Stimulation of the Salt Receptor of the Blowfly

I. *NaCl*

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ABSTRACT Application of NaCl solutions to the tip of a labellar sensillum of the blowfly elicited a repetitive neural response from the salt receptor. The response was examined with respect to reproducibility and adaptation. A threshold was observed for tests with dilute solutions. Above this, the response increased linearly with the logarithm of the molarity. The response was not significantly affected by the pH of stimulating solutions, buffered or not, between 3 and 10. Beyond this range, it was reversibly inhibited until, at greater extremes of pH, atypical stimulation independent of the presence of salt was seen. Receptor sensitivity increased with fly age. The results presented here may be due to effects at sites in the sensillum other than the receptor membrane.

Behaviorally, certain Diptera, including the blowfly, *Phormia regina* Meigen, respond to certain dissolved salts in contact with the tips of their labellar gustatory sensilla (Dethier, 1955). The anatomy of these sensilla has been investigated using light microscopy (Dethier, 1955; Stürckow, 1962; Peters, 1965), electron microscopy (Larsen, 1962; Adams, Holbert, and Forgash, 1965), and by measuring their electrical impedance (Stürckow, 1964). The sensillum studied here is a hairlike structure about 300 μ long. It has an external cuticle of high electrical resistance and low permeability to aqueous solutions except at its tip. Here, stimulating solutions have access to the interior via a pore or pores in the cuticle. Within the sensillum run the dendrites of several bipolar neurons; their cell bodies lie near its base, and neural processes lead from these towards the central nervous system. On morphological evidence, these neurons have been presumed to be primary receptor cells which respond directly to stimulating solutions applied to the sensillum.

Electrophysiological recording between the tip and the base of the sensillum during application of salt and sugar solutions reveals action potentials (spikes) elicited from at least two different neurons. One neuron, yielding large spikes and designated the "L" fiber by the original investigators (Hodgson, Lettvin, and Roeder, 1955) responds to certain salt solutions. The present paper reports an electrophysiological study of the stimulation of this fiber by NaCl. It

will be referred to here as the "salt receptor," following the terminology of Evans and Mellon (1962 *a*). The investigations have been limited to varying certain stimulating conditions, such as the concentration and pH of the stimulating solution, and observing the spike activity evoked in this receptor with the hope of elucidating the mechanism of its stimulation.

Reviews on the morphology and physiology of the contact chemoreceptors of interest here have been published by Dethier (1963), Hodgson (1964), and Wolbarsht (1965).

METHODS

The general technique was essentially that used by Evans and Mellon (1962 *b*). The isolated head of a 3 to 5 day old male imago of *Phormia regina* Meigen was mounted on a saline-filled glass capillary electrode (which served as the indifferent electrode) inserted through the foramen magnum and into a large blood sinus in the proboscis. Stimulation and recording occurred simultaneously when a similar recording electrode, filled about 0.1 sec before the onset of stimulation with fresh solution of an alkali halide (technique described by Evans and Mellon, 1962 *a*), was slipped over the tip of a large marginal labellar sensillum (type 1 as described by Evans and Mellon, 1962 *a*). The recorded potentials were amplified, displayed on a cathode ray oscilloscope, and photographed. The permanent records were then examined visually. Ambient temperature was 22 to 24°C. It should be noted that no differences in response characteristics have been seen between the isolated head preparation used here and recordings from the whole fly (Gillary, unpublished data; Hanson, 1965).

Ambient temperature, relative humidity, concentration, pH, duration of the applied stimulating solution, intervals between tests, and electric current flow through the preparation were kept constant well within an experimentally determined range where no effects were detectable. All experiments were repeated on several sensilla on each of several preparations. When using different stimuli, the order of tests was varied to insure that the results were independent of the test sequence. Because of apparent differences in sensitivity between receptors, the absolute magnitudes of responses of different receptors were never compared. Repeated tests on a given receptor were made to avoid errors due to possible variation in sensitivity with time. Only responses exhibiting normal quality and reproducibility were used. The criteria for these will be considered later. The concentration scale used throughout these studies was that of molarity. The methods have been presented elsewhere in greater detail (Gillary, 1966 *a*).

RESULTS

Qualitative Aspects of Recordings

Typical recordings during brief stimulation with 1 M (molar) NaCl have been published (Gillary, 1966 *b*; Evans and Mellon, 1962 *a*). Stimulation with a NaCl solution elicited regular trains of spikes from no more than two fibers. The constant amplitude of the spikes from each fiber was always easily distinguishable from the other. At concentrations below 0.5 M NaCl, smaller

spikes were seen in addition to the larger spikes of the salt receptor. The small spikes, whose frequency was inversely related to salt concentration, were presumably those of the water receptor described by Evans and Mellon (1962 *a*). At concentrations above 2 M, in addition to a high salt receptor response, a smaller spike was seen whose low regular frequency was a direct function of salt concentration. This spike may be of the water receptor or another fiber. For intermediate salt concentrations, only the large spike was seen. This investigation was concerned primarily with the salt receptor, which produces the large spike. Smaller spikes appearing in the records were ignored.

In a few experiments, recording was done from one sensillum while applying stimulating solutions to adjacent sensilla. The salt spikes were seen to reflect

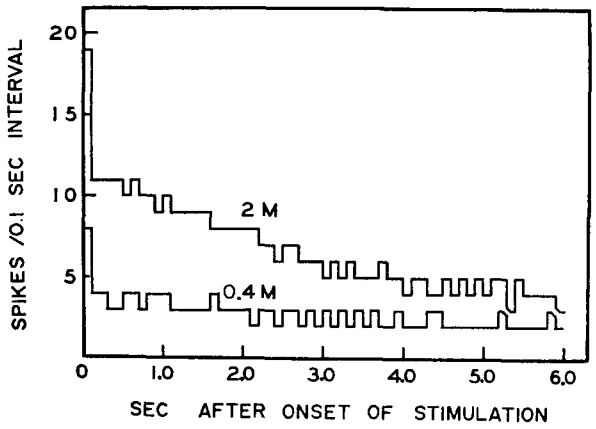

FIGURE 1. Spike frequency vs. time. Spikes were recorded from two sensilla during prolonged stimulation with 0.4 M and 2 M NaCl, respectively. Neither sensillum had been stimulated during the previous 10 min. The number of spikes in each successive interval is plotted against the time after the onset of stimulation.

only the stimulating solution in the recording electrode and bore no relation to stimulating solutions applied to adjacent sensilla. This indicates that the salt receptor spikes originated from a cell associated only with the sensillum to which the stimulating solution was applied.

The unstimulated receptor exhibits little or no "spontaneous" spike activity (Evans and Mellon, 1962 *a*). Following contact of the tip of the sensillum with a stimulating salt solution, after a short latency on the order of a few milliseconds, a train of spikes was elicited. The frequency was initially high but declined rapidly within the first 100 msec to a slowly declining or relatively steady level for high or low salt concentrations, respectively. This may be seen for typical receptors in Fig. 1. In most records, successive intervals between spikes were constant or changed gradually. Records in which the spike frequency was irregular were discarded since they are symptomatic of injury or deterioration (see also Mellon, 1961).

For convenience, the first 100 msec of recording during stimulation will be referred to as the initial phase, and the subsequent 500 msec, the steady phase, similar to terminology adopted by Evans and Mellon (1962 *b*). Contrary to their findings, however, no evidence was found confirming the independence of these two phases. Repeated tests over the full range of NaCl concentrations using intervals varying between several seconds to hours, as well as tests with other salts, never resulted in preferential adaptation of the initial phase. On the contrary, a fairly constant relationship between the two was found, the average frequency of the initial phase being approximately two to three times that of the steady phase. The variable relationship between these two phases portrayed in Fig. 2 of their paper is possibly an artifact due to amplifier blockage, which may be seen at the beginning of their published records (Evans and Mellon, 1962 *a*).

In the light of difficulties in recording all of the initial phase and the ease of recording and quantitatively treating the relatively constant spike frequency throughout the entire steady phase, only the latter phase was treated quantitatively here. "Response" will mean the number of salt receptor impulses which occur during the steady phase.

Adaptation

Adaptation, the quantitative reduction in the response by prior stimulation, was exhibited by the receptor. Solutions at various fixed concentrations, durations, and intervals between tests were repeatedly tested. Upon repeated stimulation, the response fell rapidly during the first few tests and then leveled off to a somewhat steady value (see Fig. 2). The rate of fall and decrease of level were greater for greater concentration and duration, and for shorter intervals between tests, indicating a tendency for recovery of the receptor with time while unstimulated. This was also noted by Hodgson and Roeder (1956). Prolonged application of water or 0.1 M NaCl between brief tests every half minute with concentrated salt had no significant effect on the response. The degree of adaptation seems to be directly related to the concentration of the stimulating solution or the response magnitude, and inversely to the time of recovery.

As a compromise between long intervals needed to avoid adaptational effects and short ones needed to avoid variation with time, a standard interval of 3 min between tests was used in all experiments, unless otherwise specified. Using this interval, for most receptors there was little further adaptation after the first two tests, and responses of such an adapted receptor were usually less than 20% below the initial response.

Reproducibility

The reproducibility of results obtainable was determined and limits of variability set beyond which a given experiment was said to yield nonreproducible

results. The biological material and its manipulation were kept as uniform as possible and conditions during the experiments mentioned in the Methods were kept constant well within limits which might give rise to variability in sensitivity. One or several concentrations were repeatedly tested while varying the order to insure independence of the test sequence, and the range of the responses to a given concentration was examined. The results indicate that as performed, the stimulation procedure was capable of yielding responses within 10% of the mean better than 80% of the time for normally responding recep-

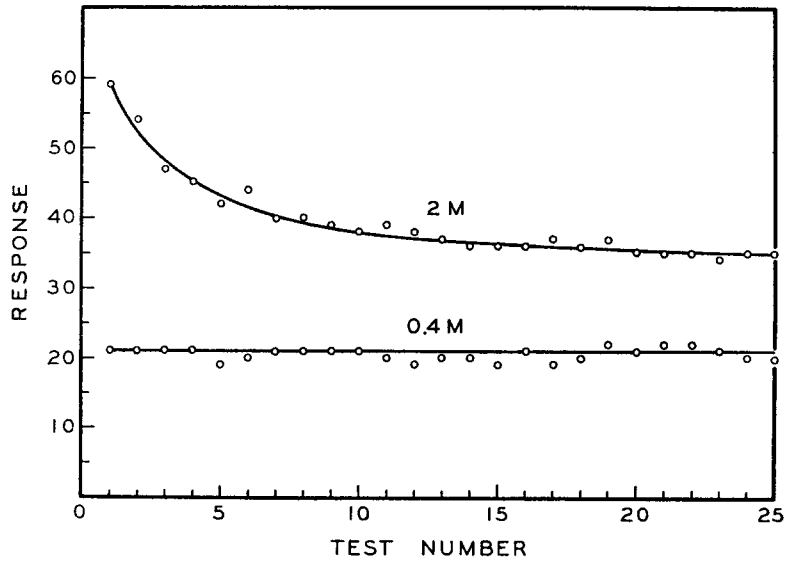

FIGURE 2. The responses of two salt receptors to repeated tests. The receptors, unstimulated for more than 10 min prior to the first test, were stimulated every 10 sec for less than 1 sec duration with 0.4 M and 2 M NaCl, respectively. (Response is defined in first section of Results.)

tors, and quite often they were within 5%. In the current investigation, a response was considered reproducible if it fell within 10% of the mean of all responses to the same applied stimulus. Experiments yielding nonreproducible responses were considered unreliable and were discarded.

Response vs. Concentration

Approximately 20 concentrations were tested ranging from 0.05 M NaCl through the saturated solution (about 5 M). Distilled water was also tested. Several types of test sequences were employed. (*a*) A series of ascending concentrations was tested. This sequence was the only one used by Evans and Mellon (1962 *b*). (*b*) Several concentrations were repeatedly tested in an irregular sequence. (*c*) Two fairly close concentrations were repeatedly tested and the responses compared.

The first type of sequence yielded fairly similar response vs. concentration relationships for all the receptors tested, but since each concentration was tested only once, reproducibility for a given receptor could not be checked. In light of the studies on adaptation, however, this sequence should yield results least distorted by adaptation. The second type of experiment in which reproducibility could be checked, had the disadvantage of requiring a large number of tests over a long period of time. As would be expected in the light of

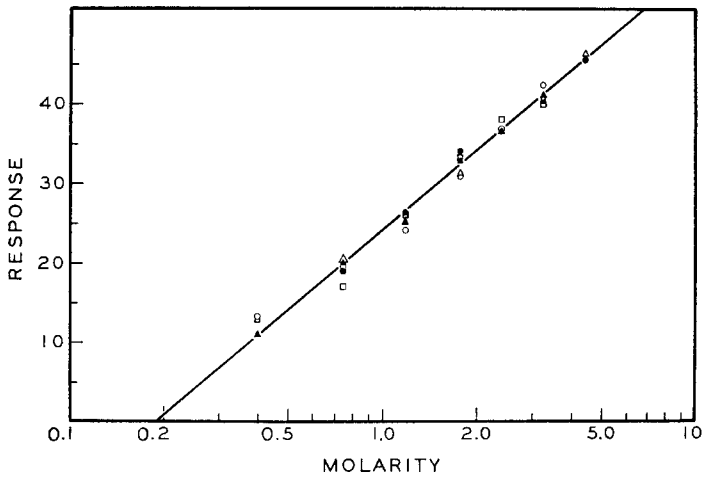

FIGURE 3. The responses of five salt receptors to ascending concentration test sequences. Tests at 0.1 M yielded 0 response. The responses of each receptor were normalized to the average least squares line as follows: Least squares lines for the responses of each of the five receptors were determined. Their averaged slopes and intercepts yielded the average line. Each response of the five receptors has been plotted in the figure so that it bears the same relationship to the average line as it did to its respective line.

time-variant preparation sensitivity, the data were less precise than those of the first method. The third method was suitable for a precise determination of the slope of a small portion of the response vs. concentration curve under conditions of practically constant adaptation. However, synthesis of the whole response vs. concentration curve necessitated using data from a large number of receptors having different sensitivities, and slopes at higher concentrations were determined from receptors more highly adapted than those tested at lower concentrations.

Results from all the methods yielded a consistent picture of the response vs. concentration curve. The receptors exhibited no activity below a threshold at approximately 0.1 M. Above this, the response curve rose at a decreasing rate with increasing concentration over the full range up to saturated salt. Within the precision of the data, the curve shape was consistent with that of a linear plot of response vs. the logarithm of molarity as in Fig. 3.

pH

Evans and Mellon (1962 *b*) recorded the response to NaCl solutions at several pH's but in keeping with their attempt to determine the pK of a presumed anionic receptor site, the pH was varied between 1 and 8. The problem seemed to warrant a more thorough investigation, including a more detailed look at the response vs. pH curve over a wider range, the effect of the presence of a buffer in the test solutions, and possible injurious effects due to application of solutions at extremes of pH.

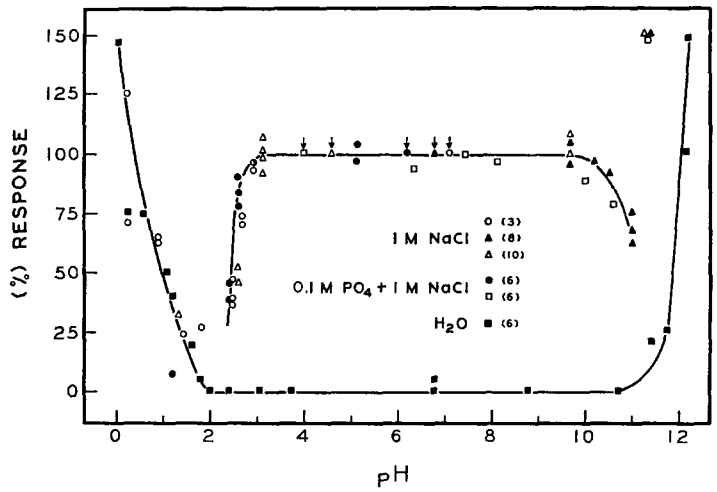

FIGURE 4. 1 M NaCl response as a function of pH. Each type of point (open circle, filled triangle, etc.) represents data from a single preparation. Each point is the average of the responses of the number of receptors tested (given in parentheses) on the preparation at a given time. The test sequence was irregular and the averaged responses were normalized to those at the pH's in the plateau region indicated by arrows. The upper curve represents the normal response to NaCl stimulation; the lower represents the response independent of NaCl. (See text for details.)

Stimulation was effected with 1 M NaCl made up in distilled water or 0.1 M phosphate. The buffer was unstimulating and did not affect the response to 1 M NaCl. The pH was adjusted with NaOH or HCl. The normal test procedure was employed throughout with these solutions.

Quantitative aspects of the response as a function of pH may be seen in Fig. 4. The pH had no measurable effect over a plateau region extending from about 3 to 10. Beyond this the response was inhibited until at more extreme values, stimulation independent of the presence of salt was seen which seemed due to the effects of pH alone. The curve seems quite symmetrical and is not detectably affected by the presence of buffer.

Between pH's of about 2 and 11, the quality of the response was normal. At

extremes where pH itself seemed to stimulate, the responses were very irregular indicating abnormal type stimulation which is characteristic of injury. However, using tests of less than 1 sec duration, reproducible responses for pH's within the plateau region were obtainable, even when tested intermittently at pH's of 1.5 and 11. This indicates some degree of reversibility of pH effects, although more prolonged or frequently repeated tests at pH extremes did result in a noticeable decrease in spike frequency and regularity to subsequent test stimulation.

The results of subsequent studies on the effects of pH on the responses of the fibers within the sensillum which respond respectively to sugars and to water indicate a picture similar to that of the salt fiber (Blaumanis, 1965). This suggests a similar mode of action of pH on all fibers.

Evans and Mellon (1962 *b*) state that inhibition of the salt receptor occurred at a higher pH when the salt concentration was less. Their data, those of Mellon (1961), and those presented here were used to determine, at various NaCl concentrations between 1 and 5 M, the low pH at which the response was inhibited to a value one-half that on the plateau region. A value of approximately minus one-half of a pH unit per unit of molarity was obtained. The significance of this, however, has not yet been assessed.

Age

While all other studies were carried out with 3 to 5 day old flies, some experiments were performed to see whether there was any relationship between the age of the fly and the response to NaCl stimulation. Responses to concentrations between 0.5 and 5 M were measured from various stages, ranging from the motionless nymph (dissected out of the pupal case) through a 16 day old imago. Under fixed experimental conditions, younger flies always yielded lower responses at each tested concentration than older ones, indicating an increase in sensitivity with age. This increase could be due to developmental changes involving any of several components of the sensillum which can affect the response (see Discussion). It will not be considered further. Qualitatively, spikes from younger stages appeared to have higher amplitude and longer duration than those recorded from older stages. This may reflect some morphological change in the sensillum.

DISCUSSION

The current studies were limited to varying certain stimulating conditions and examining the frequency of the spikes recorded. The original aim was to elucidate the process of stimulation at the cell membrane of the salt receptor. However, the sensillum under study is a rather complicated physiological structure. Before experimental results are attributed to phenomena associated

with the receptor membrane, their explanation in terms of effects elsewhere in the sensillum must be excluded.

General Scheme

It is convenient to consider the results in terms of a general scheme of three stages, suggested by properties common to many other receptor systems (Gray, 1959; Davis, 1961; Kennedy, 1962) and the particular morphology and physiology for this one.

The first is the stage of possible alteration of the applied stimulating solution before its effect at the primary receptor site. The properties of the bulk phase of this stimulating solution are precisely known. However, this solution is not necessarily the actual stimulus presented to the receptor membrane, since there is no evidence that equilibrium conditions exist. This stage could involve a pore or pores in the sensillum, extracellular space between the pore and neuron, or interfacial effects at the surface of the cell membrane.

The second stage involves the primary receptor site, which is probably associated with the receptor cell membrane in the tip of the sensillum. Presumably, the coming together of the actual stimulus and this membrane results in some electrical event, which gives rise to a receptor potential.

The third stage includes all events involved in impulse generation which are subsequent to the primary events at the receptor membrane. The initiation of propagated impulses appears to occur near the base of the sensillum (Tateda and Morita, 1959). Presumably, current caused to flow at the primary receptor site can pass through this relatively distant part of the neuron. The frequency of elicited impulses is presumably a direct function of this current, which is often called a "generator current." These impulses are recorded experimentally.

In this scheme, fibers in the sensillum other than the salt receptor have been ignored. While it is possible that electrical or chemical interaction may occur between these fibers and the salt receptor, no evidence has yet been found which supports this (Wolbarsht, 1958; Browne and Hodgson, 1962). For the present, it will be assumed that there is none.

Stage 1

Several results suggest that the first stage may significantly affect the relationship between the stimulating solution and the response. The receptor is physiologically stimulated upon application of salt solutions from about 0.1 M through several molar. Neuronal membranes can be completely depolarized by concentrations of externally applied KCl which are more than an order of magnitude below the higher tested concentrations (Horowicz, 1961; Narahashi, 1963). These higher concentrations would usually be considered in-

jurious to cell membranes. The receptor responds in a normal manner to solutions whose pH can vary over more than 7 units. Cell membranes seem to function normally within a range of external pH which is considerably less than this (Davson, 1964; Rud, 1961). The large tolerance of the receptor to pH and salt concentration suggests that the concentration of the stimulus presented to the receptor membrane may be considerably less than that of the bulk phase of the stimulating solution.

Electron micrographs (Adams et al., 1965) suggest a tiny pore (or pores) through which the stimulating solution presumably enters the sensillum, and an extracellular space around the tips of the dendrites. Other evidence (Stürckow, 1966) indicates a viscous extracellular substance within the sensillum. There is currently no reason to assume that these would not alter the concentration of a solution applied externally to the pore prior to an effect at the dendrites.

Stage 3

Several aspects of the train of impulses elicited upon stimulation of the salt receptor have certain similarities. The decline of spike frequency with time, adaptation, and the rate of rise of response with concentration are all dependent on stimulating intensity or spike frequency. In addition, these effects are seen during stimulation with many different salts (Gillary, 1966 *b*). This implies that they may be due to a common process. Furthermore, these effects are characteristic of the neural response of other receptors. This suggests that their site of origin may be common to many types of receptors.

It does not seem very likely that such general effects would originate from the primary reaction at the receptor membrane. Events at this site would be expected to be more strongly dependent on the specific nature of the stimulus. It seems more probable that these general effects arise from events subsequent to this primary reaction, such as the coupling between the primary effect and the initiation of impulses, or the actual generation of the impulse at its site of origin.

Stage 2

Since it is not known which, if any, of the experimental results are directly manifested at the primary receptor site, one can merely speculate on the possible relevance of the results to the mechanism of action of the stimulus. Two such mechanisms are stimulation via alteration of the electrochemical potential gradient across the receptor membrane, and stimulation via the binding or adsorption of ions to specific receptor sites on the membrane surface. However, neither is preferentially supported by the data.

An Adsorption Theory

A theory of stimulation of the salt receptor, based on one proposed by Beidler (1954) for the mechanism of stimulation of mammalian taste receptors, has

been proposed by Evans and Mellon (1962 *b*). An attempt will be made to evaluate the theory primarily as it is presented by the latter authors.

Briefly, the theory postulates that salts stimulate through combination with unspecified sites, that the reaction obeys the mass law and is in thermodynamic equilibrium, and that the response magnitude is proportional to the

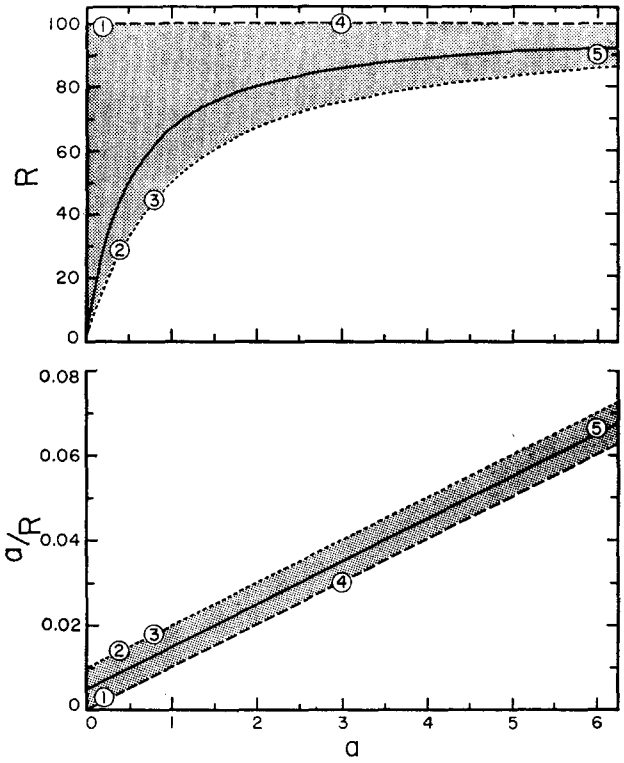

FIGURE 5. Comparison of the representation of data on coordinates of a/R vs. a with that on coordinates of R vs. a. The values of R and a and the symbols used in both plots are identical. Only the ordinates differ. In each plot, the 3 curves satisfy the equation $R/(R_m - R) = Ka$ where $R_m = 100$. For the dotted, solid, and dashed curves, K is, respectively, 1, 2, and infinity. The numbered circles represent hypothetical data.

number of occupied sites. The theory predicts that the curve of response vs. concentration obeys a Langmuir adsorption isotherm, approaching a maximum response (R_m) at higher concentrations where essentially all the receptor sites are occupied. The rate of rise of this curve is a function of the affinity between salt and binding site, indicated by an association constant (K). A plot of concentration (a) divided by response (R) vs. concentration should be linear, and from it values for R_m and K determined.

The main piece of evidence offered by Evans and Mellon in support of the theory is the apparent linearity of a/R vs. a data plots. This criterion is inadequate since this type of plot can visually distort deviations from linearity of R

vs. a data which is only roughly hyperbolic. This is illustrated in Fig. 5 in which hypothetical data are plotted using the two types of axes. It is possible to determine whether the data do or do not fit the theory only if (a) the precision of the data is known, and (b) the data are compared with hypothetical values resulting from the theory.

The fact that the frequency of the steady phase is both constant and reproducible does not imply that the presumed stimulating reactions are in thermodynamic equilibrium. Such phenomena could result from nonequilibrium conditions, such as a steady state. The degree of variability in K's for a given salt between preparations suggests that they are not equilibrium constants.

If the stimulating solution and the actual stimulus at the receptor site are different, the magnitudes of the K's and free energy changes calculated from the data would be affected by this difference. The existence of a threshold could also markedly influence the values determined for K. As indicated earlier in the Discussion, the relationship between stimulating intensity and response could be affected by stage 3 and not solely determined at the primary receptor site. This suggests that the data should not be explained solely in terms of an adsorption mechanism.

The foregoing discussion is not meant to imply that the basic mechanism of stimulation proposed in the theory is incorrect. Rather, an attempt has been made to indicate that the evidence for the adsorption theory is weak and that a potentially complex situation in the sensillum under study should not be prematurely simplified by attempting to explain all experimental results only in terms of chemical events of a particular nature at the receptor membrane.

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