Factors Influencing the Differential Sorption of Odorant Molecules across the Olfactory Mucosa

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ABSTRACT By use of a flow dilution olfactometer, tritium-labeled odorants were presented through the external naris to the bullfrog's intact olfactory sac. After stimulation the animal was frozen in liquid nitrogen. The dorsal surface and eminentia of the olfactory sac were then removed and sawed into sections perpendicular to the long axis of the mucosal surface. Each section was dissolved in a tissue solubilizer and counted in a liquid scintillation system. The amount of radioactivity in each section was used to estimate the number of odorant molecules it sorbed. For tritiated butanol there was a significant decrease in radioactivity from the section containing the external naris to that overhanging the internal naris. The steepness of the gradient was unaffected by a rather large range of stimulus flow rates, volumes, and partial pressures. Only when these parameters were pushed to extreme physical limits did this gradient change significantly. When the stimulus was presented through the internal rather than the external naris, the butanol gradient reversed its direction, decreasing from the internal to external. Unlike butanol, tritiated octane presented through the external naris was rather evenly distributed among the mucosal sections. That is, octane showed no distribution gradient across the mucosa. These results complement previous electrophysiological data that suggested a "chromatographic-like" differential sorption of odorant molecules across the mucosa.

INTRODUCTION

On the basis of recordings of the spike activity of the olfactory bulb Adrian (1950, 1954) proposed two possible mechanisms at the level of the olfactory mucosa which could underlie olfactory discrimination: (a) the receptors themselves might be selectively tuned to particular odorants; and (b) the molecules of different odorants might be distributed in different space-time patterns across the mucosa in accordance with those physicochemical properties that affect the progress of their migration. In the former mechanism each receptor would signal how closely the incoming odorant molecules might match that receptor's particular tuning. Thus odorants would be encoded by various combinations of receptors responding at different levels relative to each other. In the latter mechanism each receptor would signal the degree and rapidity with which the incoming odorant molecules reached its position along the mucosa; each odor-
ant would then be encoded by a different space-time activity pattern across the receptor sheet.

Although in the years following Adrian's work most attention has been given to the concept of selectively tuned receptors (Gesteland et al., 1963; Mathews and Tucker, 1966; Mathews, 1972; O'Connell and Mozell, 1969), the concept of the differential migration and distribution of the molecules of different odorants across the mucosa has received at least some consideration. Moncrieff (1955) prepared the heads of freshly killed sheep so that an odorized air stream could be introduced into one naris and eluted from the other. He noted that the eluted air stream remained odorless to his human observers for a period of time the length of which was dependent upon the particular odorant used. This experiment suggested that different odorants migrate across the nasal mucosa (though no specifically the olfactory mucosa) at different rates probably due to their differential sorption along the epithelial surface.

Using an electrophysiological approach, Mozell (1966, 1970) sampled the activity elicited by different odorants at two widely separated regions of the bullfrog olfactory mucosa. He simultaneously recorded the summed multiunit discharges from the two branches of the olfactory nerve which supply these two regions. The more medial nerve branch (MB) reflected the activity of a mucosal region in the roof of the olfactory sac near the external naris, where the odorized air first enters the olfactory sac. The more lateral nerve branch (LB) supplied a region overhanging the internal naris and thus reflected the activity in a mucosal region considerably farther along the flow path. The ratio of the discharge magnitude of the lateral nerve branch to that of the medial nerve branch (LB:MB ratio) was used to quantify the gradient of activity across the mucosa resulting from an artificially produced sniff of odorant drawn through the external naris; the smaller this ratio, the greater the decrease in mucosal activity from the external naris to the internal naris.

For a battery of 16 different odorants, Mozell (1970) found that each odorant produced a characteristic LB:MB ratio which was consistent from frog to frog. For several of these 16 odorants the LB:MB ratio could be somewhat modified by varying the concentration or flow rate at which the odorant was presented. However, within the concentration and flow rate ranges used, the major determinant of the ratio was the chemical itself. Thus, it appeared that different odorants did indeed establish different activity patterns across the olfactory mucosa.

What is the mechanism that underlies these different activity patterns produced by different odorants? In one approach to this question Mozell (1964) reversed the flow direction of the odorized air across the mucosa, delivering it through the internal naris instead of the external naris. He observed for four sample odorants that this maneuver reversed the nerve branches giving the larger and smaller discharges. This reversal is exactly what would be expected if the activity patterns produced by various odorants were established by the differential sorption of their molecules as they migrated along the olfactory mucosa.

However, electrophysiological recordings from olfactory nerve branches can give only indirect indication of molecular sorptive events along the olfactory
mucosa. This led Mozell and Jagodowicz (1973) to substitute the in vivo frog olfactory sac for the column of a gas chromatograph in order to obtain a more direct measurement of how odorant molecules migrate across the olfactory mucosa. The results of this study showed a rather wide range of retention times for 15 different odorants. In addition, Mozell and Jagodowicz found a strong inverse relationship between these 15 odorant retention times in the olfactory sac and the magnitudes of their electrophysiologically determined LB:MB ratios. This inverse relationship is to be expected if the LB:MB ratios do indeed depend upon molecular distribution across the mucosa. In the time of a given sniff the molecules of odorants with long retention times would presumably not migrate in large numbers much beyond the region of the external naris and would therefore produce small LB:MB ratios. On the other hand, the molecules of odorants with short retention times would reach the region of the internal naris in greater numbers, thus producing larger ratios.

Although the above data have been indicative of differential molecular distribution patterns, conclusive evidence for their existence and an appreciation of their exact topology requires a direct determination of how the molecules are actually distributed. This requirement is met here by using tritium-labeled butanol and octane molecules quantitatively to map odorant sorption patterns along the mucosa. In addition, this technique allows for a definitive determination of how these molecular distribution patterns might change as a function of stimulus flow rate, volume, and concentration. Therefore, the technique gives to the study of vertebrate olfaction an ability to quantify both the number and the location of odorant molecules across the olfactory mucosal sheet.

MATERIALS AND METHODS

Preparation

Bullfrogs (*Rana catesbeiana*) varying in weight from 550 to 850 g (supplied by Jacques Weil Company, Rayne, La.) were housed in tanks provided with continuously running tap water. The animals were used within 40 days of their arrival in the laboratory. They were anesthetized with a single subcutaneous dose of urethane (0.6 g in 1.2 ml water/100 g body weight) and kept in a moist environment until the beginning of the experiment.

Within 4 s after the introduction of the tritiated odorant into the frog's intact olfactory sac (see below), the frog was quick-frozen in a Dewar flask containing liquid nitrogen. This quick-freezing was intended to minimize any further movement of odorant molecules which could have resulted from diffusion and/or desorption. With the animal frozen solid, a no. 3 jeweler's saw was used to remove, in one piece, the roof of the olfactory sac. This piece, hereafter referred to as the dorsal surface, included the dorsal, lateral, and septal walls of the olfactory sac. In order further to section this piece and at the same time to prevent its thawing, the piece was mounted in a specially constructed steel miter box which could be maintained at a temperature below 0°C by being frequently returned to the liquid nitrogen. The dorsal surface could then be sawed into five sections (1.95 mm wide) perpendicular to the long axis of the olfactory sac (Fig. 1). These five sections were designated M1–M5, with M1 being the section cut just caudal to the external naris (also including that small piece of the floor of the olfactory sac which is part of the vestibule of the external naris) and M4 being that part of the dorsal surface overhanging the internal naris. Section M5 was then that part of the dorsal surface caudal to the internal naris.
When the dorsal surface of the olfactory sac was removed, its ventral surface (which contains the eminentia olfactoria) remained attached to the frozen carcass. The eminentia was then cut into four sections (with a no. 2 jeweler's saw) and each section was removed from the animal (Fig. 1). These four eminentia sections were designated E1–E4, with E3 being the caudolateral section facing the internal naris and E4 being the caudomedial section facing the nasal septum. By this notation, E1 and E2 were the rostrolateral and rostromedial quadrants, respectively.

**Liquid Scintillation System**

Sections M1–M5 and E1–E4 were individually dissolved in a tissue solubilizer (Soluene-350) and counted in a liquid scintillation system (Omnifluor: dry mix 98% PPO and 2% bis-MSB). Because of variable amounts of quenching, the activity of each vial was ultimately expressed in disintegrations per minute (dpm). This was accomplished by using the previously calibrated automatic external standard of the liquid scintillation counter to determine the counting efficiency of each vial. The number of molecules of a given odorant sorbed to each section could be estimated by multiplying each section's dpm by the average number of molecules per dpm for that odorant (see below).

**Stimulus**

The stimulus for these experiments was either tritiated butanol or tritiated octane (New England Nuclear, Boston, Mass.). The manufacturer determined the radiochemical purity of the tritiated butanol to be in excess of 99%. By liquid scintillation counting, the activity of the tritiated butanol was determined to be 120.2 mCi/ml. From this number the average number of butanol molecules per dpm was calculated to be $2.481 \times 10^{10}$. The activity of the tritiated octane was determined to be 352.1 mCi/ml and the average number of octane molecules per dpm was calculated to be $0.473 \times 10^{10}$.

The concentration of the tritiated stimulus was controlled by a flow dilution olfactometer (Fig. 2) similar to that described by Mozell (1966) but with some necessary adaptations to prevent the escape of radioactive molecules into the atmosphere. The internal naris cannula was connected to a negative pressure pump (Harvard infusion/withdrawal pump). When activated, the negative pump pressure diverted a sample of the radioactive...
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air stream through the external naris cannula into the frog's intact olfactory sac, thus providing an artificially produced sniff. The flow rate of the stimulus was controlled by the speed of the withdrawal pump and the volume of the stimulus by the duration of its presentation. Before stimulation 5 min were allowed for the olfactometer to reach equilibrium at the desired partial pressure.

The block diagram for the positive pressure stimulus (puffed stimulus) was similar to that seen in Fig. 2 except that the negative pressure pump was removed and the line

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**FIGURE 2.** Block diagram of the olfactometer. To produce a given odorant concentration, the compressed air was first dried and deodorized by being passed through silica gel and activated charcoal. The air was split into two independent streams (controlled by separate metering valves) and each was again dried and deodorized, this time with calcium chloride, activated charcoal, and silica gel. One stream was bubbled through the tritium-labeled odorant where, in order to approach a complete saturation at room temperature (23°C ± 1°C), the bubbles were made quite fine by forcing the air through Pyrex wool as it left the submerged inlet tube. The air, now saturated with tritiated odorant, went through a flow meter and on to the mixing chamber. The second air stream was bubbled through deodorized water and this humidified air was used to prevent dehydration of the olfactory mucosa. When the three-way valve at point A was appropriately adjusted, the nonodorized, humidified air was also directed to the mixing chamber. The resultant stream went through a second flow meter and, depending on how the three-way valves at points B and C were adjusted, flowed either to a toluene isotope trap or to the isotope filter in the hood. The three-way valves at points A and C could also be adjusted to shunt the nonodorized, humidified air stream around the mixing chamber and directly to the isotope filter in the hood. The tritiated stimulus was presented to the frog through a Teflon cannula placed in the external naris. Labeled molecules not adhering to the mucosal surface were collected in a toluene scintillation solution by way of a second cannula which was placed in the internal naris and secured there by a dental molding material (Key to Alginates).
between point D and the hood was blocked. Thus, all the flow through the olfactometer was directed into the olfactory sac, and the flow rate through the olfactometer determined the flow rate through the animal.

With the positive pressure delivery system, stimulation was terminated by shunting the air stream away from the animal. Although this immediately stopped all flow through the olfactory sac, it left a possible radiological health hazard due to the residual tritiated vapors in the cannula tubing. However, with the negative pressure system, this hazard could be reduced by removing the external naris cannula before disconnecting the animal from the withdrawal pump. Thus, with the negative pressure delivery system the stimulus was presented as a slug followed by 1.5 s of nonradioactive room air. With a 16 cm$^3$/min flow rate, this amounted to about 0.4 cm$^3$.

**Verification of the Olfactometer Output**

The use in these experiments of a tritiated labeled odorant provided an accurate technique to verify that the olfactometer delivered the number of molecules expected. By connecting the external and internal naris cannulas together (with no frog in the system), all the isotope from a given puff could be collected in the toluene scintillation solution. Consequently, at a particular partial pressure the theoretical number of molecules delivered in a particular stimulus, as determined by the ideal gas laws, could be checked against the actual number of molecules recovered as estimated from the dpm.

By use of a positive pressure delivery system, with a butanol partial pressure of 6.78 mm Hg and a net stimulus volume of 0.195 ml, the delivered number of molecules as calculated by the ideal gas laws was $4.27 \times 10^{16}$. The mean of the actual number of molecules recovered from six samples was $4.27 \times 10^{16}$, with a standard deviation of $0.10 \times 10^{16}$. The percent recovered (actual/calculated $\times 100$) was $100\% \pm 2\%$. Thus the olfactometer delivered the predicted number of molecules, and a method was available for periodically checking its performance.

After the olfactometer had been successfully cleaned of residual butanol, the system was reassembled to provide an octane stimulus. A check of the olfactometer's output showed a recovery of $100\% \pm 2\%$ of the expected molecules.

**Test for Radioactivity Losses Due to Freezing and Solubilizing**

To test the possibility that the freezing and solubilizing procedures might have led to a loss of radioactive molecules, a positive pressure radioactive stimulus was presented to the intact olfactory sac, the animal was frozen, and the dorsal surface and eminentia were removed. These pieces were not sectioned further but were instead dissolved uncut in the tissue solubilizer. With a partial pressure of 6.78 mm Hg and a stimulus volume of 0.42 cm$^3$, the theoretical number of molecules delivered to the mucosa was $9.30 \times 10^{16}$. The mean of the total number of molecules actually recovered in six animals (as determined by the sum of those found in the dorsal surface, eminentia, and toluene collection bottle) was $9.26 \times 10^{16} \pm 0.40 \times 10^{16}$. The percent recovered was $99\% \pm 4\%$. Therefore, very few, if any, of the butanol molecules were being lost because of the freezing and solubilizing procedures.

**Reverse Flow Experiment**

With seven animals the direction of flow of the tritiated butanol was reversed. That is, the external and internal naris cannulas were placed in the animal as before, but the stimulus was presented through the internal naris cannula while the negative pressure was applied to the cannula in the external naris. The other variables and sectioning techniques were the same as those described above.
RESULTS

Positive vs. Negative Pressure Butanol Delivery Systems

For the stimulating parameters noted in the lower lefthand corner of Table I, column A shows the dpm recovered per section after a positive pressure "puffed" stimulus of tritiated butanol. Each dpm is the mean for that particular section which is based on six animals. "Not sorbed" refers to those labeled

| Section           | Dorsal surface | Eminentia | Not sorbed |
|-------------------|----------------|-----------|------------|
|                   | A              | B         | C          | D           | E           | F           |
|                   | dpm/section    | % total molecules recovered | % total molecules recovered | mean surface area (nm²) | molecules/mm³ |
| M1                | 144,211        | 357.8     | 84.85±4.79 | 84.38±1.29  | 4.5±0.1     | 79.51       |
| M2                | 12,427         | 30.8      | 7.31±3.04  | 5.81±1.41   | 14.6±0.3    | 2.17        |
| M3                | 2,635          | 6.3       | 1.55±1.37  | 1.86±0.85   | 19.0±0.5    | 0.34        |
| M4                | 952            | 2.4       | 0.56±0.49  | 1.00±0.44   | 21.4±0.6    | 0.11        |
| M5                | 510            | 1.3       | 0.30±0.21  | 0.95±0.62   | 10.7±4.1    | 0.12        |
| E1                | 3,672          | 9.1       | 2.16±1.31  | 2.85±0.75   | 3.7±0.2     | 2.46        |
| E2                | 3,145          | 7.8       | 1.85±0.98  | 2.11±0.58   | 3.7±0.1     | 2.11        |
| E3                | 714            | 1.8       | 0.42±0.25  | 0.42±0.21   | 4.3±0.2     | 0.42        |
| E4                | 867            | 2.2       | 0.51±0.39  | 0.11±0.50   | 4.2±0.2     | 0.52        |
| Not sorbed        | 867            | 2.2       | 0.5       | 0.05        | 0.5         | 0.41        |

Total dpm recovered (×10⁹)

| A              | B              | C          | D           | E           | F           |
|----------------|----------------|------------|-------------|-------------|-------------|
| % total molecules recovered | % total molecules recovered | mean surface area (nm²) | molecules/mm³ |
| 0.422×10⁸ | 0.450×10⁸ | 0.500×10⁸ | 0.500×10⁸ |

% stimulus recovered

| A              | B              | C          | D           | E           | F           |
|----------------|----------------|------------|-------------|-------------|-------------|
| No. of animals | 6             | 6          | 8           | 8           | 8           |

Odorant: butanol.
Partial pressure: 0.36 mm Hg.
Flow rate: 16 cm³/min.
Volume: 0.42 cm³.
negative pressure delivery system (i.e., an artificially produced sniff), was used in eight animals. These negative pressure data (column D) are presented in the percent notation described above. As can be seen by comparing columns C and D, there is little if any difference between the mucosal distributions of butanol molecules resulting from the positive and the negative pressure delivery systems.

At the bottom of columns C and D other pertinent data are given: the total dpm recovered; the number of total molecules actually recovered; the total molecules expected as predicted by the ideal gas laws; and the percent stimulus recovered (actually recovered divided by the expected x 100).

From the data in columns C and D, there is an obvious decreasing gradient of butanol molecules from the dorsal surface section which contains the external naris (M1) to the section overhanging the internal naris (M4), and the gradient is rather steep. There is also a gradient from the rostral sections (E1 and E2) to the caudal sections (E3 and E4) of the eminentia, but this gradient is an order of magnitude less than the decrease along the dorsal surface. However, it was possible that these dorsal surface and eminentia gradients simply reflected differences in the surface areas of the various sections. To correct for differences in surface area, the number of molecules recovered from each section (column B) was divided by the mean surface area of the corresponding section (column E) (Hornung et al., 1975). This correction for surface area (column F) does not reduce the gradients.

Effect of Stimulation Parameters upon Distribution Patterns

CONCENTRATION By holding volume constant at 0.42 cm³ and flow rate at 16 cm³/min, a comparison was made of the mucosal distribution patterns established by five different butanol partial pressures (0.06, 0.36, 0.62, 0.92, and 6.78 mm Hg). The results of this concentration study have been reported in detail elsewhere (Hornung et al., 1975). Briefly, it was found that although the absolute number of butanol molecules recovered in any given mucosal section increased with partial pressure, the relative number of molecules in that section (i.e. the percentage) remained fairly constant. These percentages were essentially the same as those of column D in Table I.

VOLUME At a partial pressure of 0.92 mm Hg and a flow rate of 16 cm³/min, stimulations of 3.62, 0.42, and 0.15 cm³ were presented. These results are reported in columns A–C of Table II. The 0.42 cm³ and 3.62 cm³ volumes produced relative mucosal distributions similar to those in Table I and in the previously reported concentration study. However, as seen in Table II, with the smallest volume (0.15 cm³) the dorsal surface and eminentia gradients are clearly steeper than those produced by the largest stimulation volumes.

Although, as reported above, concentration had no effect on the butanol mucosal distribution when a 0.42 cm³ stimulation volume was used, it was possible that with a larger volume variations in concentration would produce changes in the distribution patterns. However, even with a volume of 3.62 cm³, butanol at a partial pressure of 6.78 mm Hg (the highest obtainable at room temperature) produced much the same mucosal distribution pattern (column D, Table II) as did the 0.42 cm³ stimulation at lower concentrations (e.g., column B,
Table II). On the other hand, with a stimulus volume of 15.35 cm$^3$, only 65% of the stimulus is recovered in section M1 (column E, Table II). In other words, with a 15.35 cm$^3$ stimulus and with the highest possible butanol partial pressure at room temperature, relatively more molecules of the stimulus are now found in the more caudal sections.

**FLOW RATE** At a partial pressure of 6.78 mm Hg and a stimulus volume of

| Table II | STIMULUS VOLUME EFFECTS |
|----------|--------------------------|
|          | A            | B            | C            | D            | E            |
|          | 3.62 cm$^3$ | 0.42 cm$^3$ | 0.15 cm$^3$ | 15.35 cm$^3$ |
|          | % of total molecules recovered | % of total molecules recovered | % of total molecules recovered | % of total molecules recovered | % of total molecules recovered |
| Dorsal surface | |
| M1       | 85.2±0.81  | 85.31±1.76  | 94.53±2.01  | 83.48±3.61  | 65.15±1.32  |
| M2       | 6.73±0.77  | 7.21±1.81   | 3.61±1.31   | 4.90±3.08   | 13.11±2.49  |
| M3       | 0.90±0.43  | 1.04±0.54   | 0.45±0.09   | 1.83±1.49   | 4.82±1.39   |
| M4       | 0.45±0.21  | 0.63±0.28   | 0.05±0.04   | 0.89±0.65   | 2.65±0.85   |
| M5       | 0.25±0.18  | 0.31±0.21   | 0.02±0.02   | 0.37±0.31   | 0.86±0.53   |
| Eminentia | |
| E1       | 80.80±0.73 | 1.98±0.62   | 0.92±0.07   | 3.21±2.03   | 5.28±1.74   |
| E2       | 1.84±0.61  | 1.75±0.61   | 0.40±0.11   | 3.07±2.04   | 5.38±1.33   |
| E3       | 0.25±0.15  | 0.24±0.14   | 0.01±0.01   | 0.15±0.10   | 0.46±0.20   |
| E4       | 0.13±0.09  | 0.16±0.09   | 0.01±0.01   | 0.14±0.09   | 0.65±0.40   |
| Not sorbed | 1.39±0.71  | 1.36±0.05   | 0.00±0.01   | 1.96±1.07   | 1.44±0.42   |
| Total dpm recovered (×10$^9$) | 4.06±0.42  | 0.45±0.07   | 0.15±0.04   | 29.07±2.47  | 122±20      |
| Total molecules recovered | 10.07×10$^{16}$ | 1.14×10$^{16}$ | 0.37×10$^{16}$ | 72.13×10$^{16}$ | 203×10$^{16}$ |
| Total molecules expected | 10.84×10$^{16}$ | 1.26×10$^{16}$ | 0.45×10$^{16}$ | 80.18×10$^{16}$ | 340×10$^{16}$ |
| % stimulus recovered | 93 | 91 | 84 | 90 | 89 |
| No. of animals | 8 | 8 | 6 | 4 | 3 |

Odorant: Butanol, negative pressure
Partial pressure: 0.92 mm Hg    6.78 mm Hg
Flow rate: 16 cm$^3$/min    16 cm$^3$/min
Net volume: 3.62 cm$^3$, 0.42 cm$^3$, 0.15 cm$^3$    15.35 cm$^3$, 3.62 cm$^3$

0.42 cm$^3$, butanol was presented at flow rates of 2, 4, 16, and 64 cm$^3$/min. The results are reported in columns A–D of Table III. At the slowest flow rate (2 cm$^3$/min) the dorsal surface gradient appears to be slightly steeper than with the 4 and 16 cm$^3$/min stimulations. A much greater effect in the opposite direction is found with the 64 cm$^3$/min flow rate where only 61.5% of the stimulus is recovered in section M1 (column D). Thus, with this high flow rate and high partial pressure, relatively more molecules are found in the more caudal sections.

This caudal shift would occur if the number of molecules per unit time had momentarily saturated the M1 mucosal surface. To test this possibility, three additional animals (column E) were run, with the same 64 cm$^3$/min flow rate but
at a lower stimulus partial pressure (1.69 mm Hg). In these three animals the number of molecules per unit time was reduced fourfold to the same level as a 16 cm³/min flow rate at 6.78 mm Hg (column C). Such a reduction in the stimulus concentration while still maintaining a high flow rate returned the gradient toward that seen in the lower flow rate (compare column E to columns C and D). However, the 77% recovery in section M1 is still somewhat short of the 83% reported for the 16 cm³/min flow rate. This suggests that phenomena in addition to the number of molecules per unit time are at least in part responsible for the caudal shift.

**Statistical Analysis**

Because each combination of these three stimulation parameters and two delivery systems required an individual animal in order to determine the effect on the distribution pattern, it was not feasible to test all possible combinations, especially when replication was considered important. It was therefore necessary to be selective in the choice of the combinations to be tested, often allowing the

| Section      | A            | B            | C            | D            | E            |
|--------------|--------------|--------------|--------------|--------------|--------------|
| Dorsal surface | 2 cm³/min | 4 cm³/min | 16 cm³/min | 64 cm³/min | 64 cm³/min |
| M1           | 6.78 mm Hg   | 6.78 mm Hg   | 6.78 mm Hg   | 6.78 mm Hg   | 1.69 mm Hg   |
| M2           | 2.95±0.59    | 4.45±2.12    | 6.99±2.52    | 9.96±4.18    | 6.07±1.11    |
| M3           | 2.35±0.85    | 1.69±1.82    | 1.38±1.66    | 1.93±1.23    | 1.55±0.37    |
| M4           | 0.65±0.09    | 0.92±0.31    | 1.55±0.85    | 2.29±4.36    | 0.62±0.25    |
| M5           | 0.14±0.25    | 0.12±0.11    | 0.89±0.58    | 0.58±0.32    | 0.17±0.18    |
| Eminentia    | 2 cm³/min | 4 cm³/min | 16 cm³/min | 64 cm³/min | 64 cm³/min |
| E1           | 2.49±1.18    | 2.28±0.58    | 1.76±0.99    | 4.61±1.59    | 5.61±0.98    |
| E2           | 2.35±1.00    | 2.60±0.78    | 1.85±0.89    | 4.28±2.28    | 6.11±1.47    |
| E3           | 0.40±0.06    | 0.78±0.58    | 0.17±0.10    | 1.89±0.72    | 0.93±0.38    |
| E4           | 0.49±0.27    | 0.53±0.30    | 0.09±0.13    | 1.58±1.15    | 0.88±0.32    |
| Not sorbed   | 0.16±0.03    | 0.52±0.32    | 1.49±0.93    | 1.21±1.15    | 0.97±0.06    |
| Total dpm recovered (x 10⁶) | 5.34±0.08    | 3.41±0.44    | 3.41±0.64    | 3.98±0.82    | 0.82±0.15    |
| Total molecules recovered | 8.29±10⁶    | 8.46±10⁶    | 8.46±10⁶    | 8.39±10⁶    | 2.05±10⁶    |
| Total molecules expected | 9.30±10⁶    | 9.30±10⁶    | 9.30±10⁶    | 9.30±10⁶    | 2.32±10⁶    |
| % stimulus recovered | 89   | 91   | 91   | 90   | 89   |
| No. of animals | 3 | 3 | 4 | 3 | 3 |

Odorant: Butanol, negative pressure.
Partial pressure: 6.78 mm Hg (A–D), 1.69 mm Hg (E).
Flow rate: 2 cm³/min, 4 cm³/min, 16 cm³/min, 64 cm³/min, 64 cm³/min.
Net volume: 0.42 cm³.
trends in the unfolding data to influence the subsequent choices. This made it difficult to follow any particular experimental paradigm which might be required by any particular statistical test.

To make at least some attempt at a statistical analysis, the mean percent of the stimulus recovered in section M1 was taken to reflect the entire mucosal gradient for any one treatment. A Duncan's new multiple range test (Duncan, 1955), as modified by Kramer (1956) for unequal sample size per treatment, could then be used in order to determine which means were statistically different. Over a fairly wide range of stimulation conditions the mean percent recovered in the M1 section was in the 83–85% range. The means that differed significantly from this level have been emphasized in the preceding results and are listed below:

- 61.50%, a 64 cm³/min flow rate (Table III, column D);
- 65.15%, a 15.35 cm³ vol (Table II, column E);
- 77.09%, a 64 cm³/min flow rate (Table III, column E);
- 89.09%, a 2 cm³/min flow rate (Table III, column A); and
- 94.53%, a 0.15 cm³ vol (Table II, column C).

**Reverse Flow Experiment**

The results of the reversal flow experiments are reported in column B of Table IV and can be compared to column A of the same table which gives the results of a standard direction flow study. In the reverse flow animals the gradient reverses direction. For these animals, in contrast to the standard flow direction animals, there is an increasing gradient along the dorsal surface from the section containing the external naris (M1) to the section overhanging the internal naris (M4). However, this reversed gradient is not as steep as that seen in the standard flow study.

**Octane Results**

Column A of Table V shows the distribution of octane molecules resulting from a negative pressure delivery system. As with butanol, these results are reported as the percent representing the number of molecules found in each section divided by the total molecules recovered.

A comparison of column A with column E (repeated from Table III) demonstrates that under these identical stimulation conditions the total percent of the tritiated stimulus recovered from the mucosa (sum of all dorsal surface and eminentia sections) is considerably less for octane (7.97%) than for butanol (98.51%). This same effect is reflected in the fact that for octane a large percent of the recovered stimulus is not sorbed (92.03%) by the mucosa but is instead recovered from the toluene scintillation solution trap placed between the internal naris and the withdrawal pump.

From the percent of the stimulus recovered per section there appears to be a gradient of octane molecules across the mucosa from M1 to M4, but unlike the butanol gradient, this octane gradient increases from the external to the internal naris. In addition, in its opposite direction this octane gradient is not nearly as
steep as that of butanol. However, after correction for surface area (column B) even this shallow octane gradient disappears, with most sections including those of the eminentia having approximately the same number of molecules per square millimeter. Thus, after surface area corrections there seems to be a zero gradient across the mucosa for octane.

Table V also demonstrates that with octane, unlike with butanol, there is an obvious difference in the positive and negative pressure delivery systems. That is, when the negative pressure system (column A) is used, 92.03% of the stimulus is not sorbed whereas this number drops to 40.85% with the positive pressure system (column C). Even after a surface area correction, the number of molecules per square millimeter per section is quite different for the two delivery systems (column B vs. column D). However, neither the positive nor the negative pressure delivery system, after the surface area correction is made, presents a mucosal gradient noticeably different from zero.

**DISCUSSION**

Since the check of the olfactometer output indicated that a given stimulation did indeed contain the predicted number of butanol molecules, it was somewhat surprising that only about 90% of the molecules could be found in the combined total recovered from all the mucosal sections and the scintillation solution trap.
However, when the dorsal surface and eminentia were removed and solubilized without sectioning, the percent of the stimulus recovered was almost 100%. This would seem to suggest that the missing 10% was lost in the “sawdust” produced during the sectioning.

The present study verifies that a sniff of butanol establishes a steep decreasing gradient of molecules from the external naris toward the internal naris along the dorsal surface of the olfactory sac. This is exactly what was inferred from the earlier electrophysiological (Mozell, 1970) and chromatographic (Mozell and Jagodowicz, 1973) data. Butanol gave a rather small LB:MB ratio, thus showing a sharp decline in neural activity from those medial olfactory nerve branches (MB) supplying a region near the external naris to those lateral branches (LB) supplying a region near the internal naris. This was interpreted to reflect a decreasing concentration of butanol molecules along the mucosa. In addition, the measurement of butanol’s relative retention time in the frog olfactory sac suggested that in a given sniff most of the butanol molecules would not be likely
to get far beyond the external naris. As a result of the present radioisotope study, the differential sorption of butanol molecules along the mucosa is no longer simply inferred but has now been directly and graphically confirmed.

Also directly confirmed by these radioisotope data is the interpretation of the earlier electrophysiological studies (Mozell, 1964, 1966) showing that a reversal of the flow direction of the odorized air through the olfactory sac reverses the relative discharge magnitudes recorded from the nerve branches supplying the regions near the two nares. This was interpreted by Mozell as reflecting a reversal in the spatial distribution patterns of the incoming odorant molecules. If this interpretation were correct, butanol molecules, which in a given sniff are mostly sorbed around the external naris, would be mostly sorbed around the internal naris when the flow direction was reversed. Indeed, a tritiated butanol stimulus introduced into the internal naris produced, as predicted, a decreasing gradient of butanol molecules from the dorsal surface section overhanging the internal naris (M4) toward the dorsal surface section containing the external naris (M1). Although this gradient is opposite in direction to the gradient established when butanol is introduced through the external naris, it is not as steep. In addition, there is a much greater variability in the mean percent of the stimulus recovered per section.

The increased variability and the lesser gradient observed with the reverse flow study might well reflect the difficulty encountered in pointing the internal naris cannula in the same direction for each animal. That is, unlike the external naris which can be used as a sleeve to position the cannula, the internal naris is much larger than the cannula placed in it. Therefore, in one animal the internal naris cannula might be pointing toward the eminentia whereas in another animal the cannula might be positioned toward section M3 or M4 of the dorsal surface. It would follow from this variable positioning that the mean percent of the stimulus recovered per section would show a considerable variability. It would also follow that averaging the percent of the stimulus recovered per section would lead to a reduction in the steepness of the gradient. This explanation appears reasonable since the sum of the percent of the stimulus recovered from the M3, M4, and E2 sections (those sections toward which the cannula would be most likely to be directed) was found approximately equal to the percent of the stimulus recovered from the M1 section when the flow was in the standard direction (87.25% vs. 85.33%). However, in spite of this variability these data graphically confirm that with reversed flow direction there is indeed a reversal of the mucosal distribution patterns.

One surprise in the studies using the standard flow direction was the comparatively small number of molecules sorbed by the eminentia as compared to the dorsal surface mucosa. This discrepancy persists even after correcting for differences in the surface areas of the two regions. Perhaps this discrepancy is the result of the eminentia's location in the olfactory sac. This location may be poor for the attraction of chemicals which, like butanol, are heavily sorbed around the external naris, a region of the sac into which the eminentia does not even protrude. This might also explain why the decrease in butanol molecules from the eminentia's rostral pole to its caudal region was an order of magnitude less than the decrease along the dorsal surface mucosa.
If the gradient of odorant molecules along the mucosa has an effect upon the processing of information by the olfactory system, the stability of these gradients as the stimulation parameters are varied becomes an important consideration (Mozell, 1970). Although the absolute number of butanol molecules recovered from each mucosal section was found to increase with partial pressure (Hornung et al), the relative number of molecules from section to section remained constant. This is not inconsistent with Mozell's (1970) earlier electrophysiological data which showed that over a two log unit range of partial pressures the median LB:MB ratio for butanol remained at zero. This means that within this range, the concentration necessary to produce a measurable response on the nerve branch supplying the mucosal region overhanging the internal naris (LB) was not reached, whereas the entire range produced responses on the nerve branch supplying a region near the external naris (MB). It can be argued from the steepness of the radioisotope gradient reported in the present study together with the earlier electrophysiological data that as the partial pressure is increased the number of molecules around the receptors in the lateral mucosa, though increasing, is still so small as to remain subthreshold, whereas the number of molecules around the medial receptors is always suprathreshold. Only at the highest partial pressure obtainable at ambient temperature did Mozell (1970) observe LB:MB ratios greater than zero. In terms of the present study this would suggest that this saturated vapor, although still distributed along the mucosa in the same relative proportions, now provides a large enough absolute number of molecules at the lateral receptors to yield a measurable response.

This study has shown that, as with concentration, there is a rather wide range of sniff volumes over which the butanol gradient remains constant. There are, however, limits. For example, at a volume of 0.15 cm³, the gradient shifts in such a manner that a larger percent of the stimulus is recovered in the M1 section. This might at first appear easily explicable since the odorant molecules are delivered in a stimulation volume much smaller than the 0.5 cm³ volume of the olfactory sac and would presumably not be carried much beyond the initial mucosal regions. However, this explanation is too simplistic, since with the negative pressure delivery system the stimulus was presented as a slug followed by 0.4 cm³ of room air.

There is also, at the high end of the volume continuum, a limit to the consistency of the gradients produced by butanol, but to demonstrate this limit, volume increases were combined with increases in the other stimulation parameters. Even at 6.78 mm Hg, the maximum partial pressure available for butanol at room temperature, an increase in the stimulus volume to 3.62 cm³ still did not noticeably affect the relative mucosal distribution. However, a 15.35 cm³ stimulation volume, together with the 6.78 mm Hg butanol partial pressure, does shift the mucosal gradient in such a way that a smaller percent of the stimulus is recovered in section M1. Thus it may be argued that a high volume butanol stimulation at a high partial pressure may eventually saturate the surface of the mucus in the M1 section, resulting in a shunt of relatively more molecules toward the more caudal sections.

Just as with the other stimulation parameters, there is a range of values over which flow rate was found to have little effect upon the butanol gradient. This
seems to be in agreement with Mozell's (1970) electrophysiological data which showed that for butanol, the LB:MB ratio was essentially unaffected by a variation in flow rate from 4.12 to 20.60 cm³/min. However, with the present radioisotope study, the flow rate was extended beyond the limits tested by Mozell and changes in the gradients were observed. For example, at a flow rate of 64 cm³/min the gradient shifts toward the internal naris and relatively more molecules are found in the more caudal mucosal sections.

One explanation for these data is that with a 64 cm³/min flow rate the molecules are being delivered at a faster number per unit time than the mucus can sorb and thus the gradient shifts in the direction of the internal naris. This possibility was tested, as described in Results, by matching the number of molecules per unit time delivered at two different flow rates. The results, though supporting this explanation to some extent, still suggested the possibility of additional mechanisms.

One such mechanism could involve the linear velocity of the incoming odorant molecules; the faster they enter the olfactory sac the greater the chance that they will pass by section M1 before contacting the mucosal surface.

Compared to the caudal gradient shift seen with a 64 cm³/min stimulation, an opposite effect is seen at the 2 cm³/min flow rate, the slowest used in this study. In this case the dorsal surface butanol gradient is steeper by a small but statistically significant amount than that seen with any of the higher flow rate stimulations. Since compared to the 4 and 16 cm³/min flow rates the 2 cm³/min stimulation would have both a decreased number of molecules per unit time and a decreased linear velocity, either of these phenomena or a combination of them might perhaps explain the observed rostral shift in the butanol distribution gradient.

As a first approximation to determine the contribution of each of these phenomena, the butanol mucosal distribution from a group of animals to which had been presented a 16 cm³/min stimulation with a partial pressure of 0.92 mm Hg (column B of Table II) was compared to the distribution for those animals to which was presented the 2 cm³/min stimulation at 6.78 mm Hg (column A of Table III). In these two groups of animals, although the flow rates differed, the number of molecules per unit time is approximately the same. For the 16 cm³/min flow rate at the lower partial pressure, 85% of the stimulus is recovered from the M1 section whereas with the 2 cm³/min flow rate, 89% of the stimulus is recovered from the M1 section. This small difference is statistically significant and thus appears to make the reduced number of molecules per unit time a less likely contributor to the rostral shift for the low flow rate. Perhaps, then, the linear velocity of the incoming molecules is the more likely contributor.

Not all odorants would be expected to have the same mucosal distributions as butanol. Indeed, from the previous chromatographic (Mozell and Jagodowicz, 1973) and electrophysiological (Mozell, 1970) studies one would predict that odorants with mucosal retention times shorter than butanol would, in a given sniff, have a less steep gradient whereas those with long retention times might well show gradients even steeper than those reported here.

For example, since octane produces an LB:MB ratio approximately equal to
one and has a very short retention time in the olfactory sac, it could be predicted that its molecules, in a given sniff, would be rather evenly distributed across mucosa. When the tritiated octane was delivered at one set of stimulation conditions used previously with butanol, the prediction that octane would be equally distributed across the mucosal sheet was directly confirmed since, after correction for surface area, there is a near zero gradient on both the dorsal surface and the eminentia. The only possible exception to this was seen with the negative pressure delivery system when section M5 tended to show slightly fewer octane molecules per square millimeter than the other mucosal sections. However, since section M5 is caudal to the internal naris it may, especially with the negative pressure delivery system, be somewhat peripheral to the direct flow path of odorized air through the sac.

At any rate, the radioisotope data reported here confirm Mozell's (1970) prediction that in a given sniff, odorant molecules of different molecular species are distributed differently along the olfactory mucosa. A chemical like butanol, which is apparently very strongly sorbed, will be rapidly and almost completely removed from the incoming air stream by the first few millimeters of the olfactory mucosa. On the other hand, for a chemical like octane which does not appear to be strongly sorbed, those few molecules that are sorbed are very evenly distributed across the mucosal sheet.

With octane, unlike with butanol, the choice of a positive or negative pressure delivery system affects the total number of radioactive molecules recovered per mucosal section. This difference might not reflect differences due to the delivery system per se, but may rather reflect the differences in how the animals were handled at the end of each type of stimulation. That is, with the negative pressure delivery system the stimulus is presented as a slug followed by about 0.4 cm³ of room air. Since this volume of room air is approximately equal to the volume of the olfactory sac, many of the delivered but unbound molecules filling the olfactory sac would probably be removed and collected in the toluene trap. However, with a positive pressure delivery system, which has no washout, the animal would be frozen with most of the delivered but unbound octane molecules still remaining in the air space of the olfactory sac. As a result of the freezing, these molecules would condense and freeze to the olfactory mucosal surface. Therefore, with positive pressure the mucosal sections would include many of the delivered octane molecules which with negative pressure would have been withdrawn from the sac. For butanol both the positive and negative pressure delivery systems produced the same mucosal distribution patterns, and in contrast to octane, yielded the same number of recovered molecules from the mucosa. This difference is in keeping with the observed differential attraction of these two odorants to the mucosa. That is, since, with identical stimulation conditions, the total percent of the stimulus recovered from the mucosa is much less for octane than for butanol, the relative number of molecules in the air phase would be much greater for octane. Therefore, both the washout and the condensation phenomena would have a much greater effect on the octane data.

This study shows that an odorant stimulus results in the establishment of a particular distribution pattern along the intact olfactory sac of the bullfrog.
Further, within a wide range of stimulus parameters this distribution remains fairly constant, at least for butanol. The data presented to establish these findings are likely to be gross compared to the actual variations in the molecular distributions across the mucosa. The dorsal olfactory mucosa was divided into only five sections and the eminentia into only four. A finer division would perhaps have shown finer nuances in the distribution patterns, especially in the anterior sac where the butanol distribution drops off so rapidly. In addition, in this study the mucosal distribution patterns are sampled mainly in their rostro-caudal dimension. If the dorsal surface mucosa had been sectioned parallel to the septum, the distributions might have shown a lateromedial differentiation as well. Indeed, it is quite possible that the distribution patterns are more sensitive to the stimulation parameters than this study has described, but that the resultant shifts in the patterns are so subtle as to require a finer mosaic of multidimensional sections.

These molecular distribution patterns are what Mozell (1974) predicted was necessary if a "chromatographic-like" differentiation were one of the mechanisms basic to odorant discrimination. However, even if olfactory discrimination does not depend upon these molecular distributions per se, these distributions would appear to play an important and initial role in defining the access of odorant molecules to different receptors. Thus they could have a fundamental impact upon any process which actually underlies olfactory discrimination. For example, even if a selective sensitivity of receptors is the one basic mechanism of olfactory discrimination, steep molecular concentration gradients would prevent the molecules of some odorants from reaching those otherwise appropriately tuned receptors which happen to be located at more distal points along the mucosal flow path. At the very least, different receptors along the flow path would be reached by different concentrations of the same odorant. As still another possibility, this differential sorption may have the effect of requiring the olfactory system to compensate for the uneven mucosal distribution of odorant molecules either through a series of neural mechanisms or by some manipulation of the flow rate and/or volume of the sniff. Nevertheless, regardless of the mechanisms which ultimately prove to be the basis of both quality and intensity discrimination, this initial distribution of molecules along the olfactory receptor sheet is likely to be of importance to our full understanding and appreciation of basic olfactory mechanisms.

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REFERENCES

Adrian, E. D. 1950. Sensory discrimination with some recent evidence from the olfactory organ. Br. Med. Bull. 6:350–353.
HORNUNG AND MOZELL  Differential Sorption of Odorant Molecules

Adrian, E. D. 1954. The basis of sensation, some recent studies of olfaction. Br. Med. J. 1:287-290.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics. 11:1-42.

Gesteland, R. C., J. Y. Lettvin, W. H. Pitts, and A. Rosas. 1963. Odor specificities of the frog's olfactory receptors. In Proceedings of the First International Symposium on Olfaction and Taste. Y. Zotterman, editor. Pergamon Press, New York. 19-44.

Hornung, D. E., R. D. Lansing, and M. M. Mozell. 1975. Distribution of butanol molecules along bullfrog olfactory mucosa. Nature (Lond.). 254:617-618.

Kramer, C. Y. 1956. Extension of multiple range tests to group means with unequal numbers of replications. Biometrics. 12:307-310.

Mathews, D. F. 1972. Response patterns of single neurons in the tortoise olfactory epithelium and olfactory bulb. J. Gen. Physiol. 60:166-180.

Mathews, D. F., and D. Tucker. 1966. Single unit activity in the tortoise olfactory mucosa. Fed. Proc. 25:329.

Moncrieff, R. W. 1955. The sorptive properties of the olfactory membrane. J. Physiol. 130:543-558.

Mozell, M. M. 1964. Evidence for sorption as a mechanism of the analysis of vapors. Nature (Lond.). 203:1181-1182.

Mozell, M. M. 1966. The spatiotemporal analysis of odorants at the level of the olfactory receptor sheet. J. Gen. Physiol. 50:25-41.

Mozell, M. M. 1970. Evidence for a chromatographic model of olfaction. J. Gen. Physiol. 56:60-63.

Mozell, M. M., and M. Jagodowicz. 1973. Chromatographic separation of odorants by the nose: retention times measured across in vivo olfactory mucosa. Science (Wash. D. C.). 181:1247-1249.

O'Connell, R. J., and M. M. Mozell. 1969. Quantitative stimulation of frog olfactory receptors. J. Neurophysiol. 32:51-68.