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LABELING OF SWEET TASTE BINDING SITES USING A COLLOIDAL GOLD-LABELED SWEET PROTEIN, THAUMATIN

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Abstract

Thaumatin, an intensely sweet tasting protein, was bound to colloidal gold and applied to the taste bud-bearing foliate papillae of Rhesus monkeys. Examination of thin sections of taste pores showed that gold particles were bound to merocrine secretions of Type I taste bud cells, to some cell remnants of lysed cells, and, most importantly, to small, membrane bounded blebs of cytoplasm. These blebs are thought to be shed into the pore from the tips of taste bud cell microvilli, particularly those arising from Type II cells. The binding of gold particles to microvillus tips and to the blebs suggest that this may be an important means by which taste bud cells rid themselves of taste stimulus-receptor complexes.

Key Words: Taste, sweetness, sweet taste, thaumatin, taste bud, colloidal gold, sweet receptors, taste receptors.

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Introduction

Thaumatin is an intensely sweet tasting protein isolated from the fruit of Thaumatococcus danielli, a west African plant (Van der Wel and Loewe, 1972). It is about 2000 times as sweet as the equivalent weight of sucrose. Thaumatin has a molecular weight of 22,209, has a pI of 12.0 and consists of a single polypeptide chain of 207 amino acid residues. The amino acid sequence (Iyengar et al., 1979) and 3-dimensional structure (De Vos et al., 1985) are known.

The ability of thaumatin to elicit a sweet taste response has been studied in about 40 different mammalian species (Brouwer et al., 1973; Hellekant et al., 1976; Glaser et al., 1978; Hellekant et al., 1981). These studies showed, among other things, that the sweet taste of thaumatin is not universal. It is tasted by man, old world monkeys, the great apes (chimpanzee, gibbon, orangutang) and perhaps a few other species, but not by most other mammals, e.g., dogs, hamsters, and many species of primates including prosimians and the entire group of new world monkeys, (Hellekant et al., 1976; Hellekant, 1977; Glaser et al., 1978; Hellekant et al., 1985). Behavioral and physiological evidence suggests there may be a phylogenetic division, between new world monkeys and old world monkeys, marking the point in the evolutionary scheme where the receptor for thaumatin is first expressed (Glaser et al., 1978; Hellekant et al., 1981).

The fact that sucrose and other carbohydrate sweeteners taste sweet to virtually all mammals, but thaumatin is tasted by only some, suggests that there is more than one type of sweet receptor site on taste bud cells (e.g., Hellekant, 1975). The purpose of this preliminary study was to try to localize the binding site for thaumatin on taste bud cells by applying gold-labeled thaumatin to the surface of the tongue and examining the taste pores for the presence of the marker. The rationale for this approach was based on the relatively large size and strong positive charge of the molecule, both of which might contribute to prolong its binding and slow its diffusion away from the taste bud cell receptor site and thus render it visible with this technique.

Materials and Methods

We used Rhesus monkeys (Macaca mulatta) in

these experiments. It has been shown that these old world monkeys respond to thaumatin (Glaser et al., 1978). Animals were injected with atropine to inhibit salivary secretion and then anesthetized with ketamine. The tongues were rinsed with tap water for 1 - 2 minutes and suspensions of colloidal gold-labeled proteins were applied to the foliate or circumvallate papilla region of the tongue for periods varying from 30 seconds to 5 minutes. The region was again rinsed briefly with water, the papillae were surgically removed and placed immediately in Karnovsky's solution (2% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4; Karnovsky, 1965). After biopsies were taken, animals were allowed to recover from the anesthesia. The specimens were fixed overnight, rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO_4 in the same buffer, dehydrated in alcohols and embedded in Epon-Araldite. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100 CX electron microscope.

Preparation of labeled proteins

The proteins used in these experiments included thaumatin, which tastes sweet; albumin and acetylated thaumatin (Van der Wel and Bel, 1976), both of which are tasteless, were used as controls.

5 nm and 18 nm colloidal gold particles were prepared as follows (Loftus and Albrecht, 1984; Albrecht et al., 1986): To produce the 18 nm particles, a 4% solution of $HAuCl_4$ was reduced with a 1% trisodium citrate solution. The 5 nm colloid was prepared by reduction of 1% $HAuCl_4$ with a phosphorous/ether solution. The gold particles were either conjugated to the proteins immediately or filtered through a 0.2 μm microporous filter and stored at 4°C. Thaumatin, albumin and acetylated thaumatin were conjugated to the colloidal gold as follows: the minimum pH at which each protein could stabilize the gold was determined from pH isotherms. Gold suspensions were adjusted with 0.2 N K_2CO_3 to provide a decreasing pH series. Sufficient protein to stabilize the gold was added to 0.5 ml of gold suspension at each pH. After 1 minute, 0.1 ml 10% NaCl was added. Inadequate stabilization results in aggregation of the gold particles as detected by a color change from red to blue. The minimum pH at which the proteins stabilized the gold were: thaumatin, 10.5; albumin, 6.5; and acetylated thaumatin, 6.9. The minimum amount of each protein necessary to stabilize the gold was then determined from absorption isotherms. Decreasing amounts of protein were added to 0.5 ml colloidal gold suspension at the proper pH, followed by 0.1 ml of NaCl. The minimum amounts of protein necessary to stabilize 1.0 ml gold suspension were: thaumatin, 9 μg; albumin, 130 μg; and acetylated thaumatin, 8 μg. Twenty ml gold suspension was added to a 10% excess of each protein with stirring. After 5 minute, 1 ml of freshly prepared and prefiltered (0.45 μm Millipore) 1% polyethylene glycol (M.W., 20,000 Daltons) was added to further stabilize the protein coated gold particles. The protein-labeled gold was centrifuged in polycarbonate centrifuge tubes in an angle rotor at 6,500 x g for 40 minutes for 18 nm Au, and 35,000 x g for 60 minutes for 5 nm Au. The supernatant was discarded and the concentrated gold was resuspended to 200 μl with 0.1 M Na_2HPO_4, pH 9.2. The gold-thaumatin suspension had a sweet taste to two of the authors.

Results

Monkey taste organs are bud-shaped epithelial structures, about 30 to 50 μm in diameter and about 50 to 80 μm tall. They are made up of 30 to 60 elongated epithelial cells, belonging to three categories, referred to as Types I, II, and III (cf. Farbman et al., 1985). The cells end in apical processes which project into a narrow pore, about 3 to 5 μm in diameter. Some of the processes are narrow microvilli, others are broad and club-shaped.

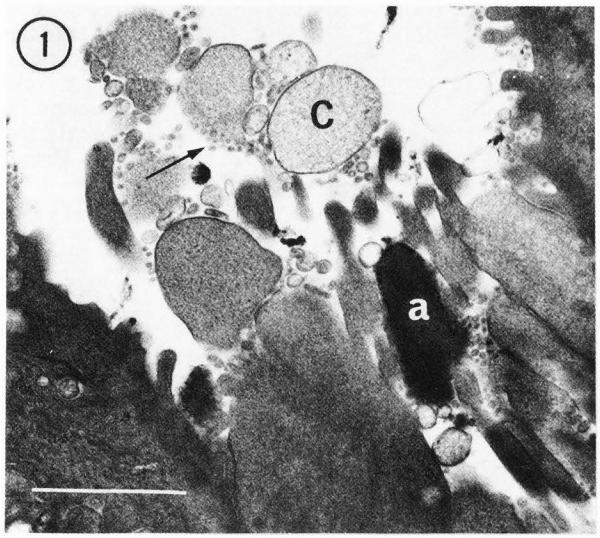
The pore also contains a number of other elements that appear to have been shed or secreted from cells (Figs. 1-7). For example, parts of the broad, club-shaped processes extending into the taste pore are pinched off and these fragments of cytoplasm are extruded into the mouth (Figs. 1,5). In some instances there was a suggestion that even larger masses of cytoplasm and, indeed, whole cells (Fig. 3), are shed into the pore. As these disintegrate, the irregular membranous structures which were once within the cytoplasm are found isolated in the pore. In addition, some small (50-100 nm) extracellular membrane bounded vesicles of cytoplasm appear to have been produced by an active apocrine secretory process or blebbing from the microvilli (Figs. 1,2,7). Finally, large, electron opaque secretory droplets produced by coalescence of secretory granules of Type I cells are seen (Figs. 1,2,4,6,7).

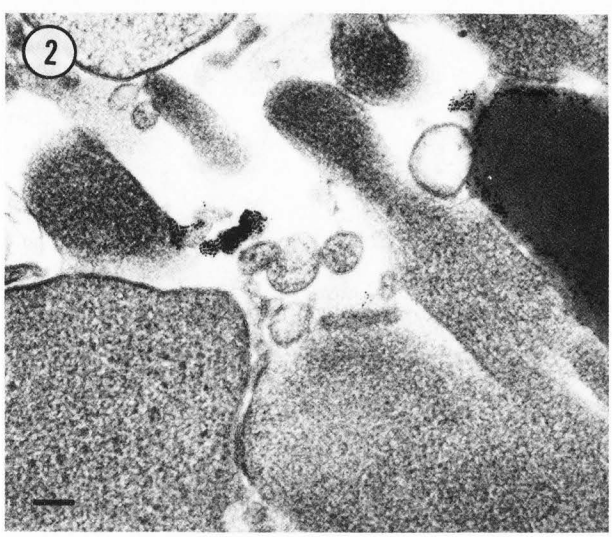
The gold-labeled thaumatin was concentrated in the region of the taste pores. Examination of the non-gustatory epithelial surface distant from the pores revealed the presence of only small amounts of gold particles; these were associated with amorphous substance on the surface, and are probably evidence of incomplete rinsing of the surface. We found many more gold particles in taste pores when thaumatin was labeled with 5 nm particles than with 18 nm particles (cf. Figs. 2 and 5).

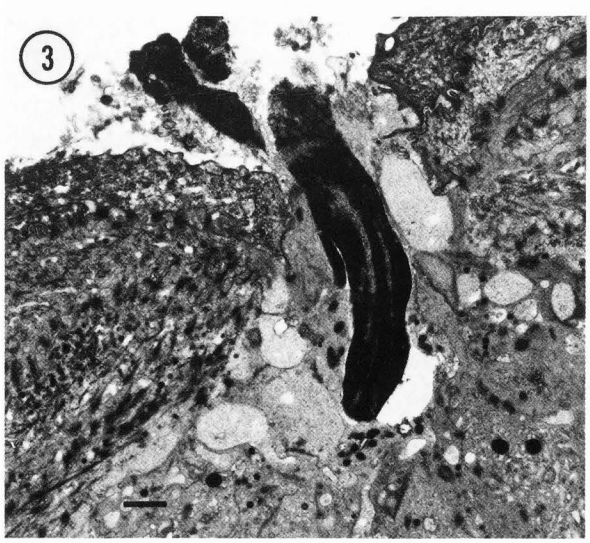
Within the pore, the label was associated mostly with the following pore elements: the small extracellular membrane-bounded elements (Figs. 1,2,7); the masses of electron opaque substance (Figs. 1,2,4,7); sometimes, when the remnants of a lysed cell were in the pore, gold particles were bound to them (Fig. 5). The membrane-bounded vesicles were frequently associated with the electron opaque secretions. We saw no evidence in any of our sections that thaumatin had entered cells.

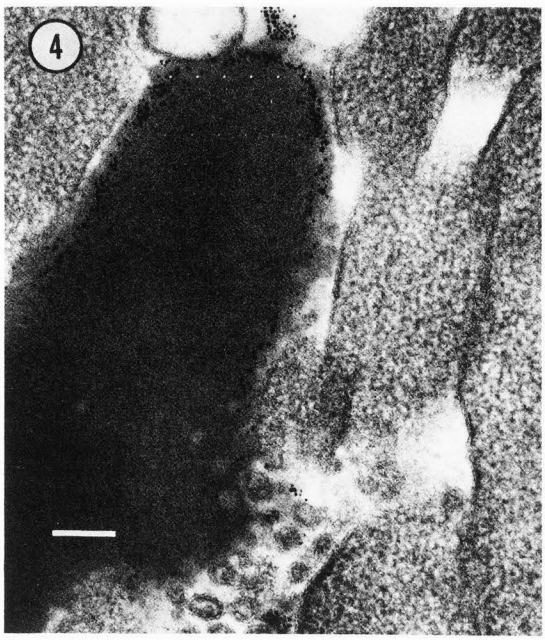
Of particular interest was the occasional finding of the gold marker associated with small blebs on the surface of microvilli, usually at the tip (Fig. 7). The gold particles were clustered in the region of the bleb and were absent over the rest of the microvillus. It was difficult to identify the cell type to which these microvilli belonged because in only a few favorable micrographs were we able to see continuity between the labeled microvillus and the apical part of the cell. In favorable sections, the labeled microvilli were traced to Type II cells. We found no label on surfaces of Type I cell microvilli. However, we were not able to rule out completely the possibility that label is associated with Type III cells.

Tissues treated with two gold-labeled control substances, albumin and acetylated thaumatin, generally contained fewer gold particles in taste pores (not shown).









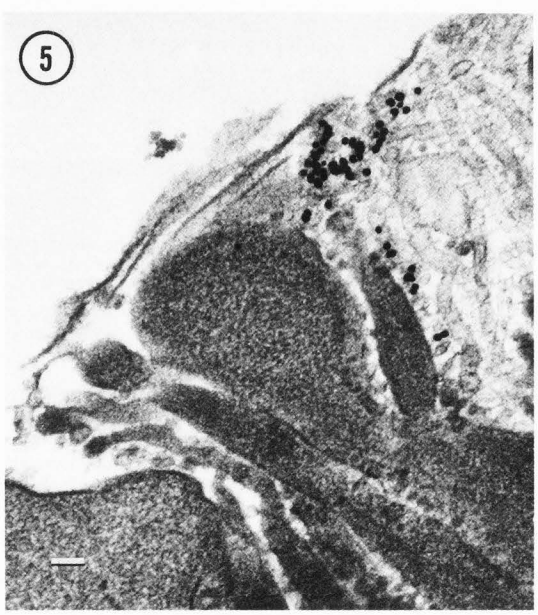


Figure 1. Contents of a taste pore include large fragments of cytoplasm (C), masses of dense, secreted substance (a) and small (50 nm) membrane bounded elements (arrow). Bar = 1 μm.

Figure 2. High magnification of area on Figure 1 showing thaumatin bound 5 nm gold marker associated with some small membrane bounded elements and the dense secreted substance. Bar = 0.1 μm.

Figure 3. A partly extruded cell in a taste pore. Bar = 1 μm.

Figure 4. A high magnification of thaumatin-labeled secreted substance and small vesicles in the taste pore. Bar = 0.1 μm.

Figure 5. This micrograph illustrates thaumatin bound 18 nm gold particles associated with membranous elements, probably smooth endoplasmic reticulum from the remnants of cell cytoplasm in a taste pore. Bar = 0.1 μm.

Discussion

It was clear from these studies that some of the material in the taste pores of Rhesus monkeys bound the gold-labeled thaumatin with high affinity -- significantly higher than the surface of the surrounding epithelium. It should be pointed out, however, that in order to demonstrate this, the tongue had to be rinsed before application of thaumatin and salivary secretion had to be inhibited to prevent non-specific binding to amorphous substance on the lingual epithelial surface. In earlier experiments, when this was not done, there was non-specific binding of thaumatin all over the tongue surface. Even in the present study, there was evidence of the gold marker where amorphous substance had not been thoroughly rinsed from the surface.

A careful evaluation of the thaumatin binding sites in the taste pore suggests that most was bound to structures that had once been intracellular, viz., the opaque, amorphous secretory product of Type I cells and the membranous elements that probably came from smooth endoplasmic reticulum of Type II cells. We regard these as non-specific and probably not directly related to the taste sensation, because it is improbable that a molecule as large as thaumatin enters taste cells before eliciting a sensation. In fact, even after 5 minutes of stimulation there was no evidence of labeled thaumatin inside taste cells.

However, thaumatin binding was also seen on small vesicles or blebs on the surface of microvilli. Binding to regions such as these is more likely to be specific and is consistent with the notion that these regions might contain receptors for the protein. The clustering of gold-labeled thaumatin on the membrane blebs suggests a cellular mechanism for disposing of the bound thaumatin, and possibly for other tastants. Our evidence suggests that the mechanism for disposing of thaumatin is segregation of the binding sites for the protein in a small region of membrane and shedding of the membrane, containing the ligand-binding site complex, as an apocrine secretion into the pore. This mechanism implies a rapid turnover of membrane and a need for a continuous supply of new membrane components. It is possible that the abundant cytoplasmic membranous elements of smooth endoplasmic reticulum in the Type II and III cells can provide the source for new membrane.

The merocrine secretory product of Type I cells is present in abundance in pores of stimulated taste buds. This corroborates previous reports that stimulation results in secretion by taste bud cells (Mattern and Paran, 1974; Brouwer and Wiersma, 1980). In the present study, it was shown that this secretory product binds gold-labeled thaumatin and this mechanism probably participates in ridding the taste bud of gustatory stimuli.

There were no substantial differences in binding of the ligand associated with time of exposure. We did see more evidence of binding to intact microvillar membrane in some of the specimens exposed for 30 seconds. It should be noted that the exposure time is not precise because a variable interval, possibly as much as 30 to 60 seconds, was required for surgical removal of the foliate papillae after application of the stimulus. The total period of time that elapsed between application of the stimulus and fixation was probably long enough for most of the structures binding the ligand to be shed into the pore. In order to demonstrate specific binding of thaumatin to those regions of the taste cell membrane important for sensation, a means of inhibiting the massive shedding of cellular debris into the pore is required. This would prolong the time of stimulus binding to the cell membrane.

From the standpoint of binding specificity, it is important to note the structures to which the gold-labeled thaumatin did not bind. The large blebs of cytoplasm that were shed by Type I cells clearly did not bind the marker. This suggests that 1) membranes of Type I cells have no functional thaumatin binding sites, and 2) these cells have no primary gustatory function. Previous workers have ascribed a secretory function to these cells, based on the presence of apical secretory granules (Farbman, 1965; Murray, 1973; Farbman et al., 1985). Our data indicate that, in addition to the merocrine secretion or exocytosis of these granules, Type I cells shed apical cytoplasm as an apocrine-type secretion; indeed, entire cells are shed into the pore.

The major points that emerge from this study are as follows: First, much more cellular debris is shed into the taste pore than had been previously supposed. Although the small, membrane bounded vesicular structures in taste pores had been described previously (Farbman, 1965; Murray et al., 1972), there was no indication of their origin or significance. Our data suggest they are shed from the microvillus tips of Type II cells, and perhaps others. Second, the taste cells may get rid of gustatory stimuli by segregating the stimulus-receptor complex in a small region of membrane at the tip of the microvillus, and expelling this as a bleb into the pore. Third, although elements in the taste pores bound thaumatin with a higher affinity than surrounding regions of oral epithelium, much of this binding was probably not directly assoicated with the mechanism of taste.

Acknowledgements

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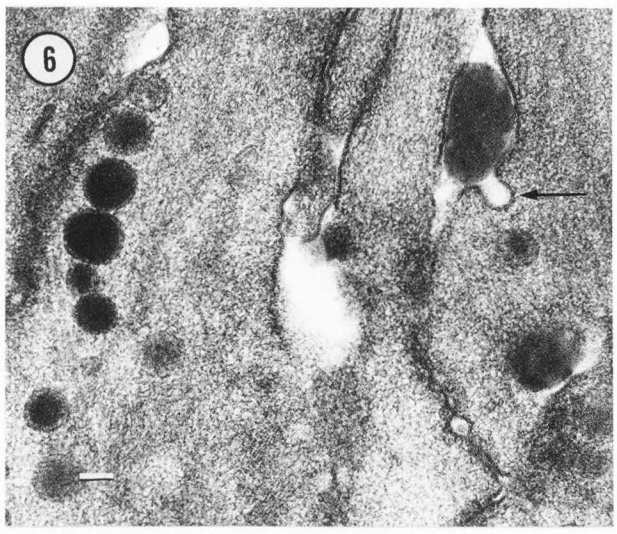

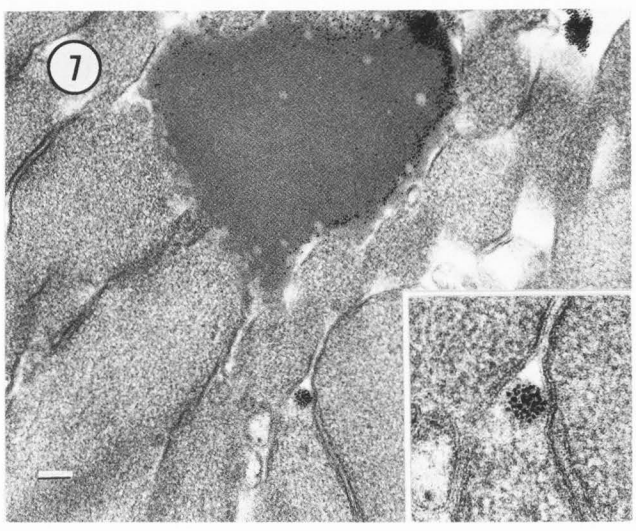

Figure 6. A mass of dense secreted substance is seen close to a segment of invaginated membrane, probably representing an exocytotic specialization (arrow). The dense substance is packaged in the dense secretory granules of Type I cells and released into the taste pore. Bar = 0.1 μm.

Figure 7. High magnification of thaumatin labeled gold particles (5 nm) associated with dense secreted substance in the pore, and with a small region at the tip of a microvillus. Bar = 0.1 μm. Inset: Higher magnification of the gold-labeled microvillus tip.

Discussion with Reviewers

J. N. Brouwer: Acetylated thaumatin has been shown, in addition to having a decreased sweet taste, to have a decreased net charge, resulting in a lower isoelectric point than that of native thaumatin (van der Wel and Bel, 1976). On the other hand, the strong positive charge of thaumatin is viewed as part of the basis of the approach in the present study, guaranteeing prolonged binding. Is acetylated

thaumatin considered to be a proper control substance, in view of its reduced positive charge?
Authors: Acetylated thaumatin was used as a control substance because it was tasteless, as noted. It might be useful to use other highly charged proteins, perhaps histones, as additional controls, but it is extremely difficult to prepare stable gold-labeled suspensions of these proteins.

J. N. Brouwer: It has been demonstrated that phosphate buffers inhibit the sweet taste of thaumatin (van der Wel, in: Criteria of Food Acceptance. I. Solms and R.L. Hall (eds.), Forster Verlag, Switzerland, 1981, p. 292). Is it conceivable that the presence of phosphate in the final suspension of gold-labeled thaumatin prevented wholly or partly the binding to thaumatin receptors in the taste cell membrane? This could explain why most of the binding was found outside the microvilli.
Authors: When thaumatin in phosphate buffer is applied to the tongue, it is tasteless, but when the tongue is then rinsed with water, the sweet taste of thaumatin is perceived. We think the thaumatin is bound to the binding sites on the tongue, even when presented in phosphate buffer, because it is detectable when the phosphate is removed by rinsing.

B. Oakley: Since you conclude that much of the thaumatin binding in the taste pit was not directly related to taste mechanisms, why are the control proteins, acetylated thaumatin and albumin, generally less evident in the taste pit?
Authors: This point is well taken. The control proteins were not totally absent in the pore. There seemed to be fewer pores that contained gold-labeled acetylated thaumatin and virtually no pores with gold-labeled albumin. We cannot explain this.

B. Oakley: The proposal that bound taste substances are often removed by shedding of receptor cell membranes into the taste pit is imaginative. Can you test this by giving the taste pit a thorough cleaning before the application of a taste solution or a non-taste solution?
Authors: We know of no way to accomplish this in view of the fact that the pores are not directly accessible - they face into the cleft between the folds of the foliate papillae.

Reviewer III: What is the evidence that "elements were shed" or "pinched off" from taste bud cells? In my experience there are always numerous profiles in the pore region which represent sections through the tips of cell processes. The authors say, "in favorable sections the labeled microvilli were traced to Type II cells". Why could this not be illustrated?
Authors: It is clear even to inexperienced observers, that the small vesicles are not connected to microvilli, and are free in the pore. Examination of serial sections have confirmed that some of the large bits of cytoplasm also are not connected to microvilli. To illustrate the connection between labeled microvillar blebs and Type II cell cytoplasm would have been cumbersome, given size limitations for illustrations.

Reviewer III: Non-gustatory surfaces are said to have only "small amounts" of deposit. This might describe all the illustrated depositions, which are not consistently found on any particular surface except that of the dense substance. Less deposition on the non-gustatory surface areas might reflect the fact that these were more effectively rinsed than the depths of the pores. One wonders also why the region was rinsed with water rather than a more isotonic solution. What eliminates the possibility of fixation artifact, especially in view of the water rinse?
Authors: As noted above, one must keep in mind the anatomy of the foliate papillae. Taste buds are in the clefts between the folds of the papillae. It is not likely that large quantities of water get into the clefts. In experiments
done after this paper was written we rinsed with a balanced Ringer-like solution containing the electrolytes of saliva, and found no difference in fixation or in binding of labeled substance.

Reviewer III: The only consistent deposition illustrated was that at the margins of the dense substances. This material is correctly recognized as the secretory product of Type I cells, but it does not require stimulation to be present. All of the hundreds of taste pores of foliate and circumvallate papillae I have examined have contained this substance. Moreover, the article referred to (Mattern and Paran, 1974) reporting secretions after stimulation was concerned with taste buds of fungiform papillae, which do not have dense substances in their pores.
Authors: With all due respect, one of us (A.I. Farbman) has also examined hundreds of taste pores, from fungiform, vallate and foliate papillae of mammalian taste buds, as well as the homologous regions of aquatic vertebrates. His experience has made it clear that what is found in the taste pore depends on how the tissue was manipulated prior to and during fixation. For example, perfusion fixation might give one kind of picture, immersion fixation another. Data acquired since this paper was written indicate that the contents of the pore can be experimentally manipulated. It is true that the Mattern and Paran study concerned fungiform papilla, but these papillae are known to contain the small vesicular secretory component, as originally documented in studies by one of us (Farbman, 1965) and described by Murray, 1973 (text reference). Both the dense substance of Type I cells and the small vesicles have been identified as being important in the binding of gold-labeled thaumatin.

Reviewer III: How permanent is the binding of the colloidal gold to the protein? What proof is there that the gold particles remain with the protein in the specimen? Non-specific binding to amorphous substance must be considered also.
Authors: The colloidal gold-protein solution was freshly made before use, and the unbound gold was separated from that bound to protein before application to the tongue. There is no absolute proof that the gold particles remain with the protein in the specimen. However, based on our experience and that of others with protein-colloidal gold interactions, it is unlikely that the gold becomes separated from the thaumatin before or during application to the tongue.

Reviewer III: The suggestion that shedding of

cytoplasm is a mechanism for dispersing of taste substances rapidly is interesting but highly speculative. No evidence is presented to substantiate this idea.
Authors: The suggestion was made as a way to explain the results. It is, as stated, a suggestion.

Reviewer III: The method of application of the taste substance did not duplicate the natural situation. It might be possible to study the early period by excising the papillae, dipping them in labeled substance and fixing them immediately. Use of OsO_4 fixative might be tried, to produce a more rapid and more stable fixation.
Authors: Application of the taste substance came as close to what might be considered physiological as we thought was possible, certainly much more than excision of the tissue would be. It is well known that aldehyde fixation is much more rapidly acting than OsO_4; hence we chose to use aldehyde fixation, followed by postfixation in OsO_4.

L. M. Beidler: Why is it suggested that Type I cells, which do not bind thaumatin, therefore have no primary gustatory function?
Authors: The absence of binding sites for thaumatin implies the absence of receptors and probably the inability to generate a potential change in response to the stimulus. This is what we mean by no primary gustatory function for Type I cells, at least insofar as their ability to respond to thaumatin.

L. M. Beidler: Why is it stated that much of the high affinity thaumatin binding in the taste pore is probably not directly assoicated with the mechanism of taste?
Authors: Much of the binding is to the secretory product of Type I cells. This probably contains glycoprotein(s) which would have a negative charge and bind the highly positively charged thaumatin. We consider this a more likely explanation for binding to this substance. The binding to the small, membrane bounded vesicles and the absence of binding to the membranes of Type I cytoplasmic fragments suggests some specificity of binding to the sites where the receptors are most probably localized, i.e., the membranes of the structures exposed to the oral environment.