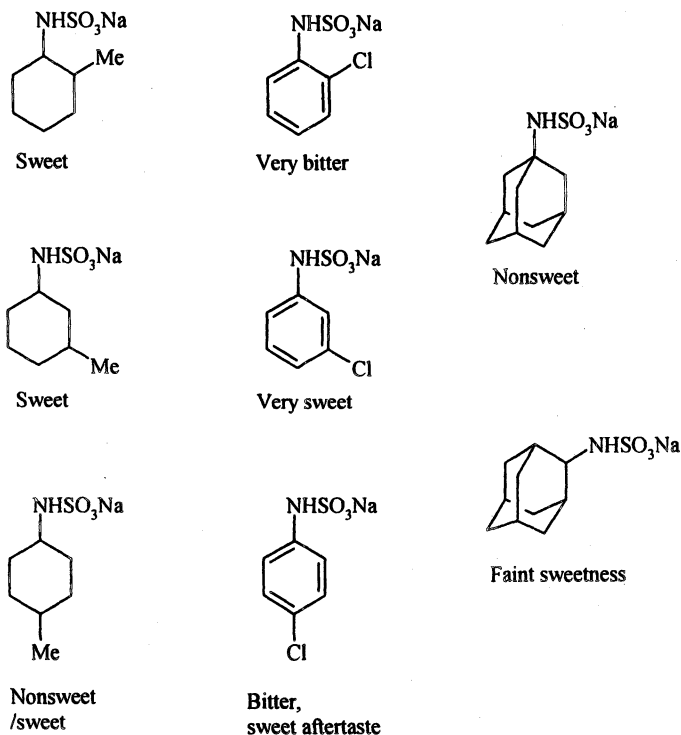
Figure 9. Tastes of structural isomers of sulfamates

For the sulfamates the effect of geometrical isomerism (Figure 10) has been examined, particularly by Unterhalt and Boschemeyer in a series of papers (*33-35*) and de Nardo, Runti and Ulian (*10*) have shown that sodium *endo*-norbornylsulfamate is tasteless and the *exo*-isomer is 'molto dolce' and is the sweetest sulfamate synthesized to date. The RS values for the two compounds are 9 and 70 respectively (*3*).

In the second part of our current work we have been examining the effect on taste of the stereoisomerism of pairs of sodium sulfamate (R)- and (S)-isomers. For the ten pairs of compounds shown in Table IV only minor differences have been observed, though some sweetness has been introduced into sodium 1-(1S)-(1,2,3,4)-tetrahydro-1-naphthalenylsulfamate compared to the (R)-isomer (see entry 8) and removed in sodium 1-(1S)-(3-methoxyphenyl)-ethylsulfamate as compared to the (R)-isomer (see entry 2).
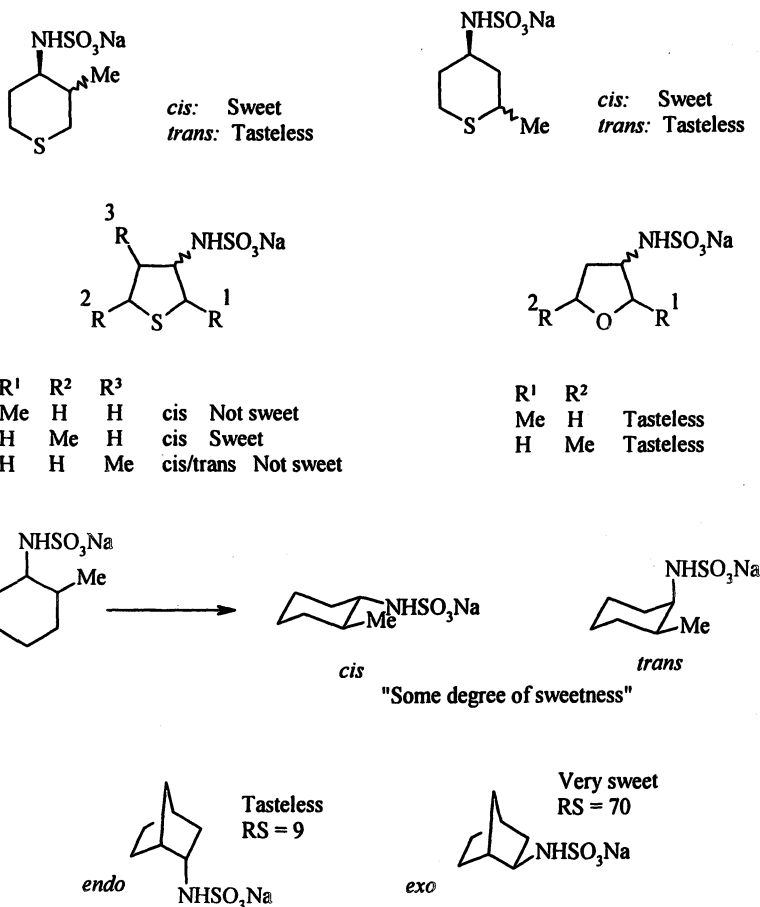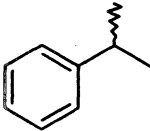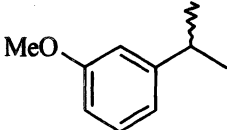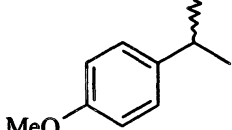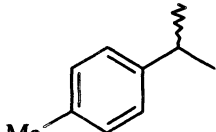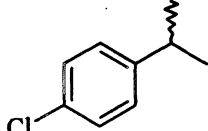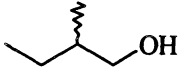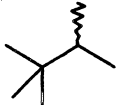
Figure 10. Taste of geometrical isomers of sulfamates
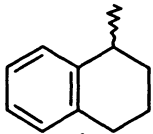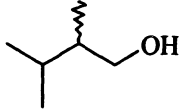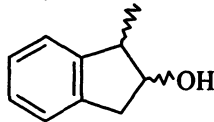
Table IV. Tastes of sulfamate stereoisomers[a]

Entry	Sulfamate Z = in ZNHSO₃Na	(R)	(S)
1		Sweet/Sour	Sweet/Bitter
2		Sweet/Bitter	Tasteless/ Bitter
3		Sweet	Tasteless/ Sweet
4		Salty	Salty/Bitter
5		Bitter/Sweet	Sweet/Bitter
6		Bitter/ Tasteless	Tasteless/ Bitter
7		Tasteless/ Sweet	Tasteless/ Sweet

[a]Bold font indicates predominant taste.

Table IV. *Continued.*

Entry	Sulfamate Z = in ZNHSO₃Na	(R)	(S)
8		**Tasteless/** **Bitter**	Sweet/Sour/ Tasteless
9		**Tasteless**	Tasteless/ **Bitter**
10		**Very Bitter**	**Bitter**

ᵃBold font indicates predominant taste.

Conclusions and Future Work

The effect of sulfamation on the taste of anilines, arylureas, L-amino acids and dipeptides has been examined. A change in the taste portfolios has been found for about half of the compounds sulfamated. The effect on the tastes on sulfamation of a series of structural, geometrical and stereoisomers has been reviewed in outline. A series of pairs of (R)- and (S)-stereoisomers have been sulfamated and the mainly slight differences in taste between these have been noted.

Future work includes further synthesis and sulfamation of ureas/thioureas and the development of structure-taste relationships (SARs) for these, synthesis of additional (R)/(S) and *cis/trans*-isomers and examination of some of the current results within the framework of recent taste receptor models.

References

1. Spillane, W. J.; McGlinchey, G. *J. Pharm. Sci.* **1981**, *70*, 933-935.
2. Spillane, W. J.; Ryder, C. A.; Curran, P. J.; Wall, S. N.; Kelly, L. M.; Feeney, B. G.; Newell, J. *J. Chem. Soc. Perkin Trans. 2*, **2000**, 1369-1374.
3. Drew, M.G.B.; Wilden, G.R.H.; Spillane, W. J.; Walsh, R. M.; Ryder, C. A.; Simmie, J. M. *J. Agric. & Food Chem* **1998**, *46*, 3016-3026.

4. Spillane, W. J.; Feeney, B. G.; Coyle, C. C. *Food Chemistry* **2002**, *79*, 15-22.

5. Spillane, W.J.; Kelly, L.M.; Feeney, B.G.; Drew, M.G.B.; Hattotuwagama, C. K. *ARKIVOC (Issue in honor of Prof. M. A. McKervey)* **2003**, 297-309.

6. Kelly, D. P.; Spillane, W. J.; Newell, J. *J. Agric. & Food Chem.* **2005**, *53*, 6750-6758.

7. Spillane, W. J.; Kelly, D. P.; Curran, P. J.; Feeney, B. G. *J. Agric. & Food Chem.* **2006**, *54*, in press.

8. Audrieth, L. F.; Sveda, M. *J. Org. Chem.* **1944**, *9*, 89-101.

9. Spillane, W. J.; Hanniffy, G. G. *J. Agric. & Food Chem.* .**2003**, *51*, 3056-3059.

10. de Nardo, M.; Runti, C.; Ulian, F. *Farmacio Ed. Sci.* **1984**, *39*, 125-132 ; [*Chem. Abs.* **1984**, *100*, 137568]

11. Tinti, J.-M.; Nofre, C. *Design of Sweeteners - a Rational Approach* In Sweeteners - Discovery, Molecular Design and Chemoreception, Walters, D. E. ; Orthoefer, F. T. ; DuBois, G. E. Ed. ACS Symposium series 450, American Chemical Society, Washington, DC, 1991; ch. 7, 88-99.

12. Tinti, J.-M.; Nofre, C. *Why Does a Sweetener Taste Sweet? - A New Model.* In Sweeteners - Discovery, Molecular Design and Chemoreception, Walters, D. E.; Orthoefer, F. T. ; DuBois, G. E. Ed.ACS Symposium series 450, American Chemical Society: Washington, DC, 1991; ch. 15, 206-213.

13. Walters, D. E. *The Rational Discovery of Sweeteners* .In Sweeteners - Discovery, Molecular Design and Chemoreception, Walters, D. E. ; Orthoefer, F. T. ; DuBois, G. E. Ed. ACS Symposium series 450, American Chemical Society Washington, DC, 1991; - ch. 1, 1-11.

14. Morini, G.; Bassoli, A.; Temussi, P. A. *J. Med. Chem.* **2005**, *48*, 5520-5529.

15. Roy, G. *Critical Reviews in Food Science* **1992**, *31*, 59-77.

16. Finzi, C.; Colonna, M. *Atti accad. Lincei, Classe sci. fis., mat. nat.* **1937**, *26*, 19-24 ; [*Chem. Abs.* **1938**, *32*, 3762[1]].

17. DuBois, G. E.; Stephenson, R. A. *J. Org. Chem.* **1980**, *45*, 5371-5373.

18. Cohn, G. *Die Organischen Geschmackstoff,* Franz Siemenroth: Berlin, 1914.

19. Cohn, G. *Geschmark und Konstitution bei Organischen Verbindungen,* Ferdinand Enke: Stuttgart, 1916.

20. Moncrieff, R. W. *The Chemical Senses*; Leonard Hill, London, 1967.

21. Verkade, P. E. . *Farmaco Ed. Sci.* **1968**, *23*, 248-291; [*Chem. Abs.* **1968**, *68*, 92869].

22. Blanksma, J. J.; Van der Weyden, P. W. N. *Rec. Trav. Chim.* **1940**, *59*, 629-632.

23. Muspratt, J. S.; Hofmann, A. W. *Justus Liebigs Annalen der Chemie* **1846**, *57*, 201-224.

24. de Koning, A. J. *J. Chem. Educ.* **1976**, *53*, 521-522.

25. Wheeler, H. L. *Amer. Chem. J.* **1895**, *17*, 697-704.

26. Blanksma, J. J.; Van den Broek, W. J.; Hoegen, D. *Rec.Trav. Chim.* **1946**, *65*, 329-332.

27. Kurzer, F. *Arylureas. I. Cyanate Method and II. Urea Method.* In *Coll. Org. Synthesis*, Rabjohn, N. Ed. vol. 4, Wiley, NY 1963; 49-54.

28. Rasmussen, C. R.; Villani, F. J.; Weaner, L. E.; Reynolds, B. E.; Hood, A. R.; Hecker, L. F.; Nortey, S. O.; Hanslin, A.; Costanzo, M. J.; Powell, E. T.; Molinari, A. J. *Synthesis* **1988**, 456-459.

29. Concagh, D. G. M.Sc Thesis, National University of Ireland, Galway Ireland, 1995.

30. Birch, G. G.; Kemp, S. E. *Chem. Senses* **1989**, *14*, 249-258.

31. Solms, J.; Vuataz, L.; Egli, R. H. *Experientia* **1965**, *21*, 692-694.

32. Mazur, R. H.; Schlatter, J. M.; Goldkamp, A. H. *J. Amer. Chem. Soc.* **1969**, *91*, 2684-2691.

33. Unterhalt, B.; Mollers, M. *Arch. Pharm. (Weinheim, Ger.)* **1991**, *324*, 525-526.

34. Unterhalt, B.; Kerckhoff, L.; Mollers, M. *Sci. Pharm.* **2000**, *68*, 101-108.

35. Unterhalt, B.; Boschemeyer, L. *Z. Lebensm. Unters.-Forsch.* **1975**, *158*, 35-36.

Chapter 35

Neoculin as a New Sweet Protein with Taste-Modifying Activity: Purification, Characterization, and X-ray Crystallography

A. Shimizu-Ibuka[1], Y. Morita[1], K. Nakajima[1], T. Asakura[2], T. Terada, T. Misaka[1], and K. Abe[1,*]

[1]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[2]Laboratory of Food Science, Atomi Junior College, Bunkyo-ku, Tokyo 112-8687, Japan

The majority of sweet compounds are of low-molecular-weight, but several proteins elicit sweet taste responses in humans. The fruit of *Curculigo latifolia* has been known to contain a protein that has both sweetness and a taste-modifying activity to convert sourness to sweetness. Recently, we have purified and re-identified the active component to reveal that it is a heterodimeric protein named "neoculin". The result of X-ray crystallographic analysis has indicated that the overall structure of neoculin is similar to those of monocot mannose-binding lectins, while there is little structural similarity between neoculin and structure-solved sweet proteins. Direct interaction between neoculin and human sweet taste receptor hT1R2-hT1R3 has been indicated by response of HEK293T

cells expressing this receptor, and by the inhibition of neoculin activity with lactisole, a hT1R2-hT1R3 blocker. Combining the results of molecular dynamics simulations and docking model generation between neoculin and hT1R2-hT1R3, we propose a hypothesis that neoculin is in dynamic equilibrium between "open" and "closed" states, and that the addition of an acid shifts the equilibrium to the "open" state for easier fitting to the receptor.

Taste is a primal sense that enables organisms to accept sweet nutrients in foods and reject bitter environmental poisons. The majority of sweet molecules are of low-molecular-weight, but six proteins—brazzein, thaumatin, monellin, curculin, mabinlin and pentadin—have been shown to elicit a sweet taste response in humans (1). Previously identified sweet proteins and taste-modifying proteins have different molecular lengths, from the 54 residues of brazzein to the 202 residues of thaumatin, with no significant similarities in their amino acid sequences (1).

Curculin, occurring in the fruit of *Curculigo latifolia* that grows in West Malaysia, is the only known protein that both elicits a sweet response and has taste-modifying activity to convert sourness to sweetness (2). Acids taste sweet in the presence of curculin. This type of taste-modifying activity is also evoked by miraculin, which has no sweetness by itself (3). Curculin was initially regarded as a homodimer consisting of two identical subunits, although the recombinant homodimer was devoid of any taste-modifying activity (2). In this chapter, we report the purification, re-identification, and crystallographic analysis of the active component, named "neoculin". We also obtained the results that indicate direct interaction between neoculin and the human sweet taste receptor hT1R2-hT1R3. These results, together with the results of molecular dynamics simulations and docking model generation, offer insights into a possible mechanism of taste-modifying activity.

Purification and Re-identification

Though the protein curculin was regarded as a homodimer consisting of two identical subunits, no successful expression of the recombinant curculin with taste-modifying activity had been reported. Why does the recombinant curculin have no activity? To answer this question, we have purified and re-identified the active component contained in *Curculigo latiforia*.

When the purified active component was subjected to SDS-PAGE and stained with CBB, it gave a main band of about 20 kDa under non-reducing

condition, and a main band of about 11 kDa and a faint band of about 13 kDa under reducing conditions. This active fraction was then submitted to two-dimentional electrophoresis in denaturing and reducing condition, resulting in the 13 kDa and 11 kDa fractions appearing at pI 4-6 and pI 7.5-9.5, respectively (data not shown). These data indicated that the active component was a 20 kDa heterodimer consisting of 13 kDa and 11 kDa subunits connected by disulfide bond(s). We named this heterodimeric protein "neoculin (NCL)". The 13 kDa fraction was referred to as "neoculin acidic subunit (NAS)" and the 11 kDa fraction as "neoculin basic subunit (NBS)".

The results of N-terminal protein sequencing indicated that the NBS had an N-terminal sequence identical to that of curculin, while NAS was an apparently new polypeptide sharing 80% amino acid identity with NBS in this N-terminal region (Figure 1).

From the analysis of proteinase-digested NAS with protein sequencer, and from the nucleotide sequences of cDNA, we determined the whole NAS sequence as shown in Figure 1 (4). NAS and NBS shared 77% amino acid identity. These neoculin subunits exhibit high degrees of amino acid sequence similarity to monocot mannose-binding lectins, such as those from garlic, daffodil and snowdrop, showing 42–46% amino acid identity. It suggests that they share a common architecture in their three-dimensional structures, though there is no known functional similarity between neoculin and these molecules. In fact, we confirmed that neoculin had no hemagglutinin activity.

X-ray crystallographic analysis

The structural basis of sweetness has been studied extensively for thaumatin, monellin and brazzein and amino acid residues important for sweetness of these proteins have been reported previously (5-11). However, no common structural features have been identified among these proteins. Since little structural information is available for the taste-modifying proteins, we have tried to analyze the three-dimensional structure of neoculin.

The neoculin crystal structure was solved by molecular replacement and refined at 2.76 Å (Figure 2A) (12). Eight polypeptide chains from A through H form four crystallographically independent heterodimers, AB, CD, EF and GH, respectively, in the asymmetric unit. The chains A, C, E and G correspond to NAS, while the chains B, D, F and H correspond to NBS. The structures of the two subunits, NAS and NBS, are very similar to each other and are superimposable (Figure 2B).

Richness of disulfide bridges is common to some sweet proteins. Thaumatin and brazzein have eight and four disulfide bonds, respectively (5,13). In the neoculin molecule, all eight cysteine residues participate in the formation of disulfide bridges, and thus there are four disulfide bonds in the heterodimer. Two

549

```
NAS          DSVILSGQTLYAGHSLTSGSYTLTIQNCNLVKYQHGRQIWASDTDG-QGSQCRLTLRSDGN
NBS          DNVLLSGQTLHADHSLQACAYTLIQNKCNLVKYQNGRQIWASNIDR-RGSGCRLTILSDGN

daffodil-ag  DNILYSGETLSPGEFINNGRYVFIMQEDCNLVLYDVDKPIWATNIGG-LDRRCHLSMQSDGN
snowdrop-ag  DNILYSGEILSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATNIGG-LSRSCFLSMQTDGN
galic-ag     RNILMNDEGLYAGQSIDVEPYHIIMQEDCNLVLYDHSTAVWTSNIDIPGKKGCKAVLQSDGN

                                                    *
NAS          IIIYDNNMVVWGSDCWGNNGTYALVLQDGLFVIYGPVLWPLGLNGCRSLN
NBS          LVLYDHNNDVVGSACWGDNGKYALVLQKGRFVIYGPVLWSLGPNGCRRVNG

daffodil-ag  LVVYSPRNNPIWASNTGGENGNYVCLQKDRNVIYGTARWATCTNIH
snowdrop-ag  LVVYNPSNKPIWASNTGGQNGNYVCILQKDRNVIYGTDRWATGTHTG
galic-ag     FVVYDAEGRSLWASHSVRGNGNYVLVLQEDGNVIYGSDIWSTNTYKG
```

Figure 1. Sequence alignment of neoculin subunits and monocot mannose-binding lectins. The residues conserved in all of the molecules are shown in black, and those conserved mainly in neoculin subunits are shown in grey. The N-terminal amino acid sequences analyzed with protein sequencer are double-underlined. N-glycosylation site in NAS is asterisked.

A
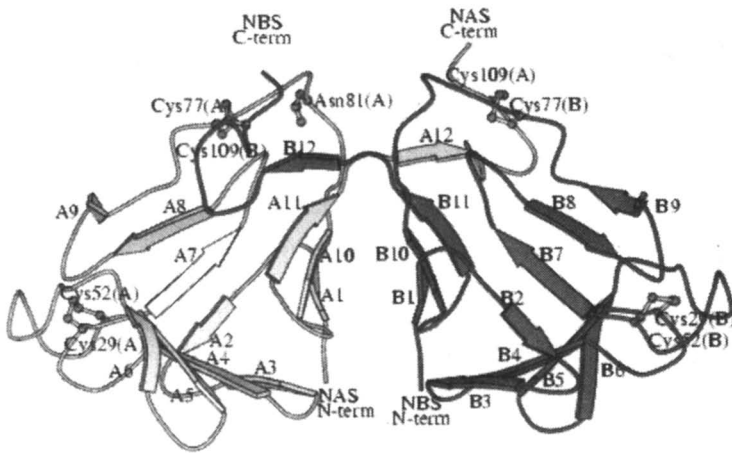
B
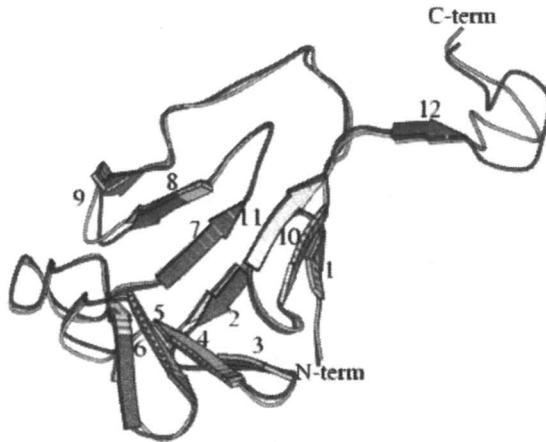
Figure 2. Ribbon diagram of neoculin. Neoculin acidic subunit (NAS) is shown
in white and neoculin basic subunit (NBS) in grey. A) Overall structure of
neoculin heterodimer. The β-strands of each subunit are labeled from B1 to
B12. The cysteine reisdues forming disulfide bonds and the sugar molecule
bound to Asn81 of NAS are shown as ball-and-stick model. B) Superposition of
the two subunits, NAS and NBS. (Reproduced with permission from ref. 12 with
minor modification.)

are intra-subunit disulphide bonds, between Cys29 and Cys52 in each subunit, while the other two are inter-subunit disulphide bridges, between Cys77 of one subunit and Cys109 of another (Figure 2A). Intra-subunit S–S bonds are also observed in the mannose-binding lectins, while inter-subunit S–S bonds are not.

Neoculin and the sweet proteins with known three-dimensional structures, namely monellin, thaumatin and brazzein, show no obvious similarity in their tertiary structures, although there is broad similarity in their richness of β-sheet structures. Meanwhile, as predicted from the high degree of amino acid sequence homology between neoculin and the mannose-binding lectins, the crystal structure of neoculin shows striking similarity to those of mannose-binding lectins, having the overall same structural topology (Figure 3A). The most pronounced structural difference between neoculin and the lectins is observed in the C-terminal regions of both subunits (Figure 3B). In both NAS and NBS, the 12th β-strand B12 is composed of only three or four residues from 100 to 102 or 103, and a subsequent large turn is fixed by an inter-subunit disulfide bond between Cys109 and Cys77. In the lectins, the corresponding regions stretch straight over the surface of another subunit. Such differences in the C-terminal regions effect the subunit-subunit interactions. The interface of two subunits is mainly composed of N-terminal β-strand B1 and C-terminal β-strands from B10 to B11 or B12 in both neoculin and the lectins. The buried surface area between two subunits of neoculin is smaller than that of the lectins, suggesting weaker interaction between the two subunits in neoculin.

The shape of the protein surface and the electrostatic potential on the surface are also significantly different between neoculin and the lectins. These differences reflect the fact that the residues protruding at the surface of the molecules are those which are less conserved between neoculin and the lectins. It is characteristic that the distribution of basic residues is non-homogeneous, especially in NBS. In NBS, there are 13 basic residues, consisting of seven arginines, three lysines, and three histidines. Six of them, His11, His14, Lys28, Arg47, Arg48, and Arg53 compose a large cluster of basicity on the surface of NBS (Figure 4). Such a large cluster of charged residues is not observed in the lectins. Since it is suggested that the basicity of broad surface regions plays a significant role in the elicitation of sweetness in some sweet proteins(9,10,14), this cluster of basic amino acids in NBS may contribute to the sweetness or taste-modifying activity of neoculin. This basic cluster includes two histidine residues, with pKa of 6.0. Since neoculin elicits only slight sweetness at neutral pH but strong sweetness at acidic pH, these histidine residues might be essential to the sweetness and/or taste-modifying activity of neoculin.

The structure also shows that three out of four basic residues located at the dimer interface of neoculin are unique to neoculin. Such location of basic amino acids is not observed in the lectins and may be the cause of the pH-dependent conformational change.

A   B
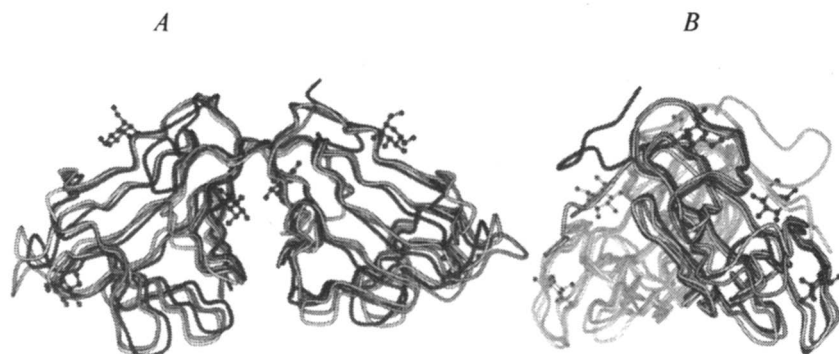
*Figure 3. Comparison between neoculin and monocot mannose-binding lectins.
A) Superposition of neoculin (dark grey), garlic lectin (light grey), snowdrop
lectin (white). Methyl-α-D-mannose molecules bound to the snowdrop lectin
are shown as ball-and-stick models. B) Superposition of neoculin and the
lectins viewed from the NBS side. NAS and corresponding subunits in the
lectins are drawn with light grey. (Reproduced with permission from
reference 12 with minor modification.)*
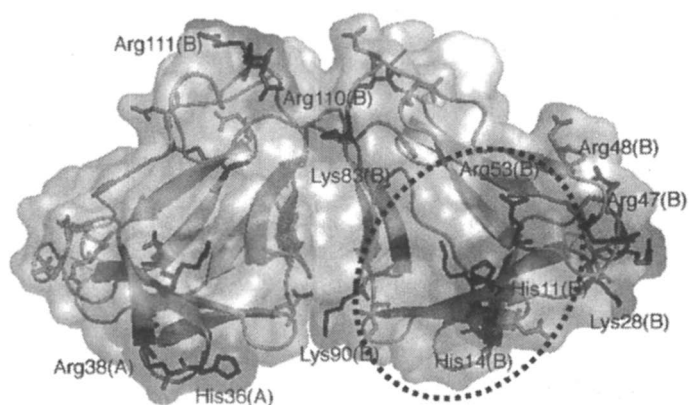
*Figure 4. Polar residues on the surface of neoculin. A large basic patch is
indicated with an oval dotted line.*

Possible pH effect on the neoculin structure

The crystal structure of neoculin solved in this study only represents the structure at neutral pH, since the protein was crystallized at pH 7.4. The neoculin heterodimer has 16 aspartates, 5 histidines, with no glutamate residues contained. Judging from the crystal structure, the titratable groups of these residues are unprotonated at neutral pH. Furthermore, it can be assumed that all of them are protonated at acidic pH (around 2.5), since they are exposed to the solvent. The results of constant-pH molecular dynamics simulations were consistent with this assumption. At neutral pH, NAS and NBS carries -3 and $+3$ charges, respectively, whereas they carry $+8$ and $+15$ as a result of protonation of the titratable groups and the C-termini. Such a shift in charge profile causes a drastic change in the electrostatic interaction between the monomers. We speculated about whether the strong repulsion between the positive charges might alter the structure of neoculin and about a possible correlation between its strong sweet taste at acidic pH and the neoculin structure. We have performed molecular dynamics simulations on the protonated and unprotonated states of neoculin to estimate the extent of the effect of the pH on the neoculin structure(12). Figures 5 shows representative structures obtained from the simulations on the unprotonated (i.e. neutral pH) and protonated (i.e. acidic pH) states, respectively. These suggest that the protonated neoculin would have a tendency to take a widely "open" conformation, while unprotonated neoculin is in a "closed" conformation, similar to the crystal structure.

Together with the observation that neoculin elicits slight sweetness even when dissolved in pure water (pH 7.0), we propose that the neoculin structure is in equilibrium between "open" and "closed" states. The equilibrium may be shifted to the "closed" state at neutral pH and to the "open" state at acidic pH, with only the neoculin molecules in the "open" state eliciting strong sweetness.

Interaction with the human sweet-taste receptor hT1R2-hT1R3

Most sweet molecules are thought to interact with the sweet taste receptor, hT1R2-hT1R3, which is known to mediate the recognition of diverse natural and synthetic sweeteners, including sweet proteins(15). Based on the docking of the sweet proteins into the models of hT1R2-hT1R3, it has been hypothesized that these proteins elicit sweet taste through binding to a large cavity of the receptor(16,17).

Direct interaction between neoculin and hT1R2-T1R3 has been indicated by two experiments. Firstly, a response to neoculin was observed when hT1R2, hT1R3, and promiscuous G protein (G16/gust25) were coexpressed in HEK-293 cells(18). Most of the neoculin-responsive cells responded also to the application of low molecular-weight sweeteners such as aspartame and saccharin (Figure 6).
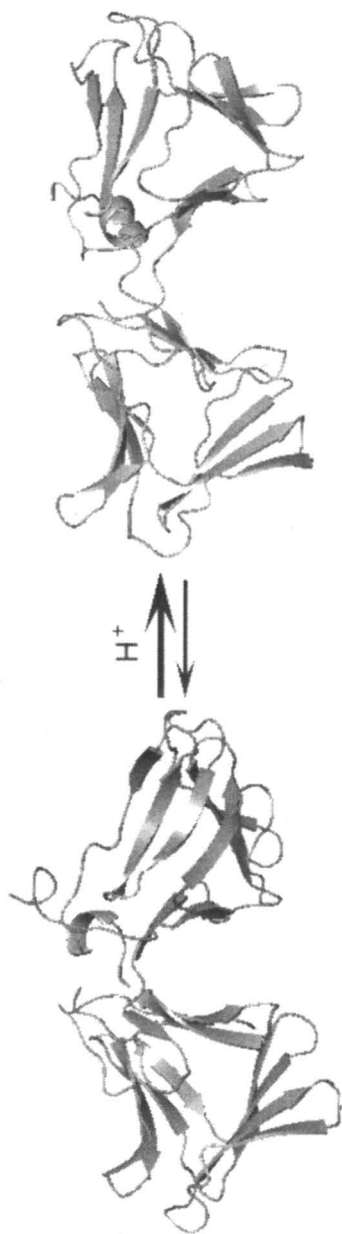
Figure 5. Representative neoculin structures under unprotonated state (left) and under protonated state (right), generated by molecular dynamics simulations.

Secondly, sweet-taste response to neoculin was inhibited in the presence of lactisole, a hT1R2-hT1R3 blocker (*18*) (Figure 6). In addition, the result of human sensory analysis showed the inhibition of taste-modifying activity of neoculin by lactisole (Figure 7). Therefore, we hypothesized that the target of neoculin, as well as of the other sweeteners, is the T1R2-T1R3 receptor and that we could gain insight into how neoculin elicits sweetness and taste-modifying activity through docking with the receptor. There is a contradiction of pH values where the sweetness is elicited between *in vivo* and *in vitro* results. This contradiction might be caused by following two factors. First, in the cell-based assay (Figure 6), the mode of receptor activation would be somehow different (i.e. G-protein coupling and additional membrane protein with supporting function, etc) from that of *in vivo*. Second, in human sensory test (Figure 7), we could not precisely measure the *in situ* pH at the taste pore. Therefore we did not compare the pH values directly.

The putative mechanism of taste-modifying activity

Prior to the generation of the docking models, we modelled the tertiary structures of the hT1R2-hT1R3 receptor based on sequence similarity to the N-terminal region of the metabotropic glutamate receptor (mGluR)(*19*), as has been performed in previous studies(*16,17,20,21*). At present, two different forms of the mGluR structure are available. One is the inactive or resting open-open form of mGluR (PDB entry: 1EWT) and the other is the active closed-open form of mGluR (PDB entry: 1EWV), whose structure is almost identical to that in complex with glutamate (PDB entry: 1EWK). Here, we modelled all possible forms of the hT1R2-hT1R3 receptor, following the method of Morini et al.(*17*), i.e. four models designated Roo_AB (T1R2 modelled on chain A and T1R3 modelled on chain B of the resting open-open form), Roo_BA (T1R2 modelled on chain B and T1R3 modelled on chain A), Aoc_AB (T1R2 modelled on chain A and T1R3 modelled on chain B of the active open-close form), and Aoc_BA (T1R2 modelled on chain B and T1R3 modelled on chain A). The representative structures obtained from the simulations on unprotonated and protonated states were docked into each model of the receptor. About 10,000 docking models were generated for each pair and we selected the best candidates on the assumption that the interaction surface area reaches a maximum when the cavities of neoculin and one of the subunits of the hT1R2-hT1R3 receptor face each other with the long axis of neoculin vertical to the long axis of the receptor. From the calculations with the neoculin model in the protonated state, nine preferable solutions were obtained. Among these solutions, neoculin was accommodated in the large cavity of hT1R2 in six solutions and in that of hT1R3 in three solutions. In contrast, no preferable solution was obtained from the calculations with the neoculin model in the unprotonated state(*12*). These results
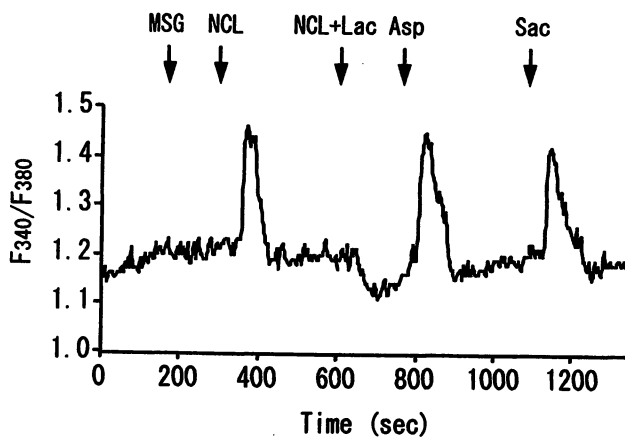
Figure 6. Line trace of the ratiometric value changes for the representative
HEK293T cell expressing hT1R2/T1R3. Black arrows indicate the time of the
application of 10 mM monosodium glutamate (MSG), 20 μM neoculin (NCL), 20
μM NCL containing 2.5 mM lactisole (NCL+lac), 10 mM aspartame (Asp), and
10 mM saccharin (Sac).
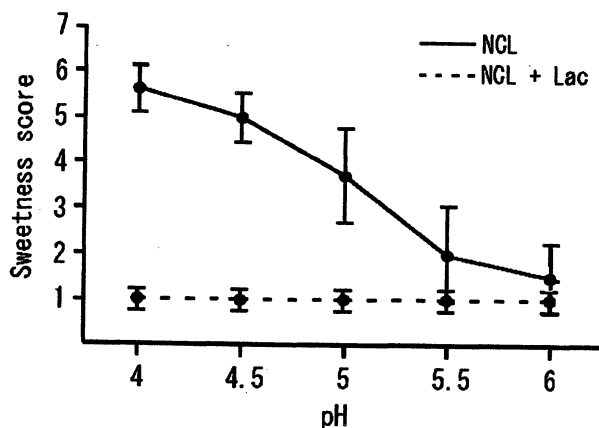
Figure 7. Evaluation of the taste-modifying activity of neoculin (NCL) in weak
acidic buffer. Panelists tasted aspartame solutions at three different
concentrations (0.1, 0.5, and 2.0 mM) and rated the sweetness on a scale from 1
to 7: 7 for >2.0, 6 for 2.0, 5 for 0.5-2.0, 4 for 0.5, 3 for 0.1-0.5, 2 for 0.1, and 1
for <0.1 mM aspartame. The sweetness score elicited by five different pH
values of 100 mM acetate buffer in the absence (solid line) or presence (dashed
line) of 2.5 mM lactisole are shown as means ± SD (n=8 or n=6, respectively).
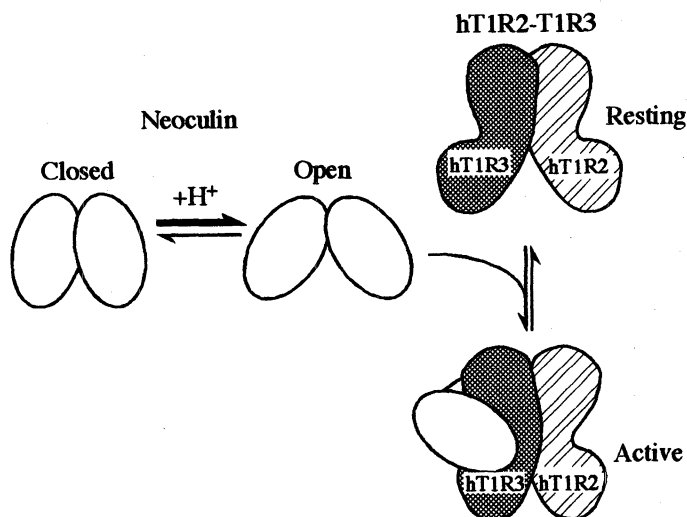
*Figure 8. Cartoon representation of possible mechanism for sweetness
elicitation and taste-modifying activity. (Reproduced with permission from
reference 12 with minor modification. Copyright 2006 Elsevier.)*

seem to be compatible with our proposal based on the molecular dynamics simulation that the conformation of neoculin is in equilibrium between "open" and "closed" states and only the neoculin molecule in the "open" conformation can elicit strong sweetness.

Combining the results of the molecular dynamics and docking simulations, we propose the following hypothesis as a possible mechanism of the taste modification by neoculin (Figure 8). The neoculin structure is in equilibrium between "open" and "closed" states. Upon lowering pH, the equilibrium is shifted to the "open" state and only the fraction adopting the "open" conformation can bind to the hT1R2-hT1R3 receptor. The binding of neoculin to the receptor in turn shifts the conformational equilibrium of the receptor to the active form. Consequently, neoculin elicits strong sweetness at acidic pH, whereas the sweetness is very weak at neutral pH. Note that it remains unclear how the change of pH affects the structure of the receptor and this was not considered in our calculations.

Conclusions

The active component in *Curculigo latifolia* to convert sourness to sweetness has been re-identified and named "neoculin". The results of our study

have suggested a possible mechanism of taste-modifying activity. Further biochemical and structural studies are required to confirm our analysis, simulation results and hypotheses based on the simulations. We have recently succeeded in producing a sufficient amount of active neoculin using recombinant *Aspergillus oryzae* (*22*). It will make possible to perform mutagenesis studies to identify the regions or amino acid residues critical for elicitation of the taste-modifying activity. To verify the hypothesis drawn from the molecular simulations, X-ray analysis of neoculin complexed with hT1R2-hT1R3 is needed, although at present it is very difficult to obtain purified hT1R2-hT1R3 sufficient for X-ray crystallography. "Non-glycemic" sweeteners are widely used by those suffering from metabolic syndromes associated with excessive carbohydrate consumption. Sweet proteins generally have strong sweetness ranging from 100~3,000 times greater than that of sucrose on a weight per weight basis, and are thus very good candidates as non-glycemic sweeteners (*1*). The high stability of neoculin, which retains its activity over a wide range of pH from 3.0 to 11.0 and at temperatures as high as 60°C, together with its favourable taste quality, would be great advantages for practical use (*23*). Further research to provide a theoretical grounding may enable the practical use of neoculin as a unique sweetener.

References

1. Kant, R. *Nutr. J.* **2005**, *4*, 5
2. Yamashita, H.; Theerasilp, S.; Aiuchi, T.; Nakaya, K.; Nakamura, Y.; Kurihara, Y. *J. Biol. Chem.* **1990**, *265*, 15770-15775
3. Theerasilp, S.; Kurihara, Y. *J. Biol. Chem.* **1988**, *263*, 11536-11539
4. Shirasuka, Y.; Nakajima, K.; Asakura, T.; Yamashita, H.; Yamamoto, A.; Hata, S.; Nagata, S.; Abo, M.; Sorimachi, H.; Abe, K. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1403-1407
5. Ogata, C. M.; Gordon, P. F.; de Vos, A. M.; Kim, S. H. *J. Mol. Biol.* **1992**, *228*, 893-908
6. Caldwell, J. E.; Abildgaard, F.; Dzakula, Z.; Ming, D.; Hellekant, G.; Markley, J. L. *Nat. Struct. Biol.* **1998**, *5*, 427-431
7. Assadi-Porter, F. M.; Aceti, D. J.; Markley, J. L. *Arch. Biochem. Biophys.* **2000**, *376*, 259-265
8. Somoza, J. R.; Cho, J. M.; Kim, S. H. *Chem. Senses* **1995**, *20*, 61-68
9. Kaneko, R.; Kitabatake, N. *Chem. Senses* **2001**, *26*, 167-177
10. Jin, Z.; Danilova, V.; Assadi-Porter, F. M.; Aceti, D. J.; Markley, J. L.; Hellekant, G. *FEBS Lett.* **2003**, *544*, 33-37
11. Tancredi, T.; Pastore, A.; Salvadori, S.; Esposito, V.; Temussi, P. A. *Eur. J. Biochem.* **2004**, *271*, 2231-2240

12. Shimizu-Ibuka, A.; Morita, Y.; Terada, T.; Asakura, T.; Nakajima, K.; Iwata, S.; Misaka, T.; Sorimachi, H.; Arai, S.; Abe, K. *J. Mol. Biol.* **2006**, *359*, 148-158

13. Kohmura, M.; Ota, M.; Izawa, H.; Ming, D.; Hellekant, G.; Ariyoshi, Y. *Biopolymers* **1996**, *38*, 553-556

14. Masuda, T.; Ide, N.; Kitabatake, N. *Chem. Senses* **2005**, *30*, 667-681

15. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4692-4696

16. Temussi, P. A. *FEBS Lett.* **2002**, *526*, 1-4

17. Morini, G.; Bassoli, A.; Temussi, P. A. *J. Med. Chem.* **2005**, *48*, 5520-5529

18. Nakajima, K.; Asakura, T.; Oike, H.; Morita, Y.; Shimizu-Ibuka, A.; Misaka, T.; Sorimachi, H.; Arai, S.; Abe, K. *Neuroreport* **2006**, *17*, 1241-1244

19. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. *Nature* **2000**, *407*, 971-977

20. Max, M.; Shanker, Y. G.; Huang, L.; Rong, M.; Liu, Z.; Campagne, F.; Weinstein, H.; Damak, S.; Margolskee, R. F. *Nat. Genet.* **2001**, *28*, 58-63

21. Walters, D. E. *Pure Appl. Chem.* **2002**, *74*, 1117-1123

22. Nakajima, K.; Asakura, T.; Maruyama, J.; Morita, Y.; Oike, H.; Shimizu-Ibuka, A.; Misaka, T.; Sorimachi, H.; Arai, S.; Kitamoto, K.; Abe, K. *Appl. Environ. Microbiol.* **2006**, *72*, 3716-3723

23. Yamashita, H.; Akabane, T.; Kurihara, Y. *Chem. Senses* **1995**, *20*, 239-243

Chapter 36

How Sweet It Is: Detailed Molecular and Functional Studies of Brazzein, a Sweet Protein and Its Analogs

Fariba Assadi-Porter[1,2], Marco Tonelli[2], James T. Radek[3], Claudia C. Cornilescu[4], and John L. Markley[1,2]

[1]Department of Biochemistry, [2]National Resonance Facility at Madison, [3]Department of Entomology, and [4]Center for Eukaryotics and Structural Genomics, University of Wisconsin–Madison, Madison, WI 53706
*Corresponding author: fariba@nmfam.wis.edu

Brazzein is a small, low-calorie, sweet protein with high stability over wide temperature and pH ranges. Brazzein has desirable taste characteristics that resemble those of carbohydrate sweeteners. Brazzein folds in a β-α-β_2 topology in which the α-helix packs against the three-stranded antiparallel β-sheet. This structure is held together by four disulfide bridges. We developed an efficient bacterial production system for brazzein that allows us to express wild-type and mutant proteins. We have designed a large number of brazzein variants for taste tests. These include mutations that affect surface charges, disulfide bridges, loops, and flexible regions. We have subjected a subset of these variants to detailed analysis by NMR spectroscopy to identify patterns of hydrogen bonds and internal mobility. The results show a correlation between these physical properties and the sweetness of the protein. These results led us to propose a multi-site binding model for the interaction between brazzein and the heterodimeric human sweet receptor, which we are continuing to test with the goal of designing more potent brazzein analogs as potential future sweeteners.

Historical background

The discovery of a number of non-calorigenic sweet proteins over the last 30 years has increased the demand for non-carbohydrate-based sweeteners with favorable taste properties. The optimal design of such sweeteners requires knowledge about structure-function relationships and the identification of chemical entities that trigger the sweetness response. Proteins with sweetness ranging from 100 to 3000 times that of sucrose on a weight basis have been identified in African and South Asian fruits and berries. Among the known, naturally occurring, sweet-tasting proteins, brazzein has properties that make it particularly attractive as a potential economic sweetener. Brazzein comes from a West African plant *Pentadiplandra brazzeana*, a climbing vine that grows in Gabon, Zaire, and Cameroon (*1*). The pulp of the ripe berries contains brazzein at about 0.2 % by weight, making the berries intensely sweet. The fruit, locally named "j'oublie", is consumed by the local population and is prized for its sweetness. Brazzein is highly stable over wide temperature and pH ranges and has taste properties that resemble those of carbohydrate sweeteners. The brazzein protein is a single polypeptide chain composed of 54 standard amino acids and contains no carbohydrate. Brazzein has been shown to elicit sweetness responses in humans by taste trials and in a non-human primate (rhesus monkey) as determined from electrophysiological recordings of signals from chorda tympani nerve fibers (*2*).

Human Taste Receptors

The human receptor appears to be a heterodimer of two conventional seven-transmembrane-helix G-coupled type receptors (T1R2/T1R3), each with an unusually large ectodomain (*3, 4*). T1R2+T1R3 receptors are the primary sweet receptors for a diverse range of sweet ligands (*3-6*). Calcium imaging assays of HEK cells transfected with hT1R2+hT1R3 respond to all sweet taste stimuli tested: sugars sucrose, fructose, galactose, glucose, etc.; amino acids glycine and D-tryptophan; sweet proteins brazzein, monellin and thaumatin; and synthetic sweeteners cyclamate, saccharin, ace K, aspartame, dulcin, neotame and sucralose (*3, 6-10*). All of these responses are inhibited by the sweet taste inhibitor lactisole, which acts on the transmembrane domain (TMD) of T1R3 (*8, 10, 11*). Different families of ligands interact with different sites on the receptor. It has been shown that T1Rs in both umami and sweet taste share a common subunit (T1R3) (*10*). In addition, Jiang et al. recently reported that the C-rich region of the extracellular domain of the T1R3 subunit of the sweet taste receptor plays an important role in differentiating responses to brazzein (*6*). Interestingly, human-specific sweet proteins are recognized only by the human T1R2/T1R3 receptors but not the rat T1R2/T1R3 receptors (*4*), as consistent

562

with the previous finding that brazzein does not excite taste fibers in the rat
(*12*). Although tentative models have been proposed for interactions of the sweet
receptor with sweet proteins and small sweet ligands (*13*), the mechanism is not
understood. The discovery and characterization of the sweet taste receptor
opens up exciting new avenues for research on the detailed mechanism of action
of sweet substances. Brazzein is an excellent candidate for experimental
investigations of the chemical and structural requirements for extracellular
triggering of a sweet response in humans and for understanding the mechanism
of the signal transduction.

Early investigations of brazzein isolated from the fruit

The amino acid sequence of brazzein was determined by peptide sequencing
(*1*). Brazzein extracted from fruit is composed of at least two species, one
fraction containing a pyroglutamyl (pGlu) N-terminus and a second, sweeter,
fraction lacking the pGlu residue (*14*).

Early variable temperature NMR studies of brazzein showed very little
change in its ^{1}H NMR spectrum over a wide range of temperature range (32–82
°C) (*15*),and the sweetness profile was shown to be undiminished after
incubation of brazzein at 100 °C for four hours (*16*).

The three-dimensional structure of pGlu-brazzein extracted from fruit was
solved by homonuclear ^{1}H NMR spectroscopy with RMSD = 1.6 Å (Figure 1)
(*17*). The protein has a highly compact structure consisting one short α-helix
and three anti-parallel β-strands held together by four disulfide bridges. No
significant sequence or structural similarity was found between brazzein and the
two other sweet-tasting proteins of known three-dimensional structure: monellin
(*18*), and thaumatin (*19, 20*). These original NMR studies of brazzein
determined that the protein adopts a cysteine-stabilized $\alpha\beta$ (CS$\alpha\beta$ fold in which
the α-helix and β-strands are stabilized by the presence of four disulfide bridges)
(*17*). The structure suggests that this evenly distributed web of disulfide bridges
stabilizes the protein by limiting the conformational entropy of its unfolded state.

Structural relationship between brazzein and other members containing CS$\alpha\beta$ fold

Other CS$\alpha\beta$ fold proteins include members of the rapeseed family of serine
proteinases inhibitors, scorpion toxins, insect defensins, plant-derived γ-thionins
and a family of antimicrobial peptides. Apart from the conserved cysteines, little
sequence identity was found between members of the different families.
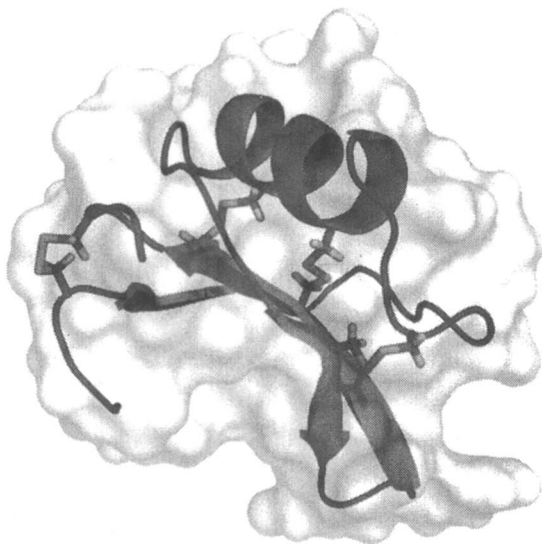
Figure 1. Backbone ribbon diagram and the surface representation of the brazzein extracted from fruit with positions of disulfide bonds are shown as determined by solution-state ^1H NMR spectroscopy (17).
(See color insert in this chapter.)

Brazzein is the only CS$\alpha\beta$ protein known to be sweet, and it has no proteinase inhibitor activity (21).

Production of recombinant brazzein, stable isotope labeled brazzein, and brazzein mutants

The protein shows great promise as a natural low-calorie sweetener. To understand the structural and chemical properties responsible for its sweetness, we have engineered a synthetic gene to express the brazzein molecule and developed the first production system for brazzein in bacteria (22). We have used this approach to discover mutants with sweet-taste properties that appear to be different than the wild-type protein (23). The sequence of the recombinant protein product is identical to the minor form of brazzein isolated from fruit, the form that lacks the N-terminal pyro-glutamate (pGlu) residue (we refer to this product, des-pGlu1-brazzein, henceforth as wild-type brazzein). Recombinant wild-type brazzein has about twice the sweetness of brazzein extracted from the fruit, which is primarily the pGlu variant.

We make the protein as a fusion with a modified form of the enzyme staphylococcal nuclease. The fusion protein is expressed in *Escherichia coli* as an insoluble product, which we solubilize, fold, and cleave to yield des-pGlu1-brazzein. The conditions we developed for folding and oxidation of the disulfides lead to a product with native structure (as determined by NMR spectroscopy) and with full activity as a sweetener (as determined by taste tests) (*22*).

Production of brazzein from *E. coli* has also enabled us to make samples labeled with stable isotopes (^{15}N or ^{13}C/^{15}N) for NMR investigations of the structure and dynamics of the protein. We recently solved a higher resolution NMR structure of wild-type brazzein by more modern methods by using ^{13}C/^{15}N-double labeling with RMSD = 0.38 Å (C. Cornilescu and F. M. Assadi-Porter, in preparation). The ^{13}C/^{15}N-double labeled brazzein was produced by our nuclease expression system. The stable isotope labeling enabled us to determine structural restraints from long-range NOEs and residual dipolar couplings required for high resolution NMR structure determination. The structure obtained from recombinant brazzein shows the same secondary structure as the fruit brazzein but with more refined backbone and side chain conformations.

Summary of results from mutagenesis of brazzein

Our first mutagenesis design was based on alanine scanning of solvent exposed residues. Our results from the variants, prepared, purified and subjected to threshold analysis by taste tests (Figure 2) (*22, 24, 25*) revealed the presence of multiple critical areas or "interaction sites": regions near Arg43 (Loop43) and the N- and C-terminal domains. Several charged residues in these regions were found to be essential to sweetness, as suggested by previous chemical modification studies (*16*). Our studies have indicated that the presence of positive charges on the surface of brazzein enhances sweetness: mutating some of these positive charges to neutral or negative charge significantly decreases the sweetness (*24, 25*). Charged residues also have been reported to be critical for the sweetness of thaumatin and monellin (*26-29*).

Multisite binding model

- ***Terminal regions.*** Both the N- and the C-terminal regions are important for sweetness in brazzein. Insertion of alanine at the N-terminus following the removal of methionine (Ala1-Asp2-sequence) increased sweetness and suggests that the presence of a hydrophobic side chain at this position enhances binding to the receptor ((*22*), and F. M. Assadi-Porter, unpublished results). At the C-terminus, deletion or addition of any residue

at position 54 diminished sweetness (deletion of Tyr54 or insertions of arginine residues at positions 55 and 56). Mutations near the C-terminus greatly altered sweetness (Asp50Ala, Cys52Ala) or changed protein stability (for example, Tyr51Ala did not fold, probably because of a clash in hydrophobic side chain packing) (Figure 2) (22).

- *Charged residues and loop regions.* Replacement of any of the charged residues near the loop regions greatly affected sweetness. Mutants Asp29Ala (Asp29Asn or Asp29Arg), His31A, Glu41Lys increased sweetness two- to four-fold. However, replacement of either Arg33 or Arg43 (at either end of β-strand III) by Ala decreased sweetness (3–4-fold for Arg33Ala but >50-fold for Arg43Ala) (Figure 2). Asp50Ala (or Asp50Asn) in the β-strand II near the C-terminus also decreased sweetness 2–3-fold. Mutants Arg33Ala and Asp50Ala, which introduce changes near the C-terminus, caused decreased sweetness. Arg33 interacts with residues near the C-terminus (including Asp50, Tyr51 hydrophobic packing) and near the N-terminus (including Lys5 and Lys6). As described below, these residues participate in backbone hydrogen bonds that may serve to transmit conformational effects of the mutations to adjacent parts of the structure (23, 30, 31).

NMR studies

For our detailed structural and dynamic analyses of brazzein variants, we chose wild-type brazzein and five mutants (two with increased sweetness and three with decreased sweetness). The ribbon-diagram in Figure 2 shows the backbone of wild-type brazzein and the positions of the five mutations (31). Four of the sites of mutation (Ala2 insertion, His31Ala, Arg33Ala, and Asp50Ala) are spatially close to one another. Two of the mutants (Ala2 insertion and His31Ala) have about twice the sweetness of wild-type brazzein; the other three mutants (Arg33Ala, Arg43Ala, and Asp50Ala) have greatly reduced sweetness (23). Arg43Ala is essentially tasteless.

Only one of the five mutants (Arg43Ala) exhibited chemical shift changes in regions remote from the site of mutation. In this mutant, changes in chemical shifts were observed in the N- and C-terminal regions, flexible 9–19 loop, as well as Arg33 loop region.

Hydrogen bonding

Thanks to a recent discovery, hydrogen bonds can now be detected and quantified through measurements of NMR spin-spin couplings. HNCO-type
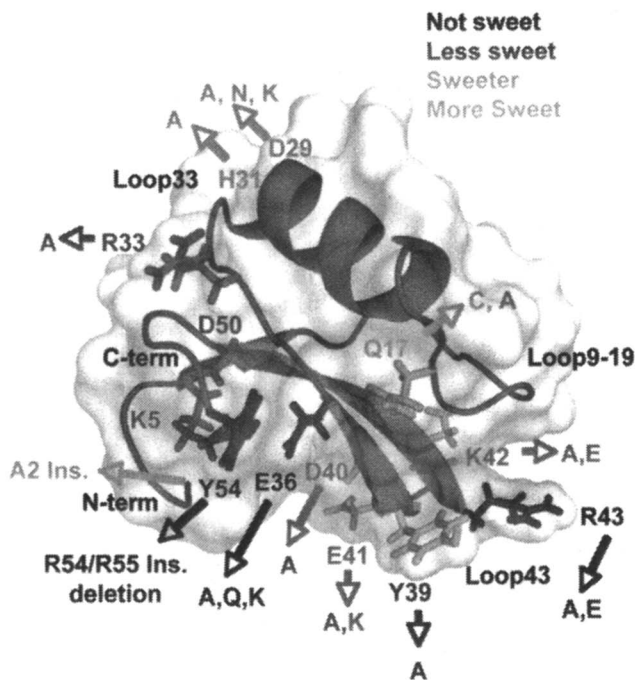
*Figure 2. Surface representation of wild-type brazzein showing a summary of
key mutations that change sweetness. Mutations that abolished sweetness are
shown in dark blue, whereas those that enhance sweetness are shown in gray.
Mutations that slightly intermediate decreased sweetness are shown in lighter
blue. Note that the side chains proposed constitute the primary sweet sites
(Loop43 and N- and C-terminal regions) are on the same face of the molecule
(22, 24, 25). (See color insert in this chapter.)*

NMR experiments provide information about trans-H-bond $^{h3}J_{NC}$ couplings, which have magnitudes usually less than 1 Hz (*32-34*). The magnitudes of the coupling constants can be converted into H-bond distances. The high sensitivity afforded by a triple-resonance cryogenic NMR probes has improved the practicality of using trans-hydrogen-bond couplings as a screen for monitoring structural changes in a protein backbone (i.e. "tight" vs. "relaxed" conformations) that result from changes in solution conditions or mutations (*35, 36*). Such measurements can be carried out much more quickly than a full structure determination typically with resolution > 0.1 Å. Changes in $^{h3}J_{NC'}$ couplings identify hydrogen bonds that are strengthened or weakened; missing peaks identify hydrogen bonds that may be broken; and new peaks indicate the presence of new hydrogen bonds will allow us to monitor subtle structural changes. We have used this approach to investigate wild-type brazzein and a series of brazzein variants with altered sweetness properties (*30, 31*).

Application of this approach to wild-type brazzein revealed 17 hydrogen bonds (Figure 3), 13 of the H-bonds were identical to those deduced from the NMR structure of fruit brazzein (*37*). Two additional H-bonds were detected, and two were found to be different from those reported earlier. Hydrogen bond patterns in brazzein indicate one α-helical stretch from residues 20–29 and three antiparallel β-strands, composed of residues 4–7, 34–40, and 44–51 all consistent with the calculated three-dimensional structure (*31*).

Analysis of the H-bonds in the six brazzein variants through measurements of trans-H-bond couplings has shown that single-site mutations can give rise to subtle structural changes (*31*). Interestingly, less sweet variants (Arg33Ala, Arg43Ala, and Asp50Ala) share the loss of two common H-bonds: Glu36 $H^N \cdots$I48 O' between β-strands II and III where they are twisted and Lys27 $H^N \cdots$Asn23 O' in the middle of the α-helix. By contrast, our results indicate that the two sweeter forms of brazzein, those with least change in chemical shifts (His31Ala and Ala2 insertion) maintain the wild-type pattern of H-bonds (*31*). Our H-bond data indicate that less sweet brazzein variants frequently have *stronger* H-bonds between β-strands than those in wild-type. However, this is not the case with sweeter variants that maintain wild-type like H-bond patterns.

Dynamics

Dynamic analysis by NMR can provide valuable information about the rigidity of proteins and the time dependence of fluctuations in molecular structures. We have carried out a preliminary analysis of ^{15}N relaxation data collected for brazzein at different NMR field strengths (600 MHz and 900 MHz). As determined by NMR relaxation measurements, mutations that decrease sweetness were found to change the flexibility of the protein (*31*). In particular, comparison of relaxation parameters from mutant Arg43Ala (non-
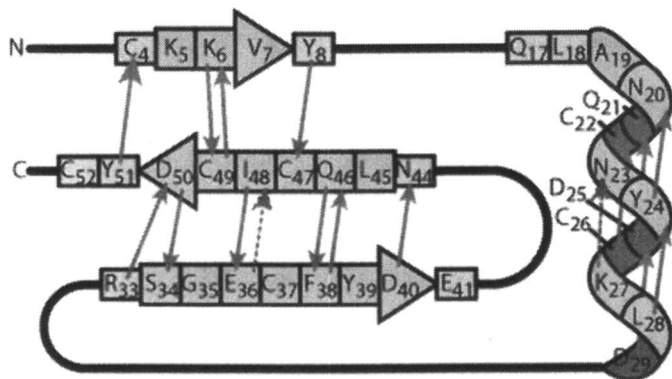
Figure 3. Hydrogen-bonds in wild-type brazzein deduced from trans-hydrogen-bond-couplings detected by NMR spectroscopy. Wild-type brazzein and two mutants with enhanced sweetness show a common pattern of hydrogen bonds, whereas all three variants with reduced sweetness have common loss of hydrogen-bonding patterns shown in dotted arrow lines (31).
(See color insert in this chapter.)

sweet analog) with those from wild-type brazzein indicates that internal mobility is decreased significantly in the loop regions of the mutant protein (Figure 4) (unpublished results). The observed loss of internal mobility can be explained in terms of the earlier trans-H-bond coupling results, which indicated that this mutation leads to a shift in one H-bond into the loop-43 region and the gain of two H-bonds between β-strands I and II (26).

Conclusions

Brazzein is a small high potency protein sweetener. In our earlier studies we had proposed a multi-site binding interaction model for brazzein with the hetrodimeric sweet receptors. The mutagenesis studies revealed the presence of at least two sites that are important for the sweetness. These sites are N- and C-termini and Loop43 region. Residues found to be important for the sweetness function are located on the same face of the molecule and separated in space by the presence of β-strands II and III. In general the presence of positive charges or introduction of hydrophobic side chains at the N-terminus enhances sweetness indicating the importance of both electrostatic and Van der Waal requirements for brazzein-sweet receptor interactions.
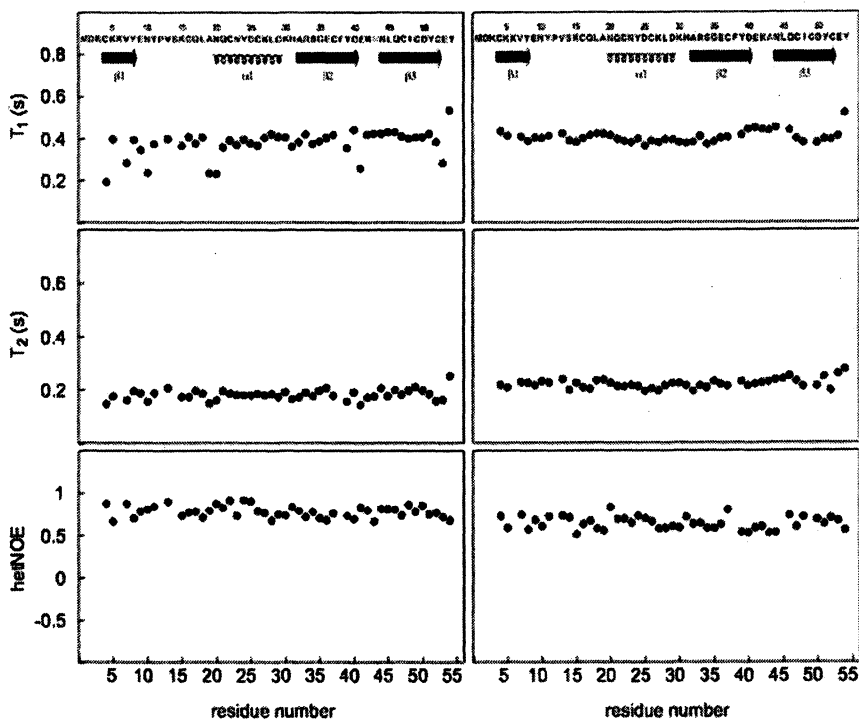
Figure 4. Comparison of relaxation parameters (longitudinal ^{15}N relaxation, T_1;
transverse ^{15}N relaxation, T_2; and ^{1}H-^{15}N nuclear Overhauser enhancement,
NOE) derived from wild-type brazzein (left panel) and Arg43Ala (non-sweet
mutant) (right panel). The internal mobility is decreased significantly in the loop
regions of the mutant protein (F. M. Assadi-Porter, and C.C. Cornilescu,
unpublished data.)

Our combined investigation of hydrogen bonding and dynamics of wild-type and mutant brazzeins have revealed a correlation between these properties and protein sweetness. These results will be important for interpreting future studies of the interaction between sweet proteins and the sweet taste receptor and for understanding how they are different from interactions of the receptor with small sweet ligands.

Acknowledgments

References

1. Ming, D.; Hellekant, G., Brazzein, a New High-Potency Thermostable Sweet Protein from Pentadiplandra brazzeana B. *FEBS Letters* **1994**, *355, (1)*, 106-108.

2. van der Wel, H.; Larson, G.; Hladik, A.; Hellekant, G.; Glaser, D., Isolation and Characterization of Pentadin, the Sweet Principle of Pentadiplandra brazzeana Baillon. *Chemical Senses* **1989**, *14*, 75-79.

3. Nelson, G.; Hoon, M. A.; Chandrashekar, J.; Zhang, Y.; Ryba, N. J.; Zuker, C. S., Mammalian sweet taste receptors. *Cell* **2001**, *106, (3)*, 381-390.

4. Li, X.; Staszewski, L.; Xu, H.; Durick, K.; Zoller, M.; Adler, E., Human receptors for sweet and umami taste. *Proc.Natl.Acad.Sci.U.S.A* **2002**, *99, (7)*, 4692-4696.

5. Damak, S.; Rong, M.; Yasumatsu, K.; Kokrashvili, Z.; Varadarajan, V.; Zou, S.; Jiang, P.; Ninomiya, Y.; Margolskee, R. F., Detection of sweet and umami taste in the absence of taste receptor T1r3. *Science* **2003**, *301, (5634)*, 850-3.

6. Jiang, P.; Ji, Q.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Margolskee, R. F.; Max, M., The cysteine-rich region of T1R3 determines responses to intensely sweet proteins. *J Biol Chem* **2004**, *279, (43)*, 45068-75.

7. Nelson, G.; Chandrashekar, J.; Hoon, M. A.; Feng, L.; Zhao, G.; Ryba, N. J.; Zuker, C. S., An amino-acid taste receptor. *Nature* **2002**, *416, (6877)*, 199-202.

8. Jiang, P.; Cui, M.; Zhao, B.; Liu, Z.; Snyder, L. A.; Benard, L. M.; Osman, R.; Margolskee, R. F.; Max, M., Lactisole interacts with the transmembrane domains of human T1R3 to inhibit sweet taste. *J Biol Chem* **2005**, *280, (15)*, 15238-46.

9. Jiang, P.; Cui, M.; Zhao, B.; Snyder, L. A.; Benard, L. M.; Osman, R.; Max, M.; Margolskee, R. F., Identification of the cyclamate interaction site within the transmembrane domain of the human sweet taste receptor subunit T1R3. *J Biol Chem* **2005**, *280, (40)*, 34296-305.

10. Xu, H.; Staszewski, L.; Tang, H.; Adler, E.; Zoller, M.; Li, X., Different functional roles of T1R subunits in the heteromeric taste receptors. *Proc.Natl.Acad.Sci U.S.A* **2004**, *101, (39)*, 14258-14263.

11. Winnig, M.; Bufe, B.; Meyerhof, W., Valine 738 and lysine 735 in the fifth transmembrane domain of rTas1r3 mediate insensitivity towards lactisole of the rat sweet taste receptor. *BMC Neurosci* **2005**, *6, (1)*, 22.

12. Danilova, V.; Hellekant, G.; Jin, Z., Effect of miraculin on behavioral and single taste fibers responses in common marmoset, Callithrix jacchus. *Chem Senses* **1998**, *23*, 550.

13. Temussi, P. A., Why are sweet proteins sweet? Interaction of brazzein, monellin and thaumatin with T1R2-T1R3 receptor. *FEBS Lett* **2002**, *526*, 1-4.

14. Ming, D. Brazzein, a New Sweet Protein from Pentadiplandra brazzeana. University of Wisconsin - Madison, 1994.

15. Caldwell, J. E.; Abildgaard, F.; Ming, D.; Hellekant, G.; Markley, J. L., Complete 1 H and Partial 13 C Resonance Assignments at 37 and 22 °C for Brazzein, An Intensely Sweet Protein. *Journal of Biomolecular NMR* **1998**, *11, (2)*, 231-232.

16. Ming, D.; Hellekant, G.; Zhong, H., Characterization and Chemical Modification of Brazzein, A High Potency Thermostable Sweet Protein From Pentadiplandra brazzeana. *Acta Botanica Yunnanica* **1996**, *18*, 123-133.

17. Caldwell, J. E.; Abildgaard, F.; Dzakula, Z.; Ming, D.; Hellekant, G.; Markley, J. L., Solution Structure of the Thermostable Sweet-Tasting Protein Brazzein. *Nature Structural Biology* **1998**, *5, (6)*, 427-431.

18. Somoza, J. R.; Jiang, F.; Tong, L.; Kang, C. H.; Cho, J. M.; Kim, S. H., Two Crystal Structures of a Potently Sweet Protein: Natural Monellin at 2.75 Å Resolution and Single-Chain Monellin at 1.7 Resolution. *Journal of Molecular Biology* **1993**, *234*, 390-404.

19. Ogata, C. M.; Gordon, P. F.; de Vos, A. M.; Kim, S. H., Crystal structure of a sweet tasting protein thaumatin I, at 1.65 A resolution. *Journal of Molecular Biology* **1992**, *228, (3)*, 893-908.

20. Ko, T. P.; Day, J.; Greenwood, A.; McPherson, A., Structures of Three Crystal Forms of the Sweet Protein Thaumatin. *Acta Crystallographica D* **1994**, *50*, 813-825.

21. Zhao, Q.; Chae, Y. K.; Markley, J. L., NMR solution structure of ATTp, an Arabidopsis thaliana trypsin inhibitor. *Biochemistry* **2002**, *41*, 12284-12296.

22. Assadi-Porter, F. M.; Aceti, D. J.; Cheng, H.; Markley, J. L., Efficient production of recombinant brazzein, a small, heat-stable, sweet-tasting protein of plant origin. *Arch Biochem Biophys* **2000**, *376, (2)*, 252-8.

23. Assadi-Porter, F. M.; Aceti, D. J.; Markley, J. L., Sweetness determinant sites of brazzein, a small, heat-stable, sweet-tasting protein. *Arch Biochem Biophys* **2000**, *376, (2)*, 259-65.

24. Jin, Z.; Danilova, V.; Assadi-Porter, F. M.; Aceti, D. J.; Markley, J. L.; Hellekant, G., Critical regions for the sweetness of brazzein. *FEBS Lett* **2003**, *544, (1-3)*, 33-7.

25. Jin, Z.; Danilova, V.; Assadi-Porter, F. M.; Markley, J. L.; Hellekant, G., Monkey electrophysiological and human psychophysical responses to mutants of the sweet protein brazzein: delineating brazzein sweetness. *Chem Senses* **2003**, *28, (6)*, 491-8.

26. Shamil, S.; Beynon, R. J., A Structure Activity Study of Thaumatin using Pyridoxyl 5'-Phosphate (PLP) as a Probe. *Chemical Senses* **1990**, *15, (4)*, 457-469.

27. van der Wel, H., Structural Modification of Sweet Proteins and its Influence on Sensory Properties. *Chemistry & Industry* **1983**, *(1)*, 19-22.

28. Morris, R. W.; Cagan, R. H.; Martenson, R. E.; Deibler, G., Methylation of the Lysine Residues of Monellin. *Proceedings of the Society for Experimental Biology and Medicine* **1978**, *157*, 194-199.

29. Kaneko, R.; Kitabatake, N., Structure-Sweetness Relationship in Thaumatin: Importance of Lysine Residues. *Chemical Senses* **2001**, *26*, 167-177.

30. Assadi-Porter, F. M.; Abildgaard, F.; Blad, H.; Cornilescu, C. C.; Markley, J. L., Brazzein, a small, sweet protein: effects of mutations on its structure, dynamics and functional properties. *Chem Senses* **2005**, 30 Suppl 1, i90-i91.

31. Assadi-Porter, F. M.; Abildgaard, F.; Blad, H.; Markley, J. L., Correlation of the sweetness of variants of the protein brazzein with patterns of hydrogen bonds detected by NMR spectroscopy. *J Biol Chem* **2003**, *278, (33),* 31331-9.

32. Cordier, F.; Grzesiek, S., Direct Observation of Hydrogen Bonds in Proteins by Interresidue 3H J NC' Scalar Couplings. *Journal of the American Chemical Society* **1999**, *121, (7),* 1601-1602.

33. Grzesiek, S.; Cordier, F.; Dingley, A. J., Scalar couplings across hydrogen bonds. *Methods Enzymol* **2001**, *338*, 111-33.

34. Cornilescu, G.; Hu, J. S.; Bax, A., Identification of the hydrogen bonding network in a protein by scalar couplings. *Journal of the American Chemical Society* **1999**, *121, (12),* 2949-2950.

35. Cordier, F.; Wang, C. Y.; Grzesiek, S.; Nicholson, L. K., Ligand-induced strain in hydrogen bonds of the c-Src SH3 domain detected by NMR. *Journal of Molecular Biology* **2000**, *304, (4),* 497-505.

36. Liu, A.; Hu, W. D.; Qamar, S.; Majumdar, A., Sensitivity enhanced NMR spectroscopy by quenching scalar coupling mediated relaxation: Application to the direct observation of hydrogen bonds in C-13/N-15-labeled proteins. *Journal of Biomolecular NMR* **2000**, *17, (1),* 55-61.

Chapter 37

Steviol Glucuronide as Excretion Product of Stevioside in Human Volunteers: Lack of Carcinogenic Properties of Steviol Glycosides and Steviol

Jan M. C. Geuns

Laboratory Functional Biology, KU Leuven, 3001 Leuven, Belgium

Absorption studies with Caco-2 cell monolayers revealed that steviol glycosides are barely absorbed by the intestines. Metabolism studies in healthy volunteers have shown that stevioside is completely degraded by bacteria of the colon into steviol and that part of this steviol is absorbed and glucuronated in the liver. The glucuronide is released in the blood and filtered by the kidneys into the urine. No accumulation of steviol glycosides or derivatives has been observed. As mutagenic effects of steviol were described, a thorough literature study has been made to evaluate possible risks when using steviol glycosides as a sweetener. The conclusion is that there are no indications that steviol glycosides used as a sweetener are not safe.

Introduction

Steviol glycosides (mainly stevioside, rebaudioside A) are sweeteners obtained from *Stevia rebaudiana* (Bertoni) Bertoni. Oral stevioside is not absorbed by the intestines or degraded by stomach juice (*1, 2, 3*). Only bacteria of the cecum (rats, hamsters, mice) or the colon of pigs and man degrade stevioside into steviol (*2-5*). As no metabolism studies were published with volunteers, and these studies are required by JECFA, FDA and EFSA, we organised research with volunteers. One of the aims of the study was to know if steviol, the aglycone of steviol glycosides, is further metabolised and/or accumulating in the body or if it is excreted in feces and/or urine.

Experimental Setup

Transport studies

Transport studies were done with Caco-2 monolayers as described (*4*). Stevioside and rebaudioside A were tested at 1 mM, steviol at 30, 100, 300 and 1000 µM concentrations in 2% DMSO.

Metabolism studies

Gellules containing 250 mg pure stevioside were administered thrice daily for 3 days with time intervals of 8 h. On the third day, blood samples (2x4 ml) were taken before and at 0.5, 1, 3, 5 and 7 h after the first dose of day 3. A 24 h urine was collected during the third day and the feces of the third day was collected on the fourth day.

To correct for losses of steviol during sample clean-up, dihydroisosteviol (Figure 1) was synthesized as internal standard (IS; *6*). Very sensitive analysis of steviol and its possible metabolites was possible after derivatisation with 4-(bromomethyl)-7-methoxycoumarin (Figure 2, detection limit between 50 and 100 pg). Sample clean-up was as described (*2*).

Steviol and its derivatives were searched for as the free form or after splitting possible glucuronides respectively sulfates by enzymatic hydrolysis with β-glucuronidase/sulfatase of *Helix pomatia* digestive juice (*2*).
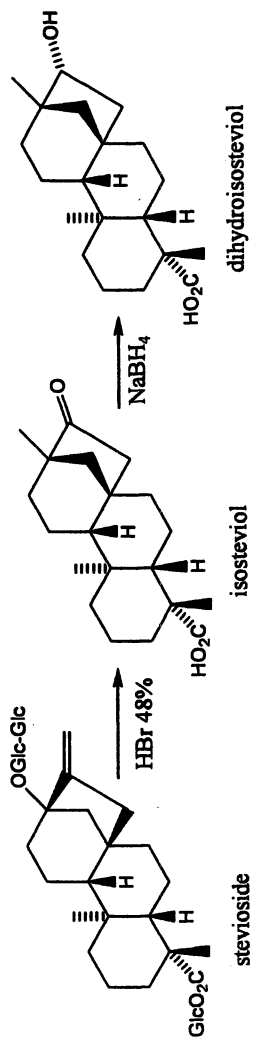
Figure 1. Breakdown of stevioside and synthesis of dihydroisosteviol.
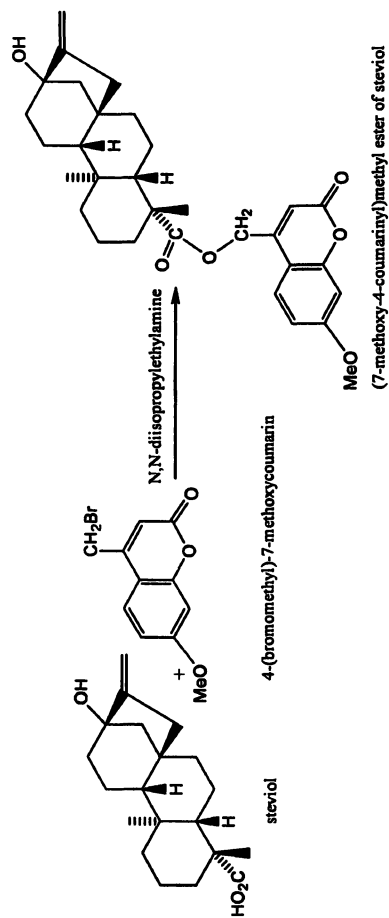
Figure 2. Derivatization of steviol with 4-(bromomethyl)-7-methoxycoumarin.

Results

Transport studies

Transport studies of stevioside, rebaudioside A and free steviol were done with Caco-2 monolayers (Figure 3).
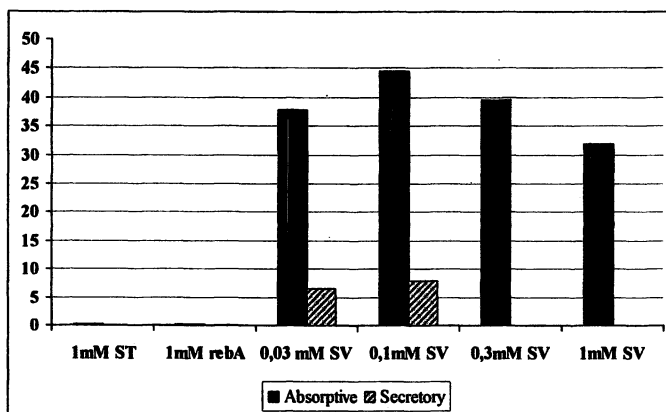
Figure 3. Permeability coefficients for 1 mM stevioside, 1 mM rebaudioside A and steviol (0.03, 0.1, 0.3 and 1 mM) (Geuns unpublished).

Absorptive transport of stevioside and rebaudioside A was below 0.16% and 0.1% respectively of the amounts administered in the apical vial. The absorptive transport of steviol was much higher (around 30%). In the secretory direction, steviol transport was significantly lower (around 5%) than in the absorptive direction (only measured for 0.03 and 0.1 mM). These results are in agreement with those obtained with everted sacs of rat intestines (7) and with human organic anion transporters (hOAT$_1$ and hOAT$_3$) expressed in *Xenopus laevis* oocytes (8).

Metabolism studies

Feces Analysis

No stevioside was detected in the feces. Free steviol was detected and was the only degradation product of stevioside found. These results are in agreement

with those of *in vitro* incubations with bacteria of human feces and of a metabolism study (*9, 10*).

Blood

No stevioside was detected in the blood after HPLC analysis and monitoring by UV at 210 nm (detection limit $\approx$ 50 ng). No free steviol was detected after derivatisation and HPLC analysis (detection limit $\approx$ 100 pg). After enzymatic hydrolysis of plasma samples, between 0.3 and 21.3 μg steviol/ml plasma was detected. After an oral dose, the steviol glucuronide present in the plasma increased to a maximum, and decreased again after it was filtered out by the kidneys (Figure 4).

The rate of metabolism in each volunteer was slightly different, giving an average steviol glucuronide concentration of about 5.65 μg steviol/ml present in the glucuronide.

Urine

No free steviol or stevioside were detected by the methods used. However, after enzymatic hydrolysis of urine extracts, between 25 and 205 mg steviol/24 h urine was released from the conjugates. There was no evidence of the occurrence of other steviol metabolites or derivatives (Figure 5).

After sample clean-up of large urine fractions, steviol glucuronide could be crystallised and fully characterised by MS, IR and ^{1}H an ^{13}C NMR (Figure 6). Its MW was 494.58, its melting point 198-199°C. It showed UV absorption at 208 nm (*2*).

It was suggested that stevioside is fully degraded by bacteria of the colon. Part of the steviol is then absorbed and transported by portal blood to the liver, where the steviol is glucuronated to steviol glucuronide. This is released into the blood and filtered by the kidneys and excreted in the urine (Figure 6).

Quantitative aspects of stevioside metabolism

The daily oral dose was 750 mg stevioside. After complete degradation in the colon, the theoretical amount of steviol formed is 300 mg (40% of the stevioside). After hydrolysis, in the urine about 102 mg steviol equivalents were found, in the blood about 102 mg (assuming the blood volume is about 7 % of the BW and the plasma volume about 56 % of the blood volume). After the first dose of the day, 34 mg steviol was found as the maximum amount in the plasma, and this value was multiplied by 3 as 3 doses were given a day (hence 102 mg/total plasma). The feces contained 23 mg. The total amount of steviol detected was 227, i.e. 76 % of the total steviol formed. This recovery is similar
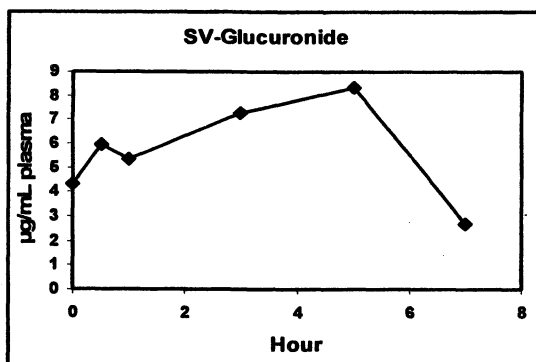
SV-Glucuronide

Figure 4. Steviol glucuronide concentration in the plasma after the first dose of day 3. Adapted with permission from reference 3. Copyright 2006 The Society for Experimental Biology and Medicine.
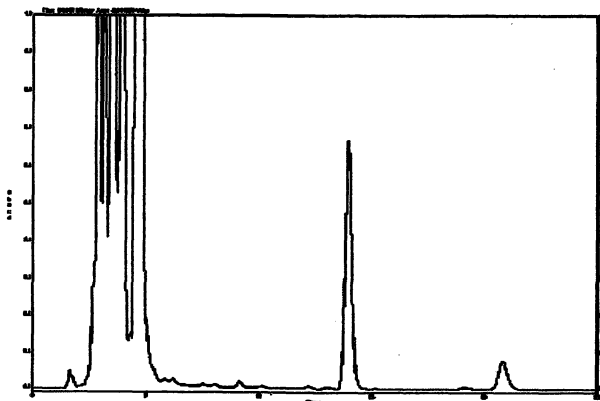
Figure 5. Example of a HPLC analysis of the derivatives of a urine fraction, with the ester of steviol around 13 min, that of IS around 23 min. Adapted with permission from reference 3. Copyright 2006 The Society for Experimental Biology and Medicine.
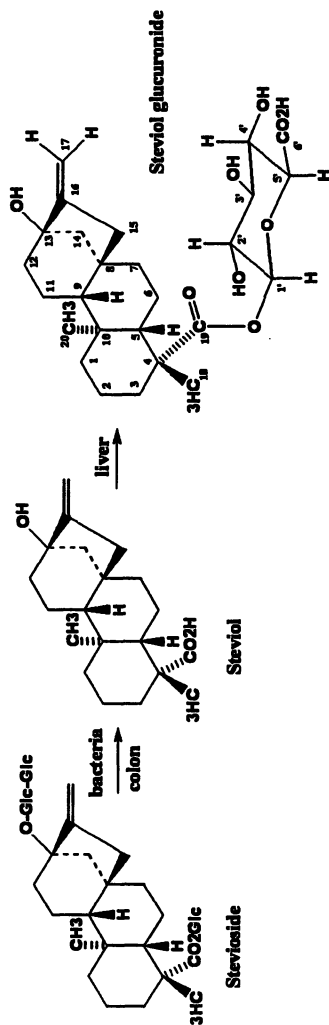
Figure 6. Route for the formation and excretion of steviol glucuronide.

to the methodological recovery found after spiking feces and blood samples with stevioside or steviol.

Comparison of the metabolism study at KULeuven and at the Milan ITB-CNR Research Institute

In the study of Milan (*10*) one single dose of 375 mg was administered and free steviol, stevioside, steviol glucuronide, steviol 16,17α-epoxide, 15-OH-steviol and 15-oxo-steviol were searched for by LC-MS Total Ion Chromatography (TIC). In the experiment done at the KULeuven (*2, 3*), 3 daily doses of 250 mg each were administered for 3 days.

Blood

No free steviol, steviol 16,17α-epoxide, 15-OH-steviol or 15-oxo-steviol compounds could be detected. Steviol glucuronide was detected in the blood plasma at a maximum concentration of 0.1 µg/ml (*10*).

Table I. Comparison of the amounts of steviol in steviol glucuronide in plasma (µg/ml) found in Milan or in KULeuven in function of time after the first (KULeuven) or single dose (Milan) of the day.

Daily Dose	*Time*					
375 mg (Milan)	*1 h*	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*
Free SV	0	0	0	0	0	0
SV-Glu	0	0	0	0.1	0.05	(0?)
750 mg (KULeuven)	*0 h*	*0.5 h*	*1 h*	*3*	*5 h*	*7 h*
Free SV	0	0	0	0	0	0
SV-Glu	5.9	8.3	5.8	2.9	6.9	3.7

In the experiment at KULeuven (Table I, *2, 3*) a maximum amount of steviol present in steviol glucuronide was found of 21.32 µg/ml, with average amounts fluctuating around 5.65 µg/ml. The higher amounts detected at KULeuven are probably due to the higher daily dose administered during a period of 3 days and by a correction for losses by use of an IS. A maximum concentration of 100 ng stevioside/ml plasma was found in 7 out of 9 volunteers in Milan (*10*). This amount was far below the detection limit of the UV detector (50 ng/10 µl injected). This result confirms the very low absorption of stevioside and rebaudioside A in transport experiments (*4, 7, 8*). The total stevioside absorbed

can be estimated to be about 110 ng/ml = 330 µg/3 l plasma. This is only about 0.0088% of the dose of 375 mg administered.

Urine

It was reported that traces of stevioside were detected in the urine of 2 volunteers (*10*). However, the amounts were too low to permit a quantification. Besides this, they found a compound with the same MW of steviol glucuronide. They found about 20 mg steviol occurring as glucuronide. At KULeuven between 25 and 205 mg steviol was found in the glucuronide (*3*). The higher amounts found are probably the consequence of the higher doses administered during a longer period of 3 days, and of the correction for losses by an IS. No other metabolites were detected (Figure 5).

In vitro studies with feces

Stevioside was completely degraded into free steviol within about 7 h. The complete degradation of rebaudioside A was much slower and lasted for about 24 h (Figure 7). In both cases, steviol was the only degradation product found. To show the quantitative conversion, steviol is fiven as stevioside or rebaudioside A equivalents respectively.These results are in agreement with those of (*3, 9*).
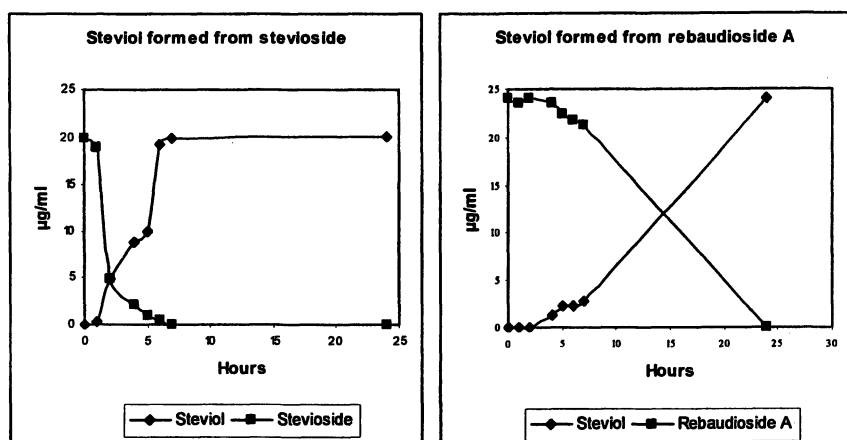
Figure 7. Degradation of stevioside and rebaudioside A by human feces incubated under anaerobic conditions (Geuns, unpublished).

Carcinogenic studies with steviol glycosides and steviol

As bacteria of the colon degrade steviol glycosides to steviol, of which a large quantity is absorbed, and as steviol showed some mutagenic activity in a few *in vitro* tests (see below), special attention is given to carcinogenic studies with steviol glycosides and free steviol.

i. Induction of gene mutations in bacteria

Steviol glycosides

Steviol glycosides did not show mutagenic effects in gene mutation essays in bacteria with or without S9 activation mix.

In recombination tests with *Bacillus subtilis* strain H17 (Rec+) and M45 (Rec-) stevioside of 95 – 98% purity was used (*11*). Filter paper discs (8 mm diam.) were damped with 20 µl of test solution and placed on plates containing the micro-organism on B-2 broth (filter discs contained 0, 20, 100, 200, 500, 1000 or 2000 µg stevioside or 0.1 µg mitomycin C as a positive control. The growth inhibition was measured by the halo of inhibition. No growth inhibition could be observed in the H17 or M45 strain traited with stevioside. However, the positive control showed a halo of inhibition of 1 (H17) and 10 mm (M45). Reversion tests were done with *E. coli* strain Wp2 (requiring tryptophane) and *S. typimurium* TA100, TA1537, 1538, TA 98 and GA 46 requiring histidine. Stevioside (95 – 98% purity) concentrations were 0, 0.1, 1, 10 and 100 mg/ml dissolved in DMSO. The positive control was 2-aminoanthracene (10 µg/ml DMSO), the negative control being DMSO. The amounts of stevioside per plate were 0, 10, 100, 1000 or 10000 µg. The plates with the positive control contained 1 µg/plate. No increase of reversions was obtained in the plates containing stevioside in the presence or absence of the S9 mix. The positive control showed a very significant stimulation.

Stevioside, rebaudioside A, B, C, dulcoside A and steviolbioside were tested at concentrations of 0, 0.1, 1 and 10 mg/l in the forward mutation assay using *S. typhimurium* TM677 carrying the R-factor plasmid pKM101 (*12*). No effects of the different steviol glycosides were detected.

Stevioside (96% purity) was tested at the concentrations of 0, 1, 2.5, 25 and 50 mg/plate by using the *in vitro* Ames test with *S. typhimurium* TA 98 and TA100 (*13*). The S9 fraction was obtained from rats, mouse, hamster and guinea pig). No effects on both tester strains were observed in the presence or absence of the metabolic activating system S9.

No stimulating effects of stevioside could be demonstrated in 6 different tests: reverse mutation assays using *S. typhimurium* and *E. coli*; forward mutation assay using *S. typhimurium* TM677; umu test with *S. typhimurium* TA1535/pSK1002; rec-assay (no growth inhibition of *B. subtilis* H17 Rec+ or M45 Rec-); streak-rec-assay (did not show any DNA-damaging to *B. subtilis*) (*14*).

Steviol

Mutagenic effects of steviol, the aglycone of stevioside, and/or its metabolites were reported in the forward mutation assay using *Salmonella typhimurium* TM677 (*12*; concentrations tested: 0, 0.1, 0.5, 1, 2.5 and 10 mg/ml; *14 – 17*). After metabolic activation, it was shown that so far unknown steviol metabolites caused mutations in *Salmonella typhimurium* TM677, i.e. transitions, transversions, duplications and deletions at the guanine phosphoribosyltransferase (*gpt*) gene (*18*). However, stevioside and even steviol were inactive in various other TA strains of *Salmonella typhimurium* with or without S9 mix at doses up to 5 mg/plate (TA 97, 98, 100, 102 and 104), in *Escherichia coli* WP2 *uvrA*/pKM101 and in the *rec*-assay using *Bacillus subtilis* even when activation S9 mix was present (99% purity, *13*; 96% purity, *14*). The direct mutagenic activity of 15-oxo-steviol was refuted by (*19*), but confirmed by (*17*). The activity of steviol in *Salmonella typhimurium* TM677 was very low and was only about 1/3000 that of 3,4-benzopyrene and that of steviol methyl ester 8,13 lactone was 1/24500 that of furylfuramide (*17*). Although a weak activity of steviol and some of its derivatives was found in the very sensitive *S. typhimurium* TM677 strain, the authors concluded that the daily use of stevioside as a sweetener is safe. Moreover, the presence in the blood of the chemically synthesized steviol derivatives after feeding stevioside is not proven at all.

ii. Induction of gene mutations in mammalian cells *in vitro*.

Steviol glycosides

Steviol glycosides (83.2% purity) did not induce gene mutations in mammalian cells *in vitro* (CHL cells) (*14*).

Five steviol glycosides (stevioside, rebaudioside A, rebaudioside C, dulcoside A and rubusoside) were assayed *in vitro* on the inhibition of Epstein-Barr Virus-Early Antigen induction (EBV-EA) using Raji cells, EBV genome-carrying human lymphoblastoid cells which were cultivated in 10% fetal

bovine serum RPMI 1640 medium (20). The indicator cells (Raji, 10^6/ml) were incubated at 37°C for 48 h in 1 ml medium containing butyric acid (4mM), TPA (32 nM) and various amounts of test compounds dissolved in 5 µL DMSO. The concentrations used were 0.32 mM, 3.2 mM, 16 mM and 32 mM, giving a molar ratio with TPA in the cell culture of 10, 100, 500 and 1000 respectively. Smears were made of the cell suspensions. The EBV-EA inducing cells were stained with high titer EBV-EA positive serum from NPC patients and detected by an indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. The EBV-EA induction was ordinarily around 35%, and this value was taken as the positive control (100%). The viability of treated Raji cells was assayed by the Tripan blue staining method. The survival rate of the cells in all the steviol glycoside treatment groups was over 80 %, except in the highest concentration (60%). Of all the steviol glycosides, stevioside had the strongest inhibitory effects on EBV-EA induction (over 95, 63 and 32 % inhibiton at 100x, 500x and 100 x mol ratio/TPA, respectively and preserved a high viability of Raji cells.

The conclusion from the experiments with mammalian cells *in vitro* is that steviol glycosides are not carcinogenic and might be chemopreventive agents of natural origine. It is not known if they have similar effects when orally administered as the amounts of stevioside consumed as a sweetener (maximal amount estimated at about 200 mg/d) will be far below the concentration of 3.2 mM, the lowest concentration having a significant effect.

Steviol

After metabolic activation of steviol (99 % purity), the aglycone of steviol glycosides, some gene mutation and chromosomal aberration was found in Chinese hamster lung fibroblasts (14). CHL cells in logarithmic phase of growth were exposed to steviol dissolved in DMSO for 3h at 37°C in the presence of S9 mix. Doses tested were: 0, 250, 300, 350 and 400 µg/ml. After treatment, the cells were washed and collected after trypsinisation. For determination of cytotoxicity, the treated cells (200-400 cells) were cultured for 7 days and the numbers of surviving colonies were counted. For determination of mutation frequency, the remaining portion of the cells was cultured in fresh medium for 7 d. After the expression time, an aliquot (2.5x10^5 cells) was transferred to fresh medium containing diphtheria toxin (0.1 Lf/ml), cultered for another 7 d and the number of diphtheria toxin resistant (DT^r) colonies was scored. Another aliquot of the cell suspension (250) was transferred to toxin-free medium to determine the plating efficiency. A dose-dependent increase in the number of mutants that were resistant to diphtheria toxin was observed. At the doses below 300 µg/ml,

no effect was observed. At 350 µg/ml the increase was 47%, at 400 µg/ml about 236%. The positive control N,N-dimethylnitrosamine at 1000 µg/ml induced a 447 % increase of mutations. The mutagenicity of steviol without the metabolic activation was not studied.

iii. Induction of chromosomal aberrations in mammelian cels *in vitro*.

Steviol glycosides

Stevioside (purity 83.2%) was not mutagenic in the chromosome aberration test using CHL cells with or without the metabolic activation (S9) (*14*). The cells were treated for 24 and 48 h (doses 0, 2, 4, 8, 12 mg/ml). Thereafter, the cells were treated with colcemid (0.2 mg/ml) for 2 h and chromosome preparations were made using a standard air-dry method. The frequency of the cells with chromosomal aberrations was scored in 100 well-spread metaphases for each dose. Types of chromosomal aberrations were classified into 5 groups: chromatid gaps including chromosome gaps, chromatid breaks, chromatid exchanges, chromosome breaks and chromosome exchanges including dicentric and ring chromosomes. Polyploid cells were also recorded. No significant increase in the frequencies of cells with chromosomal aberrations at doses up to 8 mg/ml was found. Cytotoxicity was found at the highest dose of 12 mg/ml.

The steviol glycosides (Rebaudioside A) did not induce chromosomal aberrations in the *in vitro* chromosome aberration assay using a Chinese hamster lung fibroblast cell line (CHL/IU; *21*). In the first series of experiments, 8 doses were tested in a cell growth inhibition assay: 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500 and 5000 µg/mL in the presence or absence of metabolic activation (S9). The experiment comprised short-term (6h) and continuous 24 h treatments. No cytotoxic effects were observed in any treatment group, nor were remarkable changes observed (eg. pH changes or deposition). The chromosome aberration assay was done using doses of 1250, 2500 and 5000 µg/mL, with short-term (6h) S9-, short-term S9+ and continuous 24 h treatment. There was no obvious induction of chromosomal aberrations by either short-term treatment or continuous treatment in the rebaudioside A groups. The positive control substance mitomycin C (MMC) strongly induced chromosomal aberrations in the short-term S9- and 24 h treatments as did the control cyclophosphamide (CP) in the short-term S9+ treatment (*21*).

Rebaudioside A did not induce micronucleated erythrocytes in mouse marrow cells, i.e. it is not clastogenic (*22*). The mutagenicity of rebaudioside A was studied in an *in vitro* micronucleus assay in male BDF1 mice to study the

induction of micronuclei in erythrocytes. BDF1 mice were chosen because of their common use in safety studies and micronucleus assays. Doses of 500, 1000 or 2000 mg/kg BW were administered by gavage daily for 2 consecutive days. Peripheral blood was collected 30 h after the second dose, and the frequency of micronucleated reticulocytes (MNRETs) and the percentage of reticulocytes (RETs) were calculated. The frequency of MNRETs in all rebaudioside A groups was similar to that in the negative control group, showing no statistically significant differences. No decrease of the percentage of RETs was found indicating that there was no effect on bone marrow cells. The positive control group (mitomycin C intraperitoneally at 0.5 mg/kg BW) showed a significant increase ($p<0.01$) of the frequency of micronucleated polychromatic erythrocytes in all animals.

A mixture of *Stevia* sweeteners (88.28 % purity) consisting of stevioside (51.76%), rebaudioside A (22.25 %), rebaudioside C (8.53 %), dulcoside A (2.76 %), rubusoside (1.20 %) and steviolbioside (1.51 %) was also evaluated by the *in vivo* 'comet' assay in mice (*23*). The doses used were 500, 1000 and 2000 mg/kg BW. The negative control group received physiological saline, the positive control group received 160 mg/kg methyl methanesulfonate. Mice were sacrificed 3 and 24 h after oral administration. No DNA damage was observed in stomach, colon or liver DNA. The lack of harmful effects of steviol and of the sweetener mixture in the *in vivo* comet assays in mice provides very powerful evidence that these compounds are non-carcinogenic (*24*).

Steviol

Besides steviol glycosides, also steviol was tested. The genotoxicity of steviol (>99 % purity) was evaluated using the 'comet' assay in which DNA damage is detected by DNA strand breaks and alkali-labile sites (*23*). Steviol was evaluated *in vitro* and *in vivo*. With *in vitro* experiments, 'comet' assays were done in the human lymphoblastoid cell lines TK6 and WTK-1. Since 1000 μg/mL steviol caused a marked reduction in cell survival rate, the assays were done at concentrations of 500 μg/mL or below which did not cause decreases of viable cell counts by 30% or more, regardless of the presence or absence of a rat-liver-derived metabolic activation system. No statistically significant increases in DNA damage were observed at concentrations of 500 μg/mL or below, either with or without metabolic activation. The negative control consisted of DMSO which was also used as solvent for steviol. In the positive control, cells were treated with methyl methanesulfonate in the absence of metabolic activation and with benzo[a]pyrene in its presence.

Chronic toxicity and carcinogenicity

Chronic studies with rats and hamsters

Three studies on chronic toxicity and carcinogenicity with rats were done. In addition, a chronic study with hamsters was done as well as a study on rat urinary bladder carcinogenesis.

A first study was done by (25). Male and female F344 rats were daily fed with a ration containing 0, 0.1, 0.3 or 1% of crystallised steviol glycosides (95.2% purity, containing mainly stevioside (78.3%) and rebaudioside A (17%)) for a period of 22 (males) or 24 (female rats) months (25). The amounts administered corresponded to 55 mg/kg BW (0.1%), 165 mg/kg BW (0.3%) and 550 mg/kg BW (1%). The total number of rats in the experiment was 480. At 6 and 12 months, 10 animals of each sex from each group were sacrificed for clinical and pathological tests. The middle dose induced a slight growth retardation in both sexes, but at the high dose the growth was reduced only transiently. General appearance and behavior were the same in all groups, including the control. Mortality at the end of the study in rats given stevia extracts was not significantly different from that in the controls. At 6 months, a variety of changes were found in the results of urinary, hematological and blood biochemical examinations and in organ weights, but there were no such differences at 12 months or at the end of the experiment.

At the end of the experimental period, the remaining animals were killed, and the researchers conducted biochemical, anatomical, pathological and carcinogenic tests on 41 organs following autopsy. Each of the animals was matched to a control animal that experienced exactly the same treatment, except for the stevioside. At the end of the experiment, no significant dose-related changes were found in the growth, general appearance, hematological and blood biochemical findings, organ weights, and macroscopic or microscopic observations. It was also concluded that any neoplasms that occurred were not attributable to the administration of stevioside. Even at the greatest dose of 1% (550 mg/kg BW), no significant effects were found.

The effects of a chronic oral feeding of stevioside (85% purity) during 24 months were studied in Wistar rats (26). The concentrations used were: 0, 0.2, 0.6 and 1.2% or about 0, 131, 395 and 789 mg/kg BW respectively. Each group consisted of 45 male and 45 female animals. After 6, 12 and 24 months, five male and five female rats from each group were killed for haematological and clinical biochemical tests. Growth, food utilisation and consumption, general appearance and mortality were similar in treated and control groups. The mean lifespan of rats given stevioside was not significantly different from that of the controls. No treatment-related changes were observed in haematological, urinary or clinical

biochemical values at any stage of the study. No significant differences were observed between treated and control groups of either sex in the incidences of total neoplasms or of benign or malignant tumours. Most (73%) of the tumours observed were benign, and the most frequent were adenofibromas of the mammary gland, of which 93% occurred in females. Other sites of neoplasms were the kidney, oral cavity, subcutis, mesentery, ovary, peritoneum, pituitary, colon and uterus. These types of tumours are frequently found in ageing rats of Wistar strains. The maximum NOEL of stevioside was 1.2%, and this was the greatest concentration tested by the authors. The authors suggested an ADI of stevioside for humans of 7.9 mg kg^{-1} BW (safety factor 100).

A third study was by (27). The purity of the stevioside was 95.6% and the daily doses were 0, 2.5 and 5 % of the diet. These doses corresponded to 0, 385 and 775 mg stevioside per rat or about 969 and 1120 mg/kg BW for males and females respectively (2.5% diet) and 1997 and 2387 mg/kg BW for males and females respectively (5% diet group). The concentration of stevioside in the diet did not change during the experimental period. Four week old rats (F344/DuCrj) were randomly allocated to 3 groups, each consisting of 50 males and 50 females. Throughout the experiment, rats in all groups had free access to both tap water and diet for 104 weeks. All surviving rats were killed at week 108. Body weight gains were slightly depressed in line with the dose of stevioside, in both sexes, and a decrease in the final survival rate was observed for the 5% treated males. Haematological examination of rats at week 108 did not reveal any statistically significant variation in the numbers of white blood cells, red blood cells or platelets, amounts of haemoglobin, or levels of haematocrit between the stevioside-treated groups and the control group in either sex. It was concluded that there were no significant increases in the incidence of neoplastic or non-neoplastic lesions in any organ or tissue in the stevioside-treated groups. In male animals, the number of testicular tumours had the tendency to decrease. Moreover, the incidence of adenomas of the mammary gland in the stevioside-treated female rats was significantly less than that in the controls. The severity of chronic nephropathy in males was also clearly reduced by both stevioside concentrations. The mean reason for the decrease in the final survival rate of the 5% treated males (see above) was rapid development of large granular lymphocyte type leukaemia in the final weeks of the study. However, these incidences observed in the 5% stevioside treated males were in line with background data observed with a total of 225 untreated control F344 males and must be considered incidental (27).

Male and female hamsters were daily force-fed with stevioside (90% purity) over three generations (doses: 0, 0.5, 1 and 2.5 g/kg BW) (28). No abnormalities were found in both sexes. Each female was mated and allowed to bear three litters during the period of the experiment. The young F1 and F2 hamsters

continuously receiving stevioside via drinking water until one month old and daily force-fed afterwards at the same doses as their parents showed normal growth and fertility. Histological examination of reproductive tissues from all three generations revealed no evidence of abnormality which could be linked to the effects of consuming stevioside. Stevioside at a dose as high as 2.5 g/kg BW/day neither effected growth nor reproduction in hamsters. Although the aim of this study was not to study carcinogenicity, the histological study of reproductive tissues did not reveal differences between those of treated and control groups.

In addition to the above cited 2 year chronic studies, it was reported that 5% stevioside in the diet of F344 rats did not increase the incidence of urinary bladder carcinogenesis initiated by 0.01 % N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in the drinking water during 4 weeks (29). After the initiation period of 4 weeks, 5% stevioside was added to the diet for 32 weeks. All surviving rats were sacrificed after 36 weeks, and examined histologically. Administration of 5% stevioside in the diet did not affect the incidence or extent of papillary or nodular hyperplasia in BBN-treated rats. No preneoplastic or neoplastic lesions of the urinary bladder were observed in rats treated with stevioside only. In the same study, sodium saccharin and aspartame were also studied (results not reported here).

Studies with steviol in mice, rats and hamsters

No chronic toxicity and carcinogenicity studies of steviol glycosides using mice seem to exist. However, various *in vivo* studies with mice, hamsters and rats were done in which large doses of steviol were administered. As oral steviol is easily absorbed, these studies seem very relevant. Besides this, results obtained with steviol administered by i.p. injection are also discussed (14). Results obtained with steviol glycosides in a mouse skin assay are also reported.

Four dose levels of steviol (99% purity) suspended in olive oil were administered by i.p. injection to male MS/Ae mice (125, 250, 500 and 1000 mg/kg BW) (14). Mitomycin (2 mg/kg) was used as a positive control and olive oil as negative control. All mice were killed 24 h after treatment (except the group of 500 mg/kg after 48 h). Femoral marrow cells were collected, fixed and stained with 3% Giemsa. One thousand polychromatic and normochromatic erythrocytes were scored and the numbers of micronucleated polychromatic erythrocytes (MNPCEs) and of micronucleated normochromatic erythrocytes (MNNCEs) were recorded. The numbers of micronucleated erythrocytes (MNEs) and the proportion of polychromatic erythrocytes (PCEs) relative to the total erythrocytes were evaluated by observing 1000 erythrocytes on the same slide. Steviol did not induce significant increases in the frequencies of MNPCEs,

MNNCEs or MNEs. No apparent changes were observed in the proportion of PCEs to total erythrocytes. However, at the highest dose of 1000 mg/kg BW, four out of six mice died, indicating that dose levels over the present maximum dose were unavailable for the micronucleus test. Mitomycin C induced a significant increase in the frequencies of MNPCEs and MNEs. The authors concluded that steviol did not induce micronuclei in bone marrow erythrocytes of mice.

In vivo studies of steviol (99% purity) were done by 2 independent research groups: Safety Research Institute for Chemical Compounds Co, Sapporo, Japan, and Faculty of Chemical and Biological Engineering, Aomori, Japan (*23*). The lethal dose of steviol in mice is over 2000 mg/kg BW. Therefore, steviol was administered to BDF1 or CRJ:CD1 mice as one oral dose at 250, 500, 1000 and 2000 mg/kg. Three and 24 h after gavage, DNA was assessed in the liver, kidneys, stomach, colon and testes. No statistically significant increases in DNA damage were observed in any of the organs of animals to which steviol was administered. The negative control animals received olive oil (solvent of steviol) and the positive controls received methyl methanesulfonate either orally (160 mg/kg) or intraperitoneally (80 mg/kg).

Very large doses of steviol (90% purity) intubated to hamsters (4 g/kg bw), rats and mice (8 g/kg BW) did not induce micronucleus formation in bone marrow erythrocytes of either male or female rats, hamsters or mice. However, these very large doses showed some cytotoxic effect in the female, but not the male of all treated animal species (*30*). There was no apparent change in the PCEs:NCEs (polychromatic erythrocytes:normochromatic erythrocytes) ratio of the male animals of all 3 treated species at 24, 30, 48 and 72 hour. However, steviol at the given dose can cause significant reduction of PCEs to NCEs ratio of the female hamsters at 72 h, and female rats and mice at 48 and 72 h after oral steviol. From these results, it was suggested that adverse metabolites were produced from steviol and these metabolites could reach the bone marrow, the target organ for micronucleus test. These metabolites also exhibited a slightly cytotoxic effect but no clastogenic effect to the bone marrow erythrocytes. These results are controversial to the previous report by (*14*) which showed no change in PCEs to NCEs ratio. The difference might be due to a difference in purity of steviol (90% against over 99% in Matsui's study).

In a two-stage carcinogenesis experiment in mice skin for 20 weeks, tumour formation was initiated by a single topical application of 50 μg 7,12-dimethyl-benz[*a*]anthracene (DMBA) (female ICR mice, 7 weeks old). One week after the initiation, promotion was started twice weekly by the application of 1 μg 12-O-tetradecanoylphorbol-13-acetate (TPA). When steviol glycosides (89% purity, containing stevioside (48.9%), rebaudioside A 24.4%), rebaudioside C (9.8 %) and dulcoside A (5.6%)) were applied topically 30 min before the TPA, in amounts of 0.1 or 1 mg, the number of tumours was significantly reduced (*31*).

592

In a similar two-stage carcinogenesis experiment in mice skin (specific pathogen-free female ICR, 6 weeks old), papillomas were initiated with 100 µg DMBA. One week after initiation, mice were promoted by the topical application of TPA (1 µg, 1.7 nmol) twice a week. Topical application of stevioside (85 nmol) 1 h before each promotion, delayed the formation and reduced the number of papillomas over a 15 week period (*20*). These authors also demonstrated that oral stevioside (2.5 mg/100 ml drinking water) for only 2 weeks (one week before and one week after initiation) also reduced mouse skin carcinogenesis initiated by peroxinitrite (33.1 µg, 390 nmol) and induced by TPA (1 µg) in female SENCAR mice (6 weeks old).

General Conclusion

It can be concluded that steviol glycosides did not show mutagenic activity in various gene mutation assays with bacteria, nor in mammalian cells *in vitro* or *in vivo*. Steviol showed a weak effect in the forward mutation assay using the sensitive *S. typhymurium* TM677, but not in other TA strains of this bacteria nor in *B. subtilis*. The 2 greatest concentrations of steviol tested in mammalian cells *in vitro* showed a mutagenic activity. No chromosomal aberration induced by steviol administration were detected in mammalian cells *in vitro*. Because of the positive response in **i**, and **ii** (see above), steviol has also been tested *in vivo* in mice, rats and hamsters. Four different research groups could not detect genotoxic effects of steviol although the greatest doses administered reached up to 1, 2, 4 and 8 g/kg BW depending on the research group and the animals tested. These values would suggest an ADI (safety factor 100) of steviol equivalents between 10 and 80 mg/kg BW or between 25 and 200 mg stevioside/kg BW, far above the values calculated in Table 1. That much steviol glycosides will not be required for sweetening purposes.

Chronic toxicity and carcinogenicity studies by 3 research groups using rats demonstrated the safety of stevioside uses as a sweetener. In addition, a chronic study with hamsters over 3 generations did not show the appearance of abnormal structures in the reproductive tissues. Moreover, no preneoplastic or neoplastic lesions of the urinary bladder of rats were observed after feeding stevioside. Although use of F344 rats in some studies might not be the best choice for chronic carcinogenicity studies, the decrease of the incidence of adenomas of the mammary gland is relevant, as well as the delay and decrease of the number of papillomas in the two-stage mice skin assays, both by topical and oral administration. The inhibition of the Epstein-Barr Virus Early Antigen induction by steviol glycosides might be indicative for a safe use of steviol glycosides as a sweetener. However, due to the extremely low absorption by the intestines and

the low amounts needed for sweetening purposes, the advantages of oral steviol glycosides as chemopreventive agent seem to be less relevant.

In 1999, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) had a similar conclusion:" Stevioside has a very low acute oral toxicity. Oral administration of stevioside at a dietary concentration of 2.5% to rats for two years, equal to 970 and 1100 mg kg^{-1} BW per day in males and females, respectively, had no significant effect. Reduced body-weight gain and survival rate were observed at a dietary concentration of 5% stevioside. There was no indication of carcinogenic potential in a long-term study..."(*32*).

The results of the transport experiments showed that the uptake of steviol glycosides (stevioside and rebaudioside A) was very low. The steviol glycosides reaching the colon were degraded into steviol by bacteria of the colon. Steviol was easily taken up by the colon and glucuronated by liver enzymes. The steviol glucuronide was then released into the blood stream and filtered out by the kidneys into the urine and excreted. No other metabolites of steviol were detected and there is no accumulation of steviol or steviol glucuronide in the body. Moreover, a thorough study of the literature did not reveal mutagenic or carcinogenic effects when steviol glycosides are used as sweeteners (see also part 2).

Reports have never appeared proving that the use of *Stevia* or stevioside enhances the number of cancers in populations, even after a very long time of use (eg. Paraguay, Japan: over 30 years, South-Korea: 20 years, Brazil: 17 years, China: 13 years or the USA: since 1995 admitted as a dietary supplement). However, as far as we know, an epidemiologic study has never been done.

References

1. Hutapea, A.M.; Toskulkao, C.; Buddhasukh, D.; Wilairat, P.; Glinsukon, T. *J. Clin. Biochem. Nutr.* **1997**, *23*, 177-186.
2. Geuns, J.M.C.; Buyse, J.; Vankeirsbilck, A.; Temme, E.H.M.; Compernolle, F.; Toppet, S. *J. Agric. Food Chem.* **2006**, *54*, 2794-2798.
3. Geuns, J.M.C.; Buyse, J.; Vankeirsbilck, A.; Temme, E.H.M. *EBM* **2007** (in press).
4. Geuns, J.M.C.; Augustijns, P.; Mols, R.; Buyse, J.G.; Driessen, B. *Food Chem. Toxicol.* **2003**, *41*, 1599-1607.
5. Koyama, E.; Kitazawa, K.; Ohori, Y.; Izawa, O.; Kakegawa, K.; Fujino, A.; Ui, M. *Food Chem. Toxicol.* **2003**, *41*, 359-374.
6. Minne, V.; Compernolle, F.; Toppet, S.; Geuns, J.M.C. *J. Agr. Food Chem.* **2004**, *52*, 2445-2449.
7. Koyama, E.; Sakai, N.; Ohori, Y.; Kitazawa, K.; Izawa, O.; Kakegawa, K.; Fujino, A.; Ui, M. *Food Chem. Toxicol.* **2003**, *41*, 875-883.

8. Srimaroeing, C.; Chatsudthipong, V.; Aslamkhan, A.G.; Pritchard, J.B. *J. Pharmacol. Exp. Ther.* **2005**, *313*, 321-628.

9. Gardana, C.; Simonetti, P.; Canzi, E.; Zanchi, R.; Pietta, P.G. 2003. *J. Agric. Food Chem.* **2003**, *51*, 6618-6622.

10. Simonetti, P.; Gardana, C.; Bramati, L.; Pietta, P.G. *In Proceedings of the first symposium on the Safety of Stevioside;* Geuns, J.M.C.; Buyse, J. Eds.; Euprint Editions ISBN 9074253024, 2004; pp. 51-62.

11. Okumura, M.; Fujita, Y.; Imamura, M.; Aikawa, K. *Shokahin Eiseiga Ku Zasshi* **1978**, *19*, 486-490.

12. Pezzuto, J.M.; Compadre, C.M.; Swanson, S.M.; Nanayakkara, N.P.D.; Kinghorn, A.D. *Proc. Nat. Acad. Sci. USA* **1985**, *82*, 2478-2482.

13. Klongpanichpak, S.; Toskulkao, C.; Temcharoen, P.; Apibal, S.; Glinsukon, T. *J. Med. Assoc. Thailand* **1997**, *80*, 121-128.

14. Matsui, M.; Matsui, K.; Kawasaki, Y.; Oda, Y.; Noguchi, T.; Kitagawa, Y.; Sawada, M.; Hayashi, M.; Nohmi, T.; Yoshihira, K.; Ishidate, M.; Sofuni, T. *Mutagenesis* **1996**, *11*, 573-579.

15 Compadre, C.M.; Hussain, R.A.; Nanayakkara, N.P.D.; Pezzuto, J.M.; Kinghorn, A.D. *Biomed. Environ. Mass Spectrom,* **1988**, *15*, 211-222.

16. Temcharoen, P.; Pimbua, J.; Glinsukon, T.; Rojanapo, W.; Apibal, S. *Bull. Health Sci. & Tech.* **1998**, *1*, 38-45.

17. Terai, T.; Ren, H.; Mori, G.; Yamaguchi, Y.; Hayashi, T. *Chem. Pharm. Bull.* **2002**, *50*, 1007-1010.

18. Matsui, M.; Sofuni, T.; Nohmi, T. *Mutagenesis* **1996**, *11*, 565-572.

19. Procinska, E.; Bridges, B.A.; Hanson, J.R. *Mutagenesis* **1991**, *6*, 165-167.

20. Konishima, T.; Takasaki, M. *Pure Appl. Chem.* **2002**, *74*, 1309-1316.

21. Nakajima, M. Experiment number 5001 (079-085) of the Biosafety Research Center, Foods, Drugs and Pesticides, **2000**, Experimental Toxicology Department, National Institute of Health Sciences, Japan.

22. Nakajima, M. Experiment number 5002 (079-086) of the Biosafety Research Center, Foods, Drugs and Pesticides, **2000**, Experimental Toxicology Department, National Institute of Health Sciences, Japan.

23. Sekihashi, K.; Saitoh, H.; Sasaki, Y.F. *J. Toxicol. Sci* **2002**, *27*, supplement I, 1-8.

24. Sasaki, Y.F.; Sekihashi, K.; Izumiyama, F.; Nishidate, E.; Saga, A.; Ishida, K.; Tsuda, S. *Critical Reviews in Toxicology* **2000**, *30*, 629-799.

25. Yamada, A.; Ohgaki, S.; Noda, T.; Shimizu, M. (1985) *J. of the Food Hygienic Society of Japan* **1985**, *26*, 169-183.

26. Xili, L.; Chengjiany, B.; Eryi, X.; Reiming, S.; Yuengming, W.; Haodong, S.; Zhiyian, H. *Food Chem. Toxic.* **1992**, *30*, 957-965.

27. Toyoda, K.; Matsui, H.; Shoda, T.; Uneyama, C.; Takada, K.; Takahashi, M. *Food Chem. Toxicol.* **1997**, *35*, 597-603.

28. Yodyingyuad, V.; Bunyawong, S. *Human Reproduction* **1991**, *6*, 158-165.

29. Hagiwara, A.; Fukushima, S.; Kitaori, M. *Gann* **1984**, *75*, 763-768.
30. Temcharoen, P.; Suwannatrai, M.; Klongpanichpak, S.; Apibal, S.; Glinsukon, T.; Toskulkao, C. *J. Med. Assoc. Thailand* **2000**, *83*, 101-108.
31. Yasukawa, K.; Kitanaka, S.; Seo, S. *Biol. Pharm. Bull.* **2002**, *25*, 1488-1490.
32. WHO Food Additives **1999**, *42*, 119-143.

Chapter 38

Stevioside: A Safe Sweetener and Possible New Drug for the Treatment of the Metabolic Syndrome

Jan M. C. Geuns

Laboratory Functional Biology, KU Leuven, 3001 Leuven, Belgium

Steviol glycosides used in low amounts for sweetening purposes are safe. Their absorption by the intestines is very low. They are degraded to steviol by bacteria of the colon. Part of this steviol is absorbed and transformed into steviol glucuronide that is excreted in the urine. No accumulation in the body seems to exist. No harmful effects of steviol glycosides have been published in the scientific litterature. ADI values have been suggested by calculations made from published results. High doses of steviol glycosides (750 – 1500 mg/day) may have beneficial pharmacological effects as lowering the blood pressure of hypertensive patients, lowering the blood glucose in diabetes type 2. In animal models, they have anti-carcinogenic effects. It is not proven that they have similar effects in man, as the intake as a sweetener will be very low.

Stevioside and rebaudioside A are the main sweeteners extracted from Stevia rebaudiana (Bertoni) Bertoni, although many other compounds with varying degrees of sweetness are present in the mixture extracted (*1*). As all the sweeteners are degraded to steviol by bacteria of the colon, the mixture of sweeteners should be called steviol glycosides. Stevioside and rebaudioside A taste about 300 times sweeter than sucrose (0.4% solution). Their structure is given in Figure 1. Rebaudioside A has one β-glucose unit more than stevioside.

Stevioside   Rebaudioside A
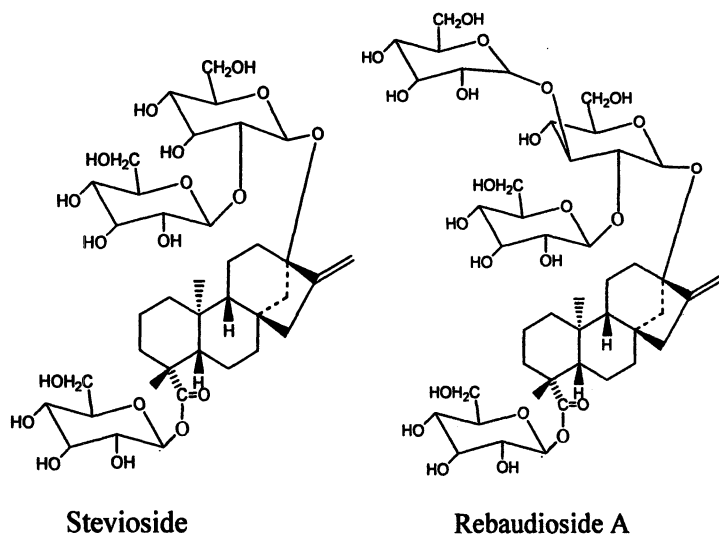
Figure 1. Structures of stevioside and rebaudioside A

The incidence of diabetes type 2, obesitas and hypertension is sharply increasing, due to too much sugar, fat and salt intake and the addition of taste enhancers (eg. glutamates). All this is accompanied by a lack of physical exercise. The yearly costs of these diseases were estimated to be 5 billion euro in Belgium and over 230 billion euro in Europe and the costs are probably about the same in the USA (Geuns, unpublished). This sum includes the money for drugs, for hospitalisation, amputations, eye diseases going to blindness, treatment of heart and blood circulation problems, special diets, dental care, costs of the medical staff and so on. This estimation of the yearly costs does not include social aspects (e.g. inability to work) and human suffering. Stevioside is a good substitute for table sugar. From the beginning, a clear-cut distinction should be made between low doses of steviol glycosides for sweetening purposes, and high doses in which case beneficial pharmacological effects might occur, but that should be administered preferably under medical surveyance.

This chapter will deal with the following points.

- Steviol glycosides used as a sweetener, in LOW doses (maximum 200-300 mg/day).
- Stevioside used in HIGH doses for its pharmacological effects. Here we will discuss effects on blood pressure, diabetes type 2 and anticarcinogenic effects.
- Steviol glucuronide will be suggested as the active principle provoking the pharmacological effects of high doses.

Steviol glycosides as a sweetener

There are several advantages in using *Stevia* or steviol glycosides as a sweetener. It is a completely natural product; steviol glycosides contain no calories; the leaves of *Stevia* can be used in their natural state; thanks to the enormous sweetening power of steviol glycosides, only small quantities need to be used; the plant is non-toxic; the leaves as well as the steviol glycosides can be cooked; they have a little bit a licorice-like aftertaste; they are stable when heated up to 200 °C; they are non fermentative and flavour enhancing, they were clinically tested and used in several countries without negative effect; they are an ideal, non-addictive sweetener for children.

Several toxicological studies have been performed as well as metabolism studies that will shortly be discussed in this chapter, as well as the ADI.

Uptake and metabolism studies

It has been shown that oral stevioside and rebaudioside A were not taken up by the human body or the uptake was extremely low (*2-4*) and none of the digestive enzymes from the gastro-intestinal tract of different animals and man were able to degrade stevioside into steviol, the aglycone of stevioside (*2,5*). Nevertheless, in feeding experiments with rats and hamsters stevioside was metabolized to steviol by the bacterial flora of the caecum. Free steviol was found in the blood of the animals with the maximum concentration occurring after 8 hours (*6,7*). Studies with roosters (*8*) and chickens (laying hens and broilers) (*9*) indicated that stevioside was rapidly eliminated from the body, largely untransformed. Bacteria isolated from the human colon were able to transform stevioside into steviol *in vitro* (*5,7,10*). *In vivo* stevioside degradation to steviol occurred by bacterial action in the colon of pigs (*3*) and humans (*11-13*). Among the selected intestinal groups, bacteroidaceae were the most efficient in hydrolyzing *Stevia* sweeteners to steviol (*10*). Steviol was the only metabolite in the feces (*3, 11-13*). Stevioside or free steviol were not detected in blood plasma, but steviol glucuronide was found in a maximum concentration of 67

µg/mL (21.3 µg steviol equivalents/mL) (*12,13*). In urine, no stevioside or free steviol were present, but steviol glucuronide was found in amounts up to 318 mg/24 h urine (205 mg steviol equivalents/24 h). No other steviol derivatives were detected (*12,13*).

ADI

Many toxicological studies have proven that steviol glycosides are safe (for review see *14*). No effects were found of steviol glycosides or steviol on carcinogenicity, on reproduction/fertility or reproductive organs. There were no teratogenic effects or effects on the embryo, as also evidenced by recent work with chicken embryo's (*15*). Steviol formed in the colon is easily excreted in urine as SV glucuronide. There is no accumulation in the body. No other harmful metabolites are formed (*12,13*). Moreover, many plant gibberellins, a group of plant hormones, are very similar to steviol and also possess a 16-methylene group and 13-hydroxyl function as also found in steviol (Figure 2). This combination is suspected of induction of mutagenic effects of steviol in S. typhimurium TM677 (*1*).
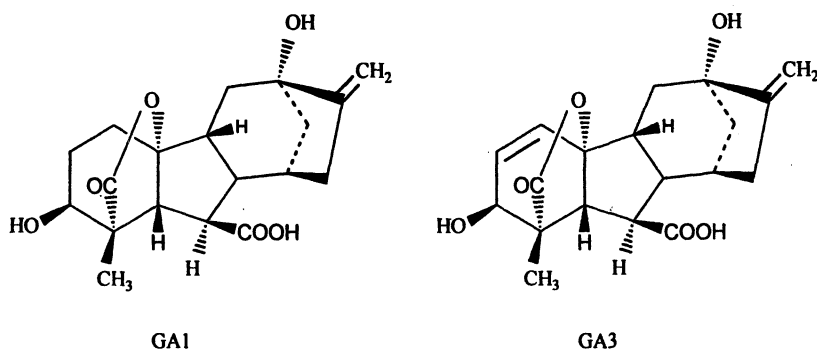
GA1   GA3

Figure 2. Examples of the structures of gibberellines (GA1, GA3) with a 16-methylene group and a 13-OH function.

No effects of steviol glycosides could be found on bio-availability of nutrients from the diet. Stevioside used as a sweetener is safe for diabetics (type 2), phenylketonuria patients (PKU) as well as for *Candida* patients. It is beneficial for persons with hypertension, is not carcinogenic, not cariogenic and no allergenicity problems are known, nor are they expected as the compounds resemble the plant gibberellins that are daily eaten by the whole world population!

There is a safe use in Japan, USA, South Korea, Brazil, Paraguay, Israel and still other countries. We summarised suggested values for the Allowable Daily

**Table I. Suggested ADI (mg/kg BW) and NOAEL (mg/kg BW).
The duration of the experiments is given in months.**

ADI (mg/kg BW)	Organism	NOAEL (mg/kg)	Duration (in months)	Reference
7.938*	Wistar rat	794	24	(16)
25	rat	2500	3	(17)
25	rat	2500	3	(18)
25	rat	2500	3	(19)
25	rat	2500	# generations	(20)
6.25**	hamster	250**		(21)
5.5*	rat F344	550	22♂/ 24♀	(22)
≈ 12.5***	man	≈ 12.5	12	(23)
≈ 25***	man	≈ 25	24	(24)
5 (2mg SVeq)	man			(25)

* The values obtained by the researchers of (16) and (22) should be considered as a minimal ADI as the authors did not test higher concentrations than 794 or 550 mg/kg BW respectively. This highest concentration was without effect.

** This ADI has been calculated for stevioside starting with the NOAEL of steviol (250 mg/kg BW/day) that was fed to the animals and that is easily taken up by the intestines and metabolised to various unknown compounds, whereas steviol glycosides are not taken up (2-4). Moreover, hamsters are known to be very sensitive to steviol, whereas other animals are not (Toskulkao et al., 1997). (Note: the mass of steviol is 40% of that of stevioside).

***The values given for man are calculated using weights obtained from the given BMI and an assumed height of 1.6 m of the Chinese volunteers. Assuming a different height provokes only minor changes in ADI. Anyhow, its value is in the range of the other values given in Table I.

Adapted with permission from reference 14. Copyright 2004 Euprint.

Intake (ADI) and no observable adverse effect levels (NOAEL) as found in the literature or calculated from published data. Only experiments that lasted at least 3 months were taken into consideration (Table I).

In the sixty-third meeting of the Joint Expert Committee for Food Additives of the WHO (JECFA; 8-17 June 2004), suggested a temporary Allowable Daily Intake (ADI) of 0-2 mg/kg BW (expressed as steviol equivalents; safety factor 200)(25). It is expressed this way because all steviol glycosides with different molecular masses are degraded to steviol by bacteria in the colon. This ADI corresponds to 5 mg stevioside or 6 mg rebaudioside A. Possibly, during the next meeting, this temporary ADI might be changed into an ADI of 0-4 mg steviol equivalents, i.e. 10 mg stevioside/kg BW. In that case, the ADI is still below the one calculated from the results with volunteers (23,24) or those obtained from experiments with animals (Table I).

Risk assessment

In many countries, the sugar consumption is around 130 g/d. To substitute for all the added sugar (worst case), about 430 mg stevioside is required, stevioside being about 300 times sweeter than sugar. However, in practice less will be used; e.g. to prepare jam, normally 1 kg sugar is added to 1 kg of fruits and 0.8 g stevioside is sufficient to be added to 1 kg of fruits instead of 3.3 g! It is difficult to substitute for all the added sugar. Furthermore, other sweeteners are already on the market. Therefore, the expected intake might be less than 100 mg/day. For a person of 60 kg, the temporary ADI allows 5 * 60 kg = 300 mg/d. The final ADI will allow more: ±10 * 60 = 600 mg/day. Moreover, in our metabolism studies, up to 750 mg stevioside was daily administered and no free steviol or its metabolites were detected in the blood or urine, meaning that the possible steviol concentration was extremely low and therefore without possible harmful effects.

Pharmacological Effects

High doses of stevioside (750 – 1500 mg/day) were shown to have several pharmacological effects. They lower blood pressure of hypertensive persons. In diabetes type 2 several effects have been detected, i.e. lowering of blood glucose levels, increase of insulin secretion, enhancing the sensitivity to insulin. Moreover, the stevioside effects are glucose-concentration dependent. In this chapter, also possible anticarcinogenic effects of stevioside will be described.

Lowering of blood pressure

Both stevioside and aqueous extracts of Stevia induce direct blood pressure reduction in hypertensive rats (26) and anaesthetized dogs (27). The effects of stevioside, when administered intravenously, were direct and dose-dependent. With 50 mg stevioside/kg, small but significant reductions of arterial blood pressure were obtained after less than 10 minutes in hypertensive rats. The maximal reduction of blood pressure (around 30%) was obtained with the highest dose of 200 mg/kg (26). Oral administration was less effective and slower than intravenous administration. A very high dose (200 mg/kg) by nasogastric administration could reduce blood pressure by 9% after 60 min in healthy mongrel dogs (27). The hypotensive effects of oral stevioside were confirmed in double blind, placebo controlled studies in Chinese hypertensive men and women taking 750 mg (23) or 1500 mg (24) of stevioside a day for one (23) or two years (24). In both studies, the systolic and diastolic blood pressure

of the stevioside group was significantly lower (about 7%). The blood pressure lowering effect persisted throughout the whole study (Figure 3) (23,24).
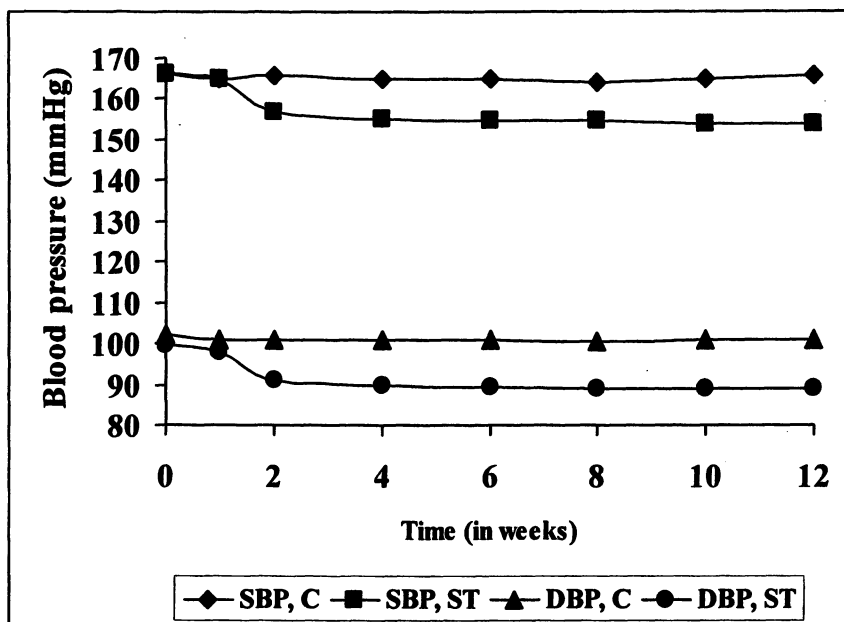
Figure 3. Lowering of systolic and diastolic blood pressure by750 mg oral stevioside(0 and 1 weeks are baseline and placebo values respectively). Adapted with permission from reference 23. Copyright 2000 Blackwell Science Ltd.

In a study of stevioside metabolism, around 10-15 mg/kg BW were administered orally to volunteers with normal blood pressures (114/74 mm Hg). No effects on blood pressure were detected (13). No effects were found on systolic or diastolic blood pressure of 3 doses of stevioside (3.75, 7.5 and 15 mg/kg BW) administered during 7, 11 and 6 weeks respectively to slightly hypertensive volunteers (140/94) (28). These results suggest that stevioside up to 15 mg/kg BW has no effects on persons with normal blood pressures. JECFA still requires double-blind placebo experiments with volunteers. Both a low and high dose (eg. 250 and 750 mg/d) have to be tested in normotensive and hypertensive volunteers (25). ·

One of the possible mechanisms proposed for the hypotensive effects of stevioside is the direct action of stevioside or its metabolites on renal function. Intravenously administered stevioside (16 mg/kg) in rats increased water, sodium and potassium excretion (29). This suggested a vasodilating effect on the kidney,

which could eventually result in blood pressure reduction. It is not known if these effects would occur after oral administration, as the uptake of stevioside is extremely low (2-4). It is further proposed (29,30) that the vasodilating action of stevioside depends on the blocking of calcium channels similar to the action of verapamil. By this mechanism, calcium influx of the smooth muscle cells is inhibited, resulting in vasodilating effects (27).

Effects on blood glucose levels

Diabetes is a chronic disease resulting from insufficient production of, or insensitivity to insulin. Then the cells of the body cannot absorb glucose from the blood resulting in elevated glucose levels.

Type 1 diabetes or insulin-dependent diabetes mellitus (IDDM) usually occurs before the age of 25 and the patient can quickly become very ill. The immune system destroys the insulin-producing β-cells in the pancreas. Thereafter, the pancreas produces little or no insulin, so nutrients cannot reach cells. The blood glucose levels are too high and the patient becomes insulin-dependent (injections).

Diabetes type 2 or non insulin-dependent diabetes mellitus (NIDDM) is the most common form of diabetes that accounts for over 90% of diagnosed diabetes. In the past, patients used to be older than 45, had overweight, hypertension and lack of physical activity. In early stages, there is often a lack of symptoms. Sometimes it is detected during routine blood screening. Patients often become insulin resistant. It can be cured by diet, weight loss, physical exercise, oral medication, and sometimes insulin is required.

During pregnancy women may develop gestational diabetes as the growing placenta starts producing hormones that prevent the insulin from working normally (in 2-5% of pregnacies). In most cases this form disappears after the baby is born.

In many countries, the occurrence of diabetes (mainly type 2) is between 5 and 10 % of the population, and, additionally, the occurrence of impaired glucose tolerance (IGT) is also between 5 and 10%. In this case, blood sugar levels are higher than normal, but not high enough to be diagnosed as diabetic (pre-diabetic state). Exceptional high percentages of diabetes are found in the population of Pima Indians (86 and 69% of women and men respectively in the group of 55-64 year old, 31) and Nauru (35% diabetics and 15% IGT). The current problem is that due to imbalanced food intake and lack of physical exercise, type 2 diabetes is occurring at very young age (from 12 years on!).

In vitro studies with incubated mouse pancreatic islets have indicated that anti-hyperglycemic effects of stevioside and steviol result from the stimulation of insulin secretion via direct action of these compounds on β-cells and the β-cell line INS-1 (Figure 4) (32). Increasing the glucose concentration from 3.3 mM to
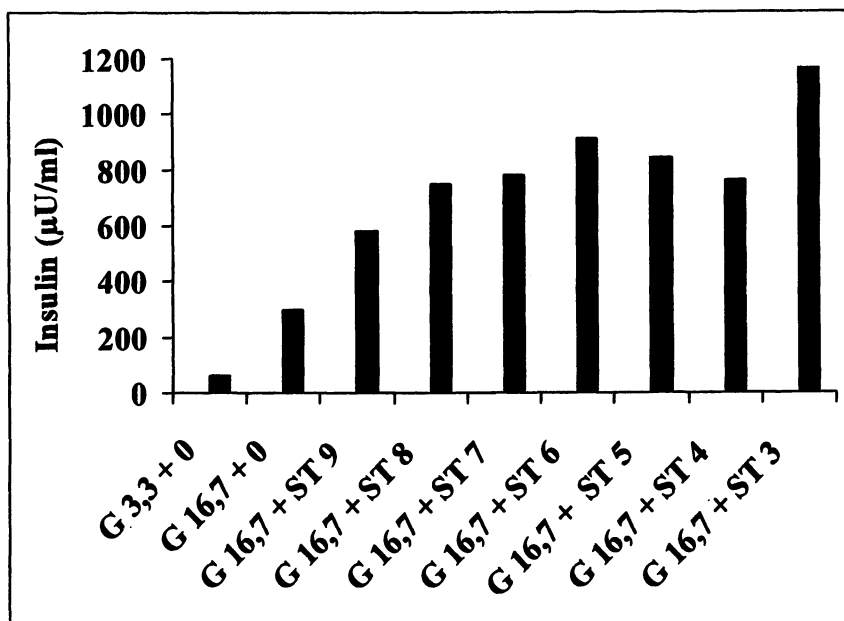
Figure 4. Insulin secretion in function of stevioside concentration by incubated mouse pancreatic islets (G: glucose in mM; ST: stevioside as – log mol/l). Adapted with permission from reference 32. Copyright 2000 WB Saunders Company

16.7 mM stimulates the release of insulin. Stevioside between 1 nM and 1 mM significantly stimulated the insulin release (Figure 4). Also in isolated rat pancreatic islets, stevioside stimulated insulin release in the presence of 7 mM D-glucose in a concentration dependent way (between 0.1 and 1 mM stevioside)(*33*).

It was also shown that the insulin release was dependent upon the glucose concentration (Figure 5)(*32*). Basal glucose levels (3.3 mM) had no effect on insulin release, whereas higher glucose levels between 8.3 and 16.7 mM significantly increased insulin release in the controls. The addition of 1 mM stevioside still increased the insulin release in a glucose dependent manner. The maximum release was obtained with 16.7 mM glucose. Pretreatment of isolated mouse islets with stevioside did not stimulate the basal insulin release and did not desensitize β-cells as does the sulphonylurea glibenclamide (*34*). Moreover, a 24 h stevioside pretreatment significantly increased the insulin content of mouse islets, while glibenclamide decreased it (*34*). Long-term human administration studies did not reveal effects of stevioside on fasting glucose concentrations in hypertensive volunteers with normal glucose levels (*23,24*),

nor in Wistar rats treated with 5.5 mg stevioside/kg BW (35). However, an unknown fraction of crude Stevia extracts at 20 mg/kg BW did reduce glycemia (34). These results are in agreement with the above observation that the insulin release is glucose dependent.
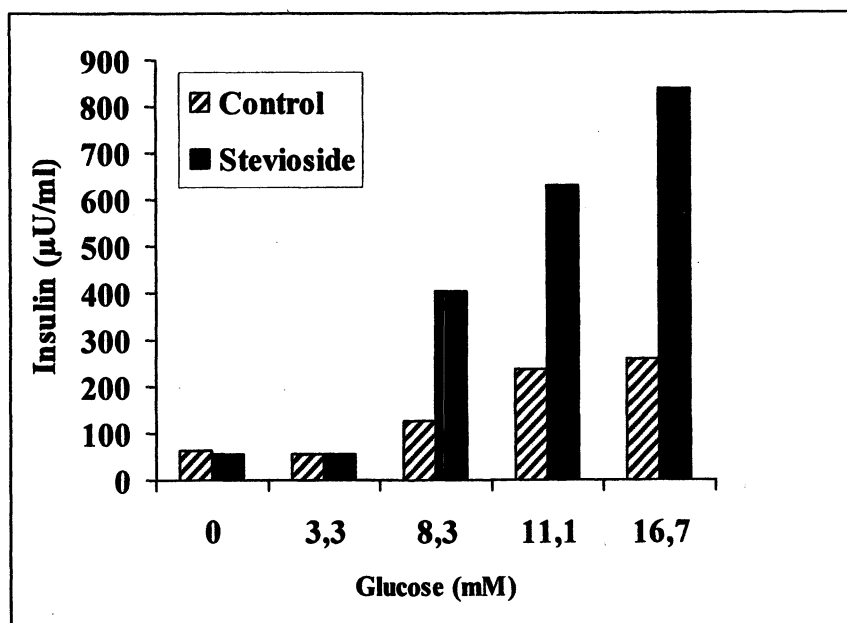
Figure 5. Insulin release by incubated mouse pancreatic islets in function of the glucose concentration. Adapted with permission from reference 32. Copyright 2000 WB Saunders Company.

The anti-hyperglycemic effect of stevioside was especially observed after a glucose load as has been observed in diabetic Goto-Kakizaki rats (36) and streptozotocin (STZ) or fructose-induced diabetic male Wistar rats (37,38), as well as in human experiments (39,40) (Figure 6).

It was shown that stevioside increased whole-body insulin sensitivity, and low concentrations (0.01 – 0.1 mM) modestly improved *in vitro* insulin action on skeletal muscle glucose transport in both lean and obese Zucker rats, indicating a potential site of action of stevioside in the skeletal muscle glucose transport system (41, 42).

A glucose tolerance test in lean Zucker rats revealed that the insulin release was decreased in rats that received 500 mg/kg BW stevioside 2 hours before the test (Figure 7A). However, the glucose level was similar as in the controls
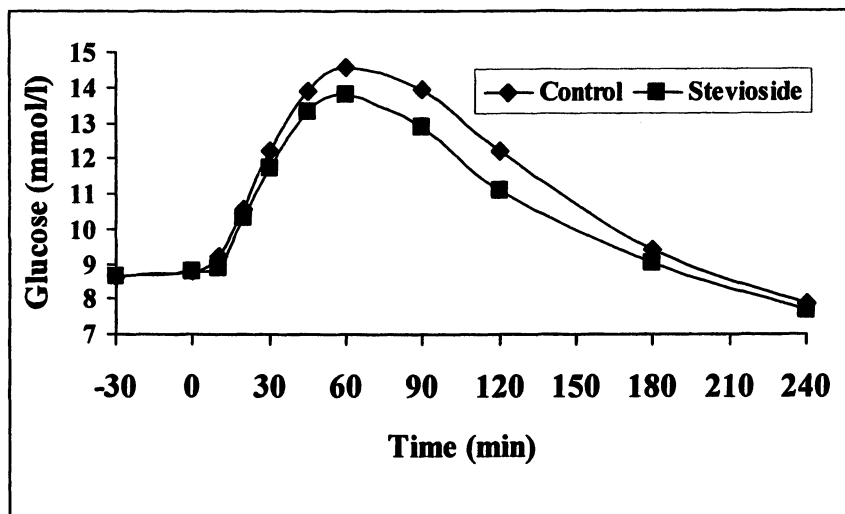
Figure 6. Decrease of blood glucose by stevioside in a glucose tolerance test with volunteers (diabetes type 2). Adapted with permission from reference 39. Copyright 2004 Elsevier Inc.

(Figure 7B), demonstrating that less insulin was more effective meaning that the insulin sensitivity had increased. This is also evidenced in obese stevioside treated Zucker rats in which both insulin and glucose levels were significantly lower, proving that the insulin sensitivity had increased, as was also shown by a halved glucose-insulin index, which is inversely correlated with insulin sensitivity (Figure 8).

In STZ-induced diabetic Wistar rats stevioside enhanced insulin secretion as well as insulin sensitivity due to a decreased phosphoenol pyruvate carboxykinase gene expression in the liver slowing down gluconeogenesis (*38*).

Stevioside decreased the release of glucagon in the α-cell line TC1-6 that had been exposed to 0.5 mM palmitate (*43*). Incubation of the cells in 0.5 mM palmitate significantly enhanced glucagon release. Stevioside dose-dependently reduced the glucagon secretion between 10^{-8} and 10^{-6} M (Figure 9).

The results of this chapter indicate that high doses of stevioside lower blood glucose levels and the effect is glucose dependent. The use of stevioside does not seem to lead to the induction of hypoglycemia accompanying the use of drugs to lower blood glucose levels. Stevioside acts by increasing the insulin release as well as the insulin sensitivity. Moreover, stevioside decreases .the glucagon release.

To change the temporary ADI into a full ADI, JECFA requires additional double-blind placebo experiments with volunteers (Type 1 and 2 diabetes) (*25*).
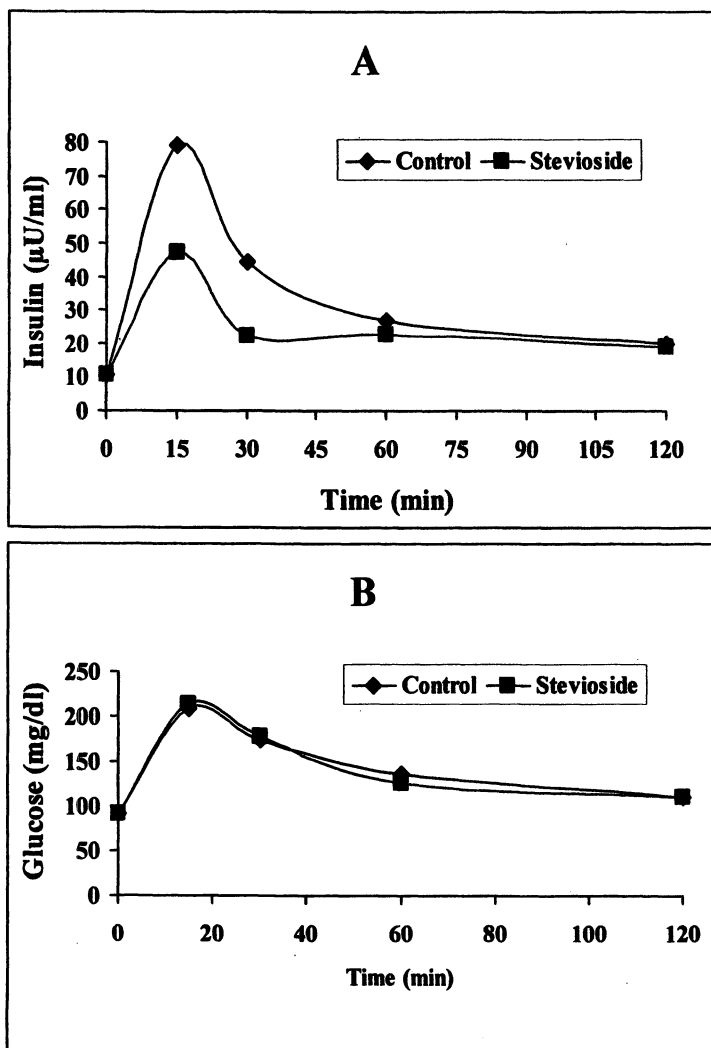
*Figure 7. Glucose tolerance test in lean Zucker rats. A: insulin release,
B: glucose levels. Adapted with permission from reference 41.
Copyright 2004 Elsevier Inc.*
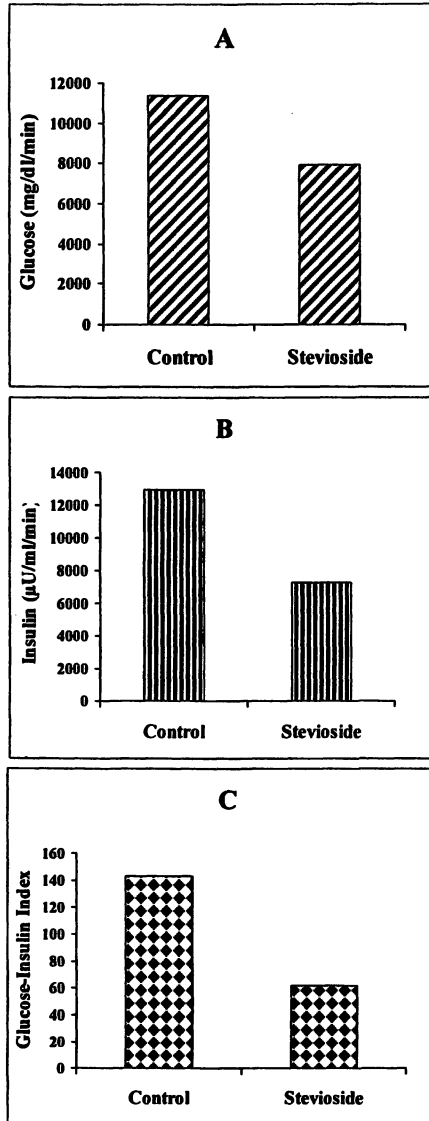
*Figure 8. Effect of stevioside on glucose level and insulin secretion in an oral
GTT in obese Zucker rats. A: IAUC glucose, mg/dL/min; B: IAUC insulin,
μU/mL/min; C: IAUC glucose-insulin index, that is inversely correlated with
insulin sensitivity. Adapted with permission from reference 41. Copyright 2004
Elsevier Inc.*
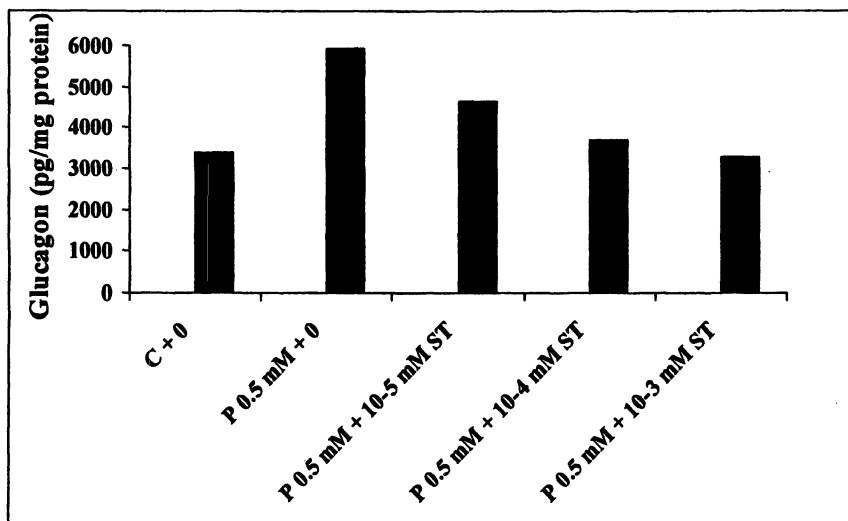
Figure 9. Glucagon secretion from α-TC1-6 cells exposed to 0.5 mM palmitate (P) in the presence of stevioside (ST; 10^{-5} tot 10^{-3} mM). Adapted with permission from reference 43. Copyright 2006 The American Physiological Society.

Both a low and high dose should be studied (e.g. 250 and 750 mg/d). Whether stevioside affects blood glucose levels in healthy volunteers needs to be investigated in further experiments looking at post-prandial effects.

Anticarcinogenic activity

In a chronic study, 95.6 % pure stevioside was administered to 4 week old F344/DuCrj rats (*44*). The daily doses were 0, 2.5 and 5 % of the diet. These doses corresponded to about 969 and 1120 mg/kg BW for males and females respectively (2.5% diet) and 1997 and 2387 mg/kg BW for males and females respectively (5% diet group). The treatment period lasted for 104 weeks. In male animals, the number of testicular tumours had the tendency to decrease. Moreover, the incidence of adenomas of the mammary gland was significantly less in the stevioside-treated female rats than in the controls. The severity of chronic nephropathy in males was also clearly reduced by both stevioside concentrations.

In a two-stage carcinogenesis experiment in mice skin for 20 weeks, tumor formation was initiated by a single topical application of 50 μg 7,12-dimethyl-benz[*a*]anthracene (DMBA) (female ICR mice, 7 weeks old). One week after the

initiation, promotion was started twice weekly by the application of 1 µg 12-O-tetradecanoylphorbol-13-acetate (TPA). When steviol glycosides (89% purity, containing stevioside (48.9%), rebaudioside A 24.4%), rebaudioside C (9.8 %) and dulcoside A (5.6%)) were applied topically 30 min before the TPA, in amounts of 0.1 or 1 mg, the number of animals developing tumors (A) and also the number of tumors per animal (B) were significantly reduced (Figure 10) (45). In a similar two-stage carcinogenesis experiment in mice skin (specific pathogen-free female ICR, 6 weeks old), papillomas were initiated with 100 µg DMBA. One week after initiation, mice were promoted by the topical application of TPA (1 µg, 1.7 nmol) twice a week. Topical application of stevioside (85 nmol) 1 h before each promotion, delayed the formation and reduced the number of papillomas over a 15 week period (46). These authors also demonstrated that oral stevioside (2.5 mg/100 ml drinking water) for only 2 weeks (one week before and one week after initiation) also reduced mouse skin carcinogenesis initiated by peroxynitrite (33.1 µg, 390 nmol) and induced by TPA (1 µg) in female SENCAR mice (6 weeks old).

Steviol glucuronide: the active principle in pharmacological effects?

Stevioside and rebaudioside A induced an increased release of insulin in isolated pancreatic isles of mouse (32,47) and rats (33). However, *in vivo* these pharmacological effects were only observed with stevioside in diabetic subjects (39), nut not with rebaudioside A in type 2 diabetic Goto-Kakizaki rats (48). It has been shown that the metabolism of rebaudioside A by the bacteria of the colon is much slower than that of stevioside (10). Moreover, in metabolism studies with volunteers, no free stevioside or steviol could be detected in the blood plasma. However, steviol glucuronide was present in concentrations up to 67 µM (12,13). These findings suggest that steviol glucuronide is the active principle in provoking the pharmacological effects of high doses of stevioside that is easily degraded whereas the degradation of rebaudioside A and hence the uptake of steviol is much slower. More research about this is still required.

References

1. Geuns, J.M.C. *Phytochemistry* **2003**, *64*, 913-921.
2. Koyama, E.; Sakai, N.; Ohori, Y.; Kitazawa, K.; Izawa, O.; Kakegawa, K.; Fujino, A.; Ui, M. *Food Chem. Toxicol.* **2003**, *41*, 875-883.
3. Geuns, J.M.C.; Augustijns, P.; Mols, R.; Buyse, J.G.; Driessen, B. *Food Chem. Toxicol.* **2003**, *41*, 1599-1607.
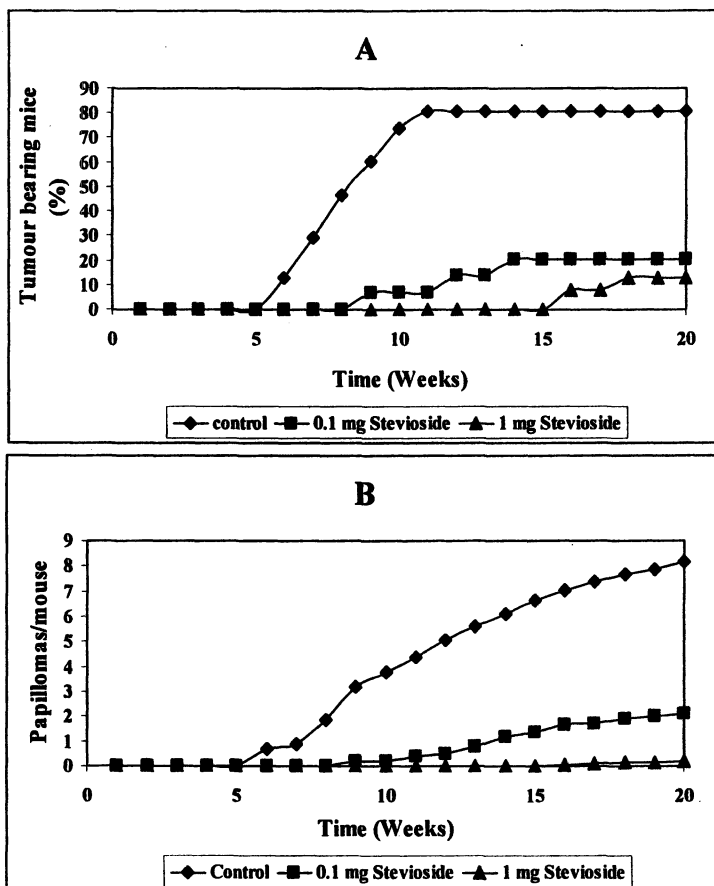
*Figure 10. Inhibitory effect of steviol glycosides on the promotion of skin
papillomas by TPA in DMBA-initiated mice. A: Percentage of tumour bearing
mice; B: Number of papillomas per mouse. Treatments: Control: TPA + vehicle;
0.1 mg stevioside + TPA; 1 mg stevioside + TPA.). Adapted with permission
from reference 45. Copyright 2004 Pharmaceutical Society of Japan.*

4. Srimaroeing, C.; Chatsudthipong, V.; Aslamkhan, A.G.; Pritchard, J.B. *J. Pharmocol. Exp. Ther.* **2005**, *313*, 321-628.

5. Hutapea, A.M.; Toskulkao, C.; Buddhasukh, D.; Wilairat, P.; Glinsukon, T. *J. Clin. Biochem. Nutr.* **1997**, *23*, 177-186.

6. Nakayama, K.; Kasahara, D.; Yamamoto, F. *J. Food Hygienic Society of Japan* **1986**, *27*, 1-8.

7. Koyama, E.; Kitazawa, K.; Ohori, Y.; Izawa, O.; Kakegawa, K.; Fujino, A.; Ui, M. *Food Chem. Toxicol.* **2003**, *41*, 359-374.

8. Pomaret, M.; Lavieille, R. *Bull. Soc. Chim. biol.* **1931**, *13*, 1248-1252.

9. Geuns, J.M.C.; Malheiros, R.D.; Moraes, V.M.B.; Decuypere, E.M.-P.; Compernolle, F.; Buyse, J.G. *J. Agric. Food Chem.* **2003**, *51*, 1095-1101.

10. Gardana, C.; Simonetti, P.; Canzi, E.; Zanchi, R.; Pietta, P.G. 2003. *J. Agric. Food Chem.* **2003**, *51*, 6618-6622.

11. Simonetti, P.; Gardana, C.; Bramati, L.; Pietta, P.G. In *Proceedings of the first symposium on the Safety of Stevioside;* Geuns, J.M.C.; Buyse, J. Eds.; Euprint Editions ISBN 9074253024, 2004; pp. 51-62.

12. Geuns, J.M.C.; Buyse, J.; Vankeirsbilck, A.; Temme, E.H.M.; Compernolle, F.; Toppet, S. *J. Agric. Food Chem.* **2006**, *54*, 2794-2798.

13. Geuns, J.M.C.; Buyse, J.; Vankeirsbilck, A.; Temme, E.H.M. *EBM* **2007** (in press).

14. Geuns J.M.C. In *Proceedings of the first symposium on the Safety of Stevioside;* Geuns, J.M.C.; Buyse, J. Eds.; Euprint Editions ISBN 9074253024, 2004; pp. 85-127.

15. Geuns J.M.C., Bruggeman V., and Buyse J.G. *J. Agric. Food Chem.* **2003**, *51*, 5162-5167.

16. Xili, L.; Chengjiany, B.; Eryi, X.; Reiming, S.; Yuengming, W.; Haodong, S.; Zhiyian, H. *Food Chem. Toxic.* **1992**, *30*, 957-965.

17. Akashi, H.; Yokoyama, Y. *Toxicological Tests. Food Industry* **1975**, *18*, 34-43.

18. Mitsuhashi H. Safety of stevioside. In Tama Biochemical Co. Ltd. Report on Safety of Stevia. pp. 1-20 (1981).

19. Aze, Y.; Toyoda, K.; Imaida, K.; Hayashi, S.; Imazawa, T.; Hayashi Y.; Takahashi, M. *Bull. Nat. Inst. Hyg. Sc.* **1991**, *109*, 48-54.

20. Yodyingyuad, V.; Bunyawong, S. *Human Reproduction* **1991**, *6*, 158-165.

21. Wasuntarawat, C.; Emcharoen, P.; Toskulkao, C.; Mungkornkarn, P.; Suttajit, M.; Glinsukon, T. *Drug Chem. Toxicol.* **1998**, *21*, 207-222.

22. Yamada, A.; Ohgaki, S.; Noda, T.; Shimizu, M. (1985) *J. of the Food Hygienic Society of Japan* **1985**, *26*, 169-183.

23. Chan, P.; Tomlinson, B.; Chen, Y.-J.; Liu, J.-C.; Hsieh, M.-H.; Cheng, J.-T. *Br. J. Clin. Pharmacol.* **2000**, *50*, 215-220.

24. Hsieh, M.H.; Chan, P.; Sue, Y.M. *Clin. Ther.* **2003**, *25*, 2797-808.

25. WHO Food Additives **1999**, *42*, 119-143.

26. Chan, P.; Xu, D.Y.; Liu, J.C.; Chen, Y.-J.; Tomlinson, B.; Huang, W.-P.; Cheng, J.-T. *Life Sci.* **1998**, 1679-1684.

27. Liu, J.C.; Kao, P.K.; Chan, P.; Hsu, Y.-H.; Hou, C.-C.; Lien, G.-S.; Hsieh, M.-H.; Chen, Y.-J.; Cheng, J.-T. *Pharmacology* **2003**, *67*, 14-20.

28. Ferri, L.A.F.; Alves-Do-Prado, W.; Yamada, S.S.; Gazola, S.; Batista, M.R.; Bazotte, R.B. *Phytother. Res.* **2006**, (in press).

29. Melis, M.S.; Sainati, A.R. *J. Ethnopharmacol.* **1991**, *33*, 257-262.

30. Melis, M.S. *J. Ethnopharmacol.* **1992**, *36*, 213-217.

31. Knowler, W.C.; Saad, M.F.; Pettitt, D.J.; Nelson, R.G.; Bennett, P.H. *Diabetes Care* **1993**, *16*, 216-227.

32. Jeppesen, P.B.; Gregersen, S.; Poulsen, C.R.; Hermansen, K. *Metabolism* **2000**, *49*, 208-214.

33. Malaisse, W.J.; Vanonderbergen, A.; Louchami, K.; Jijakli, H.; Malaisse-Lagae, F. *Cell. Signal.* **1998**, *10*, 727-733.

34. Chen, J.; Jeppesen, P.B.; Abudula, R.; Dyrskog, S.E.U.; Colombo, M.; Hermansen, K. *Life Sci.* **2006**, *78*, 1748-1753.

35. Ferreira, E.B.; de Assis Rocha Neves, F.; de Costa, M.A.D.; do Prado, W.A.; de Araújo Funari Ferri, L.; Bazotte, R.B. *Planta Med.* **2006**, *72*, 1-6.

36. Jeppesen, P.B.; Gregersen, S.; Alstrup, K.; Hermansen, K. *Phytomedicine* **2002**, *9*, 9-14.

37. Chang, J.-C.; Wu, M.C.; Liu, I.-M.; Cheng, J.-T. Increase of insulin sensitivity by stevioside in fructose-rich chow-fed rats. *Horm. Metab. Res.* **2005**, *37*, 610-616.

38. Chen, T.-H.; Chen, S.-C.; Chan, P.; Chu, Y.-L.; Yang, H.-Y.; Cheng, J.-T. Mechanism of the hypoglycemic effect of stevioside, a glycoside of Stevia rebaudiana. *Planta Med.* **2005**, *71*, 108-113.

39. Gregersen, S.; Jeppesen, P.B.; Holst, J.J.; Hermansen, K. *Metabolism* **2004**, *53*, 73-76.

40. Curi, R.; Alvarez, M.; Bazotte, R.B.; Botion, L.M.; Godoy, J.L.; Bracht, A. *Brazilian J. Med. Biol. Res.* **1986**, *19*, 771-774.

41. Lailerd, N.; Saengsirisuwan, V.; Sloniger, J.A.; Toskulkao, C.; Henriksen, E.J. *Metabolism* **2004**, *53*, 101-107.

42. Chang J-C, Wu MC, Liu I-M, Cheng J-T. Increase of insulin sensitivity by stevioside in fructose-rich chow-fed rats. *Horm. Metab. Res.* **2005**, *37*, 610-616.

43. Hong, J.; Chen, L.; Jeppesen, P.B.; Nordentoft, I.; Hermansen, K. *Am. J. Physiol. Endocrinol. Metab.* **2006**, *290*, E416-E422.

44. Toyoda, K.; Matsui, H.; Shoda, T.; Uneyama, C.; Takada, K.; Takahashi, M. *Food Chem. Toxicol.* **1997**, *35*, 597-603.

45. Yasukawa, K.; Kitanaka, S.; Seo, S. *Biol. Pharm. Bull.* **2002**, *25*, 1488-1490.

46. Konishima, T.; Takasaki, M. *Pure Appl. Chem.* **2002**, *74*, 1309-1316.
47. Abudula, R.; Jeppesen, P.B., Rolfsen, S.E.D., Xiao, J., Hermansen, K. *Metabolism* **2004**, *53*, 1378-1381.
48. Dyrskog S.E.U.; Jeppesen, P.B.; Chen, J.; Christensen, L.P.; Hermansen, K. *Rev. Diab. Stud.* **2005**, *2*, 84-91.

Author Index

Subject Index

620

*Figure 5.2. Morphology and taste cell specific immunoreactivity of cultured
taste cells and foliate papillae obtained from rat tongue. Rat taste cell cultured
on rat tail collagen type 1 coated plates were imaged after 2 days (A) Individual,
bud-type and cell clusters were observed in short term culture. Double
immunofluorescence labeling indicates cultured taste cells immunoreactive with
BrdU (green; mouse anti-BrdU 1:100, Sigma B-2531) and gustducin (red;
rabbit polyclonal, 1:500, Santa Cruz,) (B); and PLCβ2 (red; rabbit polyclonal,
1:500, Santa Cruz,). Goat anti-mouse Alexa 488 (green; 1:500, Molecular
Probe) and goat anti-rabbit Alexa 633 antibodies (red; 1:500, Molecular Probe)
were used as secondary antibody for BrdU and Gustducin and PLCβ2,
respectively. (C). Labeling with BrdU and a taste cell marker indicates
proliferation and differentiation in vitro. A small number of taste cells were
immunoreactive with NCAM antibody (mouse monoclonal, 1:500, Sigma C9672)
suggesting the presence of type III cells. Goat anti-mouse Alexa 488 (1:500,
Molecular Probe) was used as secondary antibody for NCAM staining
(D). Σχαλε βαρσ = 50 μm (A) and 80 μm (B-D)*

a.

anti-clinal 180°
(tastless)

gauch 60°
(sweet)

eclipsed 180°
(tastless)

b.

c.

Figure 6.1. a) Diol configurations and their sweet taste. b) The putative AH-B interaction with chloroform c) One of several AH-B interactions possible on a monosaccharide.
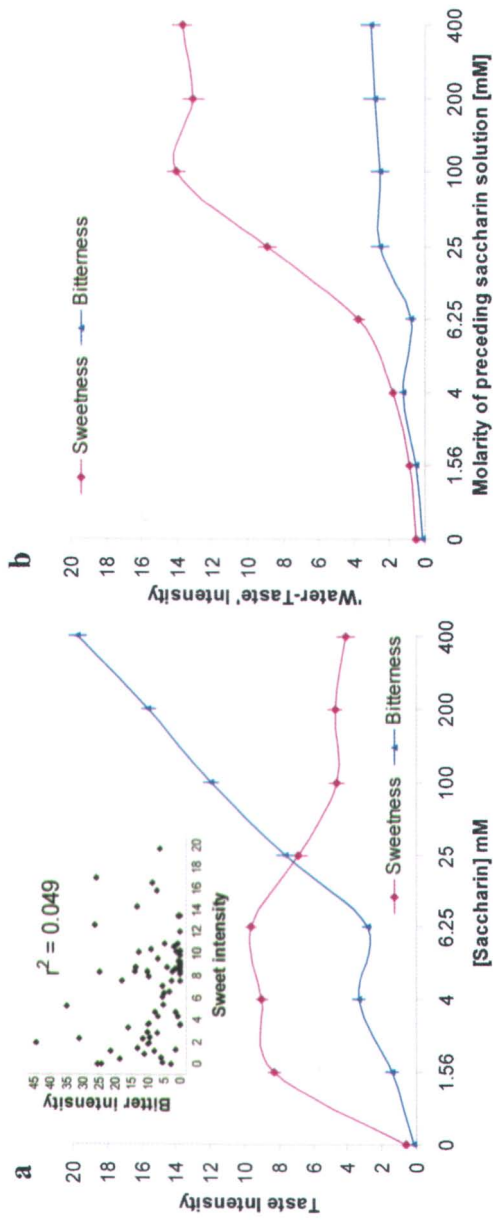
Figure 12.2. .a) Concentration- intensity curve for Na-Saccharin (second tasting), blue line indicates bitterness, pink line sweetness. n=14subjects. Inset shows the correlation of bitterness with sweetness for Na-saccharin. b) Water-taste intensity measured after exposure to different concentrations of Na-Saccharin. Adapted by permission from Macmillan Publishers Ltd: Nature [7], copyright 2006.
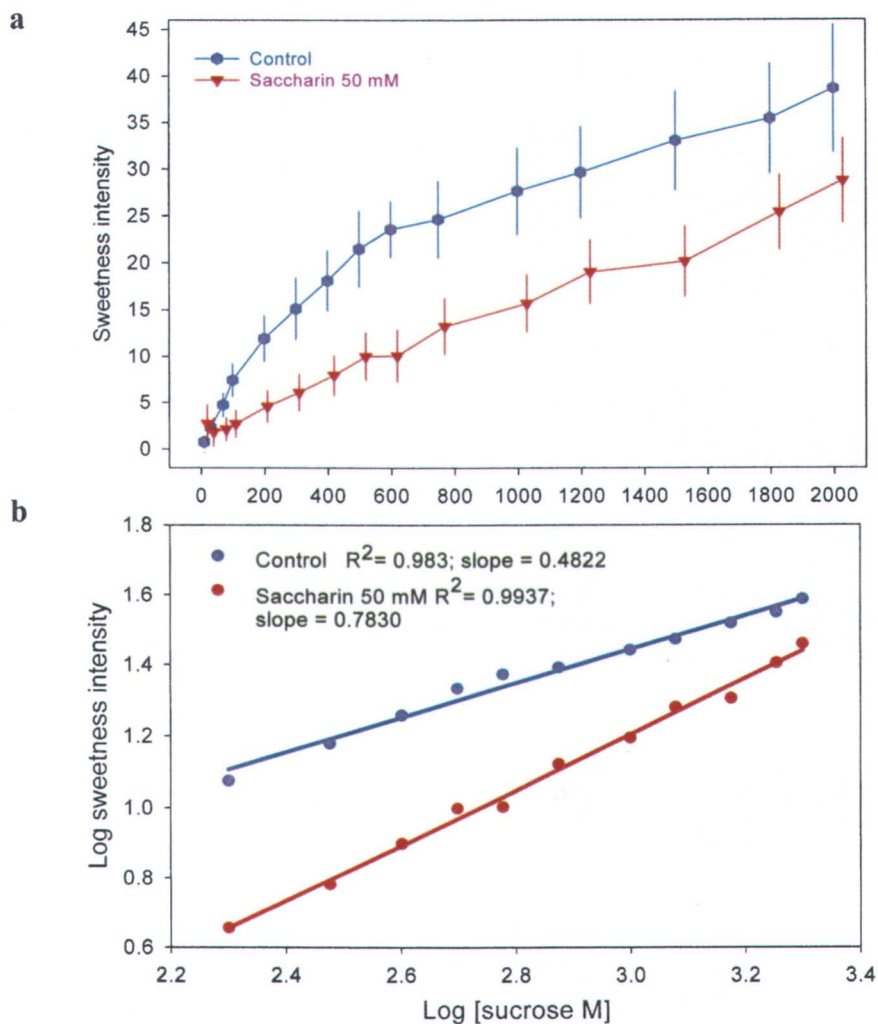
Figure 12.5. Concentration intensity curves of sucrose (control-blue line) and mixtures of sucrose with 50mM Na-Saccharin (burgundy line). a) normal space, intensity measured on a gLMS, b) log-log space, linear regression analysis. Error bars indicate SEM, n=15.
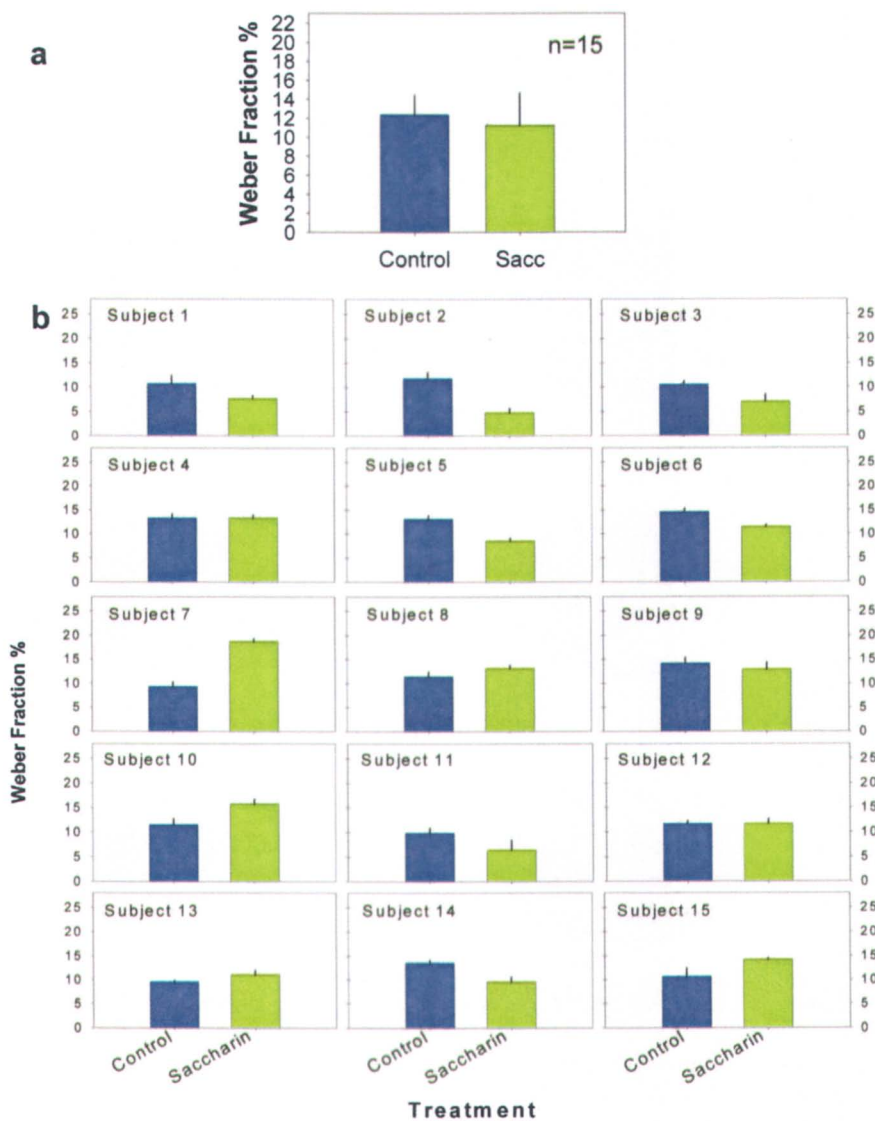
Figure 12.6. a) Weber's fractions for sucrose (blue) and sucrose + Na-saccharin (green). Standard concentration =400 mM sucrose. b) individual Weber's fractions for sucrose and sucrose (blue) + Na-saccharin (green). Error bars indicate SEM for 3 replicates.
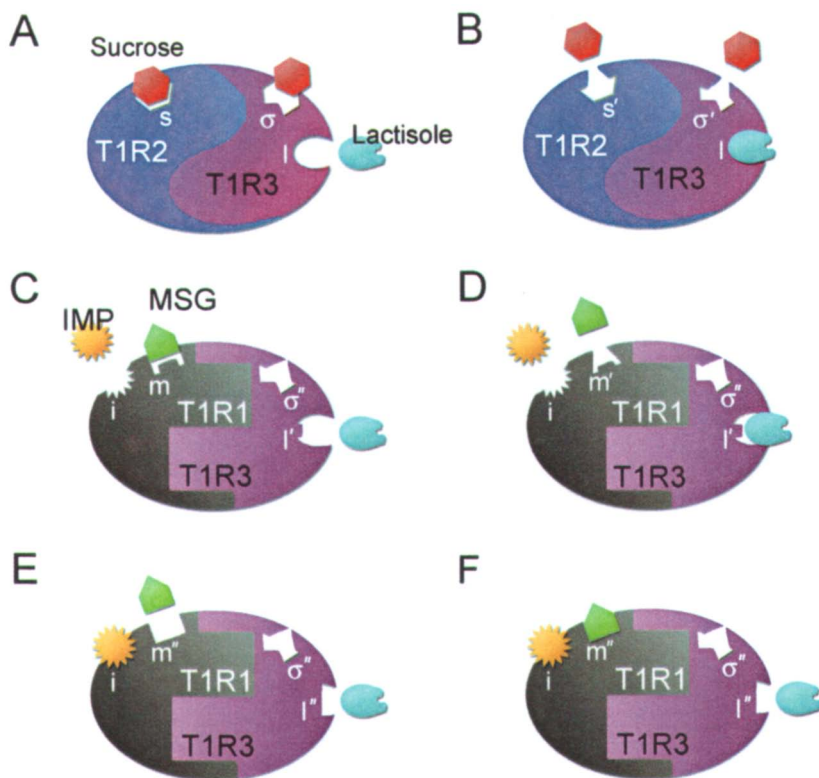
*Figure 12.9. Human sweet (T1R2(blue)-T1R3(purple)) and umami (T1R1(gray)-T1R3(purple)) taste heteromer receptor schematics: Inhibition of sucrose's (red) sweet taste by the compound lactisole (aqua) (**A & B**); inhibition of monosodium glutamate's (MSG) (green) umami taste by lactisole (**C & D**); and modulatory effects of 5'ribonucleotides, such as inosine monophosphate (IMP) (yellow), on MSG binding and IMP's blockade of lactisole's inhibition (**E & F**). Reprinted by permission from Oxford University Press: Chemical Senses, [18] copyright 2006.*
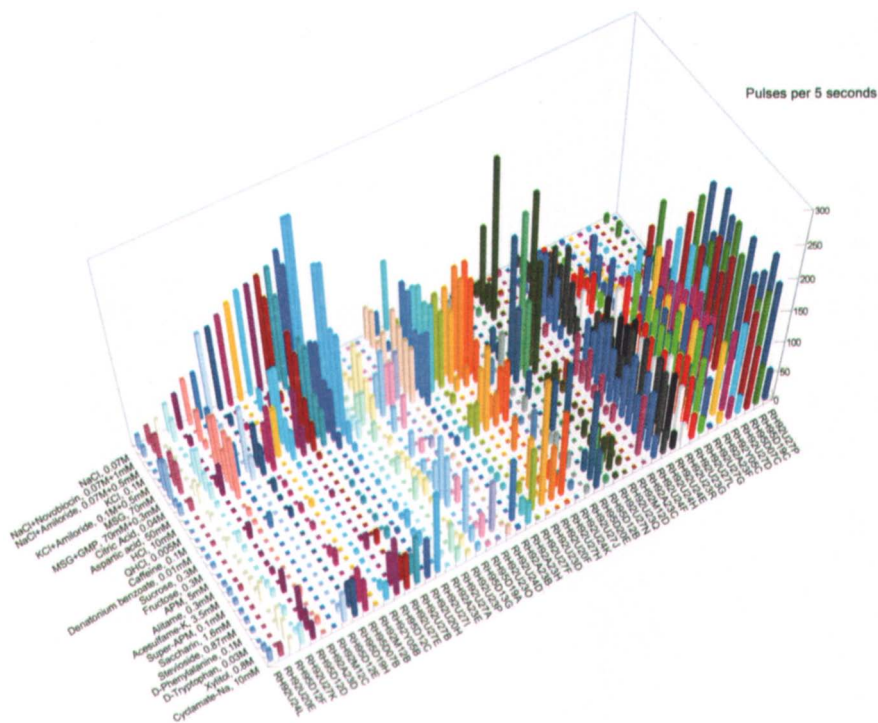
Figure 13.3. Overview of the response profiles of 51 CT single fibers. The stimuli were arranged along the X- axis in order of salt, sour, bitter and sweet. The fibers were arranged along the Y- axis in groups: NaCl, acid, Quinine hydrochloride and sucrose best fibers. MSG denotes monosodium glutamate; GMP, guanosine 5'-monophosphate.
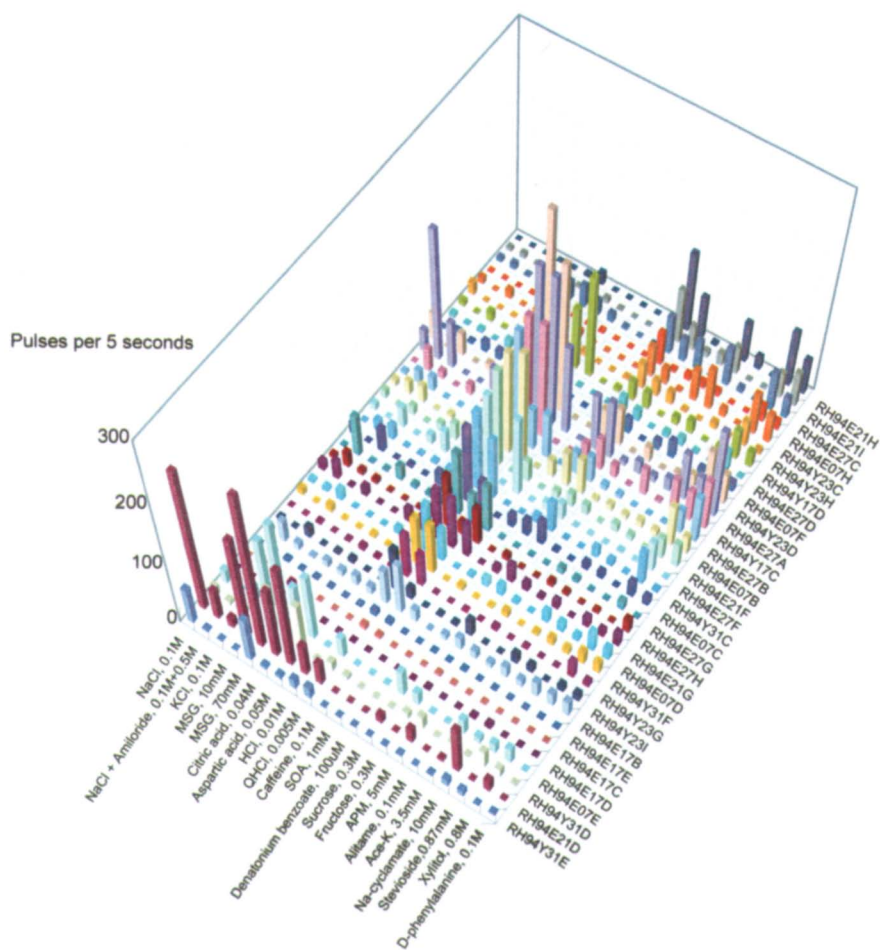
Figure 13.4. Overview of the response profiles of 33 NG single fibers. The stimuli and fibers were arranged as for the CT.
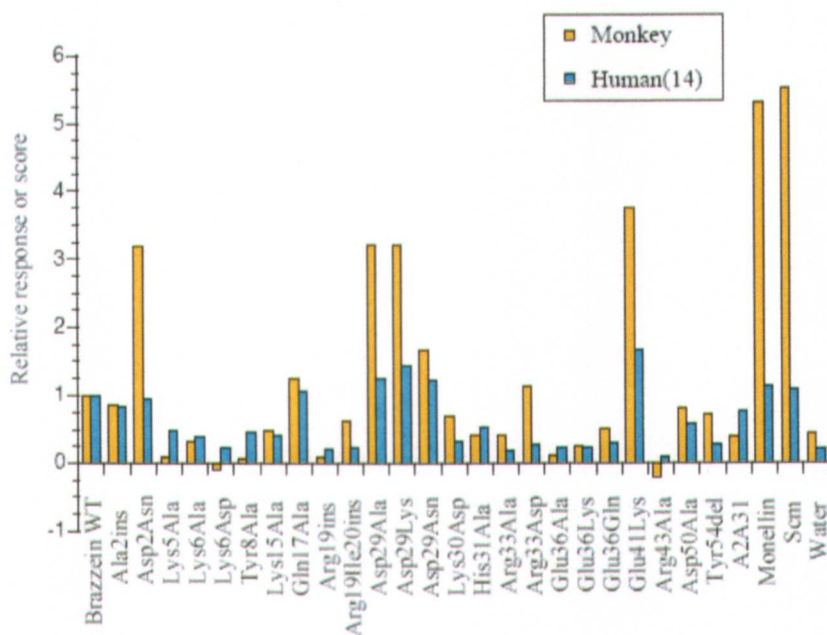
Figure 13.5. Comparison between human psychophysical and monkey electrophysiological results for brazzein, brazzein mutants, monellin, single chain monellin and water.
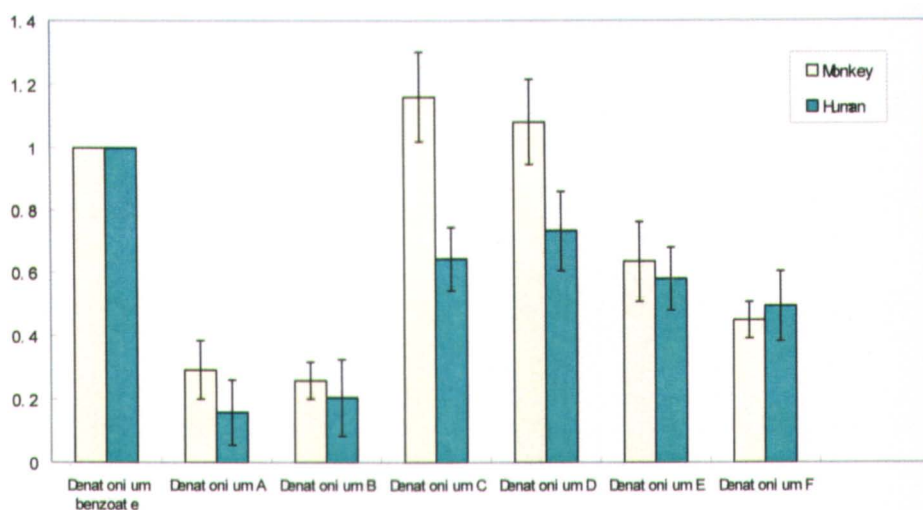
Figure 13.6. Comparison between human psychophysical and monkey electrophysiological results for denatonium benzoate, denatonium benzoate derivates and water.
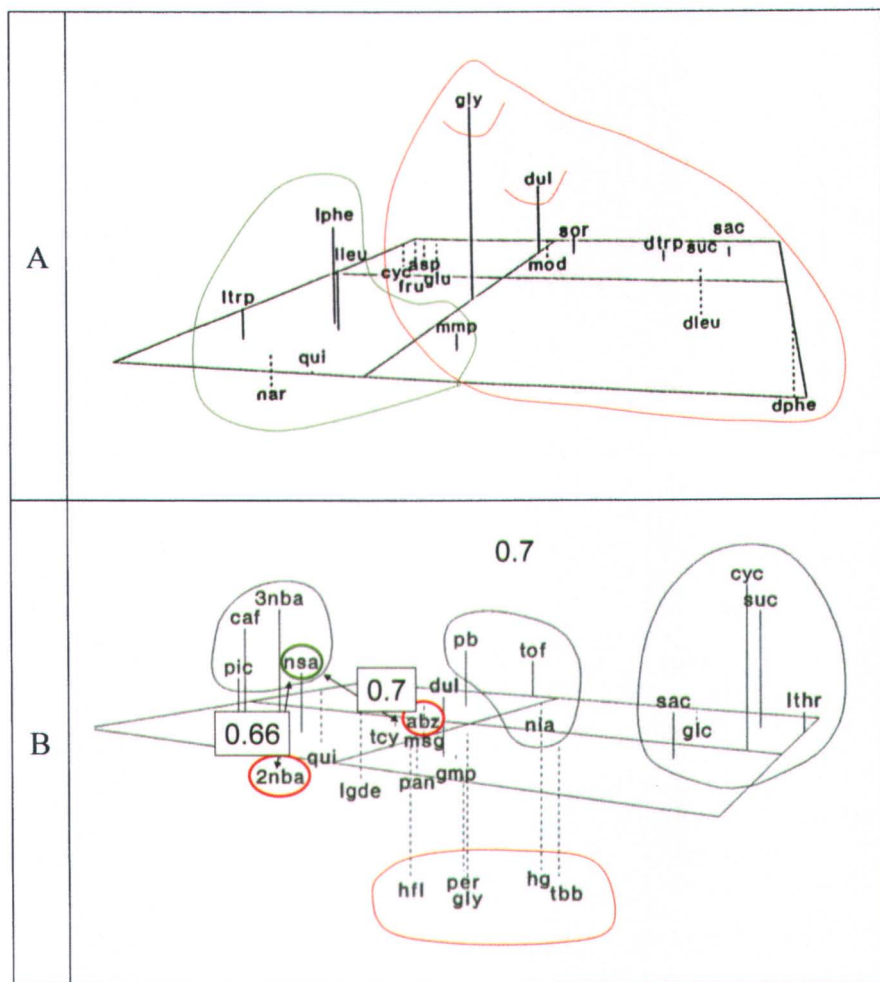
Figure 20.11. Factor Analysis of Correspondences :supra-threshold experiments I (A) and II (B and C, two different views rotating around the 3rd axis). Examples of correlations: r gly-tbb: 0.6, tbb-2nba: 0.7, 2nba-dul: 0.7, dul-per: 0.7; suc-dul: 0.5, suc-per: 0.6, suc tbb: 0.5, suc-2nba: 0.4; sac abz: 0.7, sac-nsa (sweet-bitter): 0.4, nsa-abz (bitter-sweet): 0.8, nsa-2nba (bitter-sweet): 0.66; cyc-caf: 0.13, suc-pic: 0.22. Continued on next page.
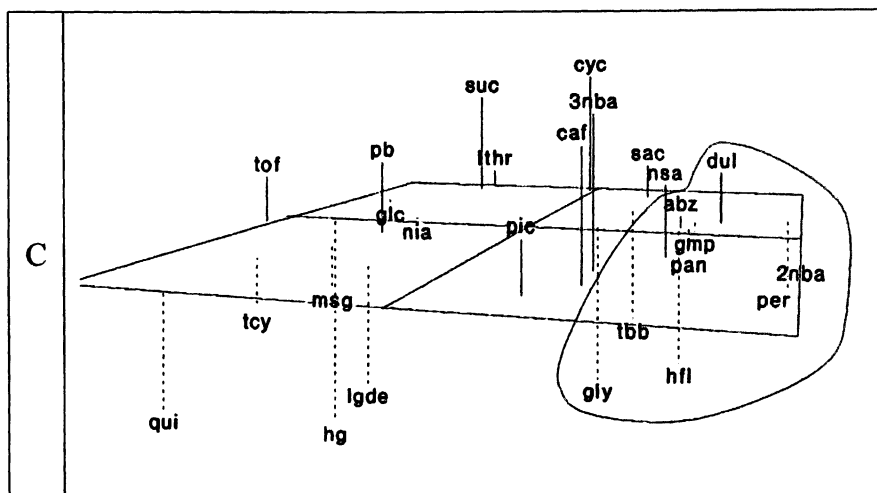
Figure 20.11. Continued.
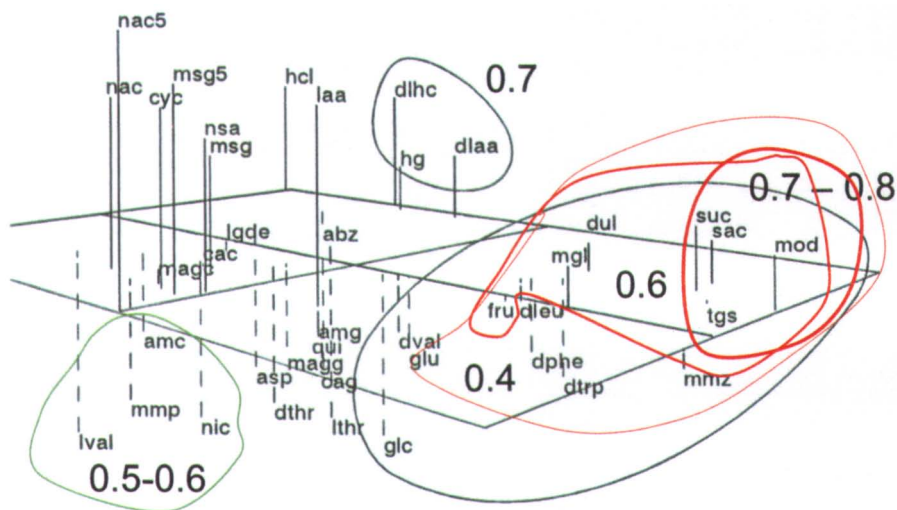
Figure 20.17. Factor analysis of correspondences, experiment I. Figures:
correlation coefficients
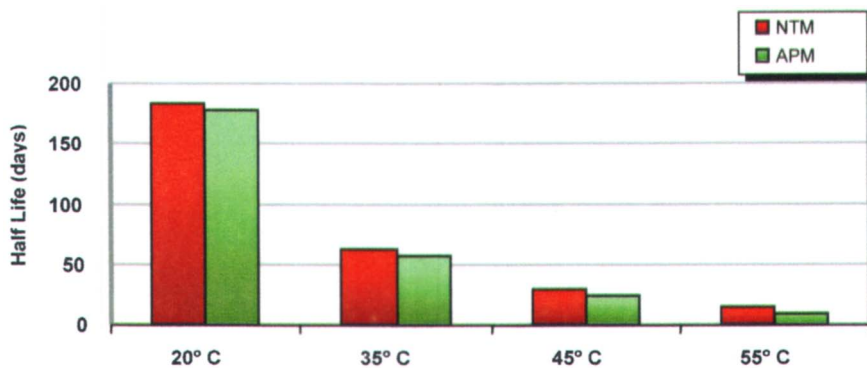
Figure 32.7. Stability of Neotame and Aspartame in Cola (pH 3.1)
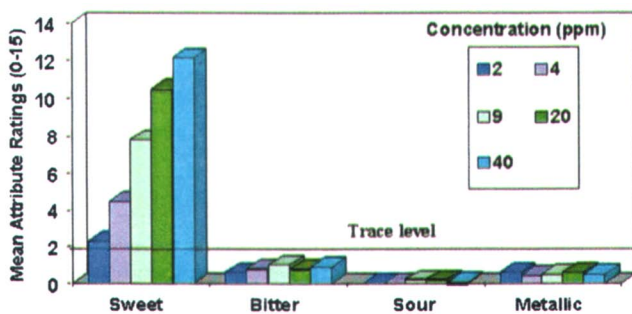
*Figure 32.11. Descriptive taste profile of neotame at various concentrations
in water*
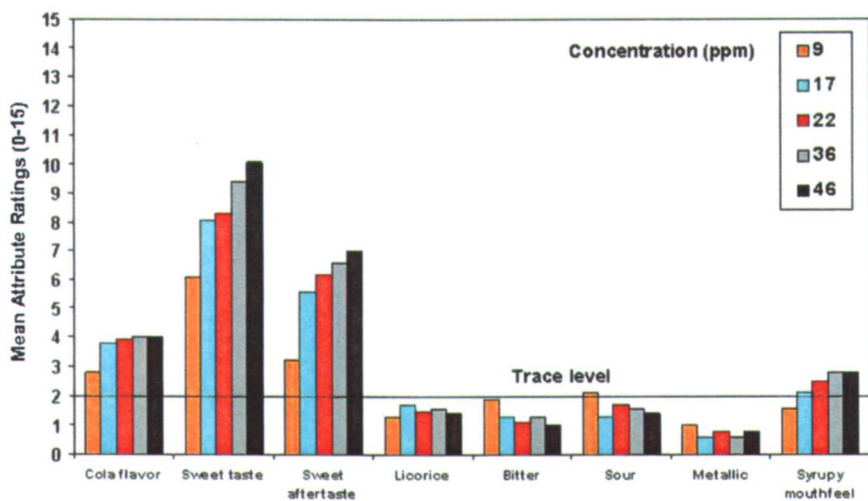
Figure 32.12. Taste profile of neotame at various concentrations in cola
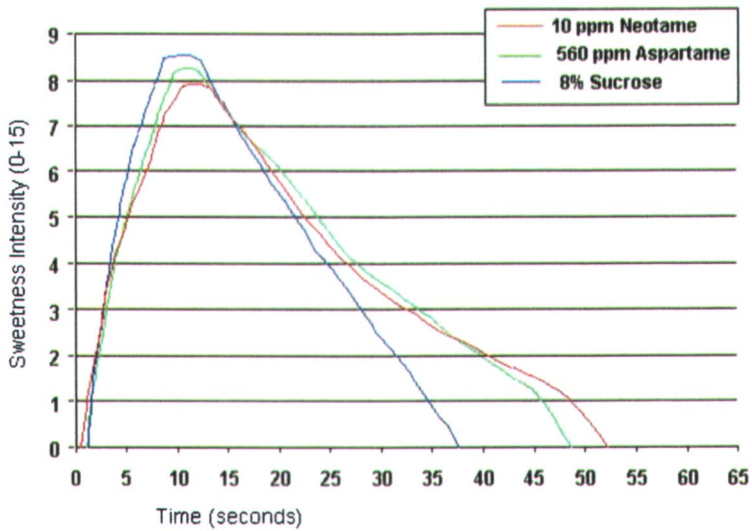
*Figure 32.13. Comparative temporal profile of neotame vs sucrose and
aspartame at isosweet concentrations in water*
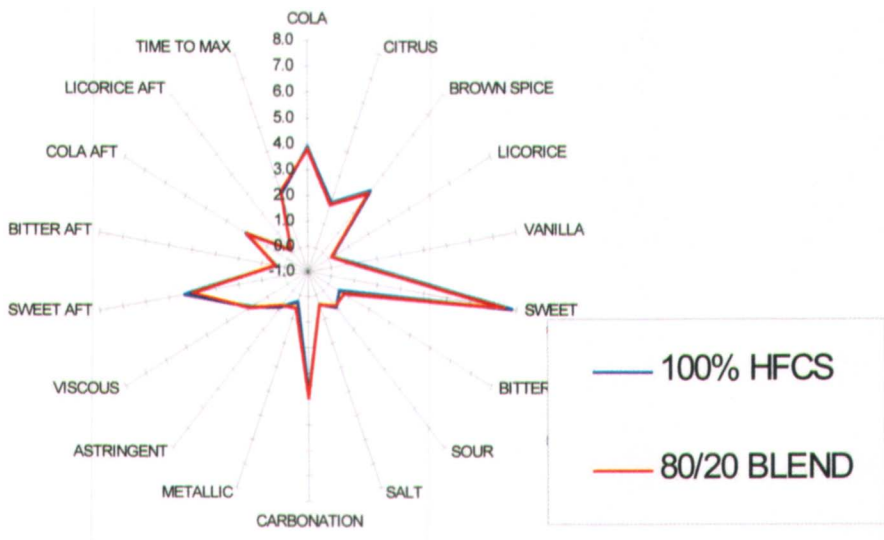
*Figure 32.14. Descriptive test results of cola beverages – 100% High Fructose
Corn Syrup*
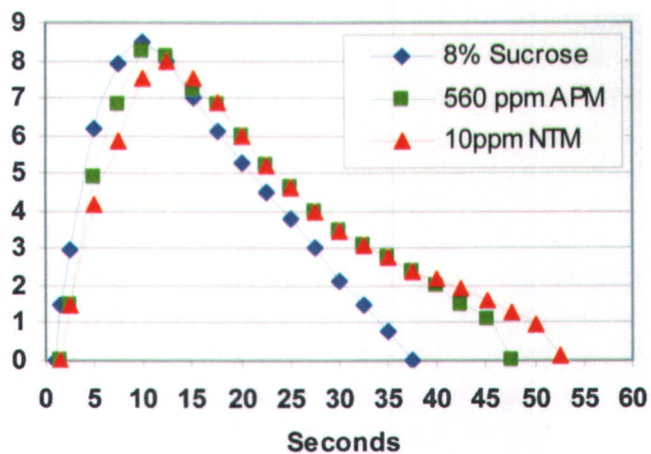
Figure 33.3. Temporal properties of neotame (NTM) compared with aspartame (APM) and sucrose [from (1)]
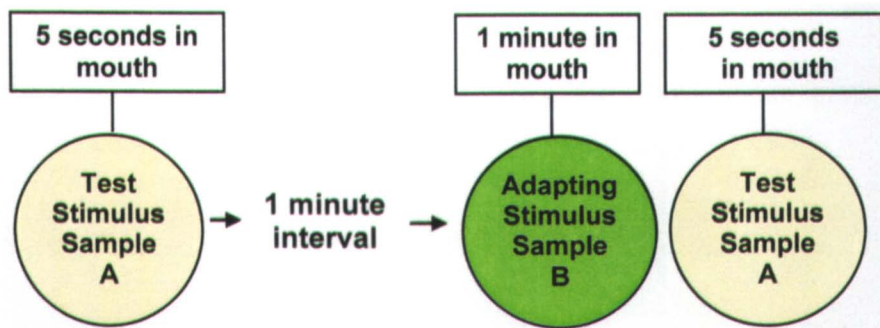
Figure 33.5. Diagram of the cross-adaptation paradigm
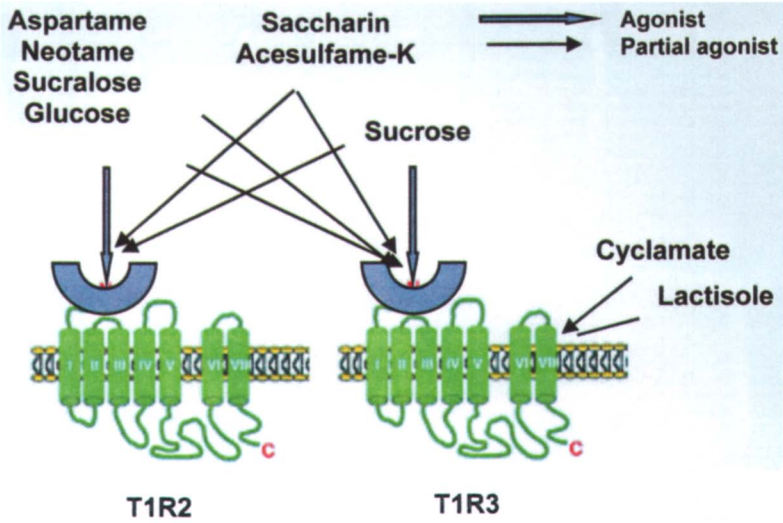
Figure 33.6. The sweet receptor dimer (T1R2 and T1R3) along with probable binding sites for various sweeteners and modifiers
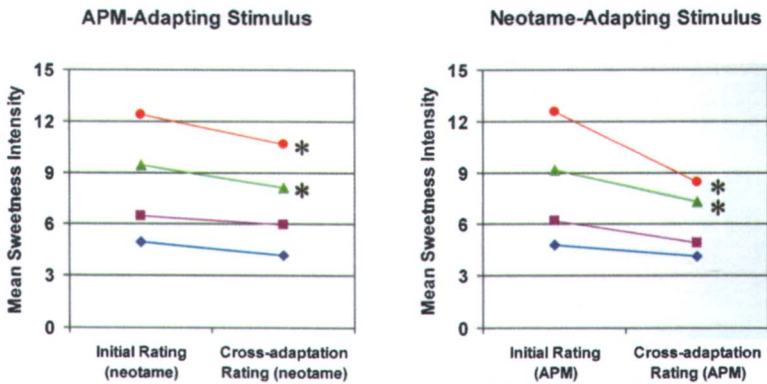
Figure 33.7a. Cross-adaptation of neotame paired with aspartame (APM)
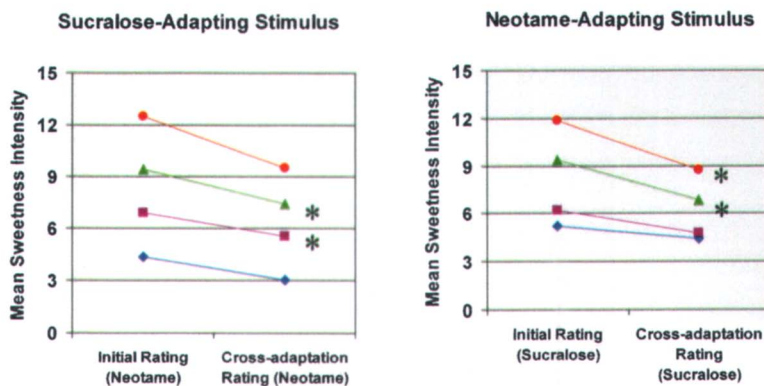
Figure 33.7b. Cross-adaptation of neotame paired with sucralose
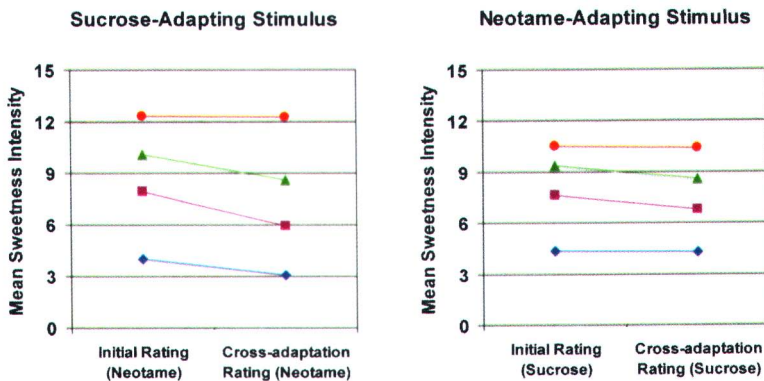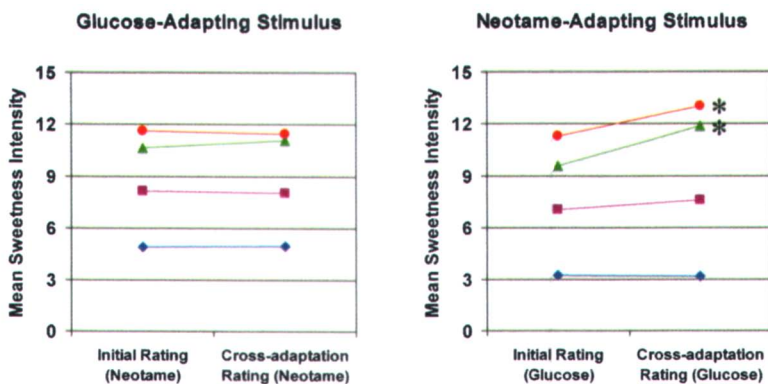

Figure 33.7c. Cross-adaptation of neotame paired with sucrose

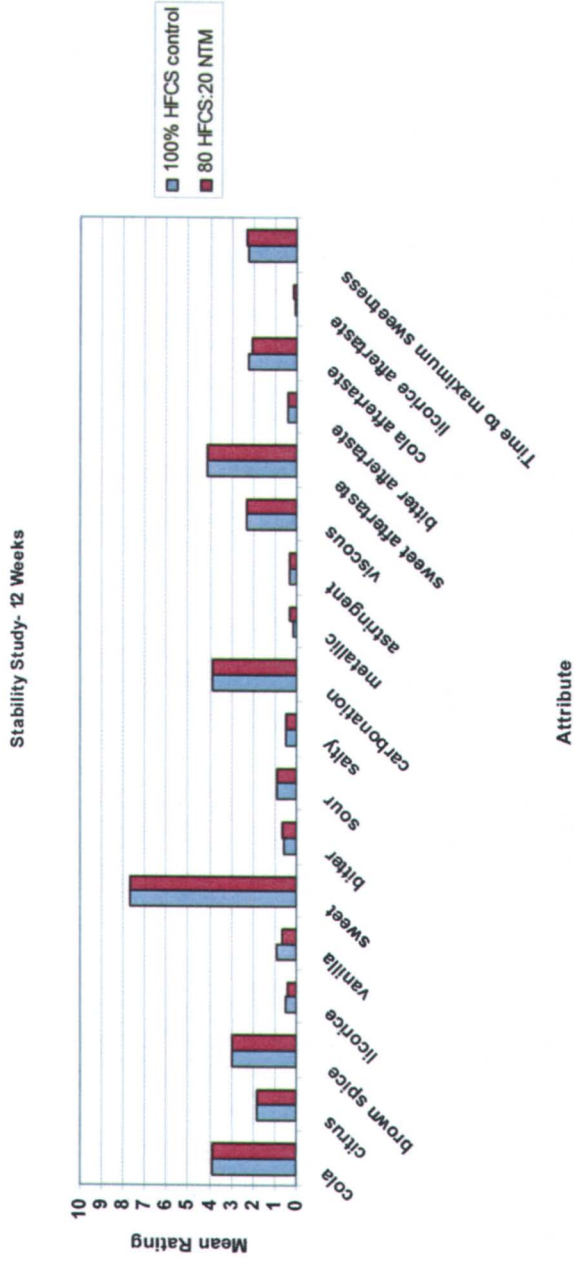Figure 33.7d. Cross-adaptation of neotame paired with glucose

Figure 33.8. Comparison of sensory profile of 100% HFCS and 80/20 HFCS55/NTM blend.
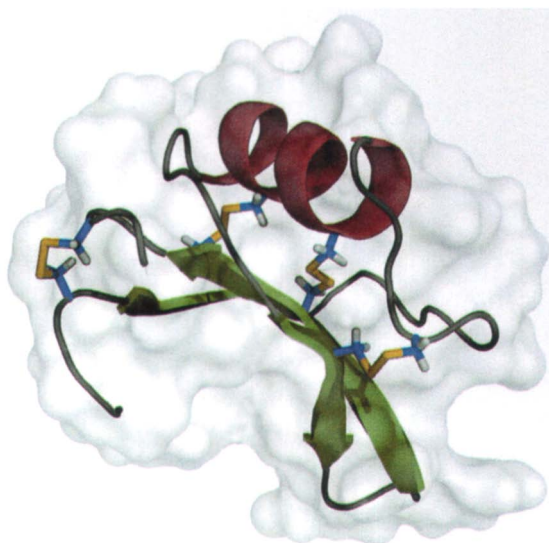
Figure 36.1. Backbone ribbon diagram and the surface representation of the brazzein extracted from fruit with positions of disulfide bonds are shown as determined by solution-state ^1H NMR spectroscopy (17).
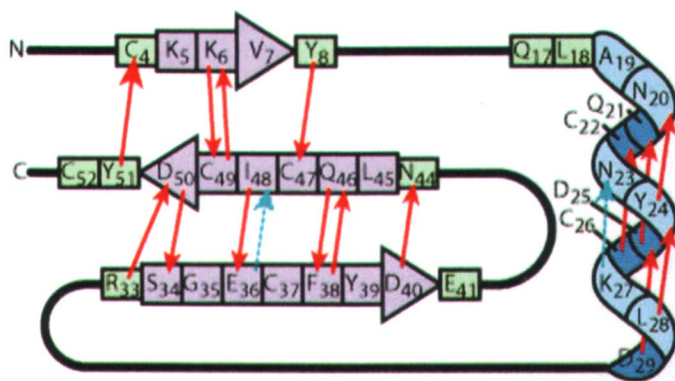

Figure 36.3. Hydrogen-bonds in wild-type brazzein deduced from trans-hydrogen-bond-couplings detected by NMR spectroscopy. Wild-type brazzein and two mutants with enhanced sweetness show a common pattern of hydrogen bonds, whereas all three variants with reduced sweetness have common loss of hydrogen-bonding patterns shown in dotted arrow lines (31).
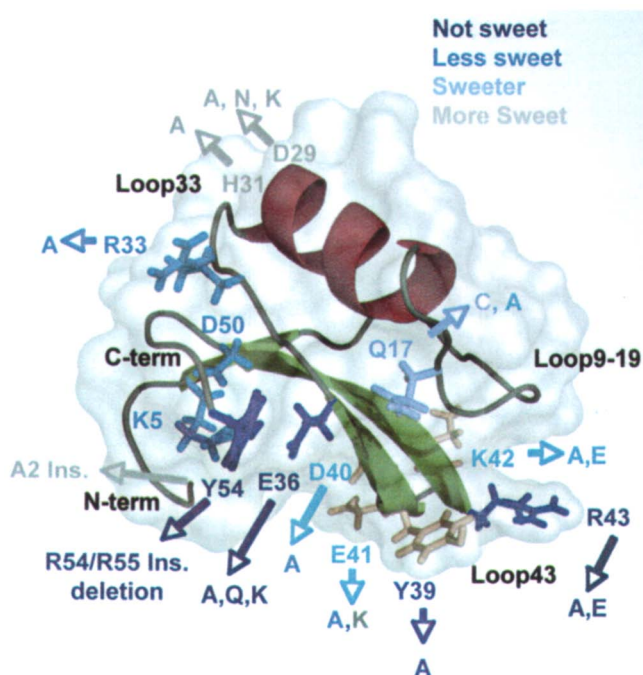
Figure 36.2. Surface representation of wild-type brazzein showing a summary of key mutations that change sweetness. Mutations that abolished sweetness are shown in dark blue, whereas those that enhance sweetness are shown in gray. Mutations that slightly intermediate decreased sweetness are shown in lighter blue. Note that the side chains proposed constitute the primary sweet sites (Loop43 and N- and C-terminal regions) are on the same face of the molecule (22, 24, 25).