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Chromatographic Separation of Odorants by the Nose: Retention Times Measured across in vivo Olfactory Mucosa

Abstract. *The column of a standard gas chromatograph was replaced with in vivo frog olfactory sac. The wide range of relative retention times as measured across the olfactory mucosa for 15 different odorants supports the concept of a chromatographic separation along the mucosa as a mechanism for distinguishing different odorants.*

The chromatographic data we report here provides direct evidence that the molecules of different odorants migrate at different rates across the olfactory mucosa. This gives further support to chromatography as one of the models for explaining olfactory discrimination at the level of the olfactory mucosa.

Mozell previously supported this chromatographic model with electrophysiological evidence only. He sampled the activity elicited by different odorants in two widely separated regions of the olfactory mucosa by simultaneously recording the multiunit discharges from the two branches of the olfactory nerve serving those regions (*1*). The more medial branch (MB) reflected the activity at a mucosal region where odorized air first enters the olfactory sac through the external naris. The more lateral branch (LB) reflected the activity farther along the flow path where the air exits from the olfactory sac through the internal naris. The ratio of the discharge magnitude recorded from the lateral branch to that recorded from the medial branch (LB/MB ratio) was used to quantify the gradient of activity across the mucosa which results from an odorant stimulation; the smaller this ratio, the sharper the decline in activity from the entrance region of the mucosa to its exit region. Mozell found that different odorants produced different LB/MB ratios, so that the analysis of odorants might depend in part on the different gradients of activity they establish across the mucosa (*2–4*). They might also be temporally differentiated because the elapsed time (that is, the latency difference) between the onset of the discharges recorded from the two nerve branches also depended on the odorant used. Two further observations influencing Mozell's conclusions were that those odorants yielding the smallest LB/MB ratios also yielded the longest latency differences (*3*) and that reversing the direction of the odorized air flow across the mucosa reversed the nerve branches giving the larger and smaller discharges (*1*).

Mozell suggested that all these observations could be explained by the same underlying mechanism—differences in the rate at which the molecules of different odorants migrate across the mucosa. As Beidler (*5*) suggested earlier in a somewhat different context, perhaps those molecules that are more strongly attracted to the mucosa migrate toward its far end less rapidly (producing longer time lapses) and in fewer numbers (producing smaller LB/MB ratios) than those with less attraction. If the olfactory mucosa can separate the molecules of different odorants by their differing abilities to migrate across it, an analogy could be made between the initial events in olfactory discrimination and those events that are fundamental to chromatography. That is, the analysis of different chemicals by chromatographic techniques likewise depends upon the phenomenon of differential molecular migration which is based upon the differential attraction of molecules to the medium through which they pass.

As one test of this analogy Mozell compared the LB/MB ratios produced by 16 different odorants to the retention times of the same odorants as measured by a standard gas chromatograph fitted with a Carbowax 20M column (*4*). With only one major exception (butanol) those odorants that took longest to migrate through a Carbowax column (that is, those having longest retention times) also had least facility to migrate across the mucosa (that is, produced the smallest LB/MB ratios).

These electrophysiological observations provide only indirect evidence of differential molecular migration patterns across the mucosa since they are made at a level several steps beyond the molecular events that are presumed to initiate them. Furthermore, even if there were, as Moncrieff (*6*) demonstrated in vitro, some chromatographic effect across the mucosa, it is possible that the mucosas of most animals are too short to allow an adequate separation of different odorants. Therefore, we decided to determine whether the molecules of different odorants do indeed migrate at demonstrably different rates across mucosas by measuring directly their relative retention times as they pass along the frog's olfactory mucosa in vivo.

We replaced the standard column of a gas chromatograph (Varian-Aerograph model 600D) with the olfactory sac of an intact frog (*Rana catesbeiana*) anesthetized with urethane. We connected the inlet port of the gas chromatograph to the frog's external naris and the frog's internal naris to the chromatograph's flame ionization detector with Teflon tubing. We also made provision to bypass the frog with this Teflon tubing, thus producing a direct connection from the inlet port to the detector. In either case we determined retention times in the usual manner by measuring the time between the injection of the odorant sample and the maximum pen deflection of the recorded chromatogram, a deflection that signals the arrival of the maximum number of odorant molecules at the

detector. We calculated the retention time for each odorant across the olfactory sac by subtracting that odorant's retention time for the Teflon tubing alone from its retention time for both the Teflon tubing and the frog olfactory sac together (see Fig. 1, inset). To convert the data to relative retention times we expressed the retention times of the odorants in each frog relative to the retention time of methyl benzoate in that frog. Converting to relative retention times is a standard practice in gas chromatography but in this experiment the conversion confers the additional advantage of adjusting the retention times in different animals to a similar scale.

We prepared saturated odorant samples by first bubbling a stream of chromatographically pure nitrogen through any one of 15 liquid odorants. We then injected 0.25-ml slugs of this saturated stream into a second, continually flowing, stream of nitrogen carrier gas that had been previously humidified to prevent the desiccation of the frog's olfactory mucosa. This stream of carrier gas moved the sample toward the detector at a flow rate of 25 ml/min, either passing through the olfactory sac or bypassing it. A flow rate of 25 ml/min meets the operational specifications for the flame ionization detector. In addition, this flow rate is about the same as that measured for air passing through a frog's olfactory sac during a normal inspiration (*4*).

Note that for the seven frogs used in the above procedure we presented each odorant at a different concentration; that is, the saturated concentration determined by the odorant's partial pressure at the ambient room temperature (22° to 25°C). However, since the retention times of some chemicals may vary with concentration (*7*), we had to run some animals in which the odorants were presented at the same concentration. Thus, to three additional frogs we presented ten odorants at the same partial pressure (0.56 mm-Hg) and one odorant, methyl benzoate, at a pressure that differed only slightly from the others (0.4 mm-Hg). We brought these odorants to equal partial pressure by the flow dilution technique (*3*).

As might be expected, since the mucosa of different frogs cannot be considered identical in shape or composition, the retention time of each odorant varies from animal to animal (Fig. 1). However, of more importance to olfactory discrimination is the variation within each animal. This was quantified by determining the range of each odorant's retention times within each animal, obtaining the mean of these ranges for each odorant among all the animals, and then determining the percentage variation of the range from each odorant's mean retention time. These variations are as follows: octane, 6 percent; nonane, 8 percent; *d*-limonene, 23 percent; heptaldehyde,

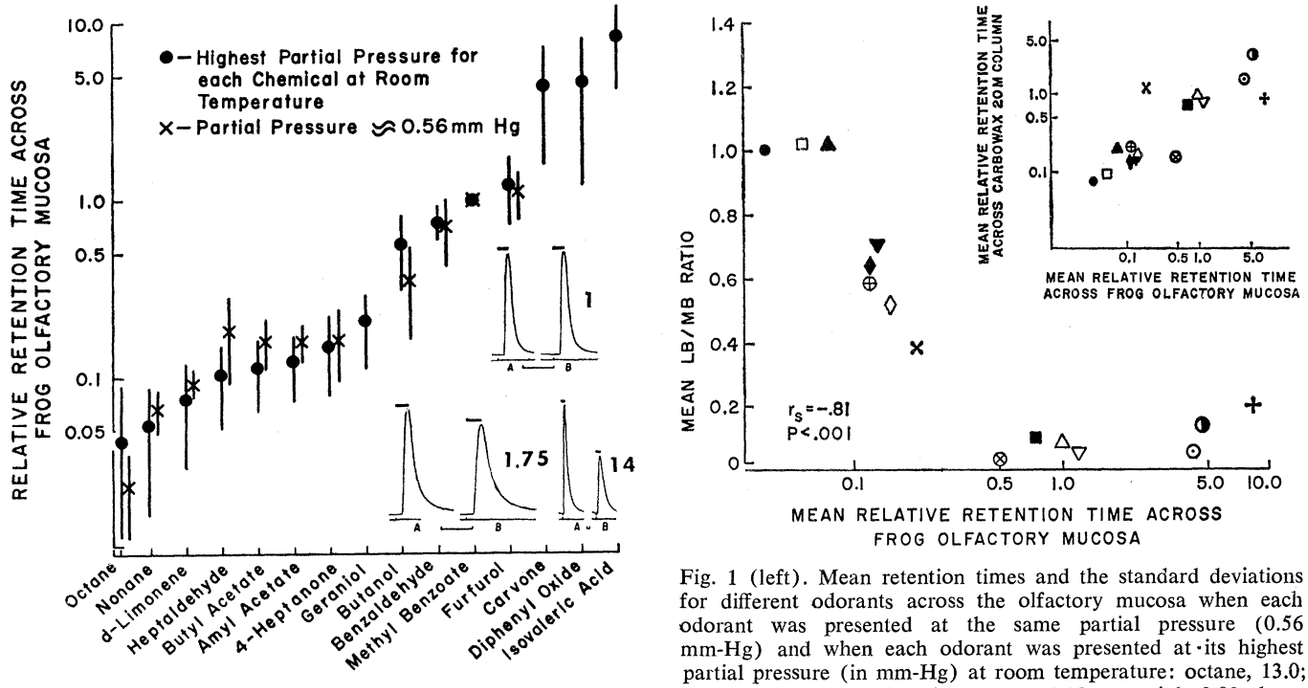

Fig. 1 (left). Mean retention times and the standard deviations for different odorants across the olfactory mucosa when each odorant was presented at the same partial pressure (0.56 mm-Hg) and when each odorant was presented at its highest partial pressure (in mm-Hg) at room temperature: octane, 13.0; nonane, 4.8; *d*-limonene, 2.05; heptaldehyde, 2.65; butyl acetate, 12.0; amyl acetate, 4.0; 4-heptanone, 1.18; geraniol, 0.03; butanol, 8.0; benzaldehyde, 0.93; methyl benzoate, 0.4; furfurol (furfuryl alcohol), 0.60; carvone, 0.12; diphenyl oxide (diphenyl ether), 0.03; isovaleric acid, 0.49. Four of the 15 odorants could not be included in the equal concentration group since their partial pressures at ambient temperature were so low that we could not obtain the nitrogen flow rates that would have been required to dilute the other odorants down to their concentration levels. The odorants are listed along the abscissa in order of their increasing relative retention times when presented at their highest partial pressures. (Inset) Retraced chromatograms of three representative chemicals: octane (top), 4-heptanone (left), benzaldehyde (right). (A) Odorant passed through Teflon tubing alone. (B) Odorant passed through both Teflon tubing and frog olfactory sac. Retention times for both these conditions represented by solid lines at the top of the chromatograms. Retention time for each odorant across the olfactory sac alone is represented by the difference in the solid lines (B minus A). Initial upward deflection of the event marker indicates odorant injections. Brackets below traces represent 1 minute. Numbers represent the relative gain of the detector amplifier for each pair of chromatograms. Fig. 2 (right). A comparison of the mean LB/MB ratios (*4*) for a group of odorants with the relative retention times of the same odorants across the olfactory mucosa. (Inset) The relationship of the relative retention times measured across a 20M Carbowax column (*4*) to that measured across the olfactory mucosa. Symbols: ●, octane; □, nonane; ◆, butyl acetate; ⊗, butanol; ▼, amyl acetate; ◇, 4-heptanone; ⊕, heptaldehyde; ▲, *d*-limonene; ■, benzaldehyde; +, isovaleric acid; ▽, furfurol; △, methyl benzoate; ×, geraniol; ⊙, carvone; ◑, diphenyl oxide.

3 percent; butyl acetate, 1 percent; amyl acetate, 2 percent; 4-heptanone, 0 percent; geraniol, 0 percent; butanol, 8 percent; benzaldehyde, 3 percent; methyl benzoate, 4 percent; and furfurol, 5 percent (*8*). These variations demonstrate that, within any one animal, odorant retention times are highly consistent in almost all cases.

Figure 1 demonstrates that, for most odorants, concentration does not appear to be a major determinant of relative retention time across the mucosa. However, there are some odorants that do show at least a suggestion of a concentration effect. This may explain why in an earlier electrophysiological study the LB/MB ratios produced by two of these odorants, butyl acetate and heptaldehyde, were observed to increase slightly with increasing concentration (*4*).

Of prime consideration in Fig. 1 is the range of different relative retention times produced by different odorants. Using all ten animals this range shows an approximate 220-fold increase from the shortest to the longest and the remaining relative retention times appear rather well distributed within these limits. This range represents an increase from a mean of 1.2 seconds to a mean of 274 seconds. Thus in spite of its small size the molecules of different odorants do appear to migrate at significantly different rates across the in vivo olfactory mucosa of frog.

Since we now have direct measurements of the facility with which molecules of different odorants migrate across the mucosa, we might ask whether the LB/MB ratios which originally led Mozell to support a chromatographic model of olfactory discrimination are in fact related to this facility. We plotted the mean LB/MB ratios (*4*) against the mean relative retention times across the mucosa determined for all ten animals (Fig. 2). There is an inverse relationship, which is given statistical emphasis by a highly significant ($P < .001$) rank order correlation coefficient ($r_s = -.81$). Thus the earlier supposition that the LB/MB ratios reflect the differential migration of molecules across the mucosa is supported by these direct measurements of the behavior of the molecules themselves. As additional evidence for this conclusion, the butanol LB/MB ratio that appeared to deviate from the general trend when plotted against its retention time on a Carbowax column (*4*) falls more nearly into its predicted position when plotted against its relative retention time along the mucosa.

From the correlation discussed above we might expect a direct relationship between the retention times previously measured across Carbowax (*4*) and those measured across the mucosa. The inset of Fig. 2 shows the strength of this relationship. Thus, some of the same properties which underlie and characterize the chromatographic behavior of a Carbowax column may also underlie and characterize the observed chromatographic behavior of the olfactory mucosa (*3, 4*).

We must emphasize that this analogy between olfaction and chromatography is made only in regard to the basic principle involved (the analysis of chemicals by the propensity of their molecules to migrate at different rates along a medium) and not to any particular set of operations currently used in any laboratory application of that principle. Presumably the nose would develop operational details, in order to take advantage of this principle, which are compatible with its own peculiarities and requirements and which would not necessarily mimic any other adaptation of the same chromatographic principle (*3, 4, 9*). For instance, as a result of respiration the carrier gas flows through the nose in two directions and is pulsatile rather than, as in a standard gas chromatograph, unidirectional and constant. Consequently, rather than using a measure based upon the time needed for molecules to travel the given distance to the one detector at the end of the column, the nose could measure, with its many detectors spread along its entire column, the distance the molecules travel and the relative number of molecules traveling that distance.

We have demonstrated that, in spite of the small size of the mucosa, the molecules of different odorants do indeed differ significantly in their ability to migrate along it. This chromatographic differentiation may be one of the mechanisms underlying olfactory discrimination. However, even if this is not so, its demonstrated existence still has major implications. For instance, the molecules of low vapor pressure odorants, which generally have long retention times, will, in a given sniff, be piled up near the entrance to the olfactory sac. They may not reach mucosal regions farther along the nasal flow path. Consequently, a large fraction of the olfactory receptors may not contact the incoming molecules of such chemicals. On the other hand, for those receptors near the entrance, which do make contact, the concentration of these molecules will be greatly increased. Such phenomena must be considered in our further attempts to understand olfactory processes.

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Recombinant Inbred Lines: Value in the Genetic Analysis of Biochemical Variants

Abstract. *Analysis of inducibility by androgens and electrophoretic mobility of kidney glucuronidase in progenitor and derived recombinant inbred mouse lines suggests that a single major regulatory gene at or near the glucuronidase structural gene on chromosome 5 determines the rate of enzyme accumulation.*

The utility of recombinant inbred (RI) lines of mice for the analysis of histocompatibility gene systems (*1*) and plasma corticosterone (*2*) has been demonstrated. We now report their special utility in the genetic analysis of a complex biochemical phenotype, the regulation of enzyme induction by a hormone.

The RI lines are produced by inbreeding the F_2 generation of a cross between two unlike progenitor inbred