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15. See reviews in R. Dingledine, Ed., *Brain Slices* (Plenum, New York, 1984); G. A. Kerkut and H. V. Wheal, Eds., *Electrophysiology of Isolated Mammalian CNS Preparations* (Academic Press, New York, 1981). CA1 cells were included for analysis if the membrane potential was stable for at least 30 minutes and if they exhibited input resistance above 20 megohm, resting potentials above 55 mV, action potentials above 65 mV, normal excitatory postsynaptic potentials and spikes during synaptic stimulation of the Schaffer-commissural fibers, and multiple spiking during depolarizing intracellular currents.

16. Since it was readily apparent that cells from the same animal often exhibited similar values of resting potential and AHP length (that is, they were not independent), values for all cells from the same animal were averaged to provide a single value per condition. Animals were then used as the statistical population; two way analyses of variance with split-plot designs were used to assess the effect of age (between groups variable) and postactivation duration (within subjects variable) on AHP amplitudes.
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Selective Deficits in the Sense of Smell Caused by Chemical Modification of the Olfactory Epithelium

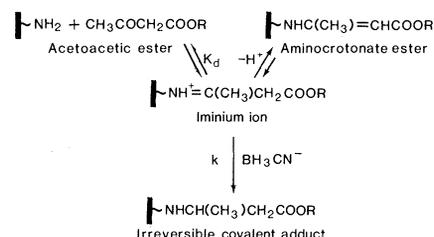
Abstract. A chemically selective procedure for covalent modification of Schiff base-forming binding sites in proteins is demonstrated *in vitro*. *In vivo* studies show that the same procedure produces a selective anosmia ("odor blindness") when applied to the olfactory epithelia of experimental animals. Surgical experiments confirm that the sense of smell is specifically affected.

The hypothesis that olfactory receptors are protein molecules is widely embraced (1). Here we report experimental findings to support that hypothesis. Our working model (2) is based upon the supposition that Schiff base-forming proteins in the olfactory epithelium bind carbonyl-containing odorant molecules. To assess this notion, we have developed a procedure to specifically and irreversibly modify proteins that bind simple ketones as iminium ions. We have probed its chemical selectivity and have explored the effects on the sense of smell of tiger salamanders (*Ambystoma tigrinum*). We have previously shown how to evaluate anosmias in this species with a behavioral assay (3).

The modification procedure is derived from well-known techniques for affinity labeling of Schiff base-forming enzymes (4). We have tested our sequence of reagents, acetoacetic ester followed by sodium cyanoborohydride (NaBH_3CN), by examining pure polypeptide proteins. As an example of a Schiff base-forming protein, the bacterial enzyme acetoacetate decarboxylase (AAD) (5) was chosen for model studies. AAD binds simple, uncharged ketones (5-7) at an active site lysine residue. Borohydride reducing agents convert reversible covalent complexes to irreversible covalent adducts (2).

Quantitative studies with pure AAD demonstrate the selectivity of irreversible modification. Nondialyzable radioactivity is incorporated into AAD after treatment with ^{14}C -labeled ethyl acetoacetate (EAA) (8) followed by

NaBH_3CN . This treatment does not radioactively label lysine-rich proteins that lack specialized Schiff base-forming



binding sites (such as bovine serum albumin or ribonuclease A), nor does it label Schiff base-forming proteins that require electrically charged substrates (such as rabbit muscle aldolase or glucosephos-

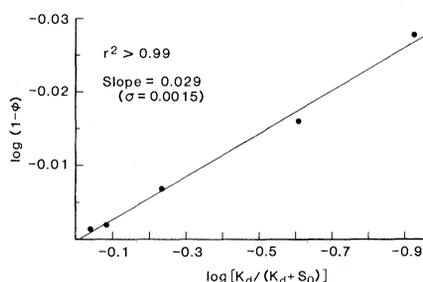


Fig. 1. Extent of modification, ϕ , of AAD as a function of initial concentration S_0 (from 0.010 mM to 0.74 mM) of ethyl [3- ^{14}C]acetoacetate. Enzyme (2.4 activity units per nanomole) and radiolabel were treated with 30 mM NaBH_3CN followed by dialysis. The slope represents the pseudo-first-order rate constant, k , for production of irreversible adduct times the dialysis residence time τ (Eq. 1). Each point represents a single determination, and the standard deviation (σ) represents the error of the slope from a least-squares fit.

phate isomerase) (9). Reaction 1 depicts a reaction mechanism for irreversible covalent modification. Acetoacetic ester binds reversibly to ϵ -amino groups of lysine residues (10) with dissociation constant K_d . An iminium ion is formed, which can be reduced by NaBH_3CN , but, unless it is in a specialized environment, the iminium ion rapidly loses a proton to form an aminocrotonate ester, which is not attacked by NaBH_3CN at $\text{pH} \geq 6$. The active site of AAD represents a specialized environment (11), where the presence of a nearby positive charge facilitates attack by NaBH_3CN (with pseudo-first-order rate constant k). In our *in vitro* studies, EAA was removed by dialysis in competition with reaction 1. Under these conditions, the extent of modification, ϕ , ought theoretically to obey Eq. 1, where S_0 represents the initial concentration of EAA and τ its residence time under dialysis conditions (2). Experimentally, Eq. 1 is obeyed, as the linear log-log plot in Fig. 1 demonstrates. The value of K_d was taken to equal the inhibition constant K_i determined from inhibition studies, 0.1 mM (2), and the graph becomes significantly curved if other values of K_d are used.

$$\phi = 1 - \left(\frac{K_d}{K_d + S_0} \right)^{k\tau}$$

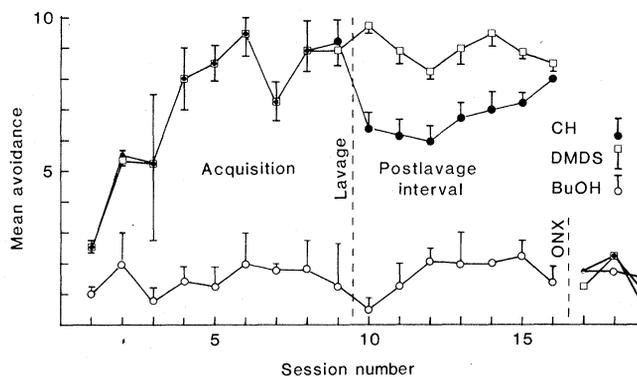
We infer, therefore, that irreversible covalent modification occurs at the same site where EAA binds as a reversible inhibitor.

When the same sequence of reagents (0.5 mM aqueous acetoacetic ester followed by 50 mM aqueous NaBH_3CN) is applied to the olfactory epithelia of tiger salamanders, the animals develop selective anosmia that lasts for approximately 1 week. Application of either reagent alone has no behavioral effect.

A group of 12 salamanders was conditioned to avoid negatively reinforced presentations of cyclohexanone (CH) or dimethyl disulfide (DMDS) (3). Responses to presentations of a third odorant, *n*-butanol (BuOH), were not reinforced, but were monitored concurrently. Training and testing were performed with odorant concentrations at 2.0 percent of vapor saturation, with one session per day comprising ten presentations of each odorant in a randomized order. Criterion learning performance was defined as ≥ 80 percent avoidance of CH and of DMDS and ≤ 20 percent avoidance of BuOH. The score for each session is expressed as the frequency of avoidance for each odorant, always given in order, CH, DMDS, and BuOH.

All animals surpassed criterion by day 9 (mean scores were 9.0, 8.8, and 1.4)

Fig. 2. Mean performance (with standard errors of the mean) of four tiger salamanders during 19 consecutive days of avoidance testing with CH and DMDS at 2.0 percent of vapor saturation, along with unreinforced trials with BuOH. Lavage with 0.5 mM EAA followed by 50 mM NaBH₃CN after testing on day 9 had no statistically significant effect on responding to DMDS, but did decrease responding to CH on days 10 to 15. Bilateral olfactory nerve sections (ONX) on day 16 caused complete loss of discrimination among odor cues. Results for two ONX animals from this treatment condition and for two ONX animals from a control group are shown for days 17 to 19 (error bars omitted for clarity).



and were assigned to three treatment conditions. One condition ($n = 4$) consisted of lavage of each olfactory sac with 100 μ l of 0.5 mM EAA; the second condition, ($n = 4$), lavage with 50 mM EAA, a treatment known to produce partial anosmia (3); and the remaining animals ($n = 4$) received lavage with 0.5 mM EAA followed by 50 mM NaBH₃CN.

Treatment with 0.5 mM EAA alone did not significantly affect performance on any of six subsequent test days (mean scores were 9.5, 9.2, and 1.5). Nasal irrigation with 50 mM EAA or with 0.5 mM EAA followed by NaBH₃CN produced response decrements for presentations of CH but not for DMDS. Effects were maximal for days 2 to 5 after lavage (mean scores were 6.4, 8.7, and 2.3; and 6.4, 8.9, and 2.0, respectively). All subjects returned to prelavage levels of responding within 8 days after chemical treatments. Results for animals given 0.5 mM EAA followed by NaBH₃CN are shown in Fig. 2.

To assess the olfactory contribution to behavior, eight animals were randomly

selected for surgical experiments. Bilateral olfactory nerve cuts (ONX) were performed on half ($n = 4$) and sham surgeries (SS) were performed on the other half ($n = 4$) (12). Although avoidance responding of SS animals was not significantly affected (mean scores over three postoperative test days were 8.6, 8.7, and 2.0), ONX abolished discriminative responding (mean scores over three postoperative test days were 1.7, 1.3, and 1.7). Thus olfaction was critical for distinguishing among the chemical stimuli at 2.0 percent of vapor saturation.

Repetition of these experiments with odorant concentrations at 2.5 percent of vapor saturation gave virtually identical results, as summarized by Table 1, except that ONX did not completely eradicate discrimination (13). Moreover, other acetoacetate esters could be used in place of EAA without altering the results. When (2-methylthio)ethyl acetoacetate, CH₃SCH₂CH₂OCOCH₂COCH₃ (MTEAA) (14), was used in place of EAA, the effects of lavage were the same (Table 1). Nasal irrigation with 50 mM NaBH₃CN alone revealed no detectable

effect (Table 1). In other experiments, 0.5 mM EAA followed by 50 mM NaBH₃CN impaired olfactory detection of cyclopentanone (at 2.0 percent of vapor saturation), while 0.5 mM EAA or 50 mM NaBH₃CN by themselves had no effect.

All of the results are consistent with the supposition that odorant molecules bind reversibly to olfactory receptors and that irreversible covalent modification can block binding at specific sites. For low acetoacetic ester concentrations (0.5 mM), production of response decrements required two steps, acetoacetic ester followed by NaBH₃CN. Neither step was effective by itself, and decrements were specific for responding to CH or cyclopentanone. We rule out damage to a spatially localized patch of receptor cells as an explanation because electrophysiological studies have shown that response to CH is uniform over the entire surface of the olfactory epithelium in this species (15). Neither do we believe that the behavioral data can be explained in terms of an overall (nonspecific) reduction of odorant molecules

Table 1. Summary of effects of lavage on avoidance responding to CH and DMDS. Each entry shows the mean number of avoidances out of ten trials (\pm standard error of the mean) for the last prelavage sessions and the first three postlavage sessions. Statistical tests for significance were performed with three-way analysis of variance with repeated measures (3). Where $P < 0.05$, Tukey b post hoc tests were used to identify significant differences among means. Table entries in italics indicate significant effects.

Stimulus	Lavage							
	50 mM NaBH ₃ CN	0.5 mM EAA	0.5 mM MTEAA	50 mM EAA	50 mM MTEAA	0.5 mM EAA + 50 mM NaBH ₃ CN	0.5 mM MTEAA + 50 mM NaBH ₃ CN	
2 percent CH								
Before	9.6 \pm 0.3*	9.0 \pm 0.5*		8.5 \pm 0.7*		9.0 \pm 0.3*		
After	9.5 \pm 0.3*	9.3 \pm 0.5*		7.2 \pm 0.2*		7.0 \pm 0.5*		
2.5 percent CH								
Before	9.2 \pm 0.4†	9.0 \pm 0.8†	9.0 \pm 0.4†	9.5 \pm 0.3†	9.3 \pm 0.3†	9.3 \pm 0.3*	9.8 \pm 0.3*	
After	9.3 \pm 0.3†	9.3 \pm 0.4†	9.3 \pm 0.5†	8.0 \pm 0.3†	6.3 \pm 0.8†	7.3 \pm 0.4*	7.0 \pm 0.5*	
2 percent DMDS								
Before	9.5 \pm 0.3*	9.5 \pm 0.3*		9.0 \pm 0.5*		9.5 \pm 0.5*		
After	9.3 \pm 0.5*	9.5 \pm 0.5*		9.3 \pm 0.4*		8.5 \pm 0.5*		
2.5 percent DMDS								
Before	9.5 \pm 0.5†	9.8 \pm 0.3†	9.0 \pm 0.3†	9.5 \pm 0.4†	9.5 \pm 0.3†	9.3 \pm 0.3*	9.5 \pm 0.5*	
After	9.5 \pm 0.5†	9.8 \pm 0.3†	9.5 \pm 0.3†	9.0 \pm 0.3†	9.0 \pm 0.5†	9.0 \pm 0.3*	9.3 \pm 0.3*	

* $n = 4$. † $n = 3$.

reaching receptors, since acquisition and lavage results were the same for two different odorant concentrations (2.0 and 2.5 percent). Finally, since response decrements were of relatively short duration (1 week), it seems unlikely that widespread physical damage is the cause of our experimental observations.

The inference that some carbonyl compounds form Schiff-base linkages with proteins in the course of olfactory detection offers the simplest explanation of our findings. *In vitro* investigations show that acetoacetic ester plus NaBH₃CN covalently modifies a model protein, AAD, which binds simple ketones reversibly. Quantitative measurements obey Eq. 1 and confirm the Schiff base-forming active site as the target of chemical blockade. Other proteins that do not bind simple ketones are not thus labeled. *In vivo* studies demonstrate analogous effects from the same chemical blockade in the noses of tiger salamanders. Ability to detect at least two ketones is selectively impaired. Surgical experiments confirm that the sense of smell is specifically affected. In sum, these results point toward irreversible covalent modification as a technique for identifying receptor sites in the olfactory epithelium.

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8. Ethyl [3-¹⁴C]acetoacetate (48.6 mCi/mmol) was purchased from New England Nuclear. Radiochemical purity ≥ 95 percent was assayed by adding a sample to unlabeled EAA, preparing the semicarbazone, and recrystallizing to constant specific activity.
9. In a typical experiment, duplicate samples were prepared by adding 0.06 to 5 μ Ci of ¹⁴C-labeled EAA to solutions of 0.05 to 0.06 mg of protein in a buffer of pH 6 to give total volumes of 0.12 to 0.13 ml. One sample was transferred to a dialysis bag, while 0.05 ml of 0.1M NaBH₃CN was added to the other, which was then transferred

- to another dialysis bag. Samples were dialyzed against buffer at 4°C. For AAD, nondialyzable radioactivity in the NaBH₃CN-treated samples was an order of magnitude greater than in the untreated samples. For other proteins, NaBH₃CN-treated and -untreated samples had the same levels of nondialyzable radioactivity.
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 12. Olfactory nerve sections (ONX) and sham surgeries (SS) were performed as double-blind studies. ONX significantly diminished responding to CH and DMDS [$F(1, 44) = 126.34$, $P < 0.001$] relative to SS. Discrimination among CH, DMDS, and BuOH was at chance level ($P > 0.25$) for ONX subjects ($n = 4$).
 13. At 2.5 percent vapor saturation, ONX showed a significant decrease relative to SS in responding to CH and DMDS [$F(1, 44) = 12.5$, $P < 0.001$], but the ONX animals still showed some ability to distinguish among odorants, with mean test scores over three postoperative days of 5.3, 5.4, and 1.3. Lavage with 0.5 mM EAA followed by 50 mM NaBH₃CN significantly decreased SS

responding to CH when compared with pre-lavage, postoperative performances [$F(1, 28) = 12.34$, $P < 0.001$], but had no statistically significant effect on ONX performances ($P > 0.25$).

14. MTEAA was prepared from commercial 2,2,8-trimethyl-1,3-dioxen-4-one and 2-methylthioethanol (Aldrich) plus trace acid with distillative removal of acetone. Product (boiling point, 88° to 89°C at 0.3 torr) was twice distilled before use as a lavage agent.
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16. Portions of this work were presented at the 13th annual meeting of the Society for Neuroscience, Boston, November 1983. We thank J. V. Connors and F. H. Westheimer for the gift of a sample of AAD, W. Silver and B. S. Gelhard for assistance in performing double-blind surgeries, and C. E. Boehm for preparing figures. This work was supported by grant NS-19424 from the National Institutes of Health.

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Diagnostic Potential for Human Malignancies of Bacterially Produced HTLV-I Envelope Protein

Abstract. *Two regions of the gene for the human T-cell leukemia virus subgroup I (HTLV-I) envelope were expressed in Escherichia coli by use of the vector pJLA16. One corresponds to the carboxyl terminal region of the major envelope protein p46, and the other corresponds to the transmembrane protein p21E. Reactivity of the expressed protein with human serum was tested by the Western blot procedure. Each of 11 sera tested that had been shown to contain antibodies to HTLV-I or HTLV-II by an enzyme-linked immunosorbent assay recognized the bacterially synthesized envelope proteins. There was no reaction detected when 17 control sera were tested. This system will be useful for large-scale seroepidemiological surveys for HTLV-I and related human retroviruses.*

Human T-cell leukemia virus subgroup I (HTLV-I) is a retrovirus causatively linked to certain adult lymphoid malignancies, notably adult T-cell leukemia-lymphoma (ATL) (1). Many isolates of this virus, identified in the United States, the Caribbean basin (2), southwestern Japan (3), Israel (2), Europe (4), and Africa (5), have been shown to be nearly identical (6). Two other isolates (HTLV-II), including one from a patient with T-cell hairy cell leukemia (7), are related to HTLV-I but differ significantly in antigen assays and in their genomes (8). A third subgroup of HTLV (HTLV-III) that is associated with the acquired immune deficiency syndrome (AIDS) has been described (9).

Antibodies that react with HTLV-I proteins have been found in the sera of ATL patients. These antibodies recognize both the *gag* core antigens and the envelope proteins of the virus (10). Viral core proteins were purified (11), sequenced (12), and used extensively in immunoassays (13); however, progress with the more important viral envelope proteins was slow. A limiting factor, therefore, in studies of the immune response to these viruses has been the difficulty in isolating the viral envelope

proteins in pure form and in quantity.

As an alternative approach, we expressed the virus envelope protein in a bacterial vector. This procedure has the advantage that only a single viral product as defined by the structure of the input DNA is made by the bacteria. HTLV-I was suitable for such an approach because the integrated proviral DNA has been cloned (14, 15) and sequenced (16). We chose to express the HTLV-I envelope by placing it into the pJLA16 derivative (17) of plasmid pJL6 (18). This plasmid contains the 13 amino terminal codons of the bacteriophage λ *cII* gene placed under the transcriptional control of the well-regulated phage λ *p_L* promoter. This plasmid has been used to express sequences from *myc*, *myb*, and *ras* oncogenes (18, 19).

Initial attempts to express the entire HTLV-I envelope were unsuccessful, possibly because this protein can interact with the bacterial cell membrane in such a way as to be toxic to the cell. Therefore, individual fragments coding for specific regions of the envelope were inserted into pJLA6 by use of polynucleotide linkers (Fig. 1). Such plasmids were introduced into *Escherichia coli* MZ1, a strain that contains a partial λ