*Journal of Chemical Ecology, Vol. 13, No. 1, 1987*

# GENERALIZATION IN OLFACTORY DETECTION OF CHEMICAL CUES CONTAINING CARBONYL FUNCTIONS BY TIGER SALAMANDERS (*Ambystoma tigrinum*)

J. RUSSELL MASON,[1] KAMALESH K. JOHRI,[2] and THOMAS HELLMAN MORTON[2]

[1]*Monell Chemical Senses Center, 3500 Market Street*
*Department of Biology*
*University of Pennsylvania*
*Philadelphia, Pennsylvania 19104*
[2]*Department of Chemistry*
*University of California*
*Riverside, California 92521*

(Received August 15, 1985; accepted January 6, 1986)

**Abstract**—Tiger salamanders generalize behaviorally between carbonyl-containing odorants (e.g., aldehydes or cycloalkanones). However, responding does not generalize from such odorants to stimulus compounds with comparable molecular shapes and dimensions but different functional groups. Discrimination between aldehydes and a ketone is temporarily impaired by two-step covalent modification of the olfactory epithelium. Two-step modification of the olfactory epithelia also impairs, but does not obliterate, olfactory detection, and generalization persists even during the period of impairment. These results are interpreted as implying the existence of carbonyl-binding, "generalist" olfactory receptors in addition to other classes of "generalist" receptors that are not affected by two-step modification. Generalization is inferred to require overlap in the response profile of more than one class of receptor.

**Key Words**—Tiger salamander, *Ambystoma tigrinum*, carbonyl function, generalist receptor, olfactory discrimination.

## INTRODUCTION

Chemoreceptors may be portrayed as falling into two categories, "specialists" and "generalists" (Schneider, 1969). Specialists represent receptors that respond to a specific stimulant molecule (and, perhaps, to a few closely related



0098-0331/87/0100-0001$05.00/0 

analogs). Pheromones, for instance, may interact with such specialized receptors. Generalists, on the other hand, represent receptors that respond to a range of molecules, which share some common feature. Animals might use such generalist receptors, for example, in detecting volatile food cues and to identify and distinguish potential hosts or prey.

Although the distinction between specialist and generalist may, in practice, be less clear-cut than it is in principle, it raises the issue of how one might ascertain experimentally whether two different molecules interact with a given receptor site. In studies of pheromone detection, it is sometimes assumed that if a molecule triggers a receptor, then the response will be the same regardless of the identity of the stimulus. By the same token, if two different molecules elicit the same response, it is often concluded that they must have interacted with the same receptor (Chapman et al., 1978). Such assumptions may not be warranted in the case of generalist receptors. We would like to draw parallels between generalist receptors and the behavioral phenomenon of generalization, which can be combined with chemical manipulation of the receptor in an effort to assess whether two different molecules bind at the same site.

In human subjects, the phenomena of adaptation and cross-adaptation have been explored in an effort to answer this type of question (Köster, 1971). However, the facts that: (1) adaptation appears to involve both central and peripheral mechanisms, and (2) the ability to recognize or distinguish odors does not seem to be necessary for the central component to function, (Eichenbaum et al., 1983) complicates the interpretation of experimental data on human beings. Behavioral experiments with animals cannot ordinarily ask the detailed sorts of psychophysical questions that can be studied with humans. But a major advantage of studying animals is that the subjects' sensory systems or CNS can be modified in the course of experimentation. This paper will discuss the application of chemical modification of the olfactory epithelium to the study of generalization.

Choice of species for experimentation was based on the following considerations. First, the test animals had to be air-breathing vertebrates for which behavioral assays had been developed. Second, the olfactory epithelium in the species of choice had to be accessible to chemical manipulation. Adult tiger salamanders (*Ambystoma tigrinum*) were chosen because they are air-breathing vertebrates that can be classically conditioned to discriminate and generalize among reagent-grade odorants (Mason et al., 1980, 1981; Mason and Stevens, 1981a,b). Also, their simple nasal cavities permit direct chemical treatment of the olfactory epithelium (Mason and Morton, 1982, 1984; Mason et al., 1984, 1985).

## METHODS AND MATERIALS

*Subjects.* Adult tiger salamanders were purchased from Charles D. Sullivan Company, Inc., Nashville, Tennessee. All of these animals had been land-

phase (air-breathing) for two or more years and were collected from two ponds about 2.5 km apart in Wilson and Sumner Counties, Tennessee (Sullivan, personal communication).

While these animals are probably all from the same breeding population, we believe that their data are representative of the species. There are several grounds for this inference. First, animals collected in other geographic locations (e.g., New Mexico, Arizona) are readily conditioned to respond to simple odorants and show discrimination and generalization among odorants similar to that reported here (e.g., Mason and Stevens, 1981a; Mason et al., 1984). Second, larval tiger salamanders that metamorphosed in our laboratory show similar response patterns to those purchased from Sullivan, Inc. (Arzt et al., 1986).

While in the laboratory, the salamanders were housed in plastic boxes in a refrigerator at 5–8°C. Refrigeration permitted the animals to be kept without weight loss and essentially disease-free for several months. Mealworms were hand-fed to animals every 14 days.

The boxes in which the salamanders were housed were lined with paper towels moistened with dechlorinated water. Liners were changed every third day. All animals were used within two months of arrival in the laboratory. Thirty minutes before each experimental session, the salamanders were removed from the refrigerator and placed in plastic tubs. Each tub contained about 100 ml of dechlorinated water. Testing occurred in a room with an ambient temperature of 23° ± 1°C.

*Chemicals.* Molecular structures of odorants used in these studies are drawn in Figure 1. Cyclohexanone, cyclopentanone, 1-butanol, ethyl acetoacetate, 1-heptene, hexanal, heptanal, 2-heptanone, and pristane (2,6,10,14-tetramethylpentadecane) were purchased commercially. Cyclohexanone and ethyl acetoacetate were redistilled at atmospheric pressure. The other odorants were used without additional purification.

Among the odorants used for generalization studies, a fluoroalkene was included as an example of an odorant molecule with molecular dimensions similar to an aldehyde, which also possesses a large permanent dipole moment, but without an aldehyde's ability to bind covalently as a Schiff base. 2-Fluoro-1-heptene was prepared from 2-bromo-1-heptene, which in turn, was prepared by reaction of 2,3-dibromopropene with *n*-butylmagnesium chloride (Lespieau and Bourguel, 1941). *N*-Bromoacetamide (1.66 g, 0.012 mol) was suspended in a mixture of 15 ml dry tetrahydrofuran and 15 ml dichloromethane and cooled in a Dry Ice–acetone slush. Excess liquid hydrogen fluoride (approximately 5 ml) was added, followed by addition of 2-bromo-1-heptene (1.77 g, 0.010 mol) in a mixture of 25 ml dry tetrahydrofuran and 15 ml dichloromethane. The reaction mixture was kept cold for 3 hr and allowed to warm slowly to room temperature. Stirring was continued for 16 hr at room temperature, and the reaction mixture was then worked up by adding 50 ml of ether and washing with water, saturated sodium bicarbonate solution, and brine. Removal of solvent afforded

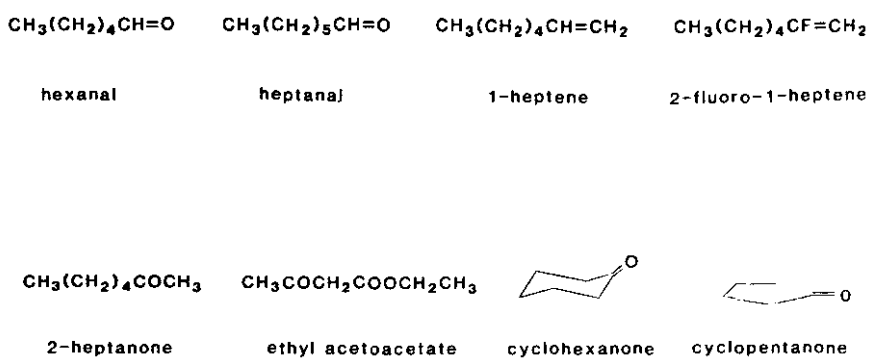


FIG. 1. Structures of odorant molecules used in the present study.

2.62 g of 1,2-dibromo-2-fluoroheptane (9.5 mmol, 95% yield), for which [$^{19}$F]NMR showed a multiplet 99 ppm upfield from $CFCl_3$; [$^1$H]NMR ($CDCl_3$): $\delta$ 4.3 (d, $J_{HF}$ = 10 Hz, 2H), 4.0 (m, 2H), 16–1.6 (m, 6H), 0.85 (t, 6 Hz, 3H).

The dibromo compound was then dehalogenated (Daub et al., 1985). A 5.0-g portion of zinc–copper couple (prepared according to Lambert et al., 1971) was added to a stirred solution of 9.0 g (33 mmol) 1,2-dibromo-2-fluoroheptane in 50 ml ether at 0°C, and the reaction mixture was allowed to warm to room temperature and stirred for 12 hr. The mixture was then filtered, the ether carefully distilled, and the residue vacuum-distilled at room temperature through a succession of three vacuum traps cooled to −23°C, −63°C, and −195°C. The first efficiently trapped 2-bromo-1-heptene and the last ether. The −63°C trap contained 1.1 g (10 mmol, 29% yield) of 2-fluoro-1-heptene: IR (neat film) 3140, 2900, 1675 cm$^{-1}$; [$^{19}$F]NMR (acetone-$d_6$): $\delta$ −94.0 (m); [$^1$H]NMR $\delta$ 4.53 (m, $J^{cis}_{H-F}$ 17.6 Hz, $J^{trans}_{H-F}$ 50.3 Hz, $J_{H-H}$ 2.4 Hz, 3H), 2.8 ($J_{H-F}$ 16.1 Hz, 2H), 1.7–1.1 (m, 6H), 0.89 (t, $J$ = 7 Hz, 3H); [$^{13}$C]NMR $\delta$ 167.0 ($J_{CF}$ = 256.3 Hz), 89.1 ($J_{CF}$ = 19.5 Hz), 31.8 ($J_{CF}$ = 26.8 Hz), 31.1, 25.7, 22.4, 13.9; mass spectrum (70 eV) *m/z* (rel intensity), 116 (11), 96 (10), 88 (10), 81 (14), 73 (24), 61 (26), 60 (22), 59 (18), 58 (19), 56 (100), 55 (22), 41 (90); $M^{\cdot+}$ 116.1003 (calc'd 116.1001). Presence of a small impurity attributed to trace 1,2-heptadiene was inferred on the basis of a variable band in the infrared spectrum at 1970 cm$^{-1}$.

*Apparatus.* The apparatus was similar to that described by Mason and Morton (1982). A flow dilution olfactometer generated volatile stimuli from neat samples of hexanal, cyclohexanone, cyclopentanone, 1-butanol, and ethyl acetoacetate, or from pristane solutions of other odorants. Stimuli were delivered in an airflow of approximately 100 ml per minute via an elliptical glass funnel that passed through the front wall of a stainless-steel conditioning chamber, as shown in Figure 2. Unless otherwise specified, odor stimuli from neat samples were presented at approximately 2.0–2.5% of vapor saturation. Salamanders

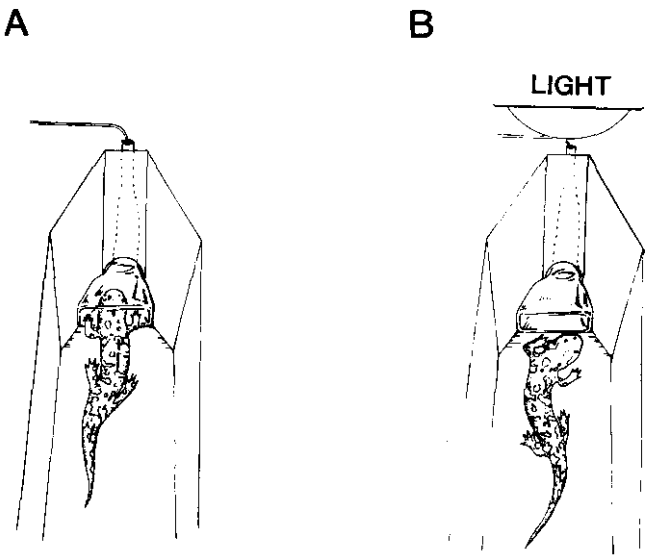


FIG. 2. Conditioning chamber for classical avoidance training of a tiger salamander. (A) Experimental animal positioned in odorant airflow. If the subject remains in this position, with its head in the glass sniffing port during presentation of odorant, a lack of avoidance is scored. (B) Experimental animal removing its head from sniffing port. If the subject removes its head during odorant presentation, an avoidance is scored. A light above the sniffing port is used as negative reinforcer. If the subject removes its head during reinforcement, an escape response is scored.

were placed in the conditioning chamber so that their heads rested in the sniffing port, and odorants were delivered to the port via separate pieces of Teflon spaghetti tubing (Mason and Stevens, 1981a). Concentrations of odorant stimuli in pristane were calculated using Raoult's Law. Specifically, for the 2% (w/v) solutions in pristane, the percent vapor saturation of the odorant at equilibrium with the solution (relative to neat odorant) was estimated to be 6%, equal to the mole fraction of the odorant in the solution, 0.06.

*Procedure.* Training involved placing the animals in the conditioning chamber with their heads resting in the sniffing port (Figure 2). Approximately 15 sec later, a 15-sec pulse of odorant–air mixture was delivered. Immediately after presentations of S+ odorant, a 300-W projector lamp was turned on to produce an aversive light reinforcer (Kuntz, 1923). The lamp was fitted with a condensing lens and was directed into the front of the conditioning chamber from 12 cm above. Although a small amount of radiant heat reaching the animal might have contributed to the aversive character of the stimulus, the light presentations did not appreciably change the temperature near the sniffing port, even though they continued for 60-sec or until the animal backed away from

the sniffing port. If backing away occurred during presentations of the S+ odorant, an avoidance response was scored. However, if backing away occurred after light onset, an escape response was scored. Unreinforced presentations (S–) of *n*-butanol or filtered air were randomly interspersed with presentations of the S+ odorant. Such presentations provided a day-to-day measure of differences in the animal's level of responsivity and an assessment of whether conditioning to the S+ odorant had occurred. Training continued until animals showed $\geq 80\%$ avoidance to presentations of S+, and $\leq 20\%$ avoidance to presentations of S−.

*Experiment 1.* Twelve salamanders were randomly assigned to two groups ($N = 6$ per group). The first group was trained to respond to hexanal (as S+), presented at 2.5% of vapor saturation. The S− was filtered air. After criterion responding was achieved (eight days), concentration–response tests were conducted over five consecutive days. For these tests, animals were presented with varied S+ odorant concentrations using a temporal forced-choice method of limits (Mason and Stevens, 1981b). Unreinforced presentations of air passed over pristane (as S−) were randomly interspersed with the S+ presentations. On each day of testing, animals were initially presented with hexanal at 2.5% of vapor saturation. Over subsequent trials, the hexanal concentration was reduced until animals failed to exhibit avoidance on two successive trials. At that point, odorant concentrations were increased until avoidance responding was again exhibited.

Following the response–concentration tests using vapors from neat hexanal, additional training trials (two days) were given using a 2.0% (w/v) hexanal solution for which the diluent was pristane, an odorless alkane of low volatility. When criterion responding was achieved, additional response–concentration trials were administered (three days) using the same procedures as those described above. After this second series of tests, three days of generalization trials were given among hexanal, 1-heptene, 2-fluoro-1-heptene, and pristane. The odorants were dissolved in pristane at a concentration of 2.0% (w/v), and volatiles above these solutions were presented (five presentations of each, in a randomized order, per session) to animals without further air dilution. All hexanal presentations during generalization were reinforced. Generalization among these odorants was followed by three days of generalization among hexanal, heptanal, 2-heptanone, and pristane. In all of these generalization tests (1) odorants were dissolved in pristane at a concentration of 2% (w/v), and (2) volatiles from these solutions were presented to animals without further air dilution.

The second group ($N = 5$) was trained to respond to 2% (w/v) hexanal in pristane, interspersed with unreinforced presentation of clean air blown over pristane. Once all subjects had reached criterion (after five days), generalization trials were run with heptanal and 2-heptanone, as described above. After three days, the animals were anesthetized, and lavage was administered to both ol-

factory sacs of each subject. Animals were anesthetized by immersion in a 0.5% aqueous solution of Tricaine. Anesthetized animals were randomly assigned to two subgroups. One subgroup ($N = 2$) was given nasal lavage with 50 mM aqueous sodium cyanoborohydride, while the other ($N = 3$) received lavage with 0.5 mM ethyl acetoacetate, followed by 50 mM sodium cyanoborohydride (Mason et al., 1985). Lavage with either ethyl acetoacetate or sodium cyanoborohydride involved injection of fluids into the olfactory sacs via the external nares. Excess fluid was absorbed at the internal nares with paper wicks. Each naris was rinsed with 100 $\mu$l of saline immediately following experimental treatments, and 2 min later, each animal was rinsed in dechlorinated water and placed in a tub containing 100 ml of dechlorinated water to recover from anesthesia. After recovery, the animals were returned to the refrigerator. On each of the six days following lavage, the animals were given generalization trials identical to those described above.

*Experiment 2.* Twelve salamanders was randomly assigned to three groups ($N = 4$ per group). Each group was trained to respond to one odorant (as S+) at 2% of vapor saturation. Group 1 was trained with cyclohexanone, while groups 2 and 3 were given training with cyclopentanone and ethyl acetoacetate, respectively. Group 3 did not exhibit acquisition of conditioned avoidance, as though insensitive to ethyl acetoacetate. When this odorant was replaced by a cycloalkanone, avoidance acquisition was observed (see below). For all three groups, unreinforced presentations of *n*-butanol (as S−) were randomly interspersed with presentations of S+ odorants.

After training to criterion, groups 1 and 2 were given generalization trials between cyclohexanone and cyclopentanone for six days. On each of these days, animals were given 20 unreinforced test trials (10 per odorant), preceded by five reinforced S+ trials interspersed with five S− presentations. These daily pretest trials served to prevent extinction and to provide a measure of each animal's level of activity.

Following generalization tests, the animals that had been presented with ethyl acetoacetate during the original training trials were randomly assigned to either the cyclohexanone (CH) or cyclopentanone (CP) group. These two groups of animals ($N = 6$ per group) were given five additional days of training, and then each group was divided into two subgroups ($N = 3$ per subgroup); CH-E, CH-C, and CP-E and CP-C, respectively. CH-C and CP-C animals were anesthetized and given a control lavage of 50 mM $NaBH_3CN$ (100 $\mu$l into each naris). CH-E and CP-E animals were anesthetized and given nasal lavages of 0.5 mM ethyl acetoacetate followed by 50 mM $NaBH_3CN$ (100 $\mu$l of each per naris). After lavage, all animals were rinsed in dechlorinated water and placed in their home cages to recover. On each of the six days following lavage, the animals were given generalization trials identical to those described above.

*Analysis.* For experiment 1, a series of two-way repeated measures analyses of variance (ANOVAs) were used to assess training periods, response-

concentration curves, and generalization tests. Tukey b post-hoc tests were used to isolate significant differences among means (Winer, 1962). For experiment 2, data from both acquisition and generalization periods were assessed using three-way analyses of variance with repeated measures on two factors (days and odorants). The independent factor in each analysis was groups.

## RESULTS

### *Experiment 1*

*Group 1.* In the first set of experiments, subjects were trained to respond to hexanal. During initial training, there were significant differences among days ($F$ (7,35) = 4.5, $P < 0.001$), and between hexanal and filtered air ($F$ (1,5) = 308.5, $P < 0.0001$). The interaction between days and stimuli was also significant ($F$ (7,35) = 4.2, $P < 0.002$), and the analysis was interpreted in terms of that effect. Tukey tests showed that avoidance responding to presentations of hexanal increased over days ($P$s $< 0.01$), while responding to filtered air remained consistently low ($P > 0.25$).

Analysis of the initial response–concentration test period (neat hexanal) revealed significant differences in responding over days ($F$ (4,20) = 8.5, $P < 0.001$) and among hexanal concentrations ($F$ (4,20) = 56.7, $P < 0.0001$; Figure 3). The interaction of these terms was also significant ($F$ (16,80) = 14.3, $P < 0.0001$), and the analysis was interpreted in terms of that effect. Post-hoc tests revealed that, over days, lower concentrations of hexanal elicited avoidance responses ($P$s $< 0.01$). Once responding stabilized, however, a concentration between 1 and 1.5% of vapor saturation was found to elicit avoidance responses 50% of the time. This concentration was operationally defined as threshold (Mason and Stevens, 1981b).

During the second training period (hexanal in pristane), the only significant difference was in responding to hexanal versus pristane ($F$ (1,5) = 783.6, $P < 0.0001$). There were no significant differences between days ($P > 0.25$), and no interaction ($P > 0.25$). Animals exhibited nearly perfect transfer of training, and exhibited 9.3 $\pm$ 1.0 responses (out of 10) to hexanal but only 0.70 $\pm$ 1.0 (out of 10) responses to pristane on the first day of training.

During the second concentration–response period, the only significant difference was among odorant concentrations ($F$ (5,25) = 55.9, $P < 0.0001$). Post-hoc tests revealed a pattern of results similar to those obtained with air dilutions of pure pristane, but a concentration estimated between 2 and 3% of vapor saturation was found to elicit avoidance responses 50% of the time. These higher threshold values were taken to indicate that the effective vapor concentration above the solutions was less than the equilibrium vapor pressure calculated for an ideal solution.

Results for unreinforced trials are summarized in Table 1. Analysis of generalization among hexanal, 2-fluoro-1-heptene, 1-heptene, and pristane re-

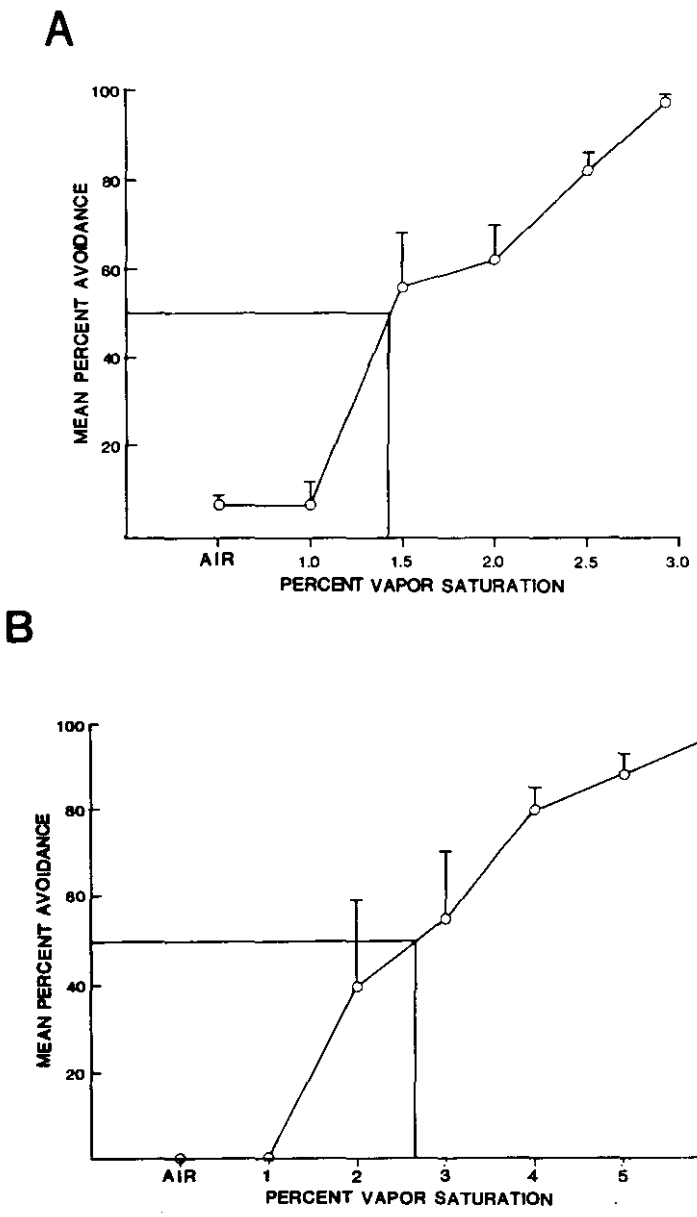


FIG. 3. Concentration–response data for hexanal. (A) Mean percent avoidance for tiger salamanders ($N = 6$) presented with various air dilutions of vapors from neat hexanal. Threshold for 50% avoidance as represented by the intersection of the horizontal and vertical lines, lies between 1.0 and 1.5% of vapor saturation. Capped vertical bars represent standard errors of the means. (B) Mean percent avoidance for tiger salamanders ($N = 6$) presented with various air dilutions of a 2.0% (w/v) solution of hexanal in pristane. From Raoult's law, the equilibrium vapor pressure over this solution is estimated to be 6% of vapor saturation. Threshold for 50% avoidance, as represented by the intersection of vertical and horizontal lines, corresponds to a value between 2% and 3% of vapor saturation. Capped vertical bars represent standard errors of the means.

TABLE 1. MEAN RESPONSES (OUT OF 5 PRESENTATIONS PER SESSION) FOR TWO GENERALIZATION EXPERIMENTS ($N = 6$ FOR EACH) BETWEEN HEXANAL AND OTHER ODORANTS[a]

| | Session | | | Session | | |
|---|---|---|---|---|---|---|
| Odorant | 1 | 2 | Odorant | 3 | 4 | 5 |
| Hexanal | 5.00 | 5.00 | Hexanal | 5.00 | 5.00 | 5.00 |
| 2-Fluoro-1-heptene | 0.00 | 0.33 | Heptanal | **3.50** | **3.33** | 3.17 |
| 1-Heptene | 1.00 | 0.33 | 2-Heptanone | 0.67 | 0.67 | 0.83 |
| Air (passed over pristane) | 0.00 | 0.00 | Air (passed over pristane) | 0.00 | 0.00 | 0.00 |

[a]Significant generalization observed only for heptanal. Entries in boldface meet significance criterion $P < 0.001$ for comparison between responding to 2-heptanone and heptanal. Responding to 2-fluoro-1-heptene, 2-heptanone, or 1-heptene failed to meet the significance criterion $P < 0.05$ when compared to air (except for 1-heptene in session 1, which failed to meet significance criterion $P < 0.02$).

vealed a significant difference among odorants ($F(3,15) = 630.0$, $P < 0.0001$). There were no differences among days ($P > 0.25$), and no interaction between days and odorants ($P > 0.25$). Post-hoc tests revealed that while animals continued to show avoidance of hexanal presentations, they failed to generalize such avoidance to the other odorants ($P < 0.01$).

Analysis of generalization among hexanal, 2-heptanone, and heptanal revealed a significant difference among odorants ($F(3,15) = 59.5$, $P < 0.0001$) but, again, no difference among days ($P > 0.25$), and no interaction between days and odorants ($P > 0.25$). Post-hoc tests revealed that animals exhibited the highest rate of avoidance to presentations of hexanal, an intermediate rate to heptanal, and almost no avoidance of 2-heptanone or pristane ($Ps < 0.01$). Also, animals failed to exhibit significant generalization of avoidance between hexanal and three other compounds with similar molecular shape and dimensions: 1-heptene, 2-fluoro-1-heptene, and 2-heptanone. As Table 1 summarizes, responding to heptanal is significantly less than responding to hexanal. We infer that these two odorants were perceived as similar, but not identical.

*Group 2.* Analysis of acquisition revealed that there were differences among days ($F(3,12) = 14.2$, $P < 0.0001$) and between responses to hexanal and pristane ($F(1,4) = 321.8$, $P < 0.0001$). The two-day interaction between days and odorants was also significant ($F(3,12) = 51.6$, $P < 0.0001$), and Tukey tests indicated that avoidance responses to hexanal presentations increased over days ($Ps < 0.01$) while avoidance responses to pristane remained consistently low.

Analysis of generalization (combined pre- and postlavage) revealed significant differences between subgroups ($F(1,4) = 17.9$, $P < 0.01$), among

odorants ($F$ (6,24) = 5.1, $P < 0.002$), and among days ($F$ (2,8) = 101.7, $P < 0.0001$). Also, there were significant two-way interactions between subgroups and days ($F$ (6,24) = 6.4, $P < 0.001$), subgroups and odorants ($F$ (2,8) = 5.4, $P < 0.05$), and days and odorants ($F$ (12,48) = 2.4, $P < 0.02$). Because the three-way interaction among subgroups, days, and odorants was significant ($F$ (12,48) = 2.95, $P < 0.05$), the analysis was interpreted in terms of that effect. Tukey tests revealed that, prior to lavage, animals exhibited high rates of avoidance responding toward hexanal and heptanal, but low rates toward 2-heptanone (Figure 4; $P$s $< 0.05$). During tests on the first three days after lavage, animals given lavages with ethyl acetoacetate followed by $NaBH_3CN$ exhibited low responding toward all odorants (relative to animals lavaged with $NaBH_3CN$ alone; $P$s $< 0.05$). Animals given lavage with $NaBH_3CN$ alone exhibited no change in responding and continued to avoid presentations of hexanal and heptanal but not presentations of 2-heptanone ($P$

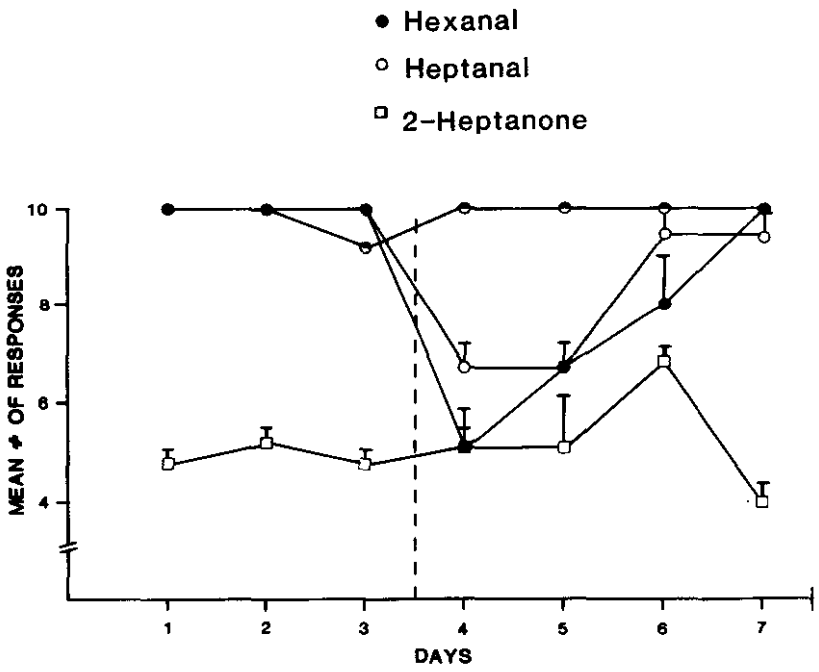


FIG. 4. Effects of chemical modification on generalization among aldehydes for subjects ($N$ = 5) trained on hexanal as S+. In postlavage sessions, half-shaded circles without error bars represent controls ($N$ = 2) that received lavage with 50 mM $NaBH_3CN$ alone. Circles with error bars represent experimental subjects ($N$ = 3) that received two-step lavage (ethyl acetoacetate followed by $NaBH_3CN$). Squares represent mean response to 2-heptanone for all five animals. Dashed line represents point at which lavage was administered.

$< 0.05$). Responding by animals given combined lavages of ethyl acetoacetate and $NaBH_3CN$ gradually increased over days $P < 0.05$), and by the fourth postlavage test, there were no differences between subgroups ($P > 0.25$).

*Experiment 2*

Analysis of the initial training period using cyclopentanone and cyclohexanone as stimuli showed that there were no differences between groups ($Ps > 0.25$), although there were significant differences among days ($F(5,30) = 18.3$, $P < 0.0001$), and between responding to S+ and S− ($F(1,6) = 1193.5$, $P < 0.0001$). The interaction between days and odorants was significant ($F(5,30) = 39.4$, $P < 0.0001$), and Tukey tests showed that while avoidance responding to S+ presentations increased over days ($P < 0.01$), responding to S− presentations remained consistently low (Figure 5). Analysis of the initial generalization period (Figure 5) showed that there were no differences between groups ($P > 0.25$), or among days ($Ps > 0.25$). The only significant effect was a two-way interaction between groups and odorants ($F(1,6) = 15.8$, $P < 0.007$). Tukey tests indicated that animals exhibited relatively higher overall levels of responding to their respective training odorant ($Ps < 0.01$). However, both groups showed high levels of responding to both cyclohexanone and cyclopentanone.

During the second training period, there were significant differences between groups ($F(3,8) = 9.3$, $P < 0.006$), between stimuli ($F(1,8) = 1048.9$, $P < 0.0001$), and across days ($F(4,32) = 3.2$, $P < 0.027$). Also, there was a significant interaction between odorant stimuli and days ($F(4,32) = 30.2$, $P < 0.0001$). Tukey tests showed that animals trained with cyclohexanone exhibited higher overall levels of responding than animals trained with cyclopentanone ($P < 0.05$). However, both groups exhibited high response levels to S+ presentations that increased across days while responding to S− presentations remained consistently low ($P < 0.01$).

Analysis of the second series of generalization tests revealed significant differences among subgroups ($F(3,8) = 39.9$, $P < 0.0001$), among odorants ($F(2,16) = 513.5$, $P < 0.0001$), and across days ($F(6,48) = 22.9$, $P < 0.0001$). Also, there were significant two-way interactions between subgroups and odorants ($F(6,16) = 20.6$, $P < 0.0001$), between subgroups and days ($F(18,48) = 5.6$, $P < 0.0001$), and between odorants and days ($F(12,96) = 16.8$, $P < 0.0001$). Because the three-way interaction between subgroups, odorants, and days was significant ($F(36,96) = 4.3$, $P < 0.0001$), the analysis was interpreted in terms of this highest order effect. Post-hoc tests indicated that CH-E and CP-E animals exhibited avoidance decrements ($Ps < 0.01$), while CH-C and CP-C animals did not ($Ps > 0.25$; Figure 6). CH-E and CP-E performance decrements were not permanent, however, and avoidance of cyclohexanone and cyclopentanone increased over days ($Ps < 0.01$). Perfor-

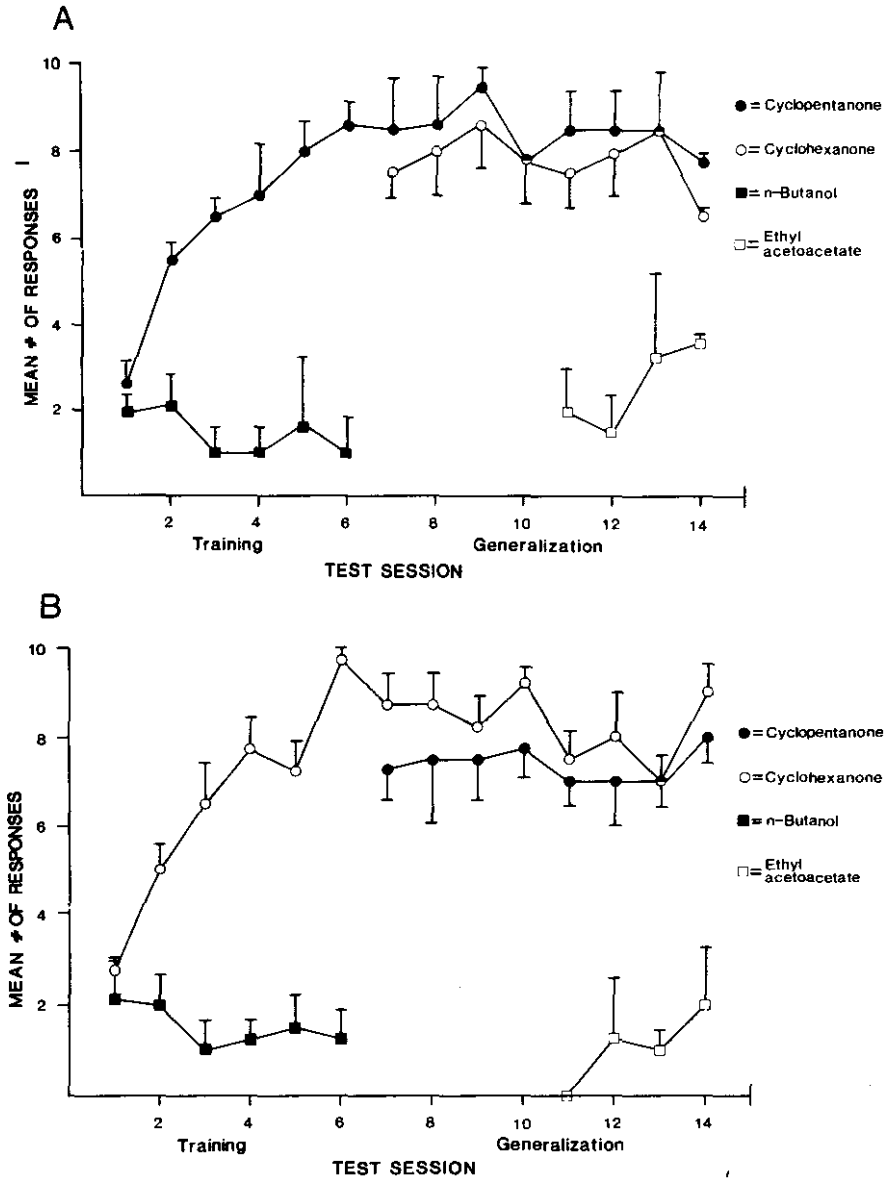


FIG. 5. Generalization between two cycloalkanones over a period of eight days. (A) Results for tiger salamanders ($N = 4$) trained on cyclopentanone (as S+) concurrently with unreinforced presentations of *n*-butanol. Responding to unreinforced presentations of cyclohexanone is significantly lower ($P < 0.01$ by ANOVA of repeated measures) than to unreinforced presentations of cyclopentanone, but generalization between the two odorants is apparent. No generalization to ethyl acetoacetate is observed. (B) Results for tiger salamanders ($N = 4$) trained on cyclohexanone (as S+) concurrently with unreinforced presentations of *n*-butanol. Responding to unreinforced presentations of cyclopentanone is significantly lower ($P < 0.01$ by ANOVA of repeated measures) than to unreinforced presentations of cyclohexanone, but generalization between the two odorants is apparent. No generalization to ethyl acetoacetate is observed.

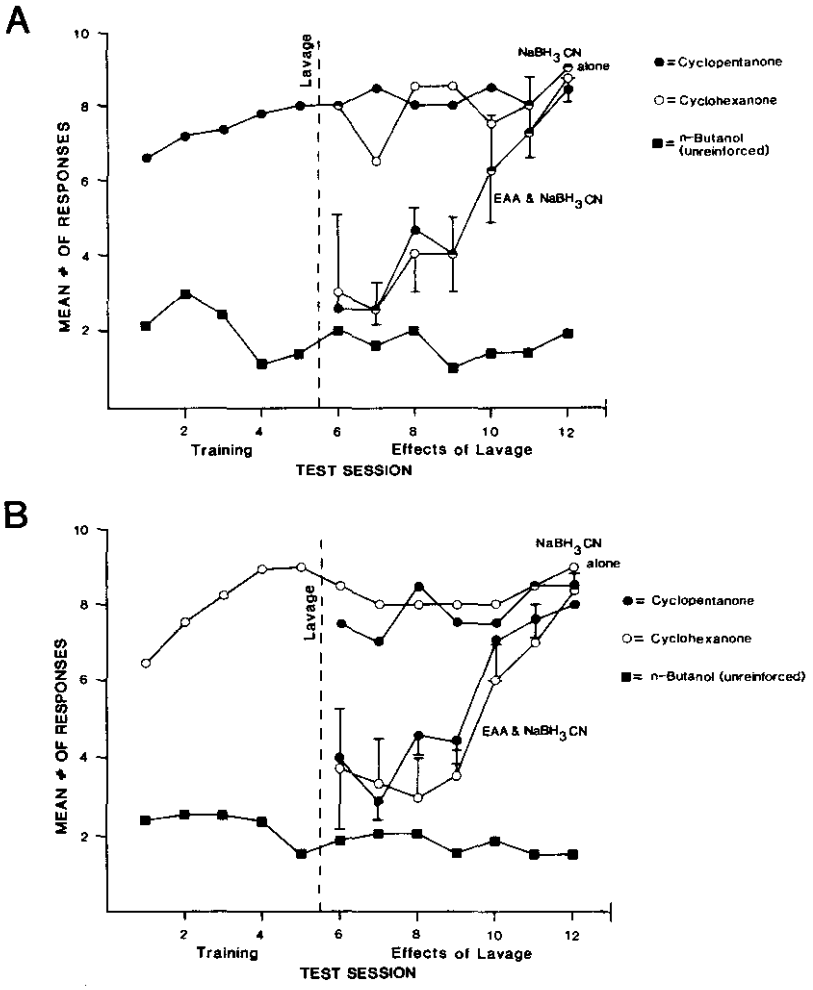


FIG. 6. Effects of chemical modification on olfactory detection and generalization for cycloalkanones. (A) Results for tiger salamanders ($N = 5$) trained on cyclopentanone (as S+) concurrently with unreinforced presentations of *n*-butanol. Lavage with 0.5 mM ethyl acetoacetate followed by 50 mM sodium cyanoborohydride was administered to three animals (represented by points with error bars), and responding to cyclopentanone and cyclohexanone in unreinforced postlavage trials dropped off significantly for four postlavage sessions. Lavage with 50 mM sodium cyanoborohydride alone was administered to two animals (represented by points without error bars). Responding to cyclopentanone and generalization to cyclohexanone were unaffected during postlavage sessions. Responding to *n*-butanol presentations were monitored throughout. (B) Results for tiger salamanders ($N = 5$) trained on cyclohexanone (as S+) with unreinforced presentations of *n*-butanol. Experimental conditions as in A, but with the roles of cyclohexanone and cyclopentanone transposed.

mance improvements were not different between CH-E and CP-E animals ($P > 0.10$).

## DISCUSSION

Tiger salamanders generalize from one aldehyde, hexanal, to its next highest homolog, heptanal. However, because presentations of the latter elicit significantly fewer avoidances than presentations of the former, we infer that these odorants are discriminable. Likewise, we infer that hexanal is discriminable from 1-heptene, 2-fluoro-1-heptene, and 2-heptanone, insofar as little or no avoidance generalization to these odorants was observed. Tiger salamanders generalize between cyclopentanone and cyclohexanone, but we infer that these two odorants are discriminable, because groups show significantly greater responding to their respective training odorant during generalization tests.

Two-step modification impairs detection of both aldehydes and of cycloalkanones. Figure 6 shows that, after lavage of the olfactory epithelium, there is no significant difference between responding to S+ and responding to the new odor. Significant impairment of detection occurs only if blocker and fixer are both applied. As shown for the controls in Figure 6, lavage with fixer by itself does not reduce the level of avoidance significantly. We have previously shown that lavage with ethyl acetoacetate alone at a concentration $\leq 1.0$ mM has no effect on responding (Mason and Morton, 1982, 1984; Mason et al., 1984, 1985). An important feature of Figure 6 is that discrimination of cycloalkanones from *n*-butanol is not completely abolished by two-step modification. Although dramatically reduced, significant levels of avoidance to both cycloalkanones persist after lavage with the blocker fixer sequence. Generalization persists, as well.

The results of two-step modification are consistent with the hypothesis that the two aldehydes and the two cycloalkanones interact with the same class of receptor. The two steps consist of a reversible covalent attachment of a site-specific ligand (blocker) followed by conversion to an irreversible linkage (via a fixer). In these experiments the blocker was ethyl acetoacetate and the fixer was sodium cyanoborohydride, which have previously been shown to label Schiff base-forming proteins specifically in vitro (Mason et al., 1985). This same procedure, when applied to the olfactory epithelium, produces a selective hyposmia to ketones (Mason et al., 1984, 1985) and aldehydes. The control animals, treated with fixer by itself, exhibit no evidence of impaired olfactory function.

Several investigators have proposed that covalent modification of specific amino acid residues can impair chemoreceptor function (Cagan, 1981; D'Ischia, et al., 1982; Mason et al., 1984, 1985). Antifeedant activity of 9$\beta$-polygodial and its 9$\alpha$-hydroxy analog, warburganal, has been attributed to a specific reaction with primary amino groups, such as lysine residues (D'Ischia et al.,

1982). Two-step modification with acetoacetic ester as blocker followed by cyanoborohydride as fixer has been shown to selectively label the active site of a Schiff base-forming enzyme in vitro and to selectively impair detection of ketone-containing odorants in vivo (Mason et al., 1984, 1985). The suggested mechanism invokes irreversible covalent attachment to especially reactive lysine residues. One advantage of using a blocker–fixer sequence is that control experiments can be performed, in which one of the steps is omitted. As Figures 4 and 6 summarize, omission of blocker prevents impairment.

When an animal is trained to avoid one carbonyl compound, it may generalize to another. Lavage with acetoacetic ester followed by cyanoborohydride impairs responding to both odorants. This suggests that the two different molecules interact with the same class of receptor. Both aldehydes and ketones interact with this class of receptors, but generalization does not occur among all simple carbonyls. For instance, animals trained on hexanal do not generalize to 2-heptanone, even though they do generalize to heptanal.

The effect of covalent modification may be agonistic rather than antagonistic. Chemical steps by which Schiff base-forming sites are blocked by two-step modification have been described (Mason et al., 1985), and it seems unlikely that a very large fraction of olfactory receptor sites can be labeled in this fashion. Since, as previously reported, increasing the dose of labeling reagent increases the duration of selective hyposmia, and not its profundity, we are led to propose the following hypothesis. When an olfactory receptor site is covalently modified, it generates a continuous output. If the covalent modification is irreversible, this output continues unabated and causes a high level of background signal, which effectively masks the response to any new odorant molecules that interact with that channel. As can be seen from Figure 6, covalent blockade did not completely obliterate responding to either cycloalkanone. This is taken to imply that the cycloalkanones also interact with receptors that are unaffected by covalent modification. As might be expected (since cyclohexanone and cyclopentanone are similar in molecular volume, dipole moment, etc.), responding to both odorants is about the same, even during the postlavage interval, where olfactory detection of ketones is greatly impaired. We infer that both cycloalkanones interact with Schiff base-forming receptor sites, but that this is not the sole basis for generalization.

Results presented here add to an increasing body of data that supports the existence of carbonyl-binding, generalist receptors in tiger salamanders. The effects of two-step modification suggest that these receptors attach odorant molecules covalently as Schiff bases. A role for other types of receptors in detection and discrimination of ketones and aldehydes is also deduced, since chemical modification impairs responding, but neither obliterates detection nor eradicates generalization. We infer that generalist olfactory receptors have the following characteristics. A given class of receptors will bind a number of odorants. At

the same time, any given molecule can interact with more than one class of receptors. For generalization to take place, two different compounds must have similar interactions with more than one receptor class. Since nature rarely presents pure compounds as stimuli, the ability to generalize, even in the presence of distractors, must be balanced against the ability to suppress false alarms and discriminate against mimics. This picture may offer an optimum strategy for dealing with natural fluctuations in the composition of olfactory cues.

*Acknowledgments*—The authors are grateful to Ms. Cheryl Boehm for assistance in performing double-blind behavioral studies and for preparing the figures. This work was supported by the National Science Foundation (CHE 85-09557) and the National Institutes of Health (NS-19424). The UCR departmental multinuclear NMR and double-focusing mass spectrometer were purchased with partial support of the NSF (Departmental Research Instrumentation grant CHE 82-03497) and the NIH (grant RR01750-01), respectively.

## REFERENCES

ARZT, A.H., SILVER, W.L., MASON, J.R., and CLARK, L. 1986. Comparison of olfactory receptor responses in land and water phase tiger salamanders. *J. Comp. Physiol. A.* 158:479–487.

CAGAN, R.H. 1981. Recognition of taste stimuli at the initial binding interaction, *in* R.H. Cagan and M.R. Kare, (eds.). *Biochemistry of Taste and Olfaction.* Academic Press, New York, pp. 175–209.

CHAPMAN, O.L., MATTES, K.C., SHERIDAN, R.S., and KLUN, J.A. 1978. Stereochemical evidence of dual chemoreceptors for an achiral sex pheromone in Lepidoptera. *J. Am. Chem. Soc.* 100:4878–4884.

DAUB, G.W., ZUCKERMAN, R.N., and JOHNSON, W.S. 1985. A synthesis of 2-fluoroalkenes. *J. Org. Chem.* 50:1599–1602.

D'ISCHIA, M., PROTA, G., and SODANO, C. 1982. Reaction of polygodial with primary amine: An alternative explanation to the antifeedant activity. *Tetrahedron Lett.* 23:3295–3298.

EICHENBAUM, H., MORTON, T.H., POTTER, H., and CORKIN, C. 1983. Selective olfactory deficients in case H.M. *Brain* 106:459–472.

KÖSTER, E.P. 1971. Adaptation and Cross-Adaptation in Olfaction. PhD thesis, Utrecht.

KUNTZ, A. 1923. The learning of a simple maze by the larvae of *Ambystoma tigrinum. Iowa Univ. Stud. Nat. Hist.* 10:27–35.

LAMBERT, J.B., KOENG, F.R., and HAMERSMA, J.W. 1971. The Tricyclo[5.1.0.$0^{3,5}$]-octan-2-ols. *J. Org. Chem.* 36:2941–2942.

LESPIEAU, R., and BOURGUEL, M. 1941. 3-Cyclohexyl-2-bromopropene. *Org. Synth. Coll.* 1:186–187.

MASON, J.R., and MORTON, T.H. 1982. Selective and reversible anosmia in tiger salamanders (*Ambystoma tigrinum*) caused by chemical treatment of the olfactory epithelium. *Physiol. Behav.* 29:709–714.

MASON, J.R., and MORTON, T.H. 1984. Fast and loose covalent binding of ketones as a molecular mechanism in vertebrate olfactory receptors. Chemical production of selective anosmia. *Tetrahedron* 40:483–492.

MASON, J.R., and STEVENS, D.A. 1981a. Discrimination and generalization among reagent grade odorants by tiger salamanders (*Ambystoma tigrinum*). *Physiol. Behav.* 27:647–653.

MASON, J.R., and STEVENS, D.A. 1981b. Behavioral determinations of thresholds for *n*-butyl acetate and *n*-butyl alcohol in the tiger salamander (*Ambystoma tigrinum*). *Chem. Senses* 6:189–195.

MASON, J.R., STEVENS, D.A., and RABIN, M.D. 1980. Instrumentally conditioned avoidance by tiger salamanders (*Ambystoma tigrinum*) to chemically pure odorants. *Chem. Senses* 5:99–105.

MASON, J.R., MEREDITH, M., and STEVENS, D. A. 1981. Odorant discrimination by tiger salamanders after combined olfactory and vomeronasal nerve cuts. *Physiol. Behav.* 27:125–132.

MASON, J.R., CLARK, L., and MORTON, T.H. 1984. Selective deficits in the sense of smell caused by chemical modification of the olfactory epithelium. *Science* 226:1092–1094.

MASON, J.R., LEONG, F.-C., PLAXCO, K.W., and MORTON, T.H. 1985. Two-step covalent modification of proteins. Selective labelling of Schiff base-forming sites and selective blockade of the sense of smell. *J. Am. Chem. Soc.* 107:6075–6084.

Schneider, D. 1969. Insect olfaction: Deciphering system for chemical messages. *Science* 163:1031–1037.

WINER, B.J. 1962. Statistical Principles in Experimental Design. McGraw-Hill, New York.