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FAST AND LOOSE COVALENT BINDING OF KETONES AS A MOLECULAR MECHANISM IN VERTEBRATE OLFACTORY RECEPTORS

CHEMICAL PRODUCTION OF SELECTIVE ANOSMIA

J. RUSSELL MASON
Monell Chemical Senses Center, Philadelphia, PA 19104, U.S.A.

and

THOMAS HELLMAN MORTON*
Department of Chimistry, University of California, Riverside, CA 92521, U.S.A.

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Abstract—Olfactory receptors undoubtedly have fast rates of association with odorant molecules. If the dissociation rate is also fast, the dissociation constant, K_d, will be comparatively large ($\geq 10^{-6}$ M). With such fast and loose binding, a receptor will recover function rapidly. If, on the other hand, the dissociation rate is slow, odorant binding will be tight, and the receptor will have slow recorvery. Previous investigators have explored tight binding ($K_d \leq 10^{-8}$ M). The present study is the first to probe the possibility of fast and loose binding in the olfactory epithelium of air-breathing vertebrates. Our approach is based on the conjecture that ketones are bound as Schiff bases (just as retinal is bound in visual pigment). As a model system, the Schiff base-forming bacterial enzyme acetoacetate decarboxylase (AAD) has been studied. Nucleophilic attack (e.g. BH_4^- reduction) of reversible AAD–carbonyl complexes produces irreversible binding to the active site in a fraction, ϕ, of the enzyme molecules. Kinetics are discussed and interpreted using the derived expression $\phi = 1 - [K_d/(K_d + S_0)]^m$, where S_0 is the initial concentration of carbonyl substrate and the exponent m is the ratio of the rate constant for attack of the protein-substrate complex to the rate constant for removal of substrate (e.g. by borohydride attack of the free carbonyl). We hypothesize a similar pathway when the olfactory epithelium of experimental animals is treated with solutions of cyclohexanone or ethyl acetoacetate, in which Schiff base linkages are attacked *in vivo* by some endogenous nucleophile. We have developed a behavioral assay for olfactory receptor inactivation and report the first example of a chemically produced selective anosmia (odor blindness).

Mechanistic questions about the sense of smell cut across traditional disciplinary boundaries. The chemical aspects of olfaction have been the subject of wide speculation, but a comparatively small number of experiments have been reported for air-breathing, vertebrate species. Many poorly understood receptor events undoubtedly take place, and the range of interactions is represented in the scheme below.

Chemists are inclined to direct their inquiries from the left hand side of the above scheme, e.g. to examine the effects of altering odorant structure[1] or to perform binding assays on fractions isolated from olfactory tissues.[2] On the other hand, investigators in the life sciences tend to approach from the right, using histological[3] and electrophysiological[4] probes, as well as behavioral studies of odor detection.[5] This paper will discuss the hypothesis that some odorants may bind covalently to receptors in the course of olfactory detection. Obviously, there are many odoriferous molecules that probably do not form covalent bonds. It is not our intention here to review the bioorganic basis of the sense of smell (which is not presently well understood), but to present new experimental results, which are consistent with covalent attachment of ketones to olfactory receptors.

We describe here a collaboration between an organic chemist and a behavioral biologist in a concerted attack on the problem from both directions. We chose a plausible chemical model, namely that odoriferous ketones bind to receptor sites as Schiff bases. We first explored the chemistry of a known protein that binds simple ketones in this fashion and found an inhibitor. We then used this inhibitor to produce a selective anosmia (or "specific odor blindness", the olfactory equivalent of color blindness) for ketones in experimental animals. This represents the first reported case of a chemically induced selective anosmia that has been detected by a behavioral assay. Though naturally occuring selective anosmias have been reported in human beings,[6] there is evidence that these are not hereditary,[7] unlike their counterparts in taste or vision. It is possible that at least some selective anosmias do not represent receptor-based phenomena.[8,9]

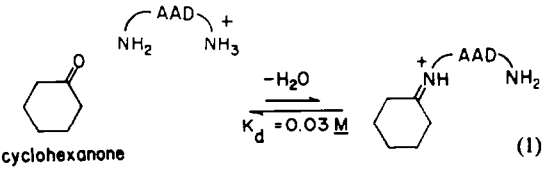
The problems of understanding olfaction in vertebrates can be contrasted with our knowledge of

vertebrate vision. Dissection of the eye reveals structures (the cornea, the lens) whose role is apparent and a colored protein (rhodopsin) in the retina that is observably bleached by light. Rhodopsin has been recognized for decades as the focus of attention for studying the chemistry of vision. Opsin, the peptide moiety, recycles through many sensory events, binding chromophoric 11-*cis*-retinal and dissociating the *trans* photoisomer repeatedly. Though the evidence is not yet entirely clear, it seems that olfactory receptor proteins also bind and dissociate odorant molecules many times and are not used up by a single sensory event.[4i]

In the eye, the functions of the retina and of the access route to the retina have been well delineated. But in the nose the ostensible clues to function are not obvious. We have only begun to discern the effects of the acess route by which stimulus reaches receptor (mucus retention, air passage restriction) as distinct from receptor phenomena. In previous studies,[8,9] we have endeavored to identify the role of the access route. Transduction still remains a complete mystery. Receptor sites have not been well characterized, and even their location in the olfactory epithelium is an article of controversy.[4d] Neither do we know how many varieties of receptor exist, whether there is only one type or just a few or very many.

Odorant binding at the receptor is very poorly understood. Unlike the retina, the olfactory epithelium does not contain a substance that can be readily identified as being chemoreceptive. Efforts have been directed towards isolating proteins from olfactory receptor tissue that bind odorants tightly,[2] i.e. with dissociation constants $K_d \leq 10^{-8}$ M. But intuition suggests that there should be receptors that bind more loosely, since tight binding requires a slow dissocation rate constant. Receptors that hold odorant molecules for such long duractions—on the order of seconds, even with a diffusion-controlled association rate—will be slow to recover function. We have previously described a model for olfactory detection using $K_d > 10^{-6}$ M and have shown that this model provides a coherent picture of olfactory response functions and accounts for many psychophysical data.[9] Senf, Menco, Punter, and Duyvestyn[10] have recently applied the Beidler taste equation to electrophysiological data on frogs and conclude that dissocation constants for a number of low molecular weight odorants are $K_d \geq 10^{-5}$ M. We therefore view a typical receptor as having both fast association and dissociation rates. At present, it is not known if there are sites that bind odorant molecules covalently. We present results here that are consistant with "fast and loose" covalent binding.

Our approach has been to probe chemical models and to develop a blocking agent. The bacterial enzyme acetoacetate decarboxylase (AAD, EC 4.1.1.4) is our chemical model. This protein was chosen for several reasons. It binds a variety of carbonyl compounds as Schiff bases on a lysine ϵ-amino group at the active site.[11] Chemists' lore has it that many ketones and aldehydes (except those that, like camphor, are sterically hindered) have an odor characteristic of their functional group. If this belief has any foundation, it suggests that there are receptors that bind the carbonyl group independently of the rest of the molecule.



(1)

The reaction of AAD with ketones is an example of fast and loose binding. The enzyme can be considered as a rigid diamine[12] and reacts with substrates in the same fashion as simple aliphatic analogues.[13] The dissociation constants for simple ketones are large. As reaction 1 shows, cyclohexanone is a poor competitive inhibitor. Nevertheless, the association rate for cyclohexanone must be fast, since there is a fast specific turnover rate constant for AAD-catalyzed exchange of an α-hydrogen with solvent,[14] $k_{cat} \approx 200\ sec^{-1}$.

Precedent provides an additional reason for examining Schiff base-forming proteins. Similar covalent binding occurs in another sensory system, namely vision. Rhodopsin consists of a simple aldehyde, retinal, bound as a Schiff base to a protein, opsin. If Schiff base formation also plays a role in olfactory binding, then it should be possible to trap protein-substrate complexes with nucleophiles. Fridovitch and Westheimer showed this with AAD *in vitro* twenty years ago,[15] and a group at Rockefeller University has recently shown that there are *in vivo* nucleophiles that trap Schiff bases non-enzymatically.[16] If a large number of Schiff base-forming receptors are occupied *in vivo* for a long period of time (by flooding with substrate, for instance), it is possible that a detectable fraction of them may be trapped by endogenous nucleophiles.

Our other reasons for choosing AAD as a chemical model are matters of convenience. The enzyme is a crystalline polypeptide with no known cofactors and no known regulatory sites. It is active as dimer subunits (mw $\approx$ 60,000), and, since it is an enzyme, the action of blocking agents is revealed by a loss of catalytic activity. While working as a Visiting Fellow in the laboratory of Professor F. H. Westheimer at Harvard University, one of us (THM) secured (with the able assistance of Mr. J. V. Connors) adequate quantities of the pure enzyme from cultures of *Clostridium acetobutylicium*.[17]

We have succeeded in producing selective anosmia in experimental animals by chemically treating the olfactory receptor epithelium. This result has special significance, because the chemical treatment was entirely devised from our model studies on AAD. Two hypotheses guided us. First, an odorant that binds fast and loose to AAD also binds fast and loose to an olfactory receptor. Second, an inhibitor that forms a tighter covalent bond to AAD will also bind to the olfactory receptor in question. This investigation proceeded by the following steps.

(1) Find a simple procedure for inhibiting AAD;

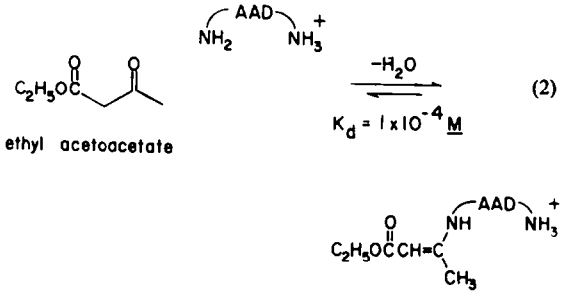
(2) Confirm the mode of binding and ascertain that nucleophilic attack renders it irreversible;

(3) Train laboratory animals to respond to two odorants, one of which is a ketone;

(4) Discover whether applying the inhibitor to an animal's receptor surface affects its ability to respond to one of the odorants.

Reasons for the choice of AAD in (1) and (2) have been presented above. The experimental animals for (3) and (4) were tiger salamanders (*Ambystoma tigrinum*). This species has been the subject of a number of anatomical[3] and electrophysiological[4b-h] studies. The olfactory epithelium of amphibians is readily accessible to direct chemical treatment, since the receptor surface is just inside the nasal openings. Anatomically, salamanders were very suitable for our experiments, unlike rats and other mammalian species, whose olfactory receptors are located deep within the skull and shielded from ready access. Tiger salamanders are the only amphibian species that has been trained to give behavioral responses to olfactory stimuli by reagent grade chemicals.[5c-e]

The ketone used for both AAD and olfactory studies was cyclohexanone. This choice was dictated by ubiquitous effect of cyclohexanone as a stimulus. Electrophysiological studies of tiger salamanders have shown that many odorants elicit different responses, depending on which portion of the olfactory epithelium is stimulated.[4f] In contrast, the response to cyclohexanone does not depend on which area of the receptor surface receives the odorant.[18] Thus, a selective anosmia to cyclohexanone cannot be attributed to damage to a special, localized patch of cells.

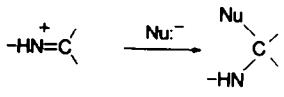


(2)

A survey of the known inhibitors of AAD did not suggest suitable candidates for our study, and we had to find a new one. Because lipid solubility was likely to be important for accessiblity to olfactory receptors, we ruled out ionic species. Because the inhibitor was to be applied to the receptor surface in a normal saline solution, water solubility was also desirable. Finally, we wanted the ordinary mechanisms of odorant clearance to function after application of the inhibitor, so moderate volatility was requisite. Previous reports had discussed the formation of aminocrotonate when acetoacetate is bound to a primary amine.[19] We reasoned that an acetoacetate ester would bind to AAD exclusively in this fashion, as shown in reaction 2, and measured a dissociation constant that is more than 100 times smaller than the K_d for cyclohexanone.

RESULTS AND DISCUSSION

Our first task was to measure the dissociation constants for the AAD complexes with cyclohexanone and ethyl acetoacetate. The observed values are those shown in reactions 1 and 2. In both cases, binding was fully reversible. Incubation of a sample of AAD in a 0.003 M solution of ethyl acetoacetate in pH 6 phosphate buffer for 20 hr at 30° was followed by dialysis, after which full activity of the enzyme was recovered. Likewise, incubation of AAD in saturated aqueous cyclohexanone (*ca* 0.05 M, with supernatant cyclohexanone) at pH 6 for days led to no loss of enzyme activity.



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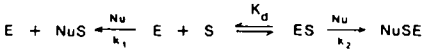


Our next task was to confirm that the mode of binding was as depicted in reactions 1 and 2. To do this, reversible enzyme complexes were treated with aqueous sodium borohydride. This reagent reduces both the unbound keto function and the Schiff base complex.[15,22] Reduction of the free substrate has no effect on the enzyme, since alcohols do not bind. But attack of the enzyme-substrate complex irreversibly fixes it as an amine, as reaction 3 depicts. The pertinent kinetic scheme is also shown. If the concentration of attacking nucleophile, [Nu], is so large that it does not change over the course of reaction, the kinetics can be described in terms of pseudo-first order rate coefficients k_1[Nu] and k_2[Nu]. If initial concentration of substrate, S_0, is much larger than the initial concentration of enzyme, E_0, then we can use the approximation $S = S_0 e^{-k_1[Nu]t}$ in the rate equations. The assumption that enzyme-substrate association and dissociation rates are fast relative to attack rates leads to the result

$$[NuSE] = E_0\left[\left(\frac{K_d + S_0 e^{-k_1[Nu]t}}{K_d + S_0}\right)^{k_2/k_1}\right] \qquad (1)$$

where NuSE represents the irreversibly trapped enzyme-substrate complex. When reaction is complete, the fraction of enzyme that has been fixed in this fashion (i.e. the extent of modification), ϕ, is

$$\phi = \frac{[NuSE]_{t=\infty}}{E_0} = 1 - \left(\frac{K_d}{K_d + S_0}\right)^m, \text{ where } m = \frac{k_2}{k_1} \qquad (2)$$

Irreversible inactivation of AAD complexes by borohydride reduction was examined by two different methods, loss of catalytic activity and incorporation of radiolabel. In a radiolabelling experiment, addition of excess borohydride to a solution of AAD in 0.01 M aqueous 1-^{14}C-cyclohexanone gave a level of non-dialyzable radioactivity that corresponds to incorporation of cyclohexanone into approx. 80% of the enzyme dimer subunits that were present (i.e. $\phi = 0.8$). Using $K_d = 0.03$ M, eqn (2) gives the result that k_2 is 6 times greater than k_1. Repetition of this procedure using bovine serum albumin in place of AAD gave a much lower ($\phi = 0.05$) incorporation of radioactivity. Neither protein incorporates appreciable radioactivity if borohydride is omitted.

In a similar experiment, addition of excess borohydride to a solution of AAD in 0.3 mM aqueous ethyl 3-^{14}C-acetoacetate gave non-dialyzable radioactivity corresponding to incorporation of ethyl acetoacetate into approximately one-seventh of the enzyme dimer subunits (i.e. $\phi = 0.14$). Using $K_d = 0.1$ mM, equation 2 gives the result that $k_2/k_1 = 0.11$. The loss of catalytic activity was too small to detect, but loss of catalytic activity was

measured when a solution of AAD in 3.7 mM unlabelled ethyl acetoacetate was treated with excess borohydride. The catalytic activity of the AAD recovered after dialysis was 75% of the initial activity (i.e. $\phi = 0.25$). This corresponds to $k_2/k_1 = 0.08$. The two independently determined k_2/k_1 ratios for S = ethyl acetoacetate agree within experimental uncertainty. These results show that ethyl acetoacetate binds to AAD covalently. The following control experiment rules out a number of alternative interpretations. The radiolabelling experiment was repeated exactly as before, but the sodium borohydride was added to the ethyl 3-^{14}C-acetoacetate solution *before* the AAD. The level of radioactivity incorporated in this experiment was negligible.

The k_2/k_1 ratios are consistent with the binding mechanisms represented in reactions 1 and 2. For S = cyclohexanone, the positive charge makes the protonated imine more electrophilic than the corresponding ketone. Hence, k_2 is nearly an order of magnitude greater than k_1. For ethyl acetoacetate bound as an uncharged aminocrotonate ester, the rate of reduction of the enamine should be slower than that of the corresponding β-keto ester. As a result, k_2 is an order of magnitude smaller than k_1.

The model studies with AAD directed our experiments with living salamanders. We presume that eqn (2) pertains here, too, but the chemical identity of the endogenous nucleophile remains unspecified. All we know is that there are nonenzymatic pathways *in vivo* for irreversibly fixing Schiff base complexes between proteins and small molecules.[16] We need not require that endogenous nucleophile attack the unbound odorant. There are other mechanisms for odorant clearance—for example, evaporation and mucus flow. Under our experimental conditions, we expect that the residence time, $\tau_{clearance}$, for odorant may be on the order of minutes or longer.[20] The appropriate exponent in eqn (2) is then $m = k_2[Nu]\tau_{clearance}$.

We believe that a behavioral assay remains the optimum method for detecting selective anosmia. Other groups have examined direct chemical treatment of the olfactory mucosa using electrophysiological assays for sensory inactivation,[21] but the proper interpretation of such results can be complicated.[23] No monitor surpasses the subject's own nervous system for telling whether the subject can or cannot smell an odorant. Our protocol for running the behavioral assay is described elsewhere in detail.[24] Briefly, each salamander was trained to avoid 2 odors, cyclohexanone and dimethyl disulfide, and trained not to avoid a third odor, n-butanol. Previous studies had shown that tiger salamanders can be trained to avoid n-butanol and that this behavior is irreversibly extinguished when the olfactory nerves are cut.[5c-e] Salamanders can therefore smell n-butanol, and their task in the present experiments was to discriminate cyclohexanone or dimethyl disulfide from n-butanol.

Subjects were trained over a period of 1–3 weeks to perform the discrimination task. Odorant (10^{-6}–10^{-5} M in air) was delivered by a Dravnieks olfactometer[25] via a glass sniffing port. Each animal was given a negative reinforcement, namely a bright light focussed onto the sniffing port, from which it escaped by withdrawing its head. When the light was immediately preceded by a puff of cyclohexanone or dimethyl disulfide, the salamanders learned to withdraw their heads before the light was turned on. Without the light as a negative reinforcement (as in the case of n-butanol as odorant) animals rarely withdrew.

Acquisition of conditioned avoidance by the first group of subjects is summarized in Fig. 1. In an experimental session, all 3 odorants were presented 8

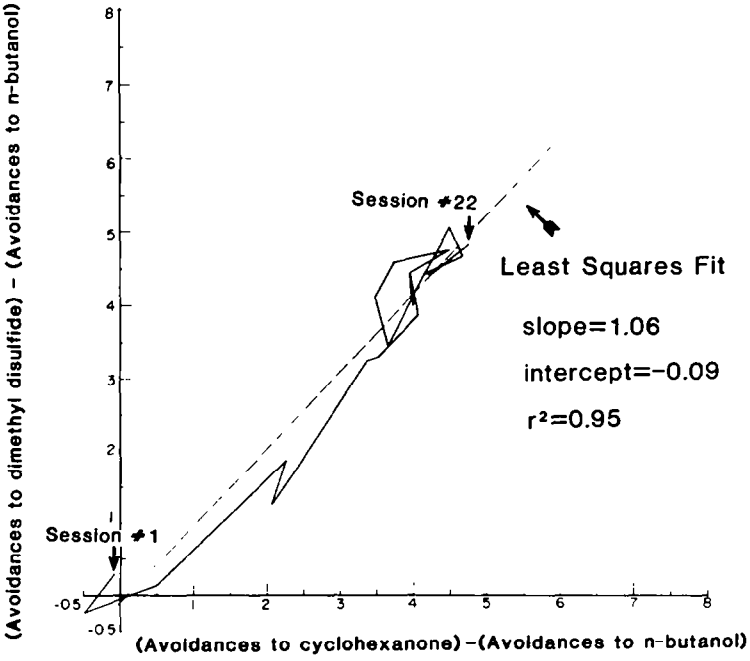


Fig. 1. Plot of acquisition data for avoidance conditioning to cyclohexanone and dimethyl disulfide of 17 tiger salamanders over 22 training sessions (1 session per day). The squiggly line represents data points connected in serial order. The dashed line shows the best linear fit of these data.

times each in random order. In each session of 24 presentations, a well-trained animal scored 0–1 avoidances to butanol and 7–8 avoidances to cyclohexanone and to dimethyl disulfide. Figure 1 shows a plot of the conditioned avoidance to cyclohexanone *vs* the conditioned avoidance to dimethyl disulfide as a function of time during training (avoidances to n-butanol are subtracted from each). This plot (shown by the squiggly line) is well fit by a straight line with slope = 1 and intercept = 0 and demonstrates that the subjects learned to avoid both odors equally. If negative reinforcement (the bright light) was then omitted, these trained animals all stopped avoiding any of the odorants, and conditioned responding completely extinguished over a period of 5–6 days. A plot of the extinction of behavior is not shown, but it, too, follows the straight line fit with slope = 1 and intercept = 0. Finally, when these animals were trained again, they reacquired conditioning, rapidly returning to pre-extinction levels of responding within one week. Once more, a plot of reacquisition (not shown) follows the same straight line fit.

The purpose of looking at extinction and reacquisition was twofold. First, it showed that the animals had been truly conditioned to avoid the negative reinforcement (the bright light) and did not exhibit aversions to the odorants themselves. Secondly, it showed that the salamanders neither learned nor forgot more rapidly with one odorant than with the other. The slope and intercept of the linear fit of the acquisition plot demonstrates this.

After conditioning, animals were then anesthetized, and lavages with aqueous solutions of odorants were administered to their olfactory sacs. In order to control for the effects of the procedure alone, anesthetized animals were given lavage with saline or given no lavage at all (results are summarized below in Figs. 4 and 5). In neither case was there a significant effect. In a separate control study, a set of animals were anesthetized, and lavage with 0.05 M n-butanol was administered. The data are displayed in Fig. 2. Once again, there was no difference between pre-and post-lavage responding.

A major difference between behavioral and chemical data is that the former are usually based on a much smaller sample size. As a consequence, behavioral data are much noisier. Statistical tools aid substantially in the interpretation. Let us choose to infer that there is no difference between two sets of data if the odds are $> 1:3$ ($p > 0.25$) that the two sets resulted from random fluctuations of a single, normally distributed group of subjects. Let us also judge that there *is* a significant difference between two mean values if the odds are $> 100:1$ ($p < 0.01$) *against* the two data sets representing random fluctuations in a normal distribution about a single mean. We assess our results using three-way analyses of variance with repeated measures on two factors (test session; odorant) and use Tukey *b* tests to isolate significant differences among means in *post hoc* comparisons.[26] For instance, in Fig. 2, there is no difference between subjects' responding to cyclohexanone and to di-

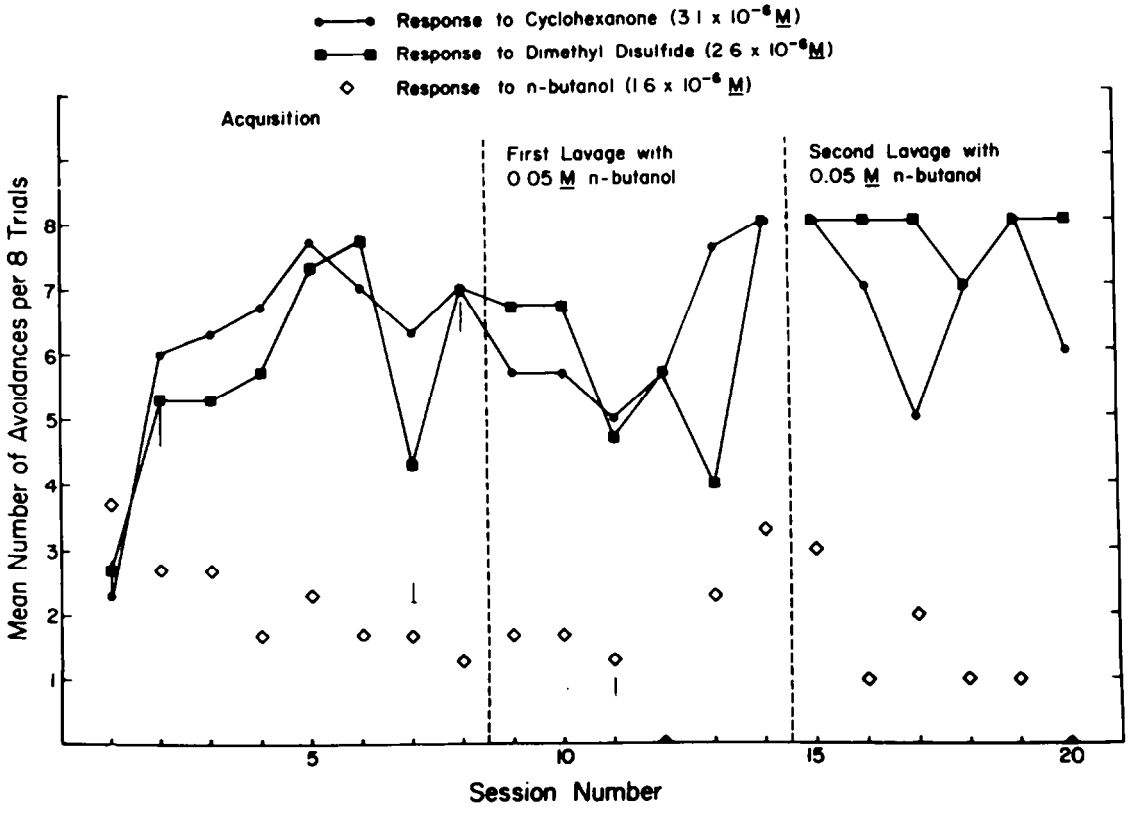
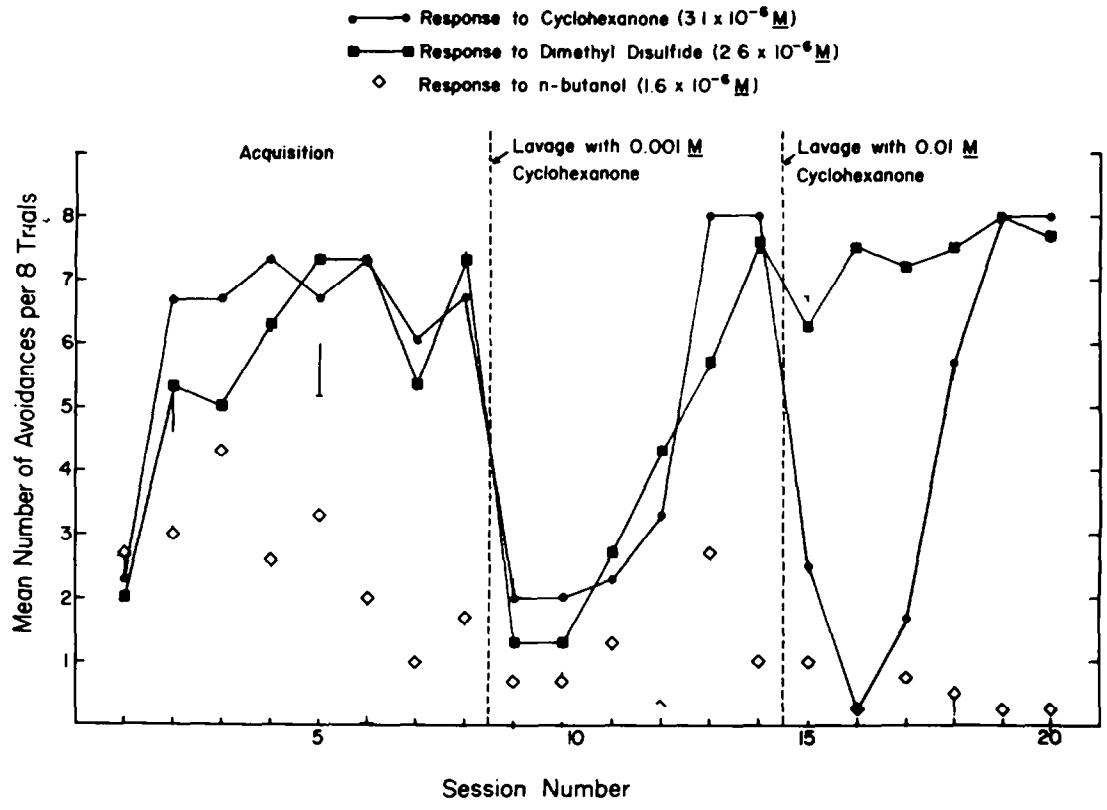


Fig. 2. Control results for effects of the lavage procedure on odor avoidance by tiger salamanders. No effect is observed when the animals are anesthetized and lavage with 0.05 M n-butanol is administered, neither right after acquisition (n = 3) nor after recovery from a previous lavage with 0.001 M ethyl acetoacetate (n = 1). These animals were in the same cohort as the subjects in Fig. 3. Sessions 9–14 correspond to 16, 40, 44, 64, 68 and 92 hr, respectively, after the first lavage.



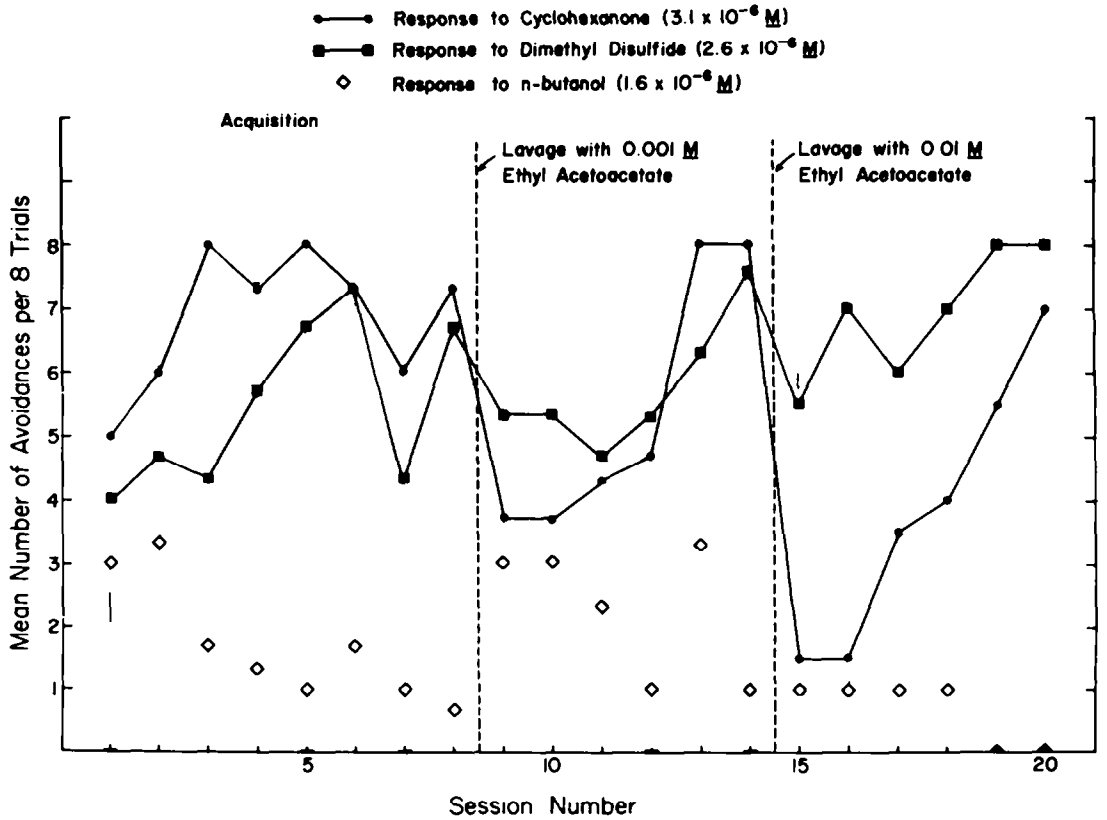


Fig. 3. Effects of lavage with cyclohexanone and ethyl acetoacetate on odor avoidance by tiger salamanders. These animals were in the same cohort as the subjects in Fig. 2. Sessions 15–20 correspond to 16, 20, 44, 64, 68 and 88 hr, respectively, after the second lavage. (a) Results for 0.001 M (n = 3) and 0.01 M (n = 4) cyclohexanone lavages. (b) Results for 0.001 M (n = 3) and 0.01 M (n = 2) ethyl acetoacetate lavages.

methyl disulfide. There is a significant difference between their responding to either of these odorants and to n-butanol. Error bars are omitted from the data shown here but have been reported elsewhere.[24]

Our observations of selective anosmia are the focus of this study. Figure 3 displays the results of two lavages that produce selective anosmia to cyclohexanone, yet have no effect on responding to dimethyl disulfide. As Fig. 3a shows, lavage with 0.001 M cyclohexanone produces no difference in responding between the two odorants, but lavage with 0.01 M causes a significant difference. On each of the three sessions (# 's 15–17) after lavage, there is a significant difference ($p < 0.01$) between responding to the two odorants. Likewise, Fig. 3(b) shows no difference after 0.001 M ethyl acetoacetate lavage, but a significant difference between responding to cyclohexanone and to dimethyl disulfide on the first two sessions (Nos. 15 and 16) after lavage with 0.01 M ethyl acetoacetate. It is worth repeating that we are concerned only with *differences* in responding to the 2 odorants and offer no interpretation for temporary *general* anosmia that appears to result from some treatments (e.g. 0.001 M cyclohexanone lavage).

If our olfactory binding model is correct, chemically induced selective anosmia should be more pronounced for odorants that bind covalently than for odorants that do not. Sulfides bind tightly to olfactory receptor tissues.[2r] The mechanism of binding is unknown, but it seems plausible that they do not form covalent linkages that are irreversibly trapped *in vivo*. Figure 4 summarizes the results from lavage with a saturated solution (0.05 M) of dimethyl disulfide. Responding to dimethyl disulfide does decrease relative to cyclohexanone, but the difference between the means is significant ($p < 0.01$) for only one session (No. 19). The response decrement is in the opposite direction (as expected) from the one caused by 0.01 M cyclohexanone or ethyl acetocetate lavage, but is not so profound.

When the lavage concentration of cyclohexanone or ethyl acetoacetate is increased, the selective anosmia to cyclohexanone lasts for a longer time. These data are plotted in Fig. 5 using the same axes as Fig. 1, with Roman numerals designating the session number after lavage. This format was chosen to illustrate the difference between general and selective anosmia. A straight line of slope = 1 and intercept = 0 is superimposed on Figs. 5(a and b). This line gave a good fit to the acquisition plot in Fig. 1. This diagonal also corresponds to the general anosmia coordinate. As general olfactory acuity fluctuates, subjects' scores will move up and down the diagonal. This is exemplified by the mean scores for controls, represented by the x's. Control subjects' performances vary widely from session to session, but the mean scores all stay fairly close to the line. In Fig. 5(b), for instance, the x's vary by as much as 6 units along the diagonal from one session to the next, but stay within 2 units of the diagonal in the direction perpendicular to it.

Displacements corresponding to general anosmia and to selective anosmias occur, respectively, in the directions indicated by the arrows in Figs 5(a). Selectively anosmic subjects' scores are displaced parallel to the coordinate axes. The open symbols in Fig. 5 represent mean scores after lavage with 0.05 M cyclohexanone (**5a**) or 0.05 M ethyl acetoacetate (**5b**). In both cases, subjects exhibit general anosmia in the first session after lavage, but then recover selectively in subsequent sessions. The distance in the x-direction away from the diagonal measures selective anosmia

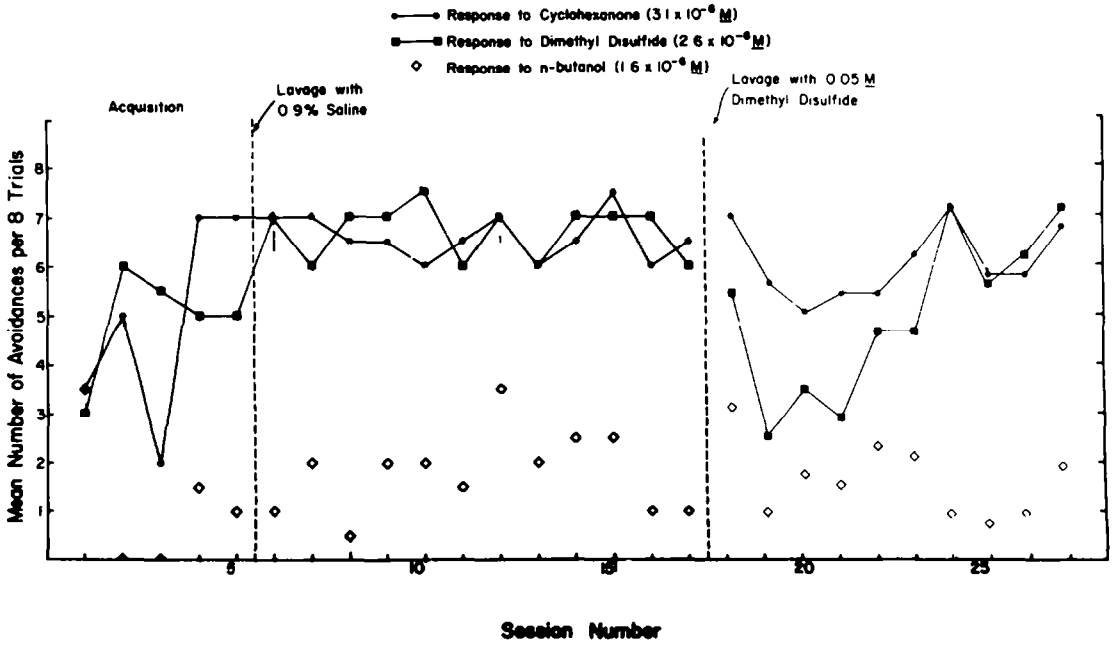


Fig. 4. Effect of dimethyl disulfide lavage compared with controls. These control subjects (n = 2) were anesthetized and given saline lavage right after acquisition. After 1 week, dimethyl disulfide lavage was administered to one of these animals as well as to four others in the same cohort that had completely recovered from other procedures. Sessions 18–27 correspond to 19, 23, 43, 47, 67, 71, 91, 115, 119 and 143 hr, respectively, after dimethyl disulfide lavage.

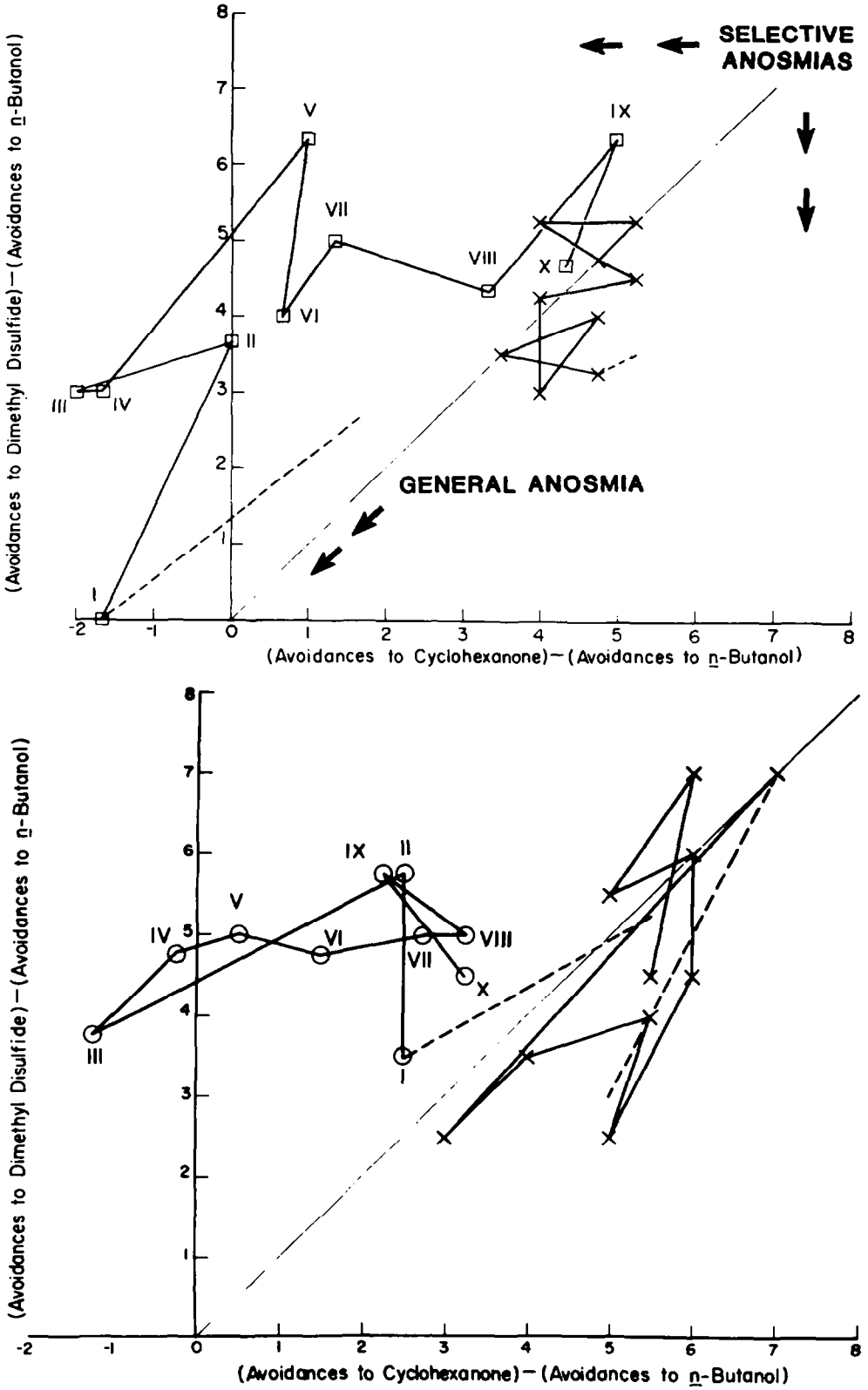


Fig. 5. Plots of odor-guided behavior for 10 sessions following lavage. × 's denote control subjects. Open symbols denote subjects given lavage with 0.05 M ketone solutions, with Roman numerals designating the session number after lavage. Solid lines connect the post-lavage sessions in sequence. Dashed lines connect the last pre-lavage with the first post-lavage session. The diagonal (slope = 1, intercept = 0) is included to illustrate the direction corresponding to equal changes in olfactory sensitivity to both odorants. (a) Contrast between the effects of 0.05 M cyclohexanone lavage (□, n = 3) and lavage with saline alone (×, n = 5). The boldface arrows indicate directions corresponding to selective anosmias (parallel to axes) and to general anosmia (along diagonal). (b) Contrast between the effects of 0.05 M ethyl acetoacetate lavage (○, n = 4) and the effect of anesthesia alone (×, n = 2).

to cyclohexanone. In Fig. 5(a) responding to cyclohexanne remains significantly lower ($p < 0.01$) than to dimethyl disulfide for sessions II–VII, corresponding to persistence of selective anosmia 85–95 hr after lavage. In Fig. 5(b) significant selective anosmia is observed for sessions II–VI and for sessions IX, corresponding to persistence of selective anosmia 110–120 hr after lavage.

A final test of the pertinence of AAD as a model for olfactory ketone receptors probed the effect of an S-alkylating agent, iodoacetamide. Iodoacetamide and iodoacetate irreversibly inactivate AAD only under illumination. In the dark, they have no effect.[27] We sought to explore the effect of lavage with iodoacetamide. Six animals were given lavage with 0.05 M iodoacetamide. Four of these animals died within 4 days, as did three animals that had been given lavage with ethyl acetoacetate prior to iodoacetamide. The two animals that survived had both been given n-butanol followed by iodoacetamide, and they did not exhibit a significant difference between pre- and post-lavage responding.

CONCLUSIONS

Chemical inactivation of olfactory detection of cyclohexanone parallels the inhibition of AAD (see note added in proof). We have developed a set of behavioral experiments for assaying the *in vivo* activity of odor receptors and can compare the effectiveness of a chemical procedure for inhibiting an enzyme with its effectiveness in blocking the sense of smell. Ethyl acetoacetate is effective in both cases, while, in the absence of light, iodoacetamide is not effective in either. Equation (2) forms a basis for quantitative interpretation of irreversible inactivation of AAD-substrate complexes of borohydride. Quantitative interpretation of the behavioral data is limited at present because we do not know the concentration, S_0, at the receptor site that corresponds to a given lavage concentration. More importantly, we do not yet know the fraction of receptors, ϕ, that must be inactivated for anosmia to be observed. We have discussed elsewhere a number of possible explanations for our behavioral results.[24] The qualitative agreement between our AAD experiments and the chemical induction of selective anosmia supports the conjecture that the binding mechanisms are chemically similar. Further experiments will test the pertinence of eqn (2) in accounting for chemical induction of selective anosmia.

EXPERIMENTAL

Materials. Reagent grade cyclohexanone (MCB) and ethyl acetoacetate (MCB) were distilled at atmospheric pressure prior to use. Reagent grade Me_2S_2 (Sigma) and n-BuOH (Eastman) were used without further purification. Iodoacetamide was purified by successive recrystallizations from hexane until colorless. Phosphate buffers were prepared using deionized water: pH 6 potassium phosphate buffer was 0.05 M phosphate adjusted to pH 5.95; the pH 9.4 buffer was 0.1 M disodium hydrogen phosphate. Lavage solns were made up in sterile irrigation saline (McGaw Laboratories). Liquid scintillation counting was run using Aquasol II cocktail (New England Nuclear), with addition of known volumes of standard 1-^{14}C-toluene for calibration of counting efficiency. Animals were anesthetized by immersion in 0.5% aqueous ethyl m-aminobenzoate methanesulfonate (Wyeth).

Studies of acetoacetate decarboxylase. AAD was isolated and purified by standard methods, and enzyme assays were performed as described by Westheimer.[17] Crystalline protein with a specific activity of 40 units/mg was observed to be essentially homogeneous by SDS–polyacrylamide gel electrophoresis ($R_f = 0.72$, relative to a BSA standard with $R_f = 0.32$). Inhibition studies on cyclohexanone were run using six substrate concentrations from 1.9 to 144 mM and five inhibitor concentrations from 36 to 360 mM in pH 6 buffer. Inhibition by ethyl acetoacetate was measured at a single substrate concentration (30 mM) and three inhibitor concentrations (3.3, 15 and 33 mM). Inhibition constants (K_d's) were obtained using double reciprocal plots and have experimental errors of approximately a factor of two.

Borohydride reduction of enzyme-substrate complexes was studied by two methods. In one study, a solution of 75 units of AAD in 1 mL of pH 6 buffer was mixed with 0.1 mL of 41 mM ethyl acetoacetate, followed by two 0.3 mL portions of 0.2 M $NaBH_4$ in pH 9.4 buffer. The sample was then dialyzed against 1.8 L of pH 6 buffer for 4 hr and assayed for catalytic activity, which, when corrected to the initial enzyme concentration, corresponded to 57 units/mL. Incubating the enzyme with $NaBH_4$ alone, followed by dialysis, did not affect its catalytic activity. Other studies were performed using incorporation of ^{14}C-label with ethyl 3-^{14}C-acetoacetate (New England Nuclear, 48.6 m Ci/mmol) or 1-^{14}C-cyclohexanone (New England Nuclear, 8.4 m Ci/mmol). For radiolabelling with ethyl acetoacetate, 0.1 mL of pH 6 buffer containing 8×10^6 dpm was mixed with a soln of 36 units of AAD in 0.1 mL of pH 6 buffer and diluted to 0.25 mL. After standing 2 hr, the solution was mixed with 0.2 mL of 0.2 M $NaBH_4$ in pH 9.4 buffer, after which sample was dialyzed for 5 days against two successive 2 L volumes of pH 6 buffer. The recovered protein contained 2.2×10^5 dpm of non dialyzable radioactivity. In a control experiment, the order of mixing was permuted: 0.1 mL $NaBH_4$ was added to 0.1 mL of ethyl 3-^{14}C-acetoacetate solution, then immediately diluted to 0.25 mL followed by addition of 0.1 mL of AAD solution (same concentrations as before). After dialysis, 3.4×10^2 dpm of nondialyzable radioactivity remained. The radiolabelled enzyme exhibited the same catalytic activity (within experimental uncertainty) as the original AAD. In a separate experiment, the effect of omitting borohydride altogether was examined. A solution of AAD with 1 mM ethyl 3-^{14}C-acetoacetate was divided into equal portions, one of which was set to dialyze immediately and the other of which treated with $NaBH_4$ prior to dialysis. The latter sample showed incorporation of 200 times more radioactivity than the former.

For radiolabelling with 1-^{14}C-cyclohexanone, two separate solutions, each containing 1.5×10^6 dpm and 12 units of AAD in 0.085 mL pH 6 phosphate buffer, were allowed to stand for 15–30 min. A 0.01 mL portion of 0.5 M $NaBH_4$ in pH 9.4 phosphate buffer was added to one of the solutions, and both solutions were separately dialyzed against three successive 200 mL portions of pH 6 buffer. Liquid scintillation counting showed incorporation of 8×10^4 dpm into the borohydride-treated sample and 450 dpm into the control. Repetition of this procedure with BSA (0.34 mg) in place of AAD showed 5×10^3 dpm incorporated into the borohydride-treated sample and 1300 dpm when borohydride was omitted. An effort to use tritiated borohydride to achieve double labelling was not successful, as we found that treatment of AAD alone with $NaBH_3T$ (8.9 Ci/mmol) resulted in incorporation of large amounts of radioactivity.[28]

Behavioral studies. The experimental subjects were 27 adult, land phase tiger salamanders purchased from Amphibians of North America, Nashville, TN. The animals were kept at 5–8° in a refrigerator between experimental sessions. The protocol for training, lavage, and testing has been described elsewhere.[24] During acquisition, one session of 24 trials was conducted per day for each animal. After lavage, subjects were tested in two 24-trial sessions each day. Lavage was administered by irrigating both olfactory sacs with 100 μL of solution. Immediately after lavage, both sacs were rinsed with 100 μL of saline, and, within 2 min, each

animal was rinsed in dechlorinated water and placed in a tub containing 100 mL of dechlorinated water to recover from anesthesia. In a double blind study, some of the treated animals' avoidances were scored by a naive observer. No effects attributable to experimenter's bias were seen. For treatments with iodoacetamide, the lavage procedure was modified by administration of 100 μL of 0.05 M iodoacetamide per sac between the odorant and saline rinse. Microscopic examination of whole head sections of treated and untreated animals did not show evidence of gross physical damage to the olfactory epithelium from the lavage procedure.

Note added in proof. Preliminary data show that, as eqn (2) predicts, selective anosmia can be induced by using a much lower concentration of ethyl acetoacetate when it is followed by lavage with a solution containing a nucleophile. For example, lavage with 5×10^{-4} M ethyl acetoacetate has no effect (*cf* Fig. 3b); neither does lavage with 5×10^{-2} M $NaBH_3CN$. But when lavage with 5×10^{-4} M ethyl acetoacetate is immediately followed by 5×10^{-2} M $NaBH_3CN$, there is a significant decrement in responding to cyclohexanone, at the same time as there is no effect on responding to Me_2S_2. [T. H. Morton and J. R. Mason, Paper presented at the 13*th Annual Meeting of the Society for Neuroscience*, Boston, 8 Nov. 1983].

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