THE TASTE BUD STRUCTURE AND WHOLE NERVE RESPONSE OF THE MUDPUPPY (NECTURUS MACULOSUS)

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ABSTRACT

THE TASTE BUD STRUCTURE AND WHOLE NERVE RESPONSE
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By

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The taste buds of the mudpuppy (Necturus maculosus) are situated on sparsely distributed elevations on the lingual surface. These are difficult to identify with a dissection microscope, hindering intracellular recording from the receptor cells of the taste organ. A scanning electron microscopic study was undertaken to reveal the surface appearance of the elevations and their taste buds at a range of magnifications. Several structures were identified whose dimension, distribution, and general appearance correspond to a description by Farbman and Yonkers (1971) of the apex of a taste bud. Solutions were applied to the whole tongue for examination of the electrophysiological responses to taste stimuli in the mudpuppy. The whole nerve activity was recorded from the lingual branch of the glossopharyngeal nerve in 12 adult mudpuppies, following chemical stimulation and rinse with distilled water. The responses to solutions of less than 1.0 molar concentration of NaCl, KCl, CsCl, $CaCl_2$, HCl, sucrose, and quinine hydrochloride were recorded and electronically integrated. The response to each concentration of each chemical was expressed as a per cent of the response to 0.1 molar

NaCl. HCl was more effective a stimulus than the other solutions over the range of concentrations compared (0.0003 M to 0.3 M). The rank order of effectiveness of the other chemicals varied with their concentration. $CaCl_2$ was found to be less effective a stimulus at all concentrations than has been reported in the frog by Kusano and Sato (1957. Jap. J. Physiol. 7:324-338).

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THE TASTE BUD STRUCTURE AND WHOLE NERVE RESPONSE OF THE MUDPUPPY (*NECTURUS MACULOSUS*)

by

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God bless Rudy, Frank, Masako, Dennis, Winnie, Chuck, Ken, Carol and Steven, Mom and Dad, and this thesis.

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I. INTRODUCTION

The taste buds of the mudpuppy, Necturus maculosus, are morphologically unique. Their large size is due to the large dimensions and great number of their constituent cells. Intracellular recording from these taste buds, therefore, should be less difficult than for animals previously investigated, e.g., cat, rat, dog, or frog.

This thesis considers two separate studies:

1. A study of the mudpuppy tongue's surface using the scanning electron microscope (SEM) at 1200-1800 X to confirm the reported size and distribution of the taste buds, correlated with examination at light microscopic magnification to better identify the buds in future experiments.

2. Experiments using the standard electrophysiological technique of whole nerve recording to discover: a) which of several chemicals are effective stimuli to the taste system of Necturus b) at which concentrations they are effective, and c) the rank order of the chemicals with respect to their effectiveness of stimulation.

II. LITERATURE REVIEW

Anatomy

Biology of the Mudpuppy

The mudpuppy is an aquatic, caudate amphibian. It is active in water as cold as 4 °C but exhibits a preference for warmer waters (18 °C). Based on the stomach contents of dissected specimens, the mudpuppy is a carnivore, consuming spawn, small fish, aquatic insects, and insect larvae. It is nocturnally active, exhibits negative phototropism, and changes its skin color in response to light, darkening on photic stimulation. At birth, larval mudpuppies are 2.3 cm long and reach a reported maximum adult length of 48.8 cm. The larvae fully mature in eight years and captive animals have lived as long as 23 years. The adult mudpuppy resembles a larval salamander or frog, especially in that it possesses bushy, external gills. This is not a purely neotenous condition, i.e., the mudpuppy is not phylogenetically more "plastic". Rather, this is an example of specialization or adaptation to its aquatic environment and an example of degenerative neoteny. Some species of Ambystoma also retain gills as adults, but unlike Necturus, they can be induced to undergo further development, losing their gills, by the administration of thyroxine. The mudpuppy's three pairs of external gills represent its primary means of respiration even though the mudpuppy also possesses lungs. Its lungs contain no true alveoli yet are well

vascularized. Normally, its pulmonary circulation is oxygenated by its gills. The mudpuppy is not of special economic importance (though edible) but has been used frequently in the biological sciences as an experimental animal owing to the unusually large size of the cells of many of its organs.

Early classification of the mudpuppy overemphasized its geographic distribution and certain differences in coloration. (The range of Necturus includes most of eastern North America, viz., the river systems of the Missippi, Susquehanna, Delaware, St. Lawrence, and Hudson rivers and the Great Lakes.) This resulted in seven different generic titles: Necturus, Menobranchus, Phanerobranchus, Triton, Proteus, Siredon, and Siren. Today, the mudpuppy is classified as a member of the suborder Protidea which has two members: the mudpuppy, Necturus, and the European blind salamander, Proteus. There are two species of Necturus: Necturus maculosus maculosus and and its dwarf derivative, Necturus punctatus. The former (maculosus maculosus) includes a sub-species, Necturus maculosus lewisi. The American Indian name for the mudpuppy is simply Tweeg. (From Eyclesheimer, 1906, Francis, 1943, Noble, 1931, Reese, 1906, Sayle, 1916, and Weichert, 1958.)

The Taste Organs of Necturus

The tongue of the mudpuppy appears smooth, lacking any obvious papillae. However, Farbman and Yonkers (1971) reported the appearance of rounded elevations or "eminences" which were "sparsely" distributed about the oral cavity, on the oral epithelium behind the

branchial arches, and on the tongue. Most of the elevations appear on the distal or secondary tongue which is separated laterally from the ontogenetically older, primary tongue. Each 250 μ diameter, round elevation contains a 20-30 μ diameter, central concavity. The concavity contains the apex of a single taste bud (Figure 1). The extension of the apex of the taste bud to the lingual surface contrasts with the arrangement found in most mammals whose buds are further below the epithelium, contacting the surface only by way of a narrow taste pore of 5 μ diameter.

The dimensions of the mudpuppy's taste buds (90-120 μ width by 100-150 μ heighth) are twice as large as those of other vertebrates (40-80 μ width by 50-80 μ heighth, Farbman and Yonkers, 1971). Their unusually large size arises both from their greater number of cells (80-100 vs. 30-80) and the larger dimensions of those cells, reported to be twice as great in diameter and length (Farbman and Yonkers, 1971).

Farbman and Yonkers identified three types of cells within a taste bud, distinguishable from adjacent epithelial cells. Basal cells, which form 10% of the total cells, are distributed about the basal periphery of a bud and do not extend to the apex (Figure 2). Light cells, 30% of the total, reach the lingual surface where they are concentrated at the center of the bud. Light cells are also distinguished by: 1) large amounts of agranular endoplasmic reticulum, channeling throughout the cytoplasm, associated with mitochondria, and 2) a deeply situated nucleus which stains intensely with toludine blue. Farbman and Yonkers consider the light cells to be

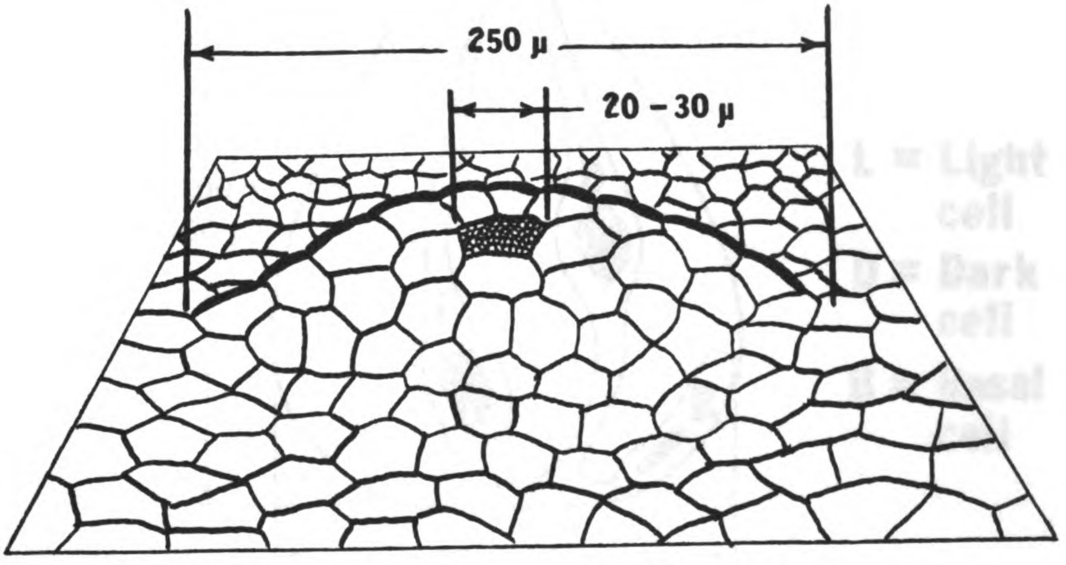


Figure 1. Lingual elevation with taste bud apex.

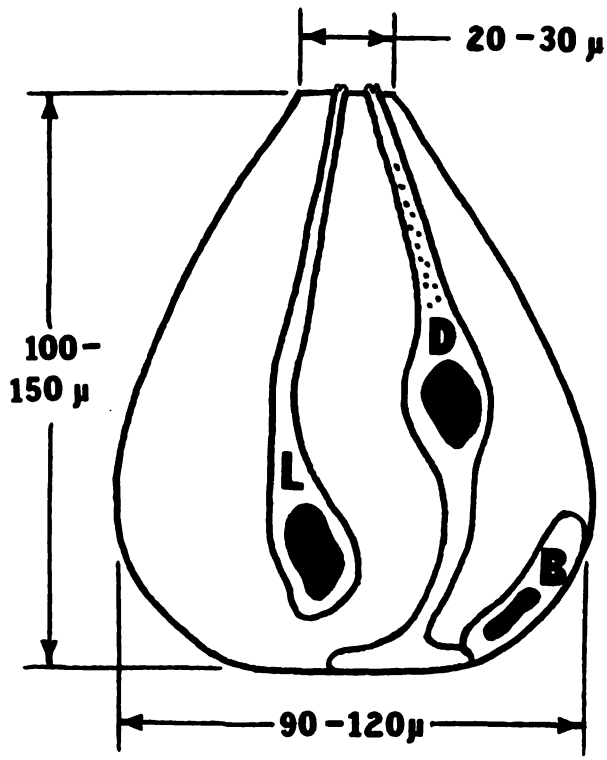


Figure 2. Taste bud of Necturus (after Farbman and Yonkers, 1971).

20 – 30 h

L = Light cell

D = Dark cell

B = Basal cell

the receptor cells of the taste bud since the agranular endoplasmic reticulo-mitochondrial system is associated with ion transport. Such a transport mechanism might contribute to the ion flux associated with the receptor cell's response to a stimulus. Dark cells, 60% of the total, are identified by small granules contained in their apical cytoplasm (0.3 to 0.4 μ diameter) which are basophillic, PAS positive, and under the electron microscope stain intensely with periodic acid and silver methanamine. A few dark cells are found among the light cells at the apex, but dark cells exclusively form the bud's apical periphery. Both light and dark cells have microvilli on their apical ends and are interconnected by the so-called tight junctions.

As Farbman and Yonkers point out, the mudpuppy should be the animal of choice for intracellular recording from taste receptor cells. Its larger bud size, with larger constituent cells, the distinct distribution of its three types of cells, and the surface exposure of the bud are all factors which should greatly reduce the difficulty in locating and inserting a microelectrode into a particular cell, especially into the purported receptor cells of the bud, the light cells.

Innervation of the Taste Buds

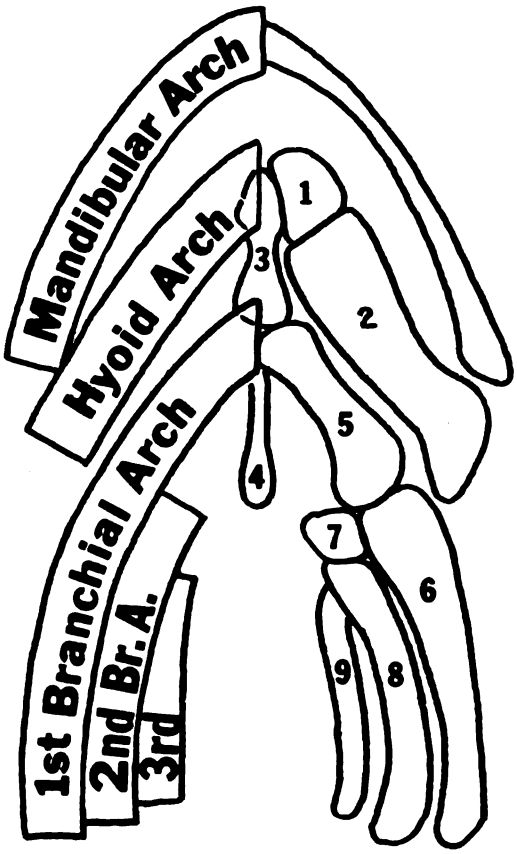
The taste bud is supported by a dermal papilla and is separated from it by a basement membrane. Below this basal lamina is a plexus of unmyelinated nerve fibers. Bundles of these fibers enter the bud through the basement membrane. They extend into the bud only as

separate fibers and do not advance as far as the apex. These small nerve fibers (0.5 - 2.0 μ) are surrounded by the dark cells (reminiscent of the Golgi cell surrounding nerve fibers in the central nervous system). They end adjacent to the light cells but these terminations lack several of the typical features of a chemical synapse. The light cells contain vesicles opposite the neural elements on occasion, but these are of slightly larger diameter (100 μ vs. 50 - 90 μ) and are not associated with the so-called synaptic membrane thickenings (Farbman and Yonkers, 1971).

The Ventral Oral-pharyngeal Skeleton

The cranial nerve innervation of the tongue is best understood after a discussion of the skeleton which supports the tongue and adjacent structures. The peripheral course and termination of the lingual afferent fibers can then be considered with this frame of reference.

The mudpuppy possesses three complete arches which form the floor of the oral-pharyngeal cavity: a boney mandible, a cartilaginous hyoid arch which supports the distal tongue, and a (first) branchial arch (Figure 3). The fishes and larval amphibians have several complete branchial arches while the adult Necturus has only incomplete vestiges of the second and third branchial arches. Figure 3, drawn from dissected specimens, essentially conforms with those of Weichert (1958), Stuart (1940), and Drüner (1901). The nomenclature is nearly identical to that of Drüner (1901), Francis (1934), and Romer (1962). Table 1, Appendix A, lists the variations



KEY

Hyoid Arch:	1 =	Hypohyal
	2 =	Ceratohyal
1st Branchial Arch:	5 =	Ceratobranchial 1
	6 =	Hypobranchial 1
2nd Branchial Arch:	7 =	Ceratobranchial 2
	8 =	Hypobranchial 2
3rd Branchial Arch:	9 =	Hypobranchial 3
Interconnections:	3 =	Basibranchial 1 (Copula)
	4 =	Basibranchial 2 (Copular stem)

Figure 3. Ventral oral-pharyngeal skeleton of Necturus. To the left are outlined the three complete arches (the mandibular arch, the hyoid arch, and the 1st branchial arch) and the vestigial 2nd and 3rd branchial arches. Their constituent cartilaginous elements are numbered and labelled in the key.

from this nomenclature among these authors.

Lingual Gustatory Innervation

Like the mammalian tongue, cranial nerves VII, IX, and X supply the mudpuppy's gustatory afferent fibers.

Facial Nerve (VII). Several anatomical considerations imply that the ramus alveolaris of cranial nerve VII is part of the taste sensory system, though electrophysiological confirmation is lacking for caudate amphibians:

1. A fasciculus communis exists in the medulla of Necturus with fibers entering from cranial nerves VII, IX, and X (Kingsbury, 1895). Strong (1895) and Kingsbury (1895) consider the fasciculus communis to be an homolog of the mammalian solitary fasciculus, the bulbar termination for gustatory afferent fibers.

2. Several authors (Strong, 1895, Francis, 1934, Norris, 1911, and Drüner, 1904) consider the ramus alveolaris to be phylogenetically related to the mammalian chorda tympani based upon the course of communis fibers and the relations of the cranial nerves to the branchial arches.

3. The ramus alveolaris also receives communis fibers indirectly from cranial nerve IX by way of a ramus communicans (Norris, 1911 and Francis, 1934).

However, the ramus alveolaris (VII) does not enter the mudpuppy's tongue, but terminates between the mandible and hyoid (Drüner, 1901). Therefore, if cranial nerve VII does contribute to the taste system of Necturus, it does not provide the major innervation of the distal tongue as the chorda tympani does in mammals. (See Figure 4.)

The Glossopharyngeal Nerve (IX) has been consistently described as part of the taste system of caudate amphibians (Coghill, 1902, Drüner, 1901, Francis, 1934, Herrick, 1894, Norris, 1911, and Strong, 1895). The ramus lingualis of the ramus post-trematicus (IX) is most

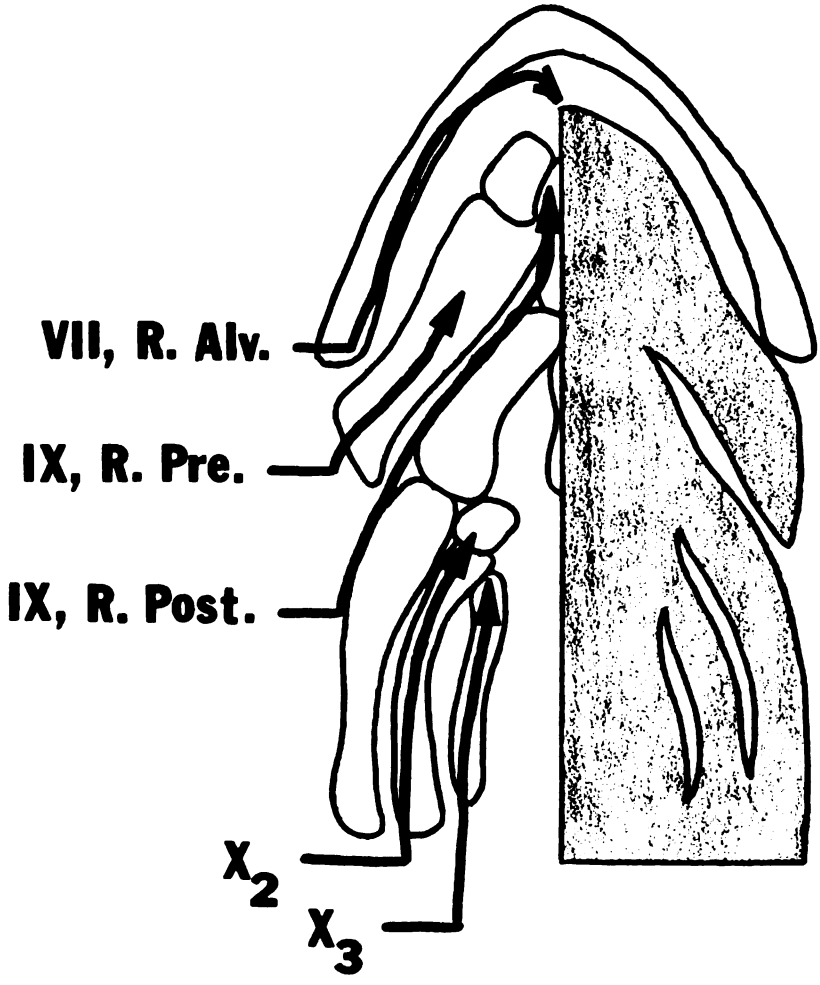


Figure 4. Gustatory innervation. Illustrated are the branches of the cranial nerves which contribute to the taste system of Necturus. The arrows show their approximate course and termination. The lateral extent of their regions of distribution is not known and should not be implied by this diagram. VII, R.Alv. is the ramus alveolaris of the facial nerve. IX, R.Pre. and IX, R. Post. are the pre- and post-trematic branches of the glossopharyngeal nerve, respectively. X_2 and X_3 are the 2nd and 3rd branchial arch nerves which in part constitute an homolog of the vagus of higher vertebrates. To the right (shaded) is an outline of the oral mucous membrane overlying the hyoid and branchial arches.

frequently mentioned. Its peripheral course is parallel to the 1st branchial arch, terminating in the oral mucous membrane above the distal hyoid (Figure 4). Less often described is the ramus pre-trematicus (IX) which terminates above the ceratohyal of the hyoid, but only as far forward as the tip of the 1st branchial arch (Drüner, 1901). Francis (1934) lists communis elements in both major branches of IX in Salamandra.

The Vagus Nerve (X) leaves the common IXth-Xth ganglion as several nerves in caudate amphibians. In Necturus, two of these nerves, the 2nd and 3rd branchial arch nerves, contribute to lingual afferent innervation. (According to Drüner, the glossopharyngeal nerve is the 1st branchial arch nerve.) Both the 2nd and 3rd branchial arch nerves terminate in the region of the ceratobranchial 2 and both contain communis elements in Salamandra (Francis, 1934).

Electrophysiology

Technological Considerations

Discovery of the electrophysiological properties of the taste system has proceeded with advances in recording technique. For example, in 1933, Hoagland, using a Matthews oscillograph to record the facial nerve responses of the catfish, Ameiurus nebulosus, observed and photographed the large action potentials arising from mechanical stimulation of its barbels. The audio equipment revealed smaller amplitude potentials occurring on chemical stimulation, but these were not greatly distinct from the oscillograph's baseline.

Moreover, a photographic record of the taste response was not obtained with the slow camera speed employed, since the baseline fluctuations obscured the small action potentials. Two years later, Pumphrey (1935), with refined dissection, electronic amplification, and photographic technique, recorded the frog's taste response from a Matthews oscillograph well enough for quantitative analysis. Zotterman (1935) was the first to record the taste response using the cathode ray oscillograph (from the cat).

Even though the frequency of action potentials can be analyzed from photographic records, it is difficult to count spikes from a whole nerve's response. In 1953 Beidler employed an electronic integrator circuit which obviated the requirement of spike counting but with certain limitations. The electronic integrator or summator responds to a train of input pulses with an output which is linearly related to both frequency and amplitude. Therefore, the experimenter cannot distinguish whether the increased integrator output is caused by: 1) greater activity from fibers which were previously discharging, 2) the recruitment of other fibers with action potentials of similar amplitude and frequency, or 3) the onset of activity in fibers of larger diameter (though perhaps firing at a slower rate). Beidler (1953) stated the assumptions required for the use of the integrator: 1) the activity is recorded from fibers of similar morphology, or 2) the fibers are equally distributed throughout the trunk (i.e., the fibers of one diameter are not confined to any particular quadrant) and therefore, their activity will be equally recorded regardless of the orientation of the nerve with respect to the recording electrode.

Whole Nerve Recording Protocol

The goals of the procedures (listed in Appendix A, Table 2) are: 1) record with the maximum signal to noise ratio, 2) to record from all gustatory fibers in a nerve trunk, 3) to chose a time constant that least alters the pattern of the response, 4) to stimulate the receptor fields of all gustatory fibers in the recorded nerve, and 5) to minimize the stimulation of other sensory modes (thermal and mechanical stimulation). A standard solution is often used to compare the responses from several taste stimuli, i.e., the magnitude of the response to a test chemical is expressed as a per cent of the response to the standard solution. Besides controlling for the stimulation of other sensory modes, this compensates for small variations in the preparation's responsiveness and allows interspecies correlation of their sensitivity to a gustatory stimulus. The frequently chosen standards are 0.1 M NaCl and 0.1 M NH_4Cl.

Several authors have questioned the effect of stimulus pH on the taste response. Appendix A, Table 3 shows that the threshold of the electrophysiological response to HCl is greater than 0.0001 M or a pH of 4, and that for most species a pH of 3 is subthreshold. Liljestrand and Zotterman (1956) observed the effect of pH as a stimulus for the cat's chorda tympani. The Ringer's solution used, which was an ineffective stimulus at tongue temperature, resulted in a response when made alkaline with NaOH above pH 11.5. Beidler (1954) observed no effect of pH on the response to 0.5 M NaCl from pH 3 to pH 11 (rat's chorda tympani). In summary, pH appears to have little effect itself or in combination with a stimulating electrolyte over

a large range, from pH 4 to 11.

The Pattern of the Whole Nerve Taste Response

The integrated neural taste response shows a typical pattern among many animals: an initial, rapid increase in activity upon stimulus application, followed by a rapid, short period of adaptation to a level still above baseline, and then a period of slow adaptation to baseline level. This pattern occurs during constant stimulation and usually results in a return to pre-stimulus levels only after rinsing. The phases of this pattern have been variously named (Beidler, 1953, Kitchell, 1961, Halpern, Bernard, and Kare, 1962) but this thesis places special emphasis on the initial transient increase in activity and follows Bernard (1964), calling this the peak transient phase.

Wide variation from the typical pattern is found. Tateda (1961) observed only a peak transient phase from the facial nerve of the catfish with stimuli less than 0.2 M. Above that concentration the peak transient was not evident, the activity rising rapidly, but followed only by gradual adaptation. Beidler (1953), in the rat, and Pfaffmann (1955), in the rat and the cat, observed the typical pattern from the chorda tympani on stimulation with NaCl and HCl while the non-electrolytes, sugar and quinine, resulted in a gradual rise followed by gradual adaptation. Halpern, Bernard, and Kare (1962) observed that the responses from the rat's chorda tympani to the different stereoisomers of alanine had different time courses: the time required to reach the maximum amplitude of the response to

a 1.0 M solution of D-alanine (170 seconds) being much longer than for L-alanine (22 seconds) or DL-alanine (30 seconds).

Therefore, since the pattern of the response may vary among animals, stimuli, and concentrations, the experimental protocol must be adaptable when the goal is to measure the maximum amplitude of the response. Beidler, Fishman, and Hardiman (1955) measured the maximum amplitude of the peak transient phase for the rabbit's responses to LiCl and NaCl while they measured the static phase (found after the peak transient phase) for the rat, quinea pig, hamster, cat, dog, and racoon, which they noted was less erratic for all stimuli.

Effectiveness of Stimuli

Several classes of stimuli have been investigated for their gustatory stimulating effectiveness. Those chemicals which evoke the four perceptual taste qualities in man (sour, salty, sweet, and bitter, elicited by HCl, NaCl, sucrose, and quinine, respectively) are effective stimuli to all vertebrates, with the exceptional lack of a significant sweet response for the cat (Pfaffmann, 1941). These have the same order of effectiveness among nearly all animals studied: (from greatest to least) quinine, HCl, NaCl, sucrose. The monovalent and divalent cations have been studied using a series of chloride salts. Appendix A, Tables 4 and 5 reveal that no consistant order is evident (Li^+ and Na^+ are both more effective than K^+ for the rat and hamster, while for the frog and catfish, the decreasing order of effectiveness is K^+, Na^+, Li^+). The monovalent cations are more

effective than the divalent cations except for the frog (for which Ca^{++} is uniquely effective). Sodium salts have been used to study the effectiveness of anions, revealing that they are less determinant of the magnitude of the taste response than are cations.

Stimulus-response curves, which present the intensity of the response versus the common logarithm of the stimulus concentration, are an alternate method for comparing the effectiveness of taste stimuli. As can be seen from Figure 5 (from Pfaffmann, 1955), the stimulus-response curves provide information which cannot be obtained from comparisons at a single concentration:

1. Threshold concentration can be extrapolated from the curves.

2. The effectiveness of the stimuli can be more accurately determined. For example, for the cat, at -1.5 Log M, NaCl, KCl, and sucrose are equally effective, while sucrose is less effective at concentrations above -1 Log M. For the rat, quinine has a much lower threshold than the other stimuli, yet its effectiveness is similar to KCl and NaCl at concentrations greater than -3 Log M.

3. Saturating levels of stimuli, when present, can be seen. The rat's sucrose responses start to plateau above 0 Log M.

Acids. The threshold for the taste response to acid is below pH 4 for most animals. At the same pH, weak acids are more effective than strong acids (Beidler, 1961, Bernard, 1964). Beidler observed that an acid's effectiveness is positively related to both the ionic strength (pH) and the concentrations of non-ionized species present in solution. A weak acid, like acetic acid, therefore, is more effective than HCl at the same pH.

Sugars. The stimulating effectiveness of the following monosaccharides is the same for the rat (Hagstrom and Zotterman, 1959) and dog (Andersen, Funakoshi, and Zotterman, 1963): D-fructose,

Figure 5. Stimulus-response curves for the cat and rat (after Pfaffmann, 1955). The graphs show the magnitude of the relative response (arbitrary units of integrator deflection adjusted to 100 for -1 Log N HCl) plotted against Log concentration of the stimulus. The aqueous solutions represent the four taste qualities (plus KCl). The magnitude of the response to water is indicated both to reveal the effect of the solvent and to allow interpretation of threshold.

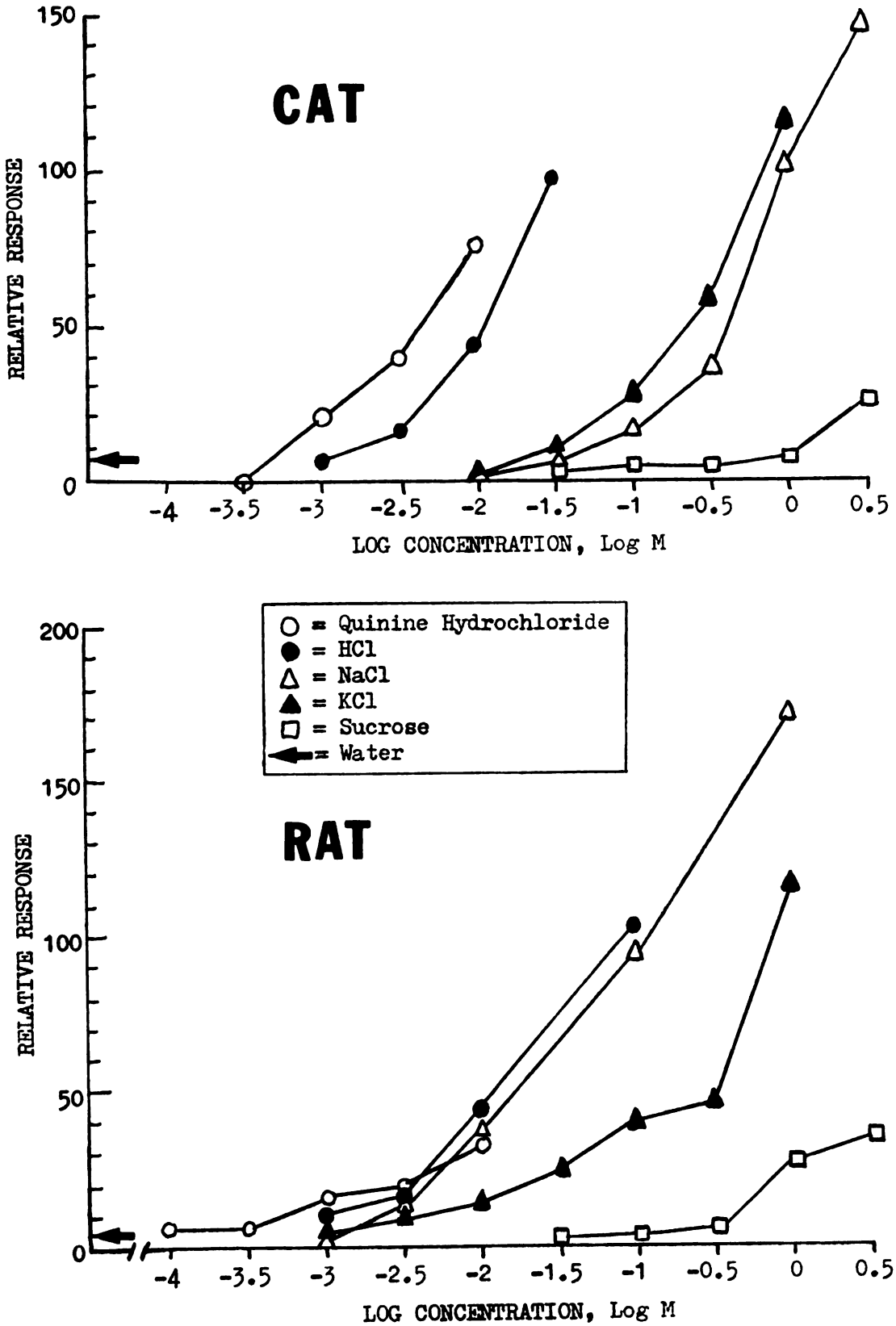

CAT
RELATIVE RESPONSE
150
100
50
0
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
0
0.5
LOG CONCENTRATION, Log M
○ = Quinine Hydrochloride
● = HCl
△ = NaCl
▲ = KCl
□ = Sucrose
◄ = Water
RAT
RELATIVE RESPONSE
200
150
100
50
0
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
0
0.5
LOG CONCENTRATION, Log M

Figure 5.

D-glucose, and D-galactose (in order of decreasing effectiveness). The disaccharides which contain these (plus a common glucose unit) are similarly ordered: sucrose, maltose, lactose. The water solubility of both the mono- and disaccharides, correlates directly with their stimulating effectiveness: (from highest water solubility and response magnitude to lowest) D-fructose, sucrose, D-glucose, lactose, D-galactose, lactose. However, as Andersen et al. (1963) point out, the physical property of water solubility itself can be only indirectly responsible and not itself the determiner of sweetness magnitude.

Water. Many species, such as the frog and the toad, exhibit a strong, positive response to distilled water. In several cases, water elicits action potentials of larger amplitude than those produced by chemical stimuli (Zotterman, 1949 and Andersson and Zotterman, 1950). Zotterman (1949), Kusano and Sato (1957), and Yamashita (1963) observed that for the frog, the stimulus-response curve of several electrolytes is U-shaped. (See Figure 6, NaCl and KCl.) The rise in the curves from -1.5 to -3.0 Log M concentration is explained by the ability for these particular electrolytes to depress the water response. Therefore, at weaker concentrations, these stimuli have less effect on the water response and the curve rises to the level of the water response. In contrast, the responses to $CaCl_2$ are greater than the magnitude of the water response for all tested concentrations. The $CaCl_2$ response curve, therefore, falls to the level of the water response at lower concentrations. The implications of the water response on the experimental protocol is considered in Appendix A,

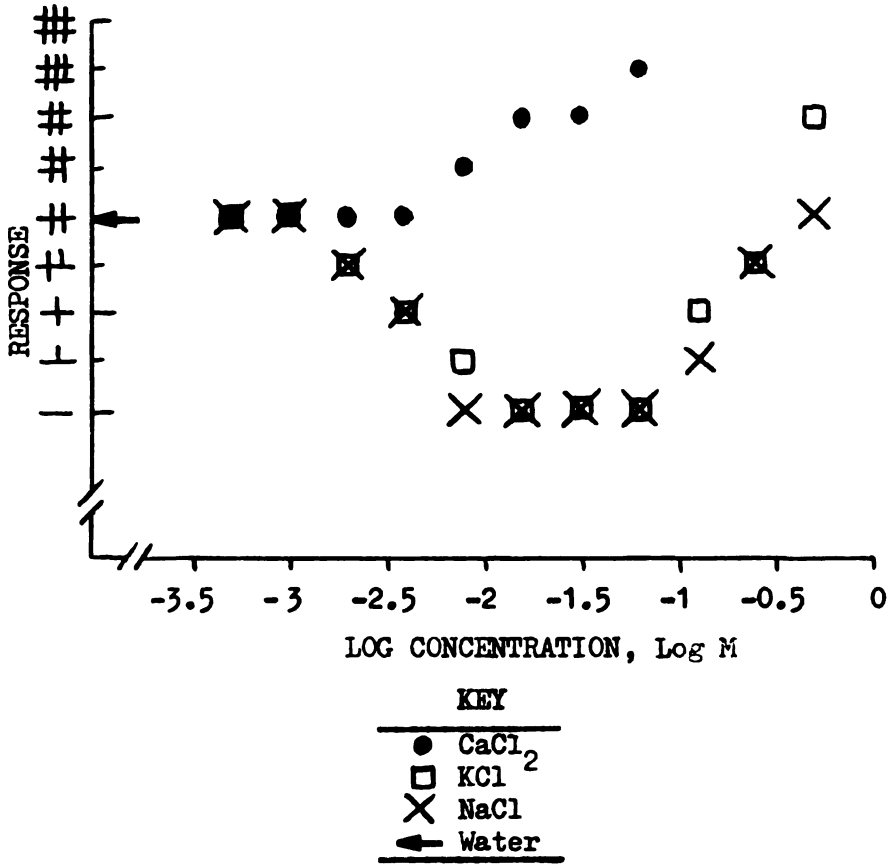


Figure 6. The water response and the influence of electrolytes. After Kusano and Sato, 1957, with response magnitude in the tally notation of these authors. The stimulus-response curves of NaCl, KCl, and $CaCl_2$ and the water response (arrow) are compared for the frog. The $CaCl_2$ curve rises above the level of the water response while the NaCl and KCl curves are U-shaped and fall below the water response level.

Table 2.

Implications about Perception

Electrophysiological experiments have been performed on the human chorda tympani. These have revealed that the magnitude of the summated whole nerve response is correlated with the perceived intensity of the taste stimulus. For example:

1. Both the electrophysiological and perceptual threshold for NaCl in the human are 0.01 M (Diamant et al., 1963).

2. Psychophysical adaptation and 95% electrophysiological adaptation occur at the same time after stimulation onset (Borg, Diamant, Oakley, Ström, and Zotterman, 1967, Borg et al., 1968, Diamant et al., 1965, Diamant and Zotterman, 1969).

3. Water at tongue temperature is not an effective perceptual taste stimulus nor a positive electrophysiological stimulus (Diamant et al., 1963 and Zotterman and Diamant, 1959).

4. The relative effectiveness of several sugars (0.5 M sucrose, fructose, maltose, galactose, lactose, glucose, 0.03 M sodium cyclamate, and 0.004 M sodium saccharide) is different among individuals while the psychophysical and electrophysiological orders of effectiveness are the same for any one person (Borg, Diamant, Oakley, Ström, and Zotterman, 1967, Borg et al., 1968, Diamant et al., 1965, Diamant and Zotterman, 1969).

These authors observe that most, if not all information about stimulus intensity is directly related to peripheral, neural activity and that even though more complex mechanisms involving the central nervous system can be imagined which would elicit perceptual intensity, none are needed.

In contrast, information about the quality of a taste stimulus has not readily been obtained from the whole nerve taste response. The gustatory response patterns of the human chorda tympani to different taste stimuli are not obviously dissimilar. Yet experiments

with gymnemic acid have demonstrated that the taste system is specific to taste quality at the level of the peripheral nervous system. This chemical, applied to the tongue, abolishes any perception of the sweet taste quality and eliminates the responses in the human chorda tympani to sucrose and sodium saccharide but not to NaCl, citric acid, or quinine hydrochloride (Borg, Diamant, Oakley, Ström, and Zotterman, 1967, Borg et al., 1968).

For Necturus, psychophysical evidence of the effects of taste stimuli is lacking. Nonetheless, it can be assumed that the electrophysiological response reflects the magnitude of its perceived gustatory sensation.

III. STATEMENT OF THE PROBLEM

Anatomical, Taste Bud Structure

According to Farbman and Yonkers (1971) the taste buds of the mudpuppy are found on the dorsal surface of the tongue in raised, round eminences with central concavities. However, these structures are sparsely distributed and could not be readily identified by several of the usual methods of examination. Serial histological sections examined with the light microscope confirmed that their distribution was sparse, only one taste bud being clearly identified in over thirty consecutive sections. Accordingly, scanning electron microscopy (SEM) was employed to examine the lingual surface in greater detail. The objective was to locate the round eminences with central concavities and to relate them to physical characteristics that could be seen with conventional, less powerful, stereoscopic viewing methods in future experiments involving stimulation or recording from a single taste bud.

Electrophysiological, Whole Nerve Recording

Whole nerve electrophysiological experiments were undertaken on the taste system of Necturus in order to find which concentrations of various chemicals were effective stimuli. Special attention was given to $CaCl_2$, realizing the frog's unique sensitivity to this

stimulus. Stimulus-response curves, derived for the chemicals, can be used later in electrophysiological studies of single fiber or receptor responses. A whole nerve electrophysiological examination is allowable and even required with knowledge of the number and distribution of the taste buds lacking. Similarly, whole tongue stimulation must be employed.

IV. METHODS

Anatomy

The tongue of a single animal was prepared for SEM in the following manner. The dorsal epithelium covering most of the distal, secondary tongue was removed in eight rectangular sections (8 X 12 mm). These were extensively washed with distilled water to remove as much mucus as possible. They were immediately fixed in a solution of 4% or 50% gluteraldehyde at 4 ^{o}C with the pH maintained at 7.4 by Sorenson's phosphate buffer. The samples were then freeze dried by cooling in isopentane which itself had been cooled in liquid nitrogen and then lyophilization of the sections completed in vacuum.

The fixed and freeze-dried sections were glued to glass cover slips, 12 mm diameter, for mounting in the SEM. The mounted sections were viewed under the light stereomicroscope to eliminate those too greatly covered with mucus (which now had a characteristic white, flaky appearance). Further examination revealed five samples with regions which appeared free of mucus as well as structures which might be discerned as the lingual eminences. The five were photographed and the areas noted for later identification under the SEM. Final preparation for viewing consisted of coating the sections with carbon, gold, and palladium. Observation under the SEM was accomplished at a range of magnifications, from 5X to 1800X. The lower power allowed correlation with the photographs from the

stereomicroscope.

After the SEM investigation, a live animal was examined as follows. The animal's tongue was extensively rinsed with water then partially dried and illuminated by a concentrated light source (fiber optic) directed at an acute angle to the lingual surface. The requisite elevations which could now be seen under the stereomicroscope were marked and the epithelium above the elevation dissected free without removing the dermal layers. Further examination of the non-fixed epithelium was by a standard Nikon binocular microscope at 400 X using transillumination and eosin staining.

Electrophysiology

Adult mudpuppies, 20 to 25 cm long and weighing 140-180 g, were anesthetised by two separate 1 ml administrations of 30gm% (w/v) urethan (ethyl carbamate solution) in amphibian Ringer's solution. The initial administration was a series of subcutaneous and intraperitoneal injections to reduce the animal's activity. The second administration, five to ten minutes later, was given intracranially via the foramen magnum and brought the animal to the surgical plane of anesthesia within 15 min. Dissection included exposure, transection, and desheathing the lingual branch of the ramus post-trematicus (IX) in the region of the lateral aspect of the hypobranchial 1 of the 1st branchial arch. Sufficient muscle was removed from this region to leave a small depression on the side of the head. The depression allowed for the placement of 22 G silver electrodes,

one on the exposed hypobranchial cartilage, and one about which the distal portion of the nerve was wrapped. The depression was then filled with mineral oil. The animal was grounded by inserting a 23 G stainless steel needle into the tissue of the lower jaw. A wire, soldered to the needle, connected the animal to the ground circuit of the preamplifier (Figure 7).

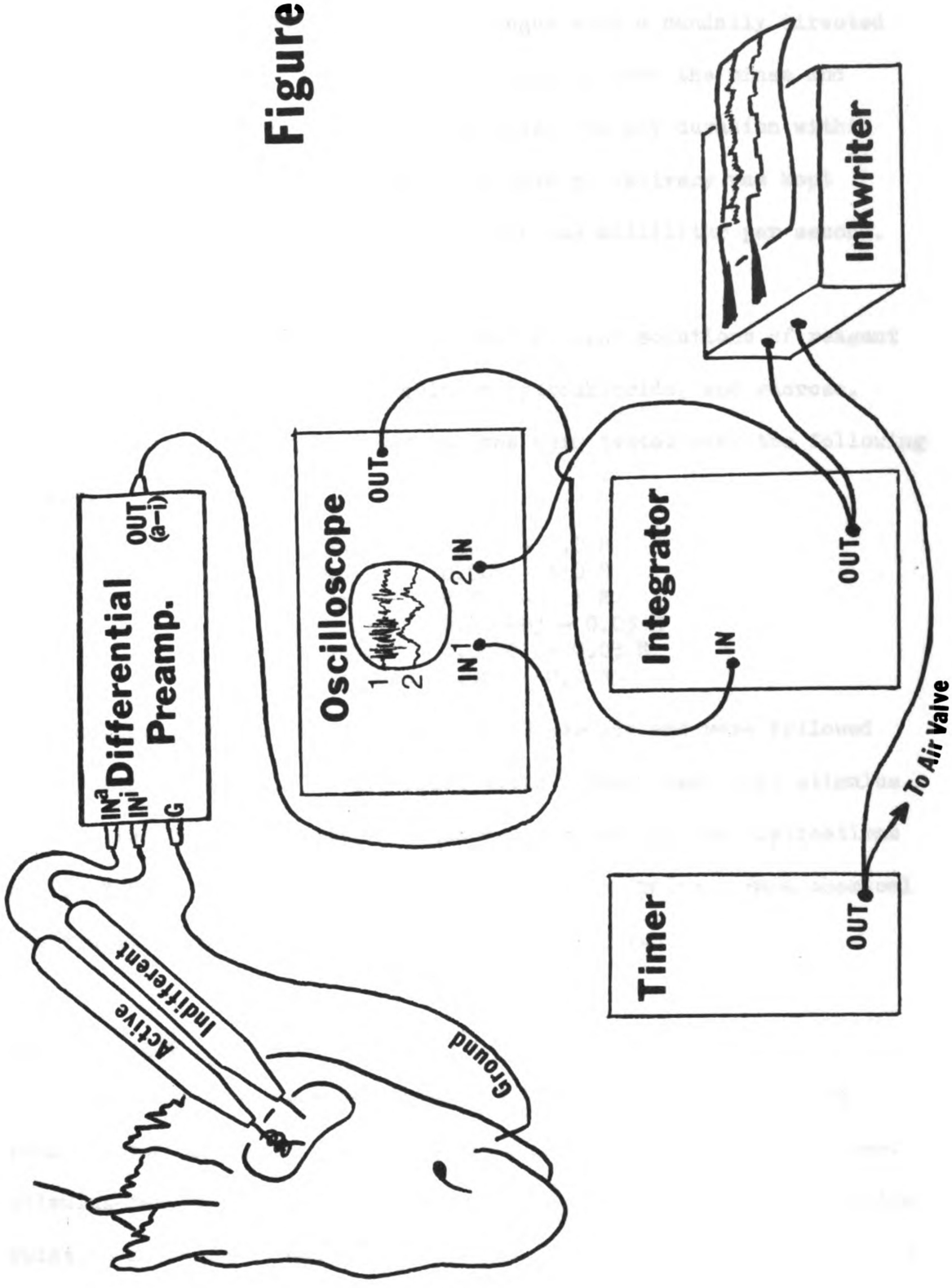
The neural activity was differentially amplified (X100) by a Grass P-15 preamplifier (Quincy, Massachusetts) with a bandwidth of 300 Hz - 3 KHz. The filtered activity was displayed on a Tektronix, Type RM 502-A, Dual-Beam oscilloscope (Beaverton, Oregon) and then fed into an electronic integrator. The integrator, of original design, was an electronic, resistance-capicitance (RC) circuit with variable capacitance (allowing the selection of one of several time constants) and with a variable gain amplifier. These features of the integrator allowed the choice of time constants of 0.05, 0.1, 0.2, 0.5, or 1.0 second as well as any greater value by the addition of external capacitance and also allowed the amplification of the summated activity, 1, 2, 5, 10, or 20 times. Time constants of 0.5 or 1.0 second were used. The summated activity was displayed on the second channel of the oscilloscope and a permanent record obtained from a Dynograph, Type 542, Amplifier-Recorder, inkwriter (Offner Electronics, Chicago, Illinois). The inkwriter also recorded the onset and offset of the stimulus.

The Stimulus Delivery System

The dual, gravity flow of pre-selected chemical solution and distilled water rinse entered a bi-directional air valve to leave by

Figure 7. Electronic equipment for gustatory whole nerve recording. Differential, AC amplification and summation of the activity from the two, 22 G silver electrodes (Active and Indifferent) is accomplished by the differential preamplifier, oscilloscope, and integrator. The electronic timer controls the air valve of the stimulus delivery system (Figure 8) and signals the delivery for display by the inkwriter.

Figure 7.


Differential Preamp.
IN a
IN i
G
OUT (a–i)
Oscilloscope
OUT
1
2
2 IN
IN 1
Integrator
IN
OUT
Inkwriter
Timer
OUT
To Air Valve
Active
Indifferent
Ground

separate outlets positioned over the animal's tongue and a waste bucket. After timer controlled intervals, the air valve changed positions, alternately flooding the tongue with a caudally directed flow of test solution or rinse. The timer allowed the rinse and stimulus periods to be independently fixed for any duration within a range from 0.2 to 300 seconds. The rate of delivery was kept minimal, above drop formation, less than one milliliter per second. (See Figure 8.)

The chemicals used as stimuli were aqueous solutions of reagent grade NaCl, KCl, CsCl, $CaCl_2$, quinine hydrochloride, and sucrose. $\frac{1}{2}$ Log dilutions of molar concentrations were tested over the following ranges.

NaCl	0.003 - 1.0 M
KCl	0.003 - 1.0 M
CsCl	0.001 - 0.1 M
$CaCl_2$	0.000003 - 0.03 M
$QHCl^2$	0.00001 - 0.03 M
Sucrose	0.001 - 0.3 M

All chemical stimuli were applied for 20 seconds and were followed by at least 40 seconds of distilled water rinse. Each test stimulus was applied three consecutive times and preceded by two applications of 0.1 M NaCl, which served as the reference stimulus. Each chemical was tested in the order of ascending concentration.

Data Analysis

The taste responses were quantified and standardized in the manner shown in Figure 9 and are then called the relative responses. Stimulus-response curves for each chemical were drawn from the median relative response at each concentration (from the interanimal median).

Figure 8. The stimulus delivery system. Panels A-C illustrate equipment which: A, allow the selection of a test solution (1, 2, or 3) and B, supply rinse (Dist. H_2O). Both liquids leave their reservoirs by gravity flow into C, where a valve driven by compressed air on signal from a timer, alternately directs one of the two liquids into the waste bucket or out of the test spout.

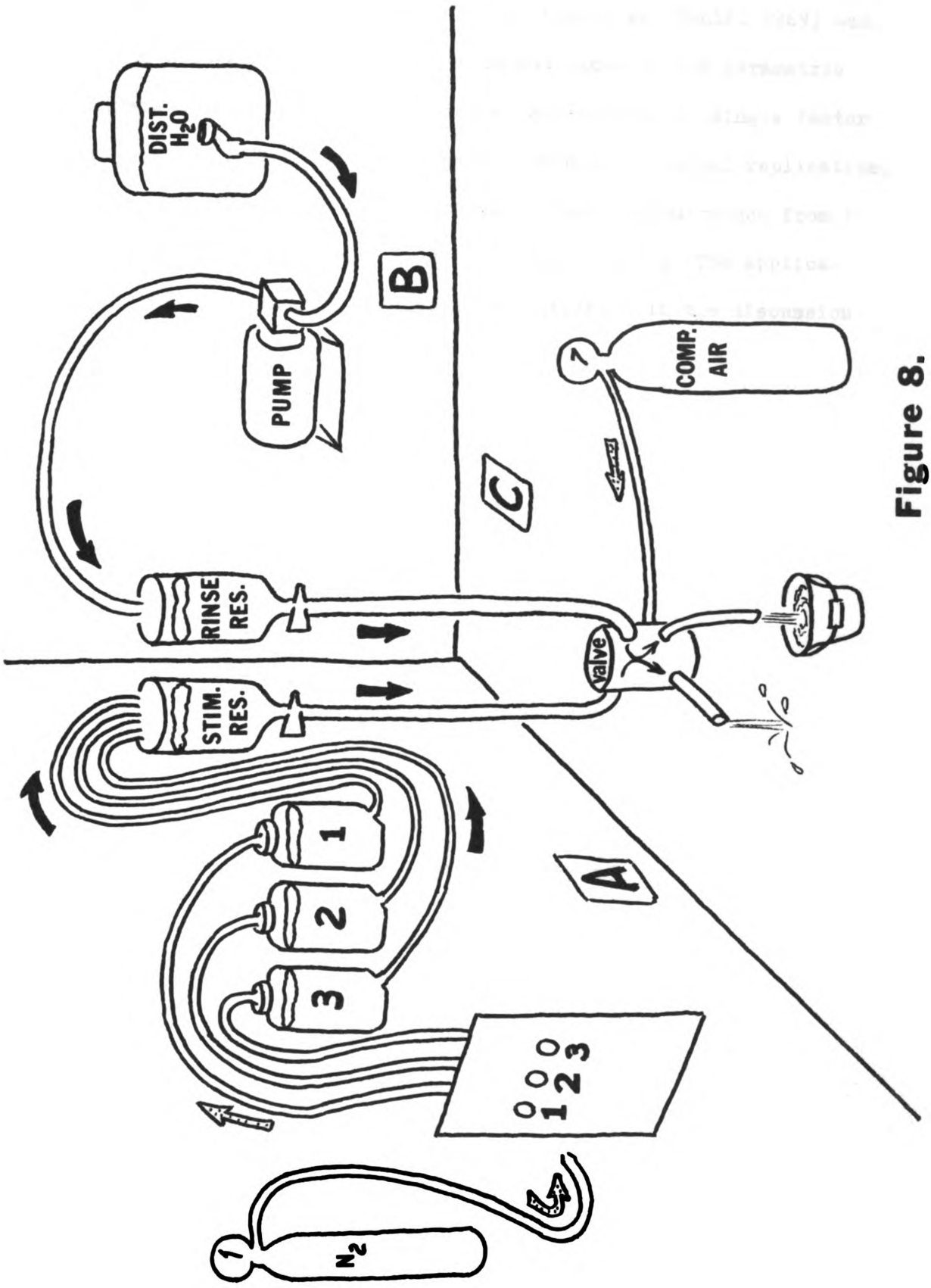
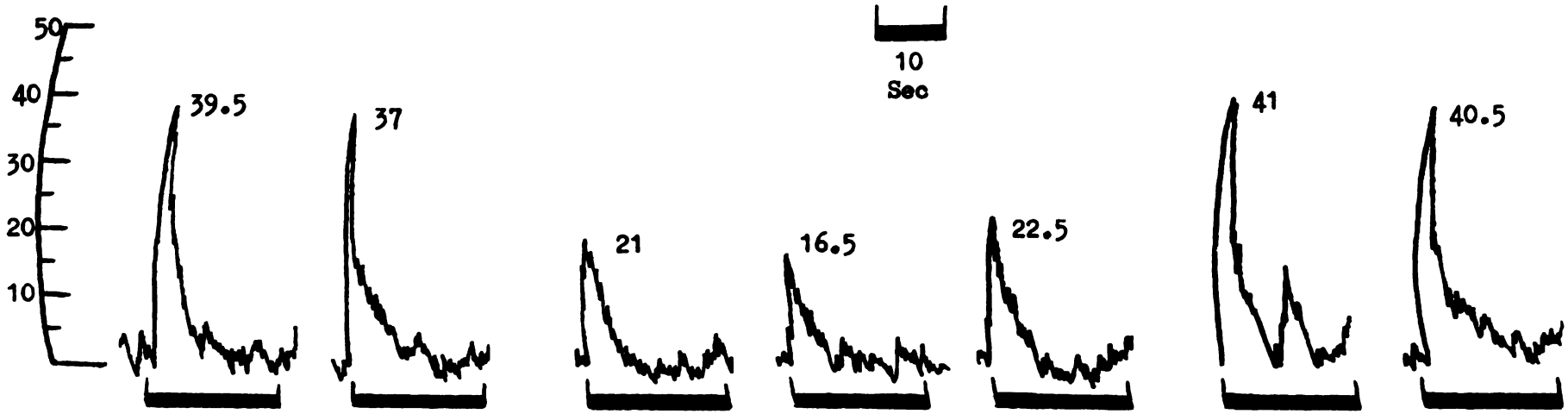

DIST. H_2O
PUMP
B
COMP. AIR
C
RINSE RES.
STIM. RES.
valve
1
2
3
A
0 0 0
1 2 3
N_2

Figure 8.

The non-parametric Kruskal-Wallis test (Sokal and Rohlf, 1969) was used in statistical analysis. It is analogous to the parametric analysis of variance for a non-nested and one-way or single factor analysis and is applicable for experiments with unequal replication. (The number of animals tested at each concentration ranged from 1 to 7 as noted on Tables 8, 9, and 10, Appendix B.) The applicability of non-parametric analysis is considered in the discussion section.

Figure 9. Calculation of the relative response. Successive traces of the integrated response to three applications of 0.003 M $CaCl_2$ are shown, preceded and followed by two applications of 0.1 M NaCl. All applications are for a period of 20 seconds. The horizontal bar marks the duration of stimulation. The rinse periods, omitted from this figure, were of at least 40 second duration. Numbers above each trace indicate response magnitude in arbitrary units. Relative response magnitude of the test stimulus is obtained by taking the median value and expressing it as a percentage of the median response to the standard stimulus, separately calculated before and after the test stimulus.



$$\text{RELATIVE RESPONSE} = \frac{\text{TEST MEDIAN}}{\text{OVERALL STANDARD MEDIAN}} \times 100 = (21.0/39.5)100 = 53.6$$

Figure 9.

V. RESULTS

Anatomy

Under the SEM, the 250 μ elevations described by Farbman and Yonkers (1971) could not be discerned on any of the sections at any magnification. One suspect area identified with the stereomicroscope was revealed as only a collection of circular depressions. Accordingly, examination was directed toward the central concavities of 20 - 30 μ diameter. Two types of structures, shown in Figures 10, 11, and 12, fulfilled the dimensional criteria. The first type (Figure 10) was observed with three other similar depressions in close proximity (within an area smaller than 200 μ). Moreover, examination of its interior revealed no discernable substructure, suggesting a duct-like opening. Figures 11 and 12 show the second type, with the requisite diameters, 29 and 22 μ respectively. These were not adjacent to other similar structures and their surface appearance reveals elements which may be cell borders and microvilli (0.1 μ diameter or less).

The search for the lingual eminences on a live animal was later successful after the tongue was extensively rinsed with water and then partially dried. The non-fixed lingual epithelium from a requisite elevation revealed a structure whose appearance closely resembled that shown in Figure 12. Its inner diameter was 28 μ and it was seen on flat epithelium, the elevation having disappeared on dissection from the dermal layers. In its center were denser

Figure 10. Structures of the lingual surface of Necturus. Sections of the tongue of the mudpuppy viewed under the SEM revealed two types of depressions. The structure below resembles a duct-like opening and has no apparent substructure to its interior. The section was fixed in 50% gluteraldehyde.

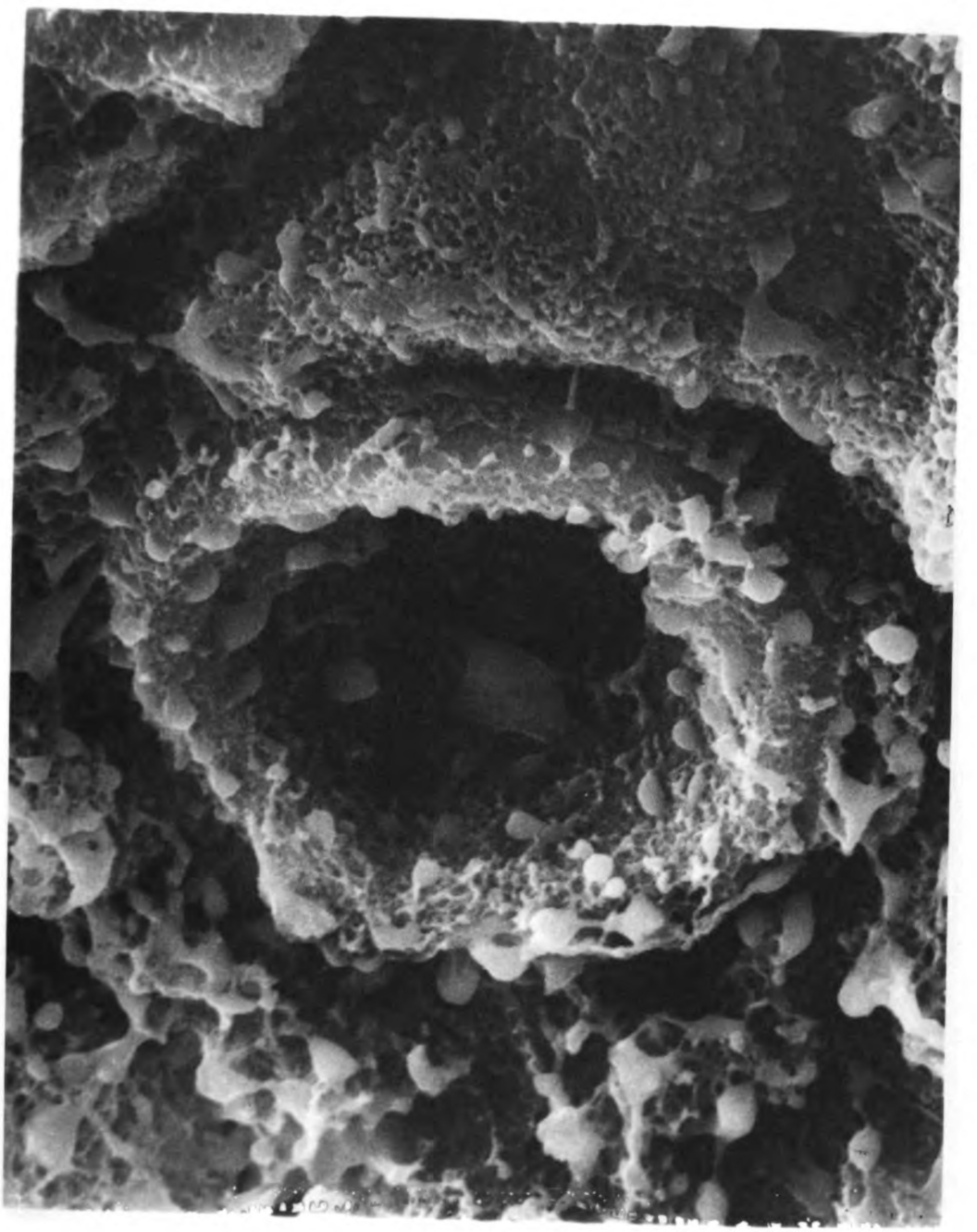


FIGURE 10.

Figure 11. Structures of the lingual surface of Necturus. Sections of the tongue of the mudpuppy viewed under the SEM revealed two types of depressions. The structure below shows considerable differentiation about its center. The section was fixed in 50% gluteraldehyde.

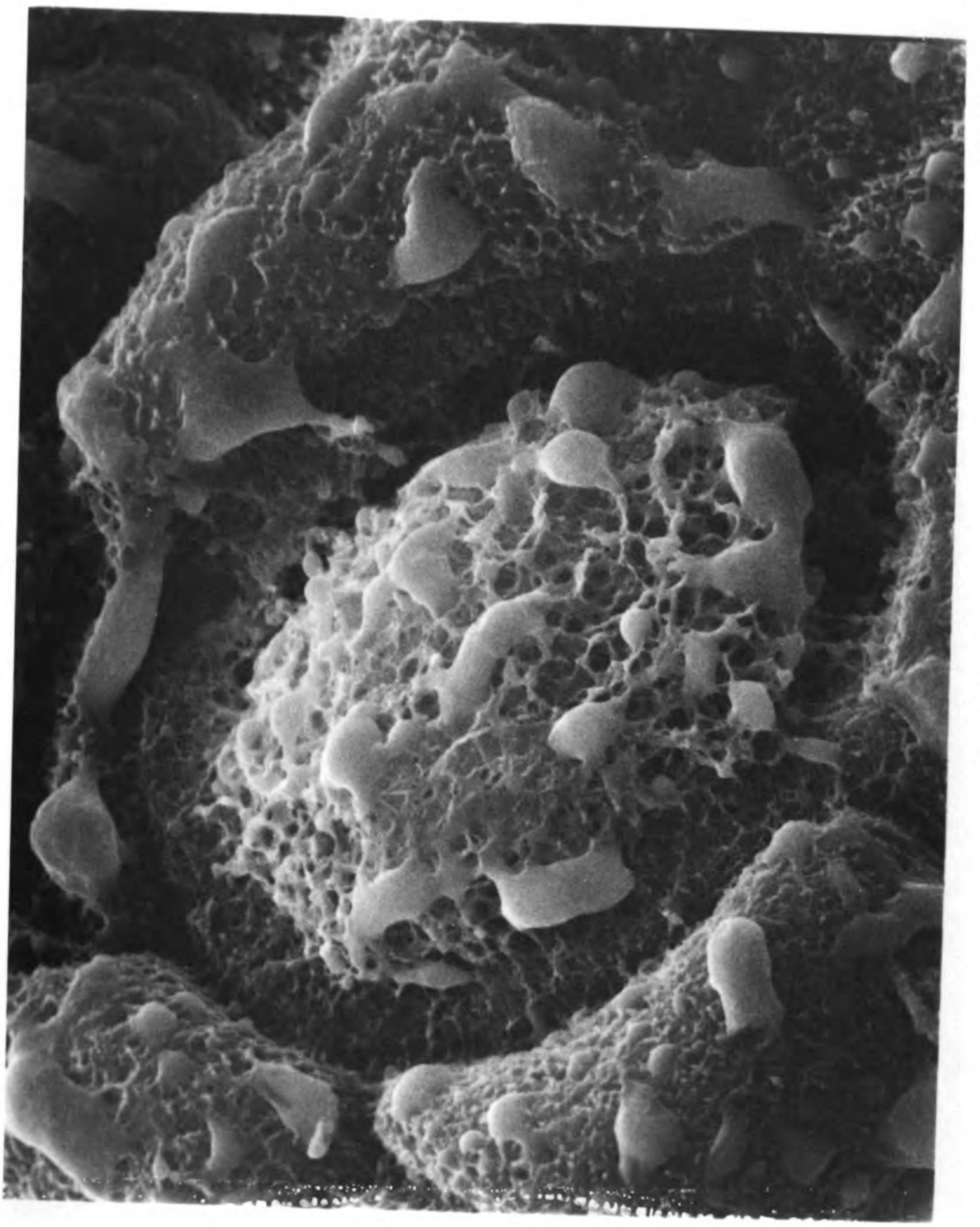


FIGURE 11.

Figure 12. Structures of the lingual surface of Necturus. Sections of the tongue of the mudpuppy viewed under the SEM revealed two types of depressions. The structure below shows considerable differentiation about its center. The section was fixed in 4% gluteraldehyde.

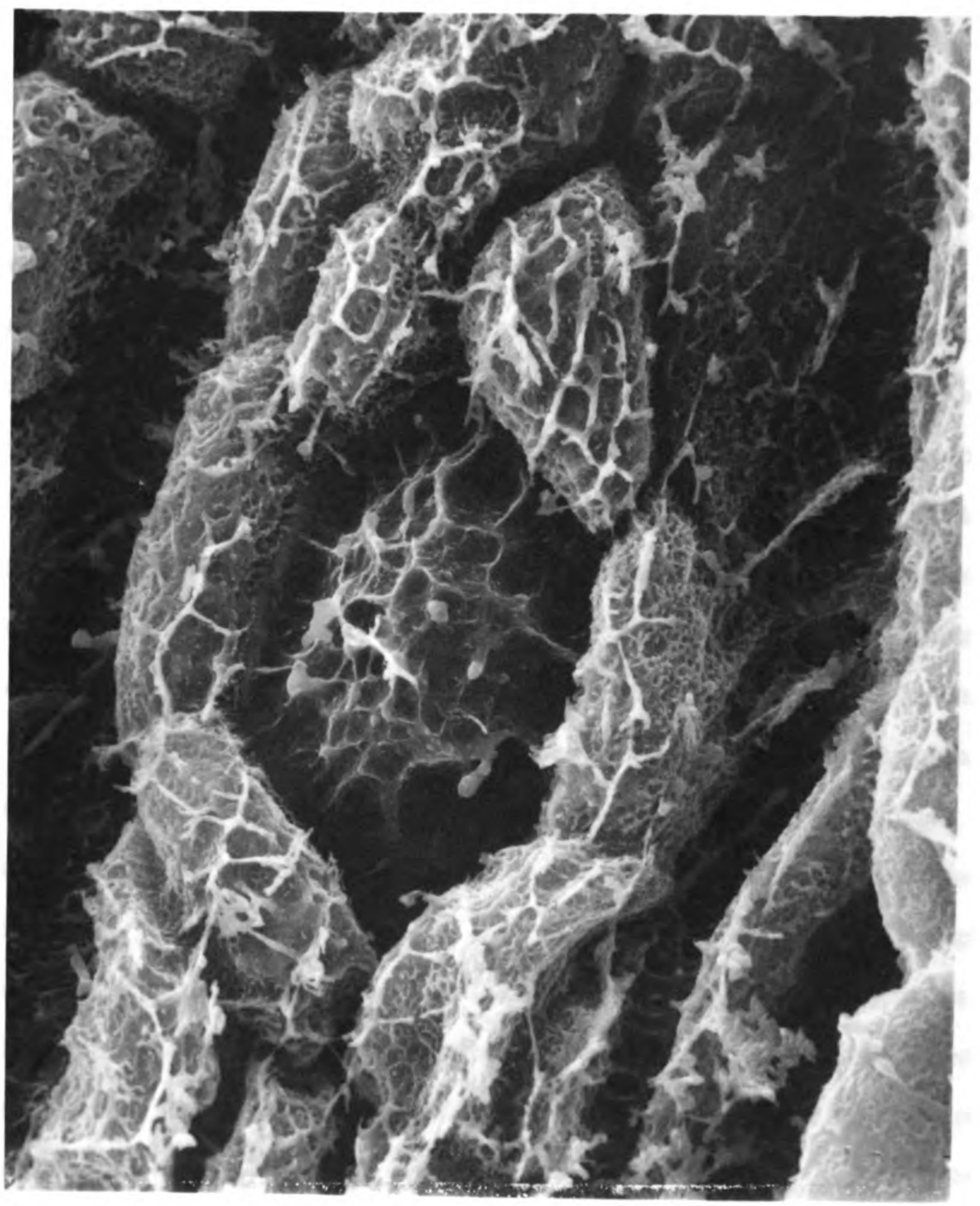


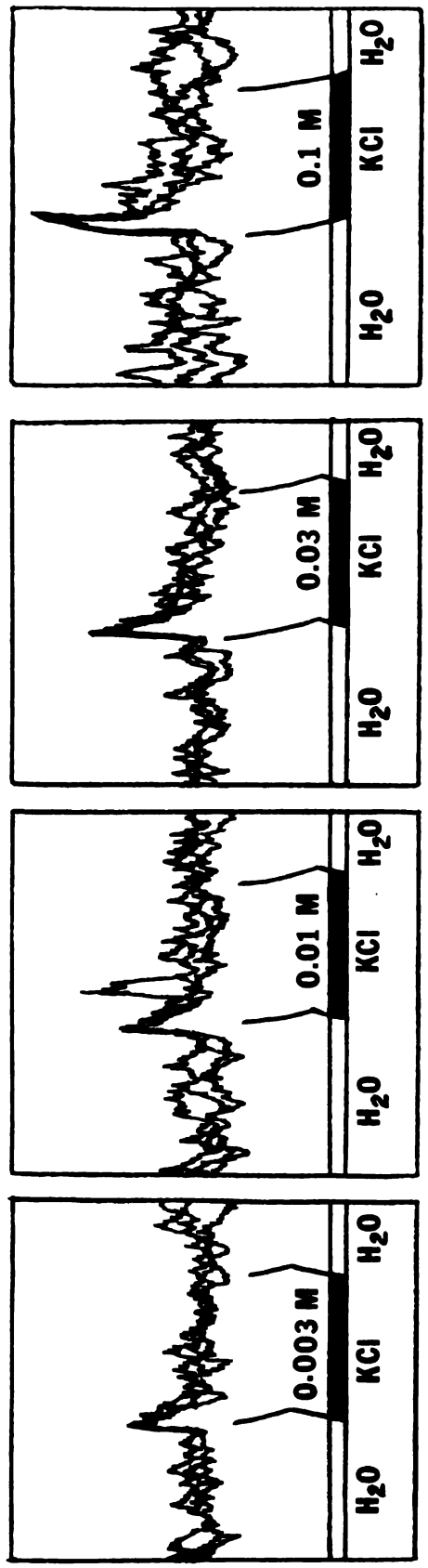
FIGURE 12.

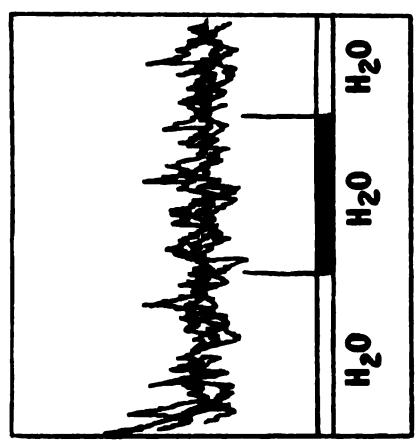
markings resembling the web-like lighter divisions seen on the central depression of Figure 12.

Electrophysiology

Responses were recorded to mechanical stimulation over the entire secondary (distal) tongue. Tactile stimulation was always more effective than gustatory. (Responses to tactile stimulation could be recorded from a fatigued preparation with no gustatory activity.) The standard test solution (0.1 M NaCl) always resulted in a consistent, positive response and indicated the gustatory sensitivity of the preparation. Suprathreshold stimuli produced a summated pattern with only a peak transient phase. This is illustrated in Figure 13 which shows overlapped records from three successive tests of KCl solutions and water. The peak transient phase is the most coincident portion of the otherwise irregular pattern and its magnitude is proportional to stimulus concentration. The application of distilled water to the dormant (non-stimulated) tongue produced a moderate peak transient phase (with a magnitude of 82.5 relative response units) which remained at a level above resting discharge during continued rinsing. This water adapted activity served as a baseline for determining gustatory thresholds. The absence of water responses to the water adapted tongue can be seen from the random character of the tracings when water is tested after a rinse (lower boxes of Figure 13). Similarly, no water response is seen after adaptation to the KCl solutions (at the onset of the water rinse

Figure 13. Superimposed traces of the integrated neural response of *Necturus* to KCl stimulation. Each panel in the upper row contains three records of the repeated application of a single concentration of a KCl solution, each preceded and followed by the application of distilled H_2O. The taste response can be seen as a consistent peak transient phase whose magnitude is proportional to stimulus concentration. Distilled H_2O produced no consistent response after prior application of H_2O (lower panel) or after the KCl stimuli (upper panels).


0.003 M
KCl
H2O
H2O
0.01 M
KCl
H2O
H2O
0.03 M
KCl
H2O
H2O
0.1 M
KCl
H2O
H2O


H2O
H2O
H2O

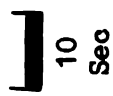

10
Sec

Figure 13.

after the KCl tests, upper boxes of Figure 13).

The stimulus-response curves of all chemicals tested are shown in Figure 14. HCl was the most effective stimulus at all concentrations. The order of effectiveness for the other solutions varies with concentration. For example, the decreasing order of effectiveness of -2.0 Log M solutions is: H^+, Na^+, Ca^{++}, K^+, quinine, Cs^+, and sucrose. At -1.5 Log M, the order is: H^+, quinine, Ca^{++}, Na^+, K^+, Cs^+, and sucrose. The order at threshold is: H^+, $Ca^{++} = K^+$, Na^+, quinine, Cs^+, and sucrose. Sucrose is least effective at all concentrations.

A modified stimulus-response curve for quinine hydrochloride, Figure 15, shows the interanimal variation of the median relative responses. A non-parametric measure of this is the interquartile range, $Q_1 - Q_3$, which is drawn about the median response to quinine hydrochloride (Q_2, QHCl in Figure 15). The water response variation is represented horizontally across the bottom of the graph.

Saturation response levels could not be determined. Greater concentrations than 0.01 N HCl or 0.3 M NaCl resulted in a relative response magnitude greater than 1,000 on the initial application. This extreme response had a different pattern. The peak transient phase was followed by a prolonged response of greater magnitude which persisted throughout the 40 second rinse period. After extreme responses for a single test series, the preparation was entirely non-responsive to further chemical stimulation. The chemical insensitivity continued despite extended rinsing (two hours) or extended rinsing and rest (cessation of all lingual stimulation). This loss of taste response affected both right and left glossopharyngeal nerves and continued

Figure 14. Gustatory stimulus-response curves for *Necturus*. These are shown for all chemicals tested (*QHCl* = quinine hydrochloride, *Sucr.* = sucrose). The interquartile range of the response to water (baseline activity) is indicated by the gray bar.

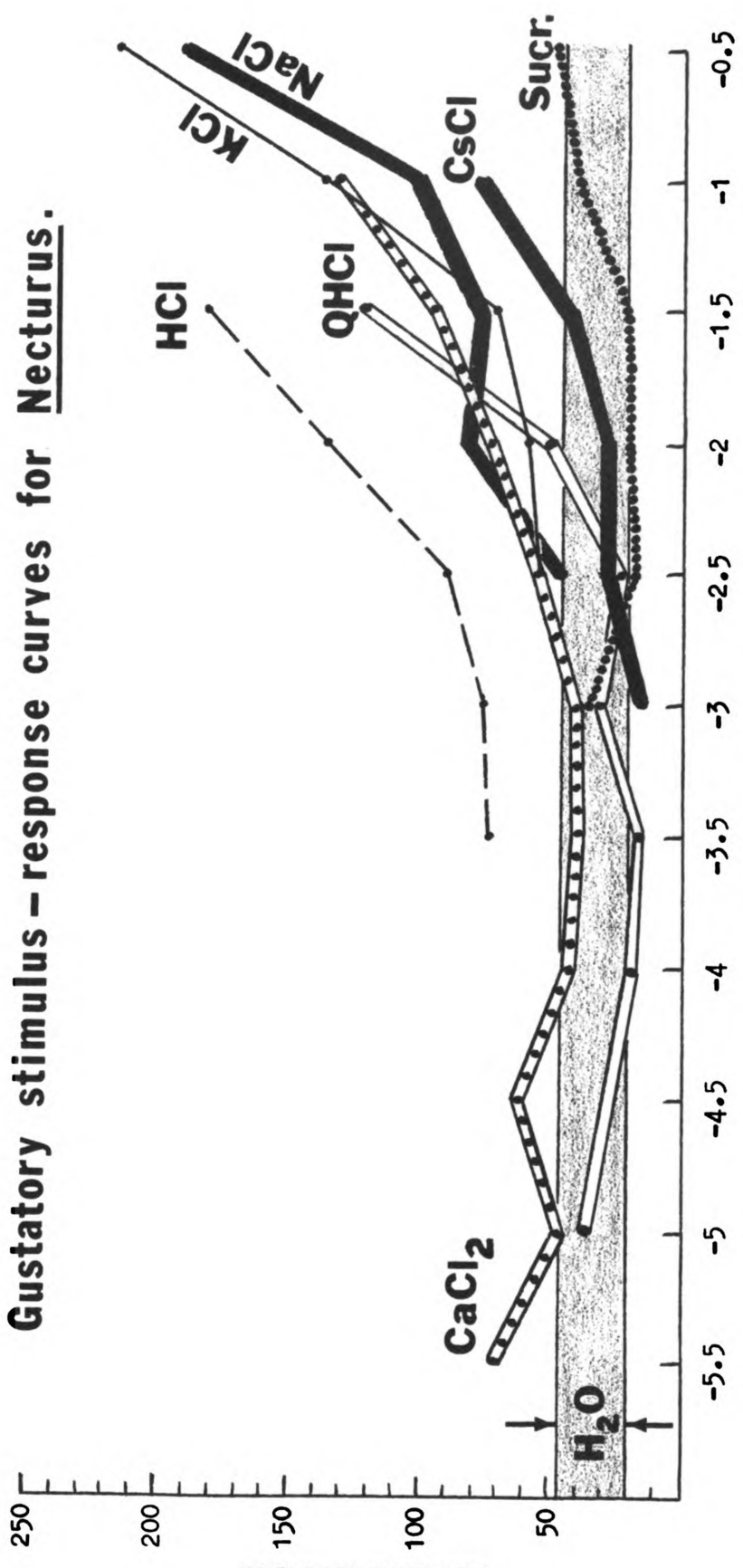

Gustatory stimulus – response curves for Necturus.
HCl
KCl
NaCl
QHCl
CsCl
Sucr.
CaCl2
H2O
RELATIVE RESPONSE
250
200
150
100
50
-5.5
-5
-4.5
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
LOG CONCENTRATION, Log M

Figure 14.

Figure 15. A modified gustatory stimulus-response curve for quinine hydrochloride (QHCl) for Necturus. The median relative response from all animals tested is shown at each concentration (Q_2, QHCl). The interanimal variation is shown as the interquartile range (Q_3 to Q_1, QHCl) at each concentration. The median response to water and its interquartile range are also shown (gray bar). For the replication at each concentration, see Tables 8-10.

A modified gustatory stimulus–response curve
for quinine hydrochloride (QHCl) for Necturus.

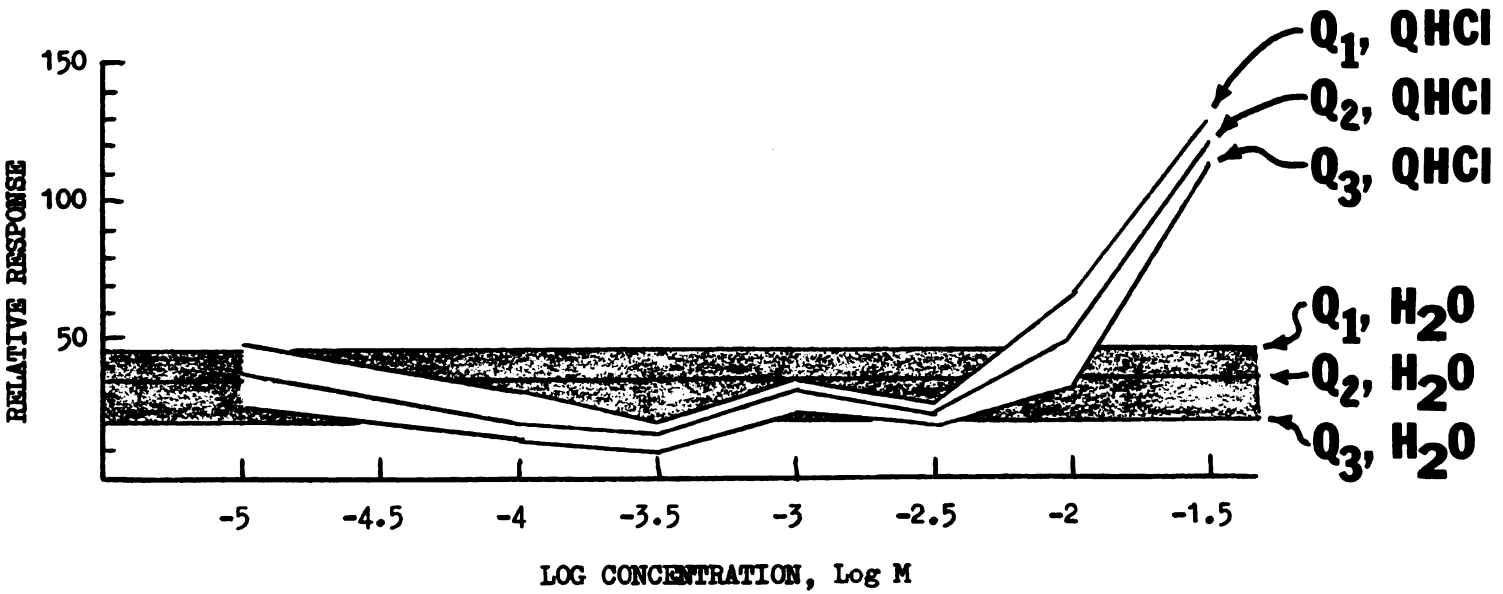


Figure 15.

despite the visible evidence of maintained blood circulation to the tongue and continued responsiveness to tactile stimulation.

VI. DISCUSSION

Anatomy

No physical characteristics of the eminences other than those described by Farbman and Yonkers (1971) were discerned which aid in their identification. The dermal layers were found to be necessary for the appearance of the elevation (and possibly the dermal papillae described by Farbman and Yonkers). The properties of the structures identified under the SEM (Figures 11 and 12), their differentiated central depression which may contain microvilli or cell borders, and their isolated distribution support the conclusion that they are the taste buds of Necturus. The later identification of an elevation with analogous central structure lends greater support to this conclusion.

Electrophysiology

Non-normality of the Taste Response

Testing the assumptions required for the use of parametric analysis revealed that non-parametric analysis was appropriate. Even though the distribution of the data was not significantly non-parametric, and the variance was not significantly heterogeneous, independence testing disallowed the use of parametric analysis. (See Appendix B.) To test the randomness or independence of the responses, a Chi-square test for goodness of fit (Sokal and Rohlf, 1969) was employed. This compared the observed frequency of the rank order of

responses within each test series with the expected equal occurence of the random permutations of rank order. The results were significant (at $p = 0.005$) leading to a rejection of the hypothesis that the responses were independent.

Effectiveness of Stimuli

Water applied to the dormant (non-stimulated) tongue was a less effective stimulus than 0.1 M NaCl (82.5 relative response units). In contrast, for the frog, Kusano and Sato (1957) and Yamashita (1963) reported that water is more effective than 0.1 M NaCl (see Figure 6). Water applied to the water-adapted tongue or to the tongue which has been adapted to an electrolyte solution is not an effective stimulus. A water response could be expected under the latter condition with the removal of an electrolyte which depresses a water response of large magnitude (such as KCl for the frog, Kusano and Sato, 1957).

Several of the Kruskal-Wallis tests (Sokal and Rohlf, 1969) were significant ($p = 0.05$) though low replication negated many others. For example, only one significant contrast involved CsCl which was tested on only one animal. On the other hand, if the replication of either stimulus was at least three animals, a difference of 45 relative response units between the contrasted stimuli resulted in a significant Kruskal-Wallis test. This power increased with greater replication. The contrasts between -2.5 Log M KCl and quinine hydrochloride, with replication of 5 and 6 animals respectively, was judged significant with a relative response difference of only 30.

HCl showed significantly greater effectiveness than the other stimuli at -3.0, -2.0, and -1.5 Log N concentrations. The pH threshold for Necturus is greater than 3.5. This resembles the findings of Konishi and Zotterman (1963) for the carp whose facial nerve responded to acetic acid of pH 3.8.

The relative effectiveness of $CaCl_2$ was less than Kusano and Sato (1957) and Yamashita (1963) reported for the frog. The frog's greater sensitivity to $CaCl_2$ may be due in part to its greater sensitivity to water. If $CaCl_2$ does not depress the water response, aqueous solutions of $CaCl_2$ may elicit activity by both the $CaCl_2$ solute and water. The order of effectiveness among the other stimuli differed with concentration. KCl was significantly more effective than quinine hydrochloride at -2.5 Log M while the opposite was true at -1.5 Log M. However, the precise order observed from the stimulus-response curves was generally not significant. Several authors found a varying order of effectiveness among several stimuli at different concentrations (Kusano and Sato, 1957 and Yamashita, 1963). Figure 5 (from Pfaffmann, 1955) shows a different order among quinine hydrochloride, HCl, KCl, and NaCl at -3.0 and -2.5 Log M concentrations for the rat. The cation's order of stimulating effectiveness for the mudpuppy (as determined by threshold from the stimulus-response curves) is compared with several other animals in Table 4, Appendix A.

Table 6, Appendix A shows the thresholds for the stimuli which in man evoke the four classic taste qualities. Though the threshold values vary greatly among the different animals, their ascending order is consistent: bitter, acid, salt, sweet. The two animals which show noteable exception, the rabbit and mudpuppy, reflect the difficulty of

using this anthropomorphic classification. Pfaffmann (1955) observed the rabbit's strong positive response to water. Accordingly, if threshold is defined with respect to the water response, the values for the rabbit are abnormally high. Furthermore, the order of effectiveness for these stimuli varies with concentration, therefore, an unusual supra-water order of threshold results: HCl, quinine hydrochloride, sucrose, and NaCl (at -2.0, -2.0, -0.5, and -0.3 Log M respectively). Accordingly, the values for the rabbit in Table 6 are the minimum effective concentrations. The rabbit and Necturus show an unusually high threshold for quinine. This may arise for several reasons: 1) their "bitter" threshold is unusually high, 2) quinine hydrochloride is a relatively ineffective stimulus among those used to represent the bitter taste quality, or 3) quinine hydrochloride may not taste "bitter" to these animals.

VII. SUMMARY

Anatomy

1. No physical characteristics other than the descriptions and dimensions given by Farbman and Yonkers (1971) could be found which further aid in locating the lingual eminences or their central concavities.

2. Observation of the eminences involves all of the following:

   a. extensively rinsing the tongue, followed by

   b. partial drying of the lingual surface,

   c. with surface illumination from a concentrated light source directed at an acute angle for best visual relief of the surface features,

   d. using the dimensional criteria established by Farbman and Yonkers (1971) to identify the surface eminence and/or central concavity.

Further histological examination of the lingual surface, using light or electron microscopic technique is needed:

1. to confirm the description of the lingual eminence,

2. to reveal whether all eminences are associated with buds, and conversely, that buds occur exclusively within an eminence,

3. to identify the number and distribution of taste buds on the lingual surface to correlate with future single fiber electrophysiology.

Histological examination should also be directed toward the lingual innervation in order to identify the number and diameter of nerve fibers:

1. which terminate within a bud,

2. which enter the sub-gemmal plexus,

3. which terminate in non-differentiated epithelium (between taste buds), and

4. which constitute the nerve trunk (ramus lingualis IX) in the region of electrophysiological recording at the branchial arch.

Electrophysiology

1. HCl is a more effective stimulus than several salts, quinine hydrochloride, and sucrose.

2. The order of effectiveness of the electrolytes and quinine varies with concentration, while sucrose is least effective at all concentrations.

3. Suprathreshold concentrations of electrolytes do not result in a saturation level of responses with increasing concentration, but elicit a response of extreme magnitude followed by loss of chemical sensitivity.

4. Among the classical four taste stimuli, Necturus, like the rabbit, has an abnormally high threshold for quinine hydrochloride.

5. Water is a relatively non-effective taste stimulus for Necturus.

6. $CaCl_2$ is a less effective gustatory stimulus for the mudpuppy than has been reported for the frog.

Whole nerve electrophysiological experimentation can be employed in future experiments to:

1. determine the role of the VIIth and Xth cranial nerves in the taste system of Necturus.

2. describe any regional taste sensitivity of the tongue and oral cavity, and

3. define the stimulating effectiveness of other stimuli (amino acids, other sugars and electrolytes).

APPENDICES

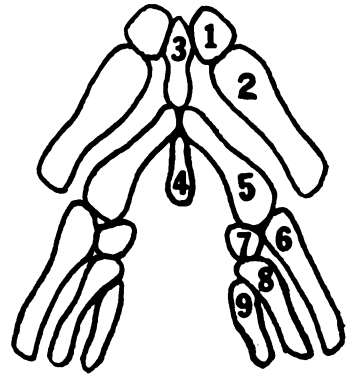
APPENDIX A

TABLES

Table 1. Visceral skeleton nomenclature. The visceral skeleton, with the mandible, defines the ventral floor of the oral-pharyngeal cavity. Despite the structural homology among the visceral skeletons of the animals listed below, the authors have applied different nomenclatures to their cartilaginous elements. These are keyed to the illustrated visceral skeleton of Necturus.

Table 1.

Visceral skeleton nomenclature.



	Drüner (1901) Necturus	Francis (1934) Salamandra	Weichert (1958) Necturus	Stuart (1940) Necturus	Romer (1962) Proteus
1.	Hypohyale	Hypo-hyal (first copular radial)	Hypohyale	Hypohyal	(Part of Ceratohyal.)
2.	Ceratohyale	Cerato-hyal	Ceratohyal	Cerato-hyal	Ceratohyal
3.	Copula	Copula	1st Basibranchial	Copula (Basibranchial)	Basihyal
4.	Copulastiel (Copular stem)	(Not present in Salamandra.)	2nd Basibranchial	(Omitted.)	Basibranchial
5.	Hypobranchiale 1	Hypobranchial I	1st Ceratobranchial	Cerato-branchial (3rd arch)	Hypobranchial I
6.	Ceratobranchiale 1	(Part of Hypo-branchial I.)	1st Epibranchial	Epibranchial	Ceratobranchial I
7.	Hypobranchiale 2	Hypobranchial II	2nd Ceratobranchial	Cerato-branchial (4th arch)	(Not labelled.)
8.	Ceratobranchiale 2	(Part of Hypo-branchial II.)	2nd Epibranchial	Epibranchial (4th arch)	Ceratobranchial II
9.	Ceratobranchiale 3	(Not present in Salamandra.)	3rd Epibranchial	Epibranchial (5th arch)	Ceratobranchial III

Table 2. Whole Nerve recording protocol. The Following procedures have been incorporated into whole nerve recording procedure to control the variables listed below.

Procedure	Effect	Reference
1. Desheathing (removal of fat, connective tissue or the epineurium).	Maximize the signal to noise ratio.	Kitchell, 1963.
2. Mineral oil emersion of nerve and electrodes.	Maximize the signal to noise ratio, lengthen the viability of the preparation.	Kitchell, 1963.
3. Optimal time constant of integration.	Observe rapid changes in the pattern of the response.	Kitchell, 1963.
4. Maintain stimulus and rinse solutions at tongue temperature.	Minimize thermal stimulation.	Kitchell, 1963.
5. Use a non-stimulating chemical or solution for rinse and solvent of test solutions.	Maximize responses to test solution.	Kitchell, 1963.
6. Apply stimuli and rinse with minimum force and constant pressure.	Minimize mechanical stimulation.	Kitchell, 1963.
7. Express responses to test solutions as a per cent of the response to the standard solution.	1. Minimize the contributions of the responses to other sensory modes. 2. Minimize the effects of small changes in the responsiveness of the preparation. 3. Allow interspecies correlations.	Beidler, Fishman, and Hardiman, 1955.

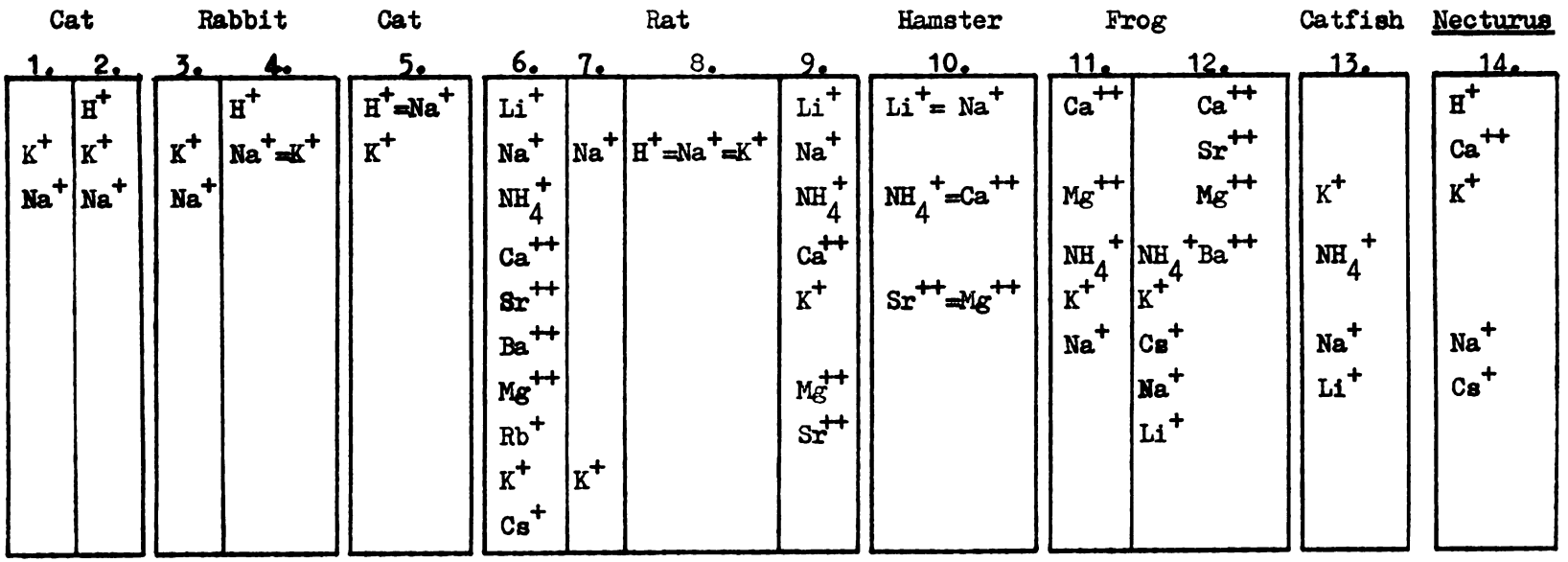
Table 3. Gustatory electrophysiological thresholds for acid. (Tested with HCl unless otherwise indicated.)

Animal	Minimum Effective Concentration*	Reference
Cat	-3.0	Pfaffmann, 1955
Rat	-3.5	Pfaffmann, 1955
Rabbit	-3.0	Pfaffmann, 1955
Calf	-3.0	Bernard, 1964
Hamster	$<$-2.0	Beidler et al., 1955
Guinea pig	$<$-2.0	Beidler et al., 1955
Dog	-2.0	Beidler et al., 1955
Carp	(acetic acid, pH = 3.8)	Konishi and Zotterman, 1963
Human	-3.0 (citric acid)	Borg, Diamant, Ström, and Zotterman, 1967, Borg et al., 1968, Diamant et al, 1963, Diamant and Zotterman, 1969

* Expressed as Log Normal concentration.

Table 4. The rank order of stimulating effectiveness of cations. The cations listed below are ordered with respect to their gustatory stimulating effectiveness for several animals. The most effective stimuli are placed at the top of each column. The vertical spacing does not signify the degree of relative effectiveness but is included for better comparison of similarly ranked ions. The letters, A-E, following each reference, indicate each author's method of determining the relative effectiveness of the taste stimuli. All cations were tested as the chloride salts.

Table 4. The rank order of stimulating effectiveness of cations.



Methods of determination:

A = 0.1 M test/0.1 M NaCl

B = Threshold comparison.

C = Comparison of responses to 0.5 M concentrations.

D = Comparison for a range of concentrations.

E = Comparison for a range of concentrations, standardized to 0.1 M NaCl response.

1. Pfaffmann, 1953--B
2. Pfaffmann, 1955--B
3. Pfaffmann, 1955--B
4. Pfaffmann, 1955--B
5. Bernard, 1964----E
6. Beidler, 1953----A
7. Pfaffmann, 1953--B
8. Pfaffmann, 1955--------B
9. Fishman, 1957----------C
10. Fishman, 1957----------C
11. Kusano and Sato, 1957--D
12. Yamashita, 1963--------C
13. Tateda, 1961----------D
14. Samanen----------B

Table 5. The rank order of stimulating effectiveness of anions. The anions listed below are ordered with respect to their stimulating effectiveness for several animals (the most effective stimuli at the top of each column). All anions were tested as the sodium salts. See Table 4 for further explanation.

Table 5. The rank order of stimulating effectiveness of anions.

Rat	Frog		Catfish	Carp
1.	2.	3.	4.	5.
$Cl^- = Br^-$	Citrate	Br^-	NO_3^-, Cl^-, Citrate, Br^-	
NO_3^-	OH^-	I^-	(Varied with concentration.)	
Citrate	Saccharide	Cl^-		Acetate
$SO_4^=$	NO_3^-	Saccharide		Cl^-
$CO_3^=$	Cl^-	SCN^-		
	Acetate	NO_3^-		
	HCO_3^-	F^-		
		HCO_3^-		
		Glutamate		

1. Beidler, 1953----------A
2. Kusano and Sato, 1957--------D
3. Yamashita, 1963----------C
4. Tateda, 1961----------D
5. Konishi and Zotterman, 1963--D

A,C,D = Methods of determination (Table 4.)

Table 6. Gustatory electrophysiological thresholds for the four classic stimuli. The electrophysiological thresholds (Log concentration) for the stimuli which evoke the four taste qualities in man are shown for several animals

Table 6. Gustatory electrophysiological thresholds for the four classic stimuli.

	-4.0	-3.5	-3.0	-2.5	-2.0	-1.5	-1.0	-0.5	0.0	
Catfish (Tateda, 1961)		H_a					N			(ø,ß)
Human (Borg, Diamant, Ström, and Zotterman, 1967, Borg et al., 1968, Diamant et al., 1963, Diamant and Zotterman, 1969.)			H_c		N		S			(ø)
Cat (Pfaffmann, 1955)		Q	H			N			S	
Rabbit (Pfaffmann, 1955)			H	N	Q		S			
Calf (Bernard, 1964)			H	N						(ø,ß)
Necturus		H		N	Q			S		
Rat (Pfaffmann, 1955)	Q	H		N				S		

LOG CONCENTRATION, Log M

KEY

S = Sweet (sucrose), H = Sour (HCl), H_a = Sour (acetic acid), H_c = Sour (citric acid), N = Salty (NaCl), Q = Bitter (quinine hydrochloride), (/) = Threshold not determined.

APPENDIX B

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

Non-normality of the Taste Response

The use of parametric analysis requires evidence that three assumptions are valid: 1) The data are normally distributed. 2) The variances within each independent variable are equal (homogeneous). 3) The results are independent (occur randomly).

The Shapiro-Wilk W-test for normality (Shapiro and Wilk, 1965) did not reveal non-parametric distribution of the responses whether analyzed 1) across several concentrations of one chemical for one animal, 2) for the same concentration of several different chemicals for the same animal, or 3) for one concentration of one chemical across several animals ($p = 0.01$, 0.05, or 0.10).

Bartlett's test for homogeneous variances (Sokal and Rohlf, 1969) revealed no differences among different chemicals at 0.003 M (comparing the variances of all animal's responses for each chemical at the above concentration).

The experimental procedure (using a series of three tests) disallowed the use of a runs test for randomness since there can be no significant number of runs in a series of three observations. However, the randomness of the responses can be compared using the following assumptions: when the three responses in a test series are of different magnitudes, (greatest, median, least, which have been abbreviated G, M, and L below), the permutations of this order should occur with

equal frequency if the responses are independent. In other words, the number of series whose responses are ordered GML, GLM, MGL, MLG, LGM, or LMG should be equal. 141 of the total of 149 tests had a distinguishable rank order of one of the above permutations. If the responses were independent, each permutation should have occurred 23.5 times. The Chi-square test for goodness of fit (Sokal and Rohlf, 1969) showed the resulting distribution of response permutations (Table 7) to be highly significant ($p < 0.005$). Therefore, non-parametric analysis was employed.

Table 7. Chi-square test for goodness of fit of response permutations. (See description above.)

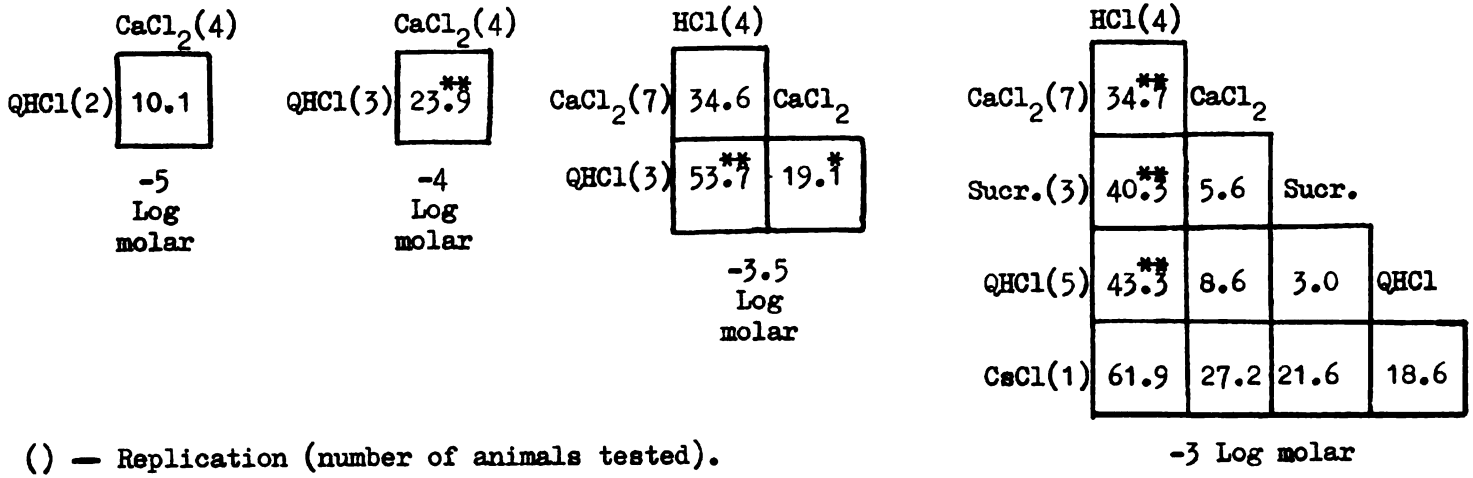
	GML	GLM	MGL	MLG	LGM	LMG	Total
Observed frequency:	54	28	16	18	13	12	141
Expected frequency:	23.5	23.5	23.5	23.5	23.5	23.5	141

Contrasts of Responses

The Kruskal-Wallis test (Sokal and Rohlf, 1969) was applied to the paired contrasts. The results are listed below in Tables 8, 9, and 10.

Table 8. Contrasts of the relative responses of Necturus to taste stimuli of -5, -4, -3.5, and -3 Log molar concentration. Displayed below by concentration are the taste stimuli (QHCl = quinine hydrochloride, Sucr. = sucrose) and their relative effectiveness. The more effective stimuli are listed at the head of each column. The difference in stimulating effectiveness (in relative response units) with each less effective stimulus is found in each box in the same column. This relative response difference is labelled significant at p = 0.05 or p = 0.10 as determined by the Kruskal-Wallis test. Parentheses indicate the replication of each stimulus.

Table 8. Contrasts of the relative responses of Necturus to taste stimuli of -5, -4, -3.5, and -3 Log molar concentration.



() — Replication (number of animals tested).

** — Significant at p=0.05 by Kruskal-Wallis test.

* — Significant at p=0.10 by Kruskal-Wallis test.

Table 9. Contrasts of the relative responses of Necturus to taste stimuli of -2.5 and -2 Log molar concentration. Displayed below by concentration are the taste stimuli (QHCl = quinine hydrochloride, Sucr. = sucrose), their relative effectiveness (in relative response units) and the significance of each contrast as determined by the Kruskal-Wallis test. See Table 8 for further explanation.

Table 9. Contrasts of the relative responses of Necturus to taste stimuli of -2.5 and -2 Log molar concentration.

	HCl(4)	$CaCl_2$	KCl	NaCl	CsCl	QHCl
$CaCl_2$(3)	34.2					
KCl(5)	34.2*	0.0				
NaCl(4)	41.5	7.3	7.3			
CsCl(1)	61.9	27.7	27.7	20.4		
QHCl(6)	64.3**	30.1	30.1**	22.8	2.4	
Sucr.(3)	68.6**	34.4**	34.4*	27.1*	6.7	4.3

-2.5 Log molar

	HCl(4)	NaCl	$CaCl_2$	KCl	QHCl	CsCl
NaCl(3)	55.8**					
$CaCl_2$(3)	66.6**	10.8				
KCl(6)	77.3*	21.5	10.7			
QHCl(3)	85.8**	30.0	19.2	8.5		
CsCl(1)	106.7	50.9	40.1	29.4	20.9	
Sucr.(2)	116.1*	60.3*	49.5*	38.8*	30.3*	9.4

-2 Log molar

()— Replication (number of animals tested).

**— Significant at p=0.05 by Kruskal-Wallis test.

*— Significant at p=0.10 by Kruskal-Wallis test.

Table 10. Contrasts of the relative responses of Necturus to taste stimuli of -1.5, -1, and -0.5 Log molar concentration. Displayed below by concentration are the taste stimuli (QHCl = quinine hydrochloride, Sucr. = sucrose), their relative effectiveness (in relative response units) and the significance of each contrast as determined by the Kruskal-Wallis test. See Table 8 for further explanation.

Table 10. Contrasts of the relative responses of Necturus to taste stimuli of -1.5, -1, and -0.5 Log molar concentration.

	HCl(3)					
QHCl(3)	60.8**	QHCl				
$CaCl_2$(3)	85.5**	24.7	$CaCl_2$			
NaCl(3)	104.8**	44.0**	19.3	NaCl		
KCl(4)	108.5**	47.7**	23.0	3.7	KCl	
CsCl(1)	139.0	78.2	53.5	34.2	30.5	CsCl
Sucr.(2)	160.8*	100.0*	75.3*	56.0*	52.3*	21.8

-1.5 Log molar

	KCl(5)			
$CaCl_2$(3)	2.7	$CaCl_2$		
NaCl(5)	34.1**	31.4	NaCl	
CsCl(1)	57.3	54.6	23.2**	CsCl
Sucr.(2)	94.7*	92.0*	60.6**	37.4

-1 Log molar

	KCl(3)	
NaCl(2)	25.5	NaCl
Sucr.(2)	168.3*	142.8

-0.5 Log molar

()— Replication (number of animals tested).

**— Significant at p=0.05 by Kruskal-Wallis test.

*— Significant at p=0.10 by Kruskal-Wallis test.

APPENDIX C

EXPERIMENTAL ANIMALS

EXPERIMENTAL ANIMALS

Adult mudpuppies (Necturus maculosus), obtained from J.R. Schettle Biologicals, Stillwater, Minnesota and Mogul-Ed, Oshkosh, Wisconsin, were held in 10 and 20 gallon (38 and 76 l) glass aquaria at 10-15 °C in aerated, charcoal-filtered water in an artificial light cycle of 16 hours per day. The animals were fed live minnows (4-6 cm long, obtained from a local bait supplier). The aquaria were cleaned with Alconox and rinsed with filtered water and the tanks refilled twice weekly.

Under these conditions, the animals developed a fungal infection identified as Saprolegnia (see Sayle, 1916 and Reichenbach-Klinke and Elkan, 1965). Gray fungal patches would appear on the animal's skin three to four weeks after shipment despite the following precautions. Infected animals were isolated from the general population, the fungus patch was scraped away with sterile applicators, the site of infection was painted with tincture of iodide, and the animals were then bathed in potassium permanganate (1 g/100 l glass distilled water for 90 min). Treated animals were then kept in filtered water to which the antibiotic, Chloromycetin, had been added (2.5 g/19 l). Nonetheless, the fungus resppeared at the initial site or elsewhere, eventually covering 1/3 to 1/2 of the animal's skin with death ensuing 2-3 weeks after the start of treatment. In addition, altering the following variables proved to be ineffective: maintaining the animals in fed or fasted condition, in full darkness or the normal artificial light cycle, and at 10-15 °C or 2-3 °C.

Successful eradication of the fungus finally occurred with the use of the antibiotic, tetracycline (610 mg/38 l). Animals have now remained free of infection using this antibiotic in the aquaria and under the conditions described in the first paragraph.

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