Unitary Responses in Frog Olfactory Epithelium to Sterically Related Molecules at Low Concentrations

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ABSTRACT Responses of receptor cells in the frog's olfactory epithelium were recorded using platinum-black metal-filled microelectrodes. Spontaneous activity varied over a wide range from 0.07 to 1.8 spikes/s. Mean interspike intervals ranged from 13.7 to 0.5 s. Excitatory responses to six sterically related compounds at low concentrations were investigated. Stimuli were delivered in an aqueous medium. Thresholds for impulse initiation varied from greater than 1 mM down to the nanomolar concentration range. Thresholds of different olfactory receptors to the same stimulus could vary by several log units. Thresholds of the same receptor cell to different stimuli could be within the same order of magnitude, or could vary by as much as 5 log units. Based upon quantitative measures of stimulus-evoked excitatory responses it appeared that some receptors did not discriminate among sterically related molecules, whereas other receptors clearly discriminated between stimuli which evoke similar odor sensations.

INTRODUCTION

Discrimination among different odors is the least understood operation performed by olfactory receptor cells. Physiological mechanisms underlying discrimination at the receptor level have been studied with single unit recording techniques (Gesteland et al., 1963; Shibuya and Shibuya, 1963; Takagi and Omura, 1963; Gesteland et al. 1965; Shibuya and Tucker, 1967; Shibuya, 1969; Altner and Boeckh, 1967; O'Connell and Mozell, 1969; Mathews, 1972; Shibuya and Tonosaki, 1972; Daval et al., 1972). From these investigations, in a variety of vertebrate species, there appears to be a general consensus that single receptor cells have relatively broad response spectra to molecules which evoke different odor sensations. Further progress in elucidating mechanisms by which receptor cells discriminate among stimuli
requires a detailed analysis of the physiological parameters of unitary responses.

The present study is aimed at investigating two physiological properties of individual olfactory receptor responses. One property is the manner by which olfactory receptors respond to near-threshold chemical stimulation. In order to evaluate such responses, detailed prior information is also required about the background spontaneous activity out of which the response may arise. The second property is concerned with investigating discriminatory responses of olfactory receptors to sterically related compounds at threshold. Analysis of this property hinges on what may be termed the problem of stimulus selection. This involves careful consideration both of the molecular characteristics of the stimulus and the organoleptic qualities of the chemical sensation evoked by the stimulus. The present study is an initial step in a systematic analysis of the physiological mechanisms involved in discrimination among carefully selected sets of chemical stimuli.

**METHODS**

**General Procedures**

Medium size grass frogs, *Rana pipiens*, were prepared as previously reported (Getchell, 1969, 1973). Animals were doubly pithed; no curare or curare-type drugs were used. They were pinned to a clean cork board covered with Parafilm. The eminentia olfactoria was exposed by dissecting away the skin, cartilage, and tissue covering the nasal cavity. A large chlorided silver plate, wrapped in saline-moistened gauze and placed in the animal's mouth, served as the ground electrode.

Platinum-black plated metal-filled microelectrodes were made according to the general technique reported by Gesteland et al. (1959). This technique was adapted by Getchell (1973) for unit receptor cell recording during aqueous perfusion of the olfactory epithelium. The electrodes were designed primarily for ease of epithelial penetration and for recording triphasic spikes from receptor cell axons. They had platinum-plated tip diameters of about 5.7 μm and resistances of about 0.25 MΩ when measured in 154 mM NaCl at 1 kHz. The spike conformation and amplitude of the recruited units were sometimes similar to those of the spontaneously active unit being investigated. Therefore, use of a window discriminator frequently gave ambiguous results and was not routinely used.

Neural activity was recorded from about 800 olfactory receptors. The activity recorded from 86 units was rigorously selected and photographed from magnetic tape which was replayed and displayed through an oscilloscope. Activity displayed by 16 units was selected from this population to analyze spontaneous activity and the remaining 70 units to investigate responses to stimuli. With the exception of Unit M (see Results) the two subpopulations did not overlap for several reasons including those now discussed. Neural activity recorded from most receptors was rejected from data analysis for a variety of reasons which included: (a) loss of the unit or changes in spike amplitude due to slippage of the microelectrode, (b) units with a low signal-
to-noise ratio, (c) units which showed bursts of spikes accompanying switching valves, (d) spikes from units showing atypical voltage conformations which indicated possible damage by the electrode, (e) units with thresholds greater than 1 mM, (f) insufficient recording time required for data analyses, (g) recruitment of units in the vicinity of the electrode tip during responses to stimuli which obscured the activity of the unit being investigated, and (h) units which were not excited by low concentrations of stimuli delivered to the olfactory epithelium. Hence, although sufficient neural activity was recorded from Unit A and Unit N (see Results) to analyze their spontaneous activity statistically, neither unit was excited by low concentrations of stimuli. Conversely, the activity recorded from receptors which responded to certain stimuli (i.e. the subpopulation of 70 units except Unit M) was not included among those units whose spontaneous activity was analyzed statistically (the 16 units above) because insufficient recording time was not obtainable or their spontaneous activity was too low to be included in the sample.

Considerable care was taken to avoid chemical contamination which could modify the recorded neural responses. All glassware was chemically cleaned, oven dried, and kept sealed before use. Quantitative chemical techniques were used in the preparation of solutions. The deionized charcoal-filtered water was routinely monitored for contaminants. Chemicals of the highest grade available were used, and stimulus purity was checked by the gas-liquid chromatograph.

**Stimulus Selection**

Selection of appropriate stimuli is a crucial factor when analyzing neural responses recorded from the olfactory system. Stimuli were chosen which were considered consistent with the aims of the investigation. Six compounds were selected on the basis of their interrelated molecular and physical characteristics and distinct sensory qualities which they evoke in man. They formed two classes: volatile compounds with characteristic odors (Class I), and certain nonvolatile compounds (Class II). Both groups were soluble in Ringer's solution. The three volatile stimuli were: nitrobenzene, benzaldehyde, and ethyl n-butyrate. Nitrobenzene and benzaldehyde, which are monosubstituted benzene derivatives (Fig. 1 a and b), elicit a characteristic bitter almond odor sensation. Subtle differences between the two also exist in that the former evokes a pungent note whereas the latter elicits a characteristically sweet odor sensation. Ethyl n-butyrate, which is the ethyl ester of butyric acid (Fig. 1 c), elicits an ethereal-fruity odor sensation reminiscent of pineapples or bananas (Arctander, 1969). These stimuli were chosen to determine if a frog's olfactory receptor could discriminate between two compounds with similar molecular characteristics which evoke the same basic odor sensation and a third with a different odor quality.

Nonvolatile compounds, e.g. certain amino acids, have been used as olfactory stimuli to investigate receptor cell responses in fish (Suzuki and Tucker, 1971; Sutterlin and Sutterlin, 1971). Effectiveness of nonvolatile compounds as stimuli for the olfactory epithelium is here confirmed for the frog. Nonvolatile stimuli used were the three isomers of tolylurea (Fig. 1 d, e, and f). They are structurally related to the benzene derivatives discussed above. The ortho- substituted derivative of methyl benzene is tasteless whereas m-tolylurea tastes bitter and the p-tolylurea tastes sweet (Beidler,
This second group of stimuli was chosen to determine if a frog's olfactory receptor could discriminate among three isomeric derivatives of benzene, each of which evokes a different sensory quality. Volatile odorous compounds which met these criteria were not available. In the absence of behavioral data, there is no evidence whether or not any of the stimuli used in this study were detected by the frog.

The lowest concentration of the compound required to stimulate the olfactory receptor, i.e. physiological threshold, was investigated. Preliminary experiments demonstrated that high concentrations, i.e. greater than 1 mM, of each stimulus evoked vigorous excitatory responses from some receptor cells. This corroborates the results of Suzuki and Tucker (1971) and Sutterlin and Sutterlin (1971) who recorded multiunit spike responses from olfactory receptors in fish. Such high concentrations were subsequently avoided as it is possible that they may cause long-lasting effects on the specificity of the receptor cell responses (for discussion see Suzuki and Tucker, 1971). In view of the fact that threshold information and unit discrimination among stimuli were the main concerns of this investigation, only stimuli at low concentrations were delivered to the olfactory epithelium.

**Nasal Perfusion and Stimulus Delivery**

The nasal perfusing medium was made up in deionized water which had passed through activated charcoal filters. It had the following composition: Na⁺ 117.6 mM, Cl⁻ 121.5 mM, K⁺ 2.5 mM, Ca²⁺ 2.0 mM, HPO₄²⁻ 1.1 mM, and 0.4 mM H₂PO₄⁻ (Takagi et al. 1968). It had a pH of 6.9, a temperature of approximately 23°C, and was aerated at least 24 h before use. The stimuli were dissolved in this chemically defined medium. The nasal perfusing technique reduced the possibility that spurious volatile compounds in the ambient environment could interact with olfactory receptors and thus initiate responses or spontaneous activity.
The nasal epithelium was continually perfused with the aerated Ringer's solution through an all glass and Teflon delivery system at approximately 0.2 ml/s. An aliquot of stimulus, approximately 0.5 ml, was introduced at about 0.2 ml/s into the perfusing medium by manually switching a stopcock. The aliquot was then pushed by the pressure head of the perfusing medium to the olfactory epithelium. The latency between switching the stopcock and arrival of the stimulus was about 6 s. The neural latency of the response could not be determined with sufficient accuracy to investigate this parameter of the receptor response as originally described by O'Connell and Mozell (1969). Because of the inherent design of the stimulus delivery system and diffusion phenomena, as determined by dye substitution techniques, the stimulus could not be introduced to the olfactory epithelium as a square pulse (see also Otto-son, 1956; Tucker and Suzuki, 1972). The leading edge of the stimulus ranged from infinitely dilute to the prepared concentration of the stimulus. Hence, it could not be precisely determined at which point in the spatial-temporal concentration gradient of the stimulus the individual receptor responded. Because of these considerations, the accuracy of the latency measurements for a given response could not be determined to greater than about 1.5 s, nor of threshold measurements to within two log steps. For these reasons stimulus markers are not indicated in the figures shown in the Results. However, despite lack of this information, it was judged that this stimulus presentation technique more closely approximated the "natural mechanism" of sniffing than delivery of a square stimulus pulse.

Of particular concern were possible deleterious effects of the perfusing medium on the physiological integrity of the epithelium. Several perfusing media, at a variety of flow rates, were tried before deciding on the one employed. The electroolfactogram (EOG) provided a physiological criterion to control for any effect of aerated Ringer's solution flowing over the epithelium. The EOG is a slow voltage change evoked by olfactory stimuli measured extracellularly at the surface of the olfactory mucosa. It is due to summed current flow through the extracellular resistance of the olfactory epithelium (Gesteland, 1971) and considered to be a summated receptor potential (Ottoson, 1956; 1971). The EOG shown in Fig. 2 a was recorded before Ringer's

![Figure 2](image-url)

Figure 2. Two EOG's evoked by vaporous puff of ethyl n-butyrate before (a) and after (b) Ringer's solution flowed over olfactory mucosa for 255 min at 0.2 ml/s, stimulus puff 1.5-s duration at 0.16 ml/s into background moist air stream delivered to epithelium at 2.56 ml/s. Note lack of voltage distortion in response.
solution flowed through the nasal cavity and was typical of the normal slow voltage change evoked by the vapour of ethyl n-butyrate. After the olfactory mucosa had been exposed to a continual flow of Ringer's solution for 255 min and had been repeatedly stimulated with aliquots of stimuli, no significant changes were observed (Fig. 2b) in the voltage characteristics of the EOG evoked by an identical vaporous puff of ethyl n-butyrate. When care was taken to reduce short circuiting due to residual Ringer's solution in the nasal cavity, only minor variations were typically observed in the EOG's voltage waveform. These and similar control experiments gave reasonable assurance that the cellular mechanisms which generate the EOG in response to an odorous substance were not altered by the ionic solution flowing over the olfactory epithelium. Since triphasic spikes were recorded from receptor cell axons with equal ease throughout an experiment reasonable assurance was given that mechanisms for initiating and transmitting spikes were not seriously affected by the perfusing technique.

An interstimulus interval of 2 min was chosen because no changes were observed in the EOG's voltage waveform when volatile odorous stimuli were delivered to the epithelium at this interval at low flow rates.

**Data Analysis**

Data analysis was directed to certain basic statistical parameters of the spontaneous activity recorded from receptor units: mean interspike intervals, standard deviations of the mean interval and the coefficient of variability (i.e. relative variability). The overall mean rate of discharge, lambda (λ), was employed as the basic measure of spontaneous activity (Griffith and Horn, 1966). The number of spikes, N, present in a 3-min continuous section were counted. The time, T, in seconds between the first and last spikes in the sequence was measured. The ratio \( \lambda = (N - 1)/T \) is the over-all mean rate of discharge and has the dimension of spikes per second. This measure reduced the variability in the calculated firing frequencies due to time segments before the first recorded spike and after the last recorded spike in the train of spontaneusly generated spikes under study. These time segments were sufficiently long in several cases to warrant use of this technique. It also provided a way to relate the very low spontaneous activity of the olfactory receptors to that of other neurons. Certain well-delineated excitatory responses evoked by stimuli were also statistically analyzed.

**RESULTS**

**Spontaneous Activity**

Discharge patterns of spikes recorded from receptor cells could be classified into two categories. The first category included those units which revealed themselves only when stimuli were delivered to the olfactory epithelium. Such spikes, presumably recorded from silent receptors, were seldom recorded in sufficient unit isolation to evaluate their response patterns. After these stimulus-evoked responses were recruited into the background spontaneous activity,
the receptor generally became silent again. The second category consisted of those receptor cells which exhibited spontaneous spike activity in the absence of experimentally introduced chemical stimuli. Although both categories of unit activity have been reported in the olfactory epithelium of several vertebrate genera (for review see Gesteland, 1971) sufficient physiological information has not been reported to evaluate the significance of spontaneous activity in a quantitative manner.

Previous work has shown that the spontaneous activity of olfactory receptor cells in poikilotherms is quite low when compared with that activity recorded in the CNS. O’Connell and Mozell (1969) and Mathews (1972) reported an average of about 3 spikes/min in the frog and tortoise epithelia, respectively. Gesteland (1971) reported that approximately 60 spikes/min was typical for the frog. Daval et al. (1972) noted that the activity ranged around 12 spikes/min in the frog maintained at 12°C. However, physiological parameters such as spike conformation, length of time for which the spontaneous activity was monitored, interspike interval information, and variance in firing frequency have not been reported. The present study therefore, is an initial step toward obtaining quantitative measures of these basic parameters.

Representative spontaneous activity recorded from 16 olfactory receptor cells was selected from the larger pool of active units. Units were chosen primarily on the basis of high signal-to-noise ratio and isolation from other active units in the vicinity of the electrode tip, which ensured that each event in the time sequence could be examined. It was unlikely that this activity was induced by exogeneous factors as judged by physiological criteria (Getchell, 1973; Getchell and Getchell, 1974). Each unit was designated with a capital letter. With the exception of Unit B, which exhibited an initially positive diphasic spike conformation, spikes were recorded from the receptor cell axon in the remaining 15 units as evidenced by their triphasic (positive-negative-positive voltage sequence) spike conformation. Although Unit P either met its demise or was lost during the 10th min of investigation, it was retained for study as an example of a spontaneously active unit with an atypically high mean firing rate. The temporal and voltage characteristics recorded from a representative spontaneously active unit, Unit A, are shown in Fig. 3.

Spontaneous activity was monitored for 3 min. The number of spikes recorded from different units ranged from 13 to 336 spontaneous discharges in 3 min. Several units were encountered whose ongoing activity was less than 13 spikes/3 min; e.g. the unit shown in Fig. 10 which spontaneously discharged 10 spikes/12 min. Hence, the spontaneous activity recorded from these very slowly discharging units did not readily lend itself to statistical treatment within the constraints of the experimental protocol, i.e. 12 interspike intervals/3 min minimum, and was arbitrarily excluded from this data analysis. The over-all mean rate of discharge, \( \lambda \), varied from 0.07 spikes/s
Figure 3. Triphasic spike (d) recorded from a spontaneously active unit, Unit A; typical unit isolation, signal-to-noise ratio and variation in interspike interval shown in a, b, and c. The unit's mean spontaneous rate of discharge was 0.42 spikes/s and mean interspike interval of 2.4 ± 2.9 s.

(Unit L) to 1.8 spikes/s (Unit P) for the slowest and most rapidly firing units, respectively, with 68% of the units discharging at less than 0.42 spikes/s. The relationship between λ and the mean interspike interval is shown graphically in Fig. 4. Although three pairs of units had the same mean rate of discharge; i.e. Units O and N at 0.10 spikes/s, Units F and H at 0.21 spikes/s, Units A and C at 0.42 spikes/s the difference in their mean interspike interval was 1.2, 3.4, and 0.3 s, respectively.

For each unit, the standard deviation of the mean interval closely approximated the mean interspike interval itself (Fig. 5) which suggests the arhythmicity of spontaneous activity recorded from olfactory receptors. The standard deviations for 12 units were equal to or greater than the unit's mean interspike interval. Hence, the coefficient of variation for these units was greater than 1.0 which precludes the interval distribution from being truly Poisson (Brown et al., 1973). In the graphic plot of the data (Fig. 5) units with coefficients of variation greater than 1.0 lie above the line of identity. Unit I had a mean interspike interval of 2.7 ± 5.8 s and showed the greatest variability in intervals which ranged from 11.6 s to about 150 ms. In contrast, Unit E had a mean interspike interval of 3.7 ± 3.1 s and showed the least variability in intervals which ranged from 14.3 s to about 230 ms. As the behavior of these two units suggests, the distribution of interspike intervals was generally not symmetrical about the mean. Rather, the distribution was characteristically skewed. The occurrence of several long interspike intervals in each sample was responsible in large measure for the standard deviations which were equal to or greater than the mean interval. Accumulation of larger amounts of interspike interval data should eventually permit a thor-
Olfactory Receptor Responses

Responses to Stimuli

Spike patterns recorded from 70 receptor cells which responded to stimuli were investigated. Single unit isolation was difficult to achieve in the olfactory epithelium. Although the extracellular electrode frequently recorded from more than one unit simultaneously, spikes from different units could occasionally be separated from one another on the bases of amplitude and voltage conformation (see Figs. 3, 6, 7). Responses to odorous stimuli were recorded from receptor cell axons as evidenced by the depth of electrode penetration and triphasic spike voltage sequence. Response profiles of a well-isolated spontaneously active unit were often obscured by recruitment of nonspontaneously active receptors during stimulation. Occasionally, an excitatory

Figure 4. Plot of overall mean firing rate ($\lambda$) and mean interval for each of 16 spontaneously active units. See text.
burst of spikes accompanied the mechanical switching of valves. This indicated a mechanical artifact induced by the movement of the electrode relative to the neuron, mechanical excitation of the unit by the electrode, or possibly a mechanosensitive unit. The technique did not allow discrimination among these alternatives. A burst of spikes occasionally accompanied the arrival of a control aliquot of Ringer's solution. Spike conformations and response patterns for both responses were indistinguishable from those evoked by chemical stimulation. Receptor units which showed either response were disregarded from further study.

Many units did not respond to any one of the six stimuli at the low concentrations tested. For example, a triphasic spike generated by a spontaneously active unit, Unit N, fired asynchronously at 0.10 spikes/s with a mean interspike interval of 9.7 ± 8.7 sec (Fig. 6a). The coefficient of variation was 0.90. The peak-to-peak amplitude of the spike varied 2.5% from the mean amplitude during the 3-min prestimulus monitoring period. The unit was not mechanically sensitive and did not respond to a control aliquot of Ringer's (b). At the highest concentrations tested (0.33 mM), the unit was not excited by identical aliquots of o-tolylurea (c), m-tolylurea (d), or p-tolylurea (e). The spike showed little change in interspike intervals during the intervening 2-min intervals between stimulations. Low amplitude spikes,
Figure 6. Unit N spontaneous activity (a); nonresponsive to control aliquot of Ringer or mechanical switching transients (b); not excited by aliquots of 0.33 mM o, m, and p-tolylurea (c, d, and e), respectively.

presumably recorded from receptor units distant from the electrode tip, were recruited into each response (c, d, and e). A spike with an intermediate amplitude was excited by the p-tolylurea (e) but not by the other two isomers at identical stimulus concentrations. Hence, the receptor unit recorded as the intermediate amplitude spike discriminated the p isomer from the o and m compounds.

When aliquots of stimuli, both volatile (see Fig. 1, Class I) and nonvolatile (see Fig. 1, Class II) were delivered to the olfactory epithelium, spikes were recorded from about 20 units which were excited by at least one stimulus from each class. Background activity (Fig. 7 a) was recorded simultaneously from several spontaneously active olfactory receptors. They did not respond to a control aliquot of Ringer’s (Fig. 7 b). Increases in spike firing frequency were recorded when aliquots of 12.5 μM ethyl n-butyrate (c) and 0.33 mM o-tolylurea (d) were delivered. The excitatory response for each lasted about 4 s and elicited about 5 spikes/s. Although unit isolation remained fairly intact at these stimulus strengths, smaller amplitude spikes were recruited into each response. An aliquot of 3.33 nM o-tolylurea also excited the unit (e) but to a lesser extent than the higher concentration. Since the aliquot of 33 nM o-tolylurea (f) evoked a dubious excitatory response, the threshold for the unit to o-tolylurea was conservatively judged to be greater than 33 nM but less than 3.3 μM (see Methods for comments on threshold information). Of the six stimuli used in this study, ethyl n-butyrate gave the lowest thresholds. One unit was encountered whose threshold to ethyl n-butyrate was greater than 0.125 nM but less than 12.5 nM. The same unit was excited by 0.4 mM benzaldehyde. Spikes were recorded from units whose typical threshold values to ethyl n-butyrate were about 1.25 nM.
No significant changes were observed in the triphasic spike conformation in the excitatory responses with either class of stimulus or to serial dilutions of the stimulant. Normal spike patterns in the excitatory response (see Fig. 8b and d) were evoked by the nonvolatile stimuli. High concentrations, that is, greater than 1 mM, of the nonvolatile stimuli were not required to evoke excitatory responses from the receptors. Low concentrations did not cause irreversible changes in the EOG evoked by a puff of a vaporous stimulus.

**Figure 7.** Multiunit responses of olfactory receptors to aliquots of volatile (c, 12.5 μM ethyl n-butyrate) and nonvolatile (d, 0.33 mM; e, 3.3 μM; f, 33 nM o-tolylurea) stimuli; background activity (a) and control aliquots (b).

**Figure 8.** Threshold determination and responses of Unit M to near-threshold concentrations (0.33 mM) of o-tolylurea (b) and p-tolylurea (d); no excitatory responses evoked at 3.3 μM for p-isomer (c) or the o-isomer (d); spontaneous activity shown in a.
Hence, the tolylureas were judged to be adequate physiological stimuli for the frog's olfactory epithelium.

Spikes recorded from a spontaneously active unit, Unit M, had an overall mean rate of discharge $\lambda$ of 0.20 spikes/s with a mean interspike interval of 5.2 ± 4.4 s and a coefficient of variation (CV) of 0.85 (Fig. 8 a). An aliquot of 0.33 mM $o$-tolylurea excited (Fig. 8 b) the olfactory receptor, whereas 3.3 $\mu$M did not (Fig. 8 c). Hence, the threshold for Unit M was judged greater than 3.3 $\mu$M but less than 0.33 mM. During the excitatory response the unit's firing frequency was increased to 3.77 spikes/s with a mean interspike interval of 0.21 ± 0.06 s. The variation in interspike intervals (CV) was decreased to 0.28 in response to the stimulus. Likewise, a 0.33 mM aliquot of $p$-tolylurea excited the receptor cell (Fig. 8 d), whereas a 3.3 $\mu$M aliquot did not (Fig. 8 e). In response to the $p$ isomer, the unit's firing frequency $\lambda$ was increased to 3.72 spikes/s while the mean interspike interval was decreased to 0.22 ± 0.08 s. The variation in interspike intervals (CV) was greater for the $p$-tolylurea, i.e. 0.36, than the $o$ isomer during the excitatory response.

A sequential interspike interval analysis of the excitatory responses recorded from Unit M is shown graphically in Fig. 9. The excitatory response evoked by $o$-tolylurea consisted of five interspike intervals (closed squares) in which there was an approximately exponential decrease in the first four interspike intervals lasting about 800 ms. In contrast, the excitatory response evoked by $p$-tolylurea consisted of eight interspike intervals (open squares) during which there was an exponential decrease in the first three interspike intervals.
lasting about 600 ms. Each initial response was followed by erratic but generally increasingly longer intervals. Each response was preceded by an interspike interval (dashed lines) whose duration was within 1 SD from the mean interspike interval calculated for the spontaneous activity.

Other responses were recorded from units which were excited by either one or all three of the tolylurea isomers at similar concentrations. In general, most units excited by the tolylureas had thresholds greater than 3.3 μM but less than 0.33 mM.

Both nitrobenzene and benzaldehyde had physiological thresholds intermediate between those obtained for the tolylurcas and ethyl n-butyrate. Ten spikes were recorded in 12 min from a spontaneously active olfactory receptor (Fig. 10). The mean interspike interval during spontaneous activity was 74.5 ± 48.1 s with a coefficient of variation of 0.65. The vigorous excitatory response evoked by a 4.5 μM aliquot of benzaldehyde increased the rate of discharge λ to 7.2 spikes/s. The mean interspike interval during the response was 0.13 ± 0.05 s. Likewise, a 3.7 μM aliquot of nitrobenzene excited the receptor cell (e) increasing its rate of discharge λ to 13.3 spikes/s with a mean interspike interval of 0.06 ± 0.02 s. Variation (CV) in intervals was 0.38 in the response evoked by benzaldehyde and somewhat less, i.e. 0.33 in that evoked by nitrobenzene. Aliquots of 0.45 nM benzaldehyde (d) and 37 nM nitrobenzene (f) did not excite the receptor cell. Hence, the threshold was judged to be at an intermediate value between the micro- and nanomolar quantities.

**Figure 10.** Threshold determination and excitatory responses to near-threshold concentrations of volatile stimuli, 4.5 μM (c) and 45 nM (d) benzaldehyde, 3.7 μM (e), and 37 nM (f) nitrobenzene. Spontaneous activity (a) less than 2 spikes/min, no response to control aliquot (b).
Fig. 11 describes the sequential interspike interval analysis for the excitatory responses evoked by benzaldehyde (open circles) and nitrobenzene (closed circles) shown in Fig. 10. The responses evoked by benzaldehyde lasted about 1.5 s and consisted of 12 interspike intervals. A nearly exponential decrease in the first four intervals lasted about 400 ms and was followed by a series of intervals which were longer than the shortest interval in the initial response. The response evoked by nitrobenzene was considerably different in profile in that the initial rapid decrease in the first three intervals lasted about 250 ms. It was followed by a rather constant rate of discharge lasting about 900 ms and then slowly increasing interspike intervals. The interspike interval which preceded the active responses (dashed lines) was longer than the sum of the intervals in each response. Before the response evoked by benzaldehyde it was greater than 1 SD from the mean interspike interval calculated for the spontaneous activity whereas before the response evoked by nitrobenzene it fell within 1 SD.

Spikes were recorded from other olfactory receptors which were similarly excited by micromolar quantities of benzaldehyde and nitrobenzene. Three units were clearly excited by nanomolar quantities of these stimuli. Hence, thresholds for different receptors to the same stimulus may vary from milli- to nanomolar quantities. A single unit was encountered which was excited
by a 4.5 μM aliquot of benzaldehyde but not nitrobenzene at concentrations between milli- and nanomolar quantities. Details of the interval patterns for each unit evoked by different stimuli were generally dissimilar although their responses shared the same general characteristics to those shown in Fig. 11.

It was of interest to investigate the parameters of excitatory responses evoked by a stimulus as it grew out of the spontaneous activity at different concentrations. The spontaneous activity of a unit was monitored for 2.5 min and had an overall mean rate of discharge $\lambda$ of 0.29 spikes/s. The activity was very asynchronous as evidenced by a mean interspike interval of 3.38 ± 5.93 s and a coefficient of variation of 1.75. An aliquot of 0.45 μM benzaldehyde evoked an excitatory response (open triangles, Fig. 12 a) during which the firing frequency $\lambda$ increased to 2.92 spikes/s. Interspike intervals during the response were quite variable in length ranging from about 100 to 480 ms. The mean interspike interval was 0.28 ± 0.21 s. Compared with the spontaneous activity the relative variability (CV) was reduced to 0.75. When the concentration of benzaldehyde was increased by two log units to 45 μM the firing frequency $\lambda$ during the excitatory response (closed triangles, Fig. 12 b) was increased to 4.24 spikes/s. The mean interspike interval was further reduced to 0.21 ± 0.04 s. The relative variability in intervals (CV) was reduced by nearly a factor of 4 to 0.19. Hence, when stimuli were delivered to olfactory receptors at low concentrations there was typically a 10-fold

Figure 12. (A) Sequential interspike interval analysis of excitatory responses recorded from a receptor to 0.45 μM benzaldehyde (open triangles) and 45 μM benzaldehyde (closed triangles). (B) Interspike interval analysis from Fig. 9, 0.33 mM o-tolylurea (closed squares) and 0.33 mM p-tolylurea (open squares); and from Fig. 11, 3.7 μM nitrobenzene (closed circles) and 4.5 μM benzaldehyde (open circles). See text for discussion.
increase in the mean rate of discharge \( \lambda \) over that of the spontaneous activity by the lowest concentration which was then nearly doubled by a 2-log step increase in concentration. Increasing concentrations of the stimulus not only decreased mean interspike intervals in the responses but they were also accompanied by a reduced variability in the intervals as evidenced by the decreased coefficient of variations when compared with spontaneous activity.

The characteristics of excitatory responses evoked by stimuli are summarized in relation to Fig. 12a and b where the sequential interspike intervals obtained from the responses shown in Figs. 9 and 11 are replotted with the same coordinates as Fig. 12a. Excitatory responses had durations which ranged from 10.6 s for \( \sigma \)-tolylurea to 2.74 s for 0.45 \( \mu \)M benzaldehyde. The response was preceded by an interspike interval which was typically of longer duration than the response and generally was within 1 SD of the mean interspike interval calculated for the unit's spontaneous activity. A unit's overall mean rate of discharge \( \lambda \) was typically increased by at least a factor of 10 over that of the spontaneous activity with near-threshold stimulus concentrations. The relative variability of interspike intervals was reduced by at least a factor of 2 during excitatory responses when compared with that of spontaneous activity. Each response was initiated by a nearly exponential decrease in interspike intervals during the initial two to four intervals which ranged from about 250 to 800 ms in total duration. This probably reflected the initial rapid invasion of generator currents triggered by the stimulus into the site of spike initiation. The times should be viewed with caution since the spatial-temporal dispersion of stimuli in Ringer's solution during stimulus delivery was not a step function. With the exception of the response evoked by nitrobenzene the initial intervals were followed by intervals of generally longer duration.

**DISCUSSION**

The question of stimulus discrimination by olfactory receptor cells is clearly complex. Specific molecular recognition mechanisms of stimulus molecules by olfactory receptors are not unlike those suggested for other systems involving specific molecular interactions (e.g., chemical synapses, hormonal activity, immunological systems, drug actions). As a working hypothesis, it is assumed that there are molecular receptors, or active sites of a receptor molecule, in the receptor cell membrane which interact with the stimulus molecule (Gesteland et al., 1965; O'Connell and Mozell, 1969; Beets, 1971; M. L. Getchell and Gesteland, 1972; Polak, 1973). The molecular interaction of the stimulus and receptor molecule, if coupled with ion conductance mechanisms across the cell membrane, may result in electrical signals which are recorded as responses by extracellular microelectrodes. When spontaneous spike activity is being recorded from a receptor cell axon and no change in
that activity is elicited by the stimulus, three interpretations are possible. They are: The threshold to elicit spike electogenesis was not reached; the membrane was not chemically irritable; or there were no molecular mechanisms on that particular receptor cell to detect the stimulus. It is critical to distinguish between the second and third possibilities when specific molecular mechanisms, other than those involved in general chemo-irritability, are invoked to account for the discriminatory capabilities of an olfactory receptor among stimuli. In the present study, to rule out nonstimulus-specific mechanisms related to the second possibility, only low concentrations of stimuli known to evoke specific chemical sensations were used.

The results support the hypothesis that olfactory receptors discriminated among stimuli on the basis of differential thresholds. Thresholds to stimuli used in this study ranged 7 log units, i.e. from milli- to nanomolar quantities. In general, excluding those units which were only excited by millimolar concentrations of stimuli, the highest threshold values (about 3.3 µM) were obtained with the tolylureas while the lowest (about 1.25 nM) were obtained for ethyl n-butyrate. Most receptors excited by benzaldehyde and nitrobenzene had thresholds between 37 nM and 4.5 µM. No units were encountered which had thresholds less than 37 nM to these two stimuli. O'Connell and Mozell (1969) estimated threshold values of about 25 µM for four volatile esters when delivered in the vapour phase to the frog's olfactory epithelium. The differences between thresholds found in these studies is probably not related to the method of stimulus delivery, i.e. vapor phase vs. fluid phase, but rather to the specific stimuli used in each study and different methods of sampling unit activity. Thresholds for the same unit to different stimuli could be within the same order of magnitude or could vary from 2 to 5 log units.

Stimulus-related responses recorded from olfactory receptors have been classified as excitatory, inhibitory, or no change in spontaneous activity (Gesteland et al., 1963, 1965) when judged by subjective criteria. Daval et al. (1972), Holley et al. (1974), and Blank (1974) reported that inhibitory responses were recorded from frog olfactory receptors by estimating the “response magnitude” which is the total number of spikes in the response minus the background spontaneous activity. Such “inhibitory responses” could not be reliably evaluated in this study using quantitative techniques due to low spontaneous firing frequencies as evidenced by long mean interspike intervals which ranged from 0.5 to 13.7 s (Fig. 4) and its asynchronous character as suggested by wide first standard deviations from the mean interval (Fig. 5). For a more detailed discussion of this question see Getchell and Getchell (1974).

A second discriminatory mechanism was deduced by comparing the excitatory responses recorded from a receptor cell evoked by two different stimuli.
When a receptor was excited by benzaldehyde, it was usually excited by nitrobenzene at similar concentrations (see Fig. 10). Both responses would be classified as "excitatory" which suggests that the receptor did not discriminate between these two stimuli. The statistical parameters and sequential interspike interval analysis (Fig. 11) indicated that the two responses were dissimilar. This would suggest that the receptor cell discriminated between nitrobenzene and benzaldehyde even though both compounds evoke the same basic odor sensation in man. Ortho- and para-tolylurea evoke different sensations in man. Both compounds evoked excitatory responses from Unit M at similar concentrations with the overall mean rate of discharges λ during the response differing by less than 100 ms. Although the number of interspike intervals in each response was different the mean intervals were nearly identical, i.e. 0.21 ± 0.06 s and 0.22 ± 0.08 s for the ortho- and para-isomers, respectively. These results indicated that the receptor did not discriminate between the two isomers. Thus, distinctive spike patterns evoked by different stimuli which are recorded from an olfactory receptor are postulated to convey at least a fraction of the informational content of the stimulus molecule to the central nervous system.

Since the compounds employed in this study elicited responses recorded from olfactory receptor cells one should be able to predict discrimination among stimuli knowing the response evoked by a given stimulus. Based on the molecular characteristics of the stimulus (see Fig. 1), it was initially predicted that a receptor would easily discriminate between the ortho- and para-isomers, whereas discrimination between ortho- and meta-, and between para- and meta- would be more difficult. If it was assumed that different molecular receptors in the receptor cell membrane were activated by different compounds which evoke distinctly different sensory qualities in a chemosensory system, it was predicted that receptors would easily discriminate among the three isomers. When a unit was "excited" by one isomer of tolylurea it could not be predicted with any reasonable assurance whether or not the unit would be excited by either or both of the other isomers. When a unit was excited by nitrobenzene it was usually excited by benzaldehyde which was initially predicted knowing the molecular characteristics of the stimuli and similar odor qualities evoked in man. If a unit was excited by either compound it could not be predicted whether or not the unit would be excited by ethyl n-butyrate. The inability to predict responses, even in general terms, knowing the response to one compound, was most unsatisfying. This may reflect an overlapping spectrum of molecular receptor site types on a single cell for different compounds with dissimilar molecular characteristics which evoke distinctly different sensory qualities, e.g., nitrobenzene and ethyl n-butyrate or m-tolylurea and p-tolylurea. In addition, it may represent the activation of different active sites on a receptor molecule for compounds with similar molecular
characteristics which evoke a similar sensory quality, e.g., nitrobenzene and benzaldehyde.

Although the quantitative analyses of excitatory responses reported here suggest that specific information was extracted from the stimulus by olfactory receptors, it is premature to conclude which specific molecular receptor site types the stimulus interacted with to initiate the response. It remains to be determined if specific spike patterns recorded from olfactory receptors in response to odor stimulation can be correlated with the molecular characteristics of the stimulus or the sensation evoked in man. Clearly, further detailed quantitative information concerning the physiological parameters of spontaneous activity and odor-evoked responses is required before a critical investigation of questions concerned with the molecular and neural mechanisms of stimulus discrimination.

The author thanks Ann Flitcraft and Harry Webster for their enthusiastic laboratory assistance. Valuable discussions with Dr. M. L. Getchell, Dr. D. Moulton, and Dr. G. M. Shepherd were appreciated.

The author is a University of Pennsylvania Plan Scholar.

The research was supported by NSF Grant GB-28016.

Received for publication 10 October 1973.

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