batches were pooled, dried in vacuo, and stored in the dark at 4 °C. Optical and NMR spectra and rechromatography by HPLC indicated that the final product was stable when so stored. At the present time, accurate extinction coefficients for the metal-free methyl ester have not been obtained and the quantity of the ester can be estimated only from the intensity of NMR spectra. On the basis of this, the overall yield from the heme content of the terminal oxidase was estimated to be on the order of 10–20%. Major losses occurred in the initial acid–acetone extract and during the demetallization procedure.

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Two-Step Covalent Modification of Proteins. Selective Labeling of Schiff Base-Forming Sites and Selective Blockade of the Sense of Smell in Vivo[1]

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Abstract: Covalent modification by two-step labeling is illustrated by a blocker–fixer sequence, in which a protein forms a reversible complex with a blocker, which is then converted to an irreversible adduct by the fixer. When the Schiff base-forming enzyme, acetoacetate, decarboxylase (AAD), is used as an in vitro model, the blocker–fixer sequence is exemplified by ethyl acetoacetate (EAA) as the blocker and borohydride as the fixer. When reaction or removal of unbound blocker competes with protein labeling (e.g., with $NaBH_4$ as fixer), the quantitative expression for the extent of labeling is $\phi = 1 - (1 + \beta)^m$, where β is the ratio of the initial blocker concentration, S_0, to the dissociation constant for the reversible protein–blocker complex. This relationship is tested and demonstrated both for removal of the blocker by chemical reaction and for removal by dialysis. The value of the exponent m is a function of the reaction rate constants. The blocker–fixer sequence EAA–$NaBH_3CN$ is shown to be specific for Schiff base-forming sites. Application of this blocker–fixer sequence to the olfactory epithelia of tiger salamanders selectively impairs detection of ketone-containing odorants. When a behavioral assay is used, decrements in responding to cyclopentanone or cyclohexanone are observed at the same time as responding to cyclopentanol, ethyl butyrate, or dimethyl disulfide is unaffected. Dose-response studies show an increase in duration (but not profundity) of selective hyposmia with increasing fixer concentration. On the other hand, the data show a much smaller increase in duration (and no increase in profundity) when the blocker concentration is increased. These results conform to expectations based on the expression for ϕ above. They are consistent with the supposition that olfactory ketone receptors with bound ligand behave as do rod cells in the retina in the dark: with a small molecule covalently attached by a Schiff base linkage and with continual secretion of neurotransmitter.

Covalent modification of proteins is a widely used tool for examining the action of enzymes.[2] Recently, this technique has been extended to the study of receptors in tissue samples. In vivo applications have been less widely probed, for most investigations have not required that the subject survive the chemical treatment. This paper describes the development of a two-step affinity labeling procedure suitable for treating olfactory epithelia of living animals. The two reagents used are a blocker, which binds reversibly to proteins, and a fixer, which converts the reversible complex to an irreversible adduct. Because the experimental subjects remain alive, their sense of smell can be assayed following treatment in order to assess whether the ability to detect specific classes of compounds has been impaired. This paper presents kinetic analyses for two-step labeling schemes, experimental tests using model proteins in solution, and results of application of a two-step labeling procedure to the noses of live tiger salamanders (*Ambystoma tigrinum*).

The perception of odors is an opportune field for chemical investigation.[3] There is no complete catalogue of olfactory sensations. Electrophysiological data (much of which has been recorded from salamanders)[4-6] do not reveal obvious patterns of coding nor is it known how many varieties of receptor sites exist. Snyder and co-workers have recently isolated an odorant-binding

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protein from bovine olfactory epithelia.[7] Identification of this protein as a neuronal receptor is by no means certain, as it also occurs in respiratory epithelia and appears to bind several unrelated odorants.

Olfaction in vertebrate species warrants special scrutiny, for nearly all volatile compounds (with the exceptions of very hydrophilic molecules, such as ethylene glycol, or the lower alkanes) appear to elicit responses. This stands in contrast to pheromone receptors, which respond only to specific compounds within a narrow range. Despite many attempts to elucidate them, the molecular bases of binding and transduction in the vertebrate olfactory system are poorly understood. Human beings can distinguish some pairs of enantiomers, in particular, (+)- and (−)-carvone.[8] This suggests that these ketones are bound at receptor sites, but the further implications are by no means obvious. But, if human experience is any guide, the sense of smell is quite general, for the ability of organic chemists to recognize carbonyl groups, sulfur-containing compounds, and amines by smell alone is documented.[9]

We wish to suggest an analogy between the sense of smell and visual receptors. The visual pigment, rhodopsin, is a covalent complex of a comparatively small molecule (11-*cis*-retinal) and a protein (opsin). Although rod cells in the retina are very different from olfactory neurons, it is worth noting that in the dark (with bound retinal), rod cells are depolarized. That is, their ion channels are open and they secrete neurotransmitter continually. Exposure to light isomerizes the retinal moiety; ion channels then close and secretion of neurotransmitter abates.[10]

Two aspects of vertebrate visual transduction are perhaps germane to the sense of smell: (1) visual receptors are actually chemoreceptors that respond to one isomer of a *covalently* bound ligand; (2) neurotransmitter is secreted in the dark, when 11-*cis*-retinal is bound, and curtailed once the ligand is photoisomerized (and after it is subsequently hydrolyzed off, to be replaced by a new ligand during regeneration of the receptor). We speculate that there may exist olfactory receptors whose stimulated condition resembles the condition of rod cells in the dark.

It is reasonable to suppose that olfaction employs a variety of receptor sites. Electrophysiological studies of single receptor neurons give a result that "strongly suggests that different types of receptive site types responsive to a given odorant can coexist on a given receptor cell".[6b] We theorize that some of these may bind odorant molecules covalently. Likely candidates for covalent binding would include receptors for ketones and aldehydes. In the visual system, retinal is bound as a Schiff base to the ϵ-amino group of a lysine residue. This paper explores the possibility of an analogous mechanism in the olfactory epithelium.

We have tested this hypothesis. As described in a previous communication, the sequence of reactions I–III (shown in the Discussion Section below) blocks Schiff base-forming binding sites selectively, and such blockade affects odorant detection in a selective fashion.[11] If rod cells are taken as a model, irreversible attachment of a ligand to a receptor should cause opening of ion channels and continual secretion of neurotransmitter. In other words, ketone receptors (if they exist and if they behave like rod cells) ought to be continuously stimulated by an irreversible, covalently bound affinity label, and a high background signal should result. Not only will other ketone-containing odorants be prevented from binding at modified sites, but the high background signal might also interfere with detection of ketone molecules that bind at unmodified ketone receptors.

Let us contrast two possible models for the effects of covalent blockade of olfactory binding sites. If, on the one hand, the principal effect of covalent modification is to prevent other molecules from binding to a receptor, then the extent of impairment will depend on the fraction of sites that are blocked. If, on the other hand, the principal effect of covalent modification is to create a high level of neurotransmitter, detection of the ketone functional group will be swamped by high background signal. In this latter case, only a small fraction of ketone receptors need to be covalently modified in order for selective impairment to be observed. When this fraction is sufficiently large, further increases will not increase the extent of impairment. In either case, if there is a high degree of redundancy in olfactory detection, other classes of receptors will have to bear the burden of detecting ketone-containing odorants. Hence, detection of a ketone should not be obliterated but will simply be impaired due to loss of one of several pieces of information about that odorant. At the same time, detection of odorants that do not contain a carbonyl group ought not to be affected.

This paper will discuss the following experiments: (a) model studies of proteins in vitro to demonstrate the specificity of a two-step covalent modification procedure, (b) selective deficits in the sense of smell resulting from application of this two-step procedure to the olfactory epithelium of tiger salamanders, and (c) dose-response relationships in vivo compared with experimental results in vitro.

Experimental Section

Except where otherwise specified, reagents were purchased from Aldrich or Sigma Chemical Co. and used without further purification. Rabbit muscle aldolase (E.C. 4.1.2.13) and yeast phosphoglucose isomerase (E.C. 5.3.1.9) from Boehringer Mannheim Biochemicals, BSA from Pentex Corp., and RNAse A (E.C. 3.1.27.5) and polylysine hydrobromide from Sigma were used as received. Crystalline acetoacetate decarboxylase (AAD) was prepared by J. V. Connors in the laboratory of F. H. Westheimer at Harvard University and was assayed to have a specific activity of 40 units/mg.[14] Ethyl [3-^{14}C]acetoacetate (48.6 mCi/mmol) was purchased from New England Nuclear. Radioisotopic purity ≥95% was assayed by adding a known quantity to a preweighed portion of freshly distilled, unlabeled ethyl acetoacetate, followed by conversion to the semicarbazone,[28] whose specific radioactivity after four

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Table I. Radiolabeling Data for AAD plus Ethyl [3-^{14}C]Acetoacetate with 30 mM Borohydride and 30 mM Cyanoborohydride as Fixers ($\beta = S_o/K_I$). Fixer Omitted in Controls. Values of ϕ based on 1.0 nmol of AAD per Sample

S_o, mM	dpm in control	30 mM $NaBH_4$			30 mM $NaBH_3CN$		
		$1/(1+\beta)$	dpm	ϕ	$1/(1+\beta)$	dpm	ϕ
0.01	20	0.909	569	0.0049	0.909	372	0.0033
0.02	49	0.826	928	0.0077	0.826	533	0.0045
0.07	39	0.610	2970	0.0267	0.588	1710	0.0156
0.3	98	0.246	8500	0.0781	0.246	4000	0.0362
0.7	594	0.119	11930	0.1024	0.119	7480	0.0622

Table II. Radiolabeling Data for AAD plus Ethyl [3-^{14}C]Acetoacetate with 15 and 60 mM Borohydride as Fixers ($\beta = S_o/K_I$). Fixer Omitted in Controls. Values of ϕ based on 1.0 nmol of AAD per Sample

S_o, mM	dpm in control	15 mM $NaBH_4$			60 mM $NaBH_4$		
		$1/(1+\beta)$	dpm	ϕ	$1/(1+\beta)$	dpm	ϕ
0.02	32	0.775	741	0.0074	0.806	918	0.0092
0.04	72	0.685	1051	0.0102	0.725	1350	0.0133
0.1	96	0.433	2188	0.0217	0.488	3515	0.0355
0.3	330	0.221	4395	0.0422	0.234	6490	0.0640
1	710	0.079	6510	0.0603	0.095	9935	0.0959

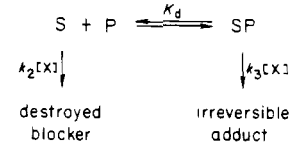
successive recrystallizations from ethanol–water was measured by liquid scintillation counting (LSC). LSC was performed on a Beckman Model 1800 using Aquasol II (New England Nuclear) as a cocktail. Reported radioactivity levels were internally calibrated by addition of known quantities of radiolabeled toluene (New England Nuclear). Experiments using sodium [^{3}H]cyanoborohydride were performed with a preproduction sample (specific activity stated to be 5.5. mCi/mg) provided by New England Nuclear. 2-(Methylthio)ethyl acetoacetate was prepared by reacting 2,2,8-trimethyl-1,3-dioxen-4-one with 2-(methylthio)ethanol in the presence of trace *p*-toluenesulfonic acid with continuous removal of acetone by distillation. After washing the reaction mixture with sodium bicarbonate, product was purified by two successive fractional distillations under reduced pressure (bp 88–89 °C/0.3 torr): NMR (90 MHz, CCl_4) δ 1.87 (s, $CH_3C{=}C$, 11% enol), 2.06 (s, SCH_3), 2.15 (s, CH_3CO, 89% keto), 2.58 (t, J = 7 Hz, CH_2S), 3.30 (s, keto CH_2CO), 4.18 (t, J = 7 Hz, CH_2O), 4.86 (s, enol C=CH), 11.91 (s, enol OH).

The lack of reactivity of ethyl acetoacetate with cyanoborohydride at pH 6 was assessed by adding a solution of sodium cyanoborohydride to three 0.02 M solutions of ethyl [3-^{14}C]acetoacetate to make them 0.03 M in cyanoborohydride, followed by addition to unlabeled ethyl acetoacetate and preparation of the semicarbazone. The cyanoborohydride–acetoacetic ester reaction mixtures were allowed to stand for 5, 15, and 30 min before workup, and the radioactivity remaining in the semicarbazones after four recrystallizations corresponded to recovery of approximately half of the initial radioactivity, slightly more than was recovered when the procedure was repeated in the absence of cyanoborohydride. When sodium borohydride was added to a 0.02 M solution of ethyl [3-^{14}C]-acetoacetate to give an initial borohydride concentration $\approx$ 0.04 M, addition of the reaction mixture to unlabeled ethyl acetoacetate followed by preparation and recrystallization of the semicarbazone showed that <1% of the initial radioactivity remained after four recrystallizations.

Radiolabeling of proteins was performed by addition of 50 μL of ethyl [3-^{14}C]acetoacetate solution to a solution of protein in 200 μL of buffer (potassium phosphate used for BSA, AAD, RNAse, and polylysine; triethanolamine used for aldolase and phosphoglucose isomerase). For each sample, quantities of solutions were carefully weighed to measure the values of S_0 (initial concentration of blocker) and P_0 (total protein concentration). Three sets of samples were used, with fixer omitted for one set and variations of borohydride fixer in the other two sets. All samples were transferred to dialysis bags (MW 10–12 000 cutoff, except for RNAse A, for which MW 2000 cutoff dialysis tubing was used) and set to dialyze at 4 °C against buffer. At least three successive dialyses against buffer were performed before the contents of the dialysis bags were assayed for radioactivity. Results of two experiments with AAD are summarized in Tables I and II, where ϕ designates the extent of modification and K_I the inhibition constant for ethyl acetoacetate.[12]

For studies with polylysine, polymer corresponding to an average of 230 monomer units (MW 57 000) was used. Three solutions containing 0.22 mg were made up and ethyl [3-^{14}C]acetoacetate was added to each to make final concentrations of 0.6 mM. One solution was dialyzed with no further treatment; sodium borohydride was added to another and sodium cyanoborohydride added to the third to give a final concentration of 10 mM. All three solutions were dialyzed, after which LSC showed them to contain 12 500, 12 000, and 14 500 dpm of nondialyzable radioactivity, respectively.

Scheme I



For a buffered solution (pH 6) of 0.12 mg of RNAse A treated with 0.2 mM ethyl [3-^{14}C]acetoacetate plus 0.04 M sodium cyanoborohydride, nondialyzable radioactivity incorporated into the protein corresponded to 110 dpm, as compared to 105 dpm incorporated into an identical sample for which cyanoborohydride had been omitted. For comparable experiments performed with 0.05-mg samples of aldolase or phosphoglucose isomerase, levels of nondialyzable radioactivity incorporated into cyanoborohydride-treated samples were 190 and 90 dpm, respectively, as compared to 370 and 125 dpm incorporated into identical samples from which cyanoborohydride had been omitted. For studies with sodium [^{3}H]cyanoborohydride, samples of AAD and BSA were treated with cyanoborohydride alone and with 0.4 mM unlabeled ethyl acetoacetate plus tritiated cyanoborohydride. All samples were allowed to stand at room temperature for 14 h before dialysis. After dialysis, the BSA samples (0.2 mg apiece) were found to have incorporated 3.8 μCi ([^{3}H]cyanoborohydride alone) and 4.0 μCi ([^{3}H]cyanoborohydride plus ethyl acetoacetate). Corresponding samples of AAD were found to have incorporated 0.75 and 0.9 μCi of tritium, respectively, and an AAD sample incubated with ethyl acetoacetate plus tritiated cyanoborohydride for 24 h incorporated 1.0 μCi. Further dialyses of these protein samples under denaturing conditions (4 M guanidine HCl at pH 10) did not substantially reduce the quantity of incorporated radioactivity.

For behavioral assays at Monell, adult land-phase tiger salamanders (raised at UCR from larvae purchased from bait shops or purchased as adults from Amphibians of North America, Nashville, TN) were trained and tested by using an airflow dilution olfactometer, as previously described,[20] with odorant concentrations at 2% of vapor saturation. The experiments summarized in Figure 2 were performed by two persons using a double-blind methodology, with a technician training and testing animals' performances in 8 trials per session per animal for cyclopentanone, cyclopentanol, and ethyl butyrate (for a total of 24 trials per session, presented in a randomized order). Other experiments were performed by a single investigator using 10 trials per session with cyclohexanone, dimethyl disulfide, and *n*-butanol (for a total of 30 trials per session in a randomized order).

Results

Chemical Studies. In one approach to covalent modification of proteins, a reversibly formed complex, SP, is attacked by another agent, X, to form an irreversible adduct. Scheme I depicts a general case where protein P and blocker S form reversible complex SP, for which the dissociation constant is K_d. Agent X attacks SP with a second-order rate constant k_3. This reagent also attacks unbound S irreversibly to destroy it with second-order rate constant k_2. Once it has reacted with X, blocker does not interact at all with P or SP.

We refer to such a two-step labeling procedure as a blocker–fixer sequence. The blocker, S, binds reversibly. Once fixer, X, is added, irreversible covalent modification results. This technique has been used to affix reporter groups at enzyme active sites.[13] Photoaffinity labeling (where X = light) represents another set of examples.

Intuition suggests that the extent of modification (which we shall symbolize as ϕ) ought to vary in the following ways. The value of ϕ should increase if the initial concentration of blocker, S_0, is increased or if the dissociation constant K_d is decreased. The extent of modification should decrease if k_2 is magnified relative to k_3. We have established a quantitative relationship based on eq 1.[12] This expression represents the integrated rate equation

$$[\text{irreversible adduct}] = P_0 - P_0\left(\frac{K_d + S_0e^{-t/\tau}}{K_d + S_0}\right)^{k_3/k_2} \quad (1)$$

$$[\text{irreversible adduct}]_{t=\infty}/P_0 = 1 - \left(\frac{K_d}{K_d + S_0}\right)^{k_3/k_2} \quad (2)$$

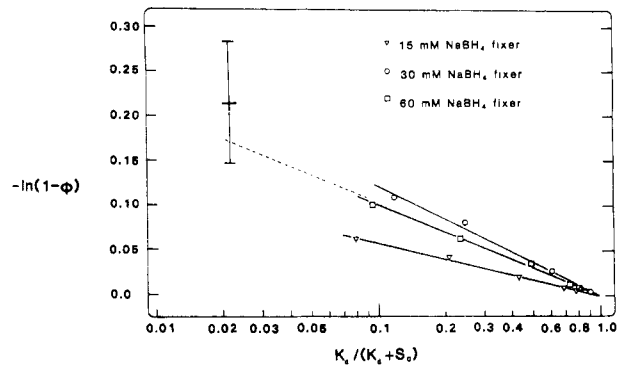


Figure 1. Extent of modification (ϕ) of acetoacetate decarboxylase (AAD) as a function of initial blocker concentration (S_0) at three different fixer ($NaBH_4$) concentrations. Solid lines correspond to least squares analyses of data based on incorporation of radioactivity from ethyl [3-^{14}C]acetoacetate as blocker (open symbols). The data point represented by + corresponds to a measurement based on enzymic catalytic activity using unlabeled ethyl acetoacetate as blocker and 50 mM $NaBH_4$ as fixer; error bars represent the experimental uncertainty of this assay, and the dashed line represents an extrapolation corresponding to the experimental conditions.

provided that three conditions are met: (1) the initial concentration of blocker, S_0, is much greater than the initial concentration of protein, P_0; (2) the concentration of fixer, X, is sufficiently high that the rates of attack are well approximated by using the pseudo-first-order rate constants $k_2[X]$ and $k_3[X]$; (3) the association and dissociation rates for S + P $\rightleftarrows$ SP are much faster than the rates of attack by X.

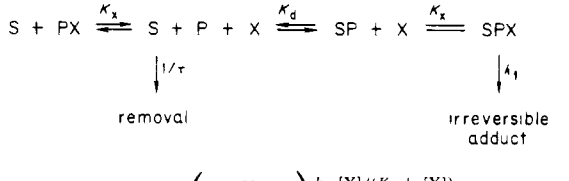
When the reaction has gone to completion, the extent of modification, ϕ, is given by eq 2. A plot of log $(1 - \phi)$ vs. log $[K_d/(K_d + S_0)]$ should therefore be linear, with slope equal to the ratio of rate constants, k_3/k_2. The linearity of such plots for the Schiff base-forming enzyme acetoacetate decarboxylase (AAD, E.C. 4.1.1.4, from *Clostridium acetobutylicum*[14]) demonstrates this relationship.

The activity site of AAD is believed to contain two amine functions in close proximity.[15-17] At pH 6, only one is protonated. The other, an ϵ-amino group of a lysine residue, forms Schiff bases reversibly with ketones and aldehydes, which act as competitive inhibitors. The enzyme also exhibits noncompetitive inhibition with a variety of anions.[18] The system where P is AAD and X is a borohydride reagent therefore provides a suitable set of experiments to test the validity of eq 2. We have previously shown that AAD is irreversibly labeled by treatment with ethyl acetoacetate as the blocker followed by $NaBH_4$ as the fixer.[12] Moreover, we have shown that permutation of the order of reagents—acetoacetic ester plus $NaBH_4$ followed by AAD—leads to negligible labeling of the enzyme.

Values of ϕ were measured for the reversible inhibitor S = ethyl acetoacetate (K_I = 0.1 mM).[12] The extent of modification was determined by using ^{14}C labeling. Selectivity of radiolabeling was assessed by examining polylysine and bovine serum albumin (BSA) in place of AAD. The first series of studies was performed by using X = BH_4^- as the fixer. For both BSA and polylysine, treatment with ^{14}C-substituted acetoacetic ester incorporated the same level of radioactivity regardless of whether borohydride was present or absent. For AAD, on the other hand, omission of the reducing agent diminished the level of incorporation by more than an order of magnitude. Figure 1 shows how ϕ varies with S_0 for X = sodium borohydride. For purposes of comparison, one value of ϕ determined by assay of catalytic activity is included. Experimental uncertainty in assaying enzymic catalysis was approximately 5%, resulting in the large error bars drawn in Figure 1 for that experimental point.

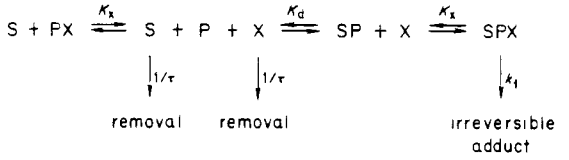
Each of the three plots in Figure 1 is linear ($r^2 \geq 0.99$) for $K_d = K_I$. Substituting different values for K_d introduces substantial curvature, and the data are poorly fit by other, naive relationships (e.g., log ϕ vs. log S_0). The observed fit of eq 2 for $K_d = K_I$ is taken to corroborate our inference that acetoacetic ester becomes

Scheme II



$$\phi = 1 - \left(\frac{K_d}{K_d + S_0}\right)^{k_1\tau[X]/(K_x + [X])} \quad (3)$$

Scheme III



irreversibly bound at the same site where it binds as a reversible inhibitor. The slopes in Figure 1 are considerably less than unity. According to eq 2, this means that BH_4^- reduces free blocker much faster than AAD-bound blocker.

Each line in Figure 1 corresponds to a different fixer concentration. Although the functional form of eq 1 is confirmed by the linearity of each plot, the slope of the three lines are different from one another. Equation 2 predicts that the slope ought to be equal to the exponent, k_2/k_1, independent of [X] when applied to Scheme I. An alternative mechanism, represented by Scheme II, also yields the functional form of eq 1 and 2 but with an exponent that can depend upon fixer concentration.

In Scheme II, X plays the role of a noncompetitive inhibitor (dissociation constant K_X) as well as of fixer. Formation of the irreversible adduct results from reaction of a ternary complex, SPX, with first-order rate constant k_1. For purposes of generality, the reaction that destroys unbound blocker has been simply represented as a "removal" step, whose rate constant $(1/\tau)$ is the reciprocal of the residence time (τ) of the blocker. The pertinent exponent in the expression for ϕ becomes $k_1\tau[X]/(K_X + [X])$ for Scheme II, as shown in eq 3. (Equation 2 corresponds to eq 3 in the limit where $K_x \gg [X]$, $k_3 = k_1/K_x$, and $\tau = 1/k_2[X]$.) When X = BH_4^- and S = ethyl acetoacetate, the residence time τ is equal to $1/k_2[X]$, and the value of ϕ should *decrease* with increasing concentration of fixer. This is the result observed in going from 30 mM [BH_4^-] to 60 mM [BH_4^-], and the decrease in slope from 0.53 to 0.43 is consistent with a value of K_X on the order of 0.1 M.

The fact that the slope for 15 mM $NaBH_4$ is *less* than for the other two concentrations does not conform to any of the expressions above. We surmise, however, that this discrepancy is due to a pH difference. Addition of $NaBH_4$ solution to 0.05 M pH 6 buffer causes a precipitous rise in pH. For 30 mM $NaBH_4$, the pH reaches a plateau in the range 8.0–8.2 within 2 min. For 60 mM $NaBH_4$, the plateau is approximately 0.5 pH unit higher. But for 15 mM $NaBH_4$, the plateau is in the range pH 6.9–7.0. With the lowest concentration of $NaBH_4$, the removal step (S $\rightarrow$ ethyl 3-hydroxybutyrate) would be expected to be substantially faster under these more acidic conditions.

Sodium borohydride is far too reactive to be suitable for in vivo applications. Therefore, sodium cyanoborohydride was explored. This reagent is quite specific for reducing protonated imines, and a substantial degree of selectivity was expected.[19] For in vivo applications, physical (or physiological) removal of the reagents competes with their labeling activity. Although cyanoborohydride does not react with unbound acetoacetic esters, kinetics analogous to Schemes I and II will operate nevertheless. The specific model for in vivo modification is represented by Scheme III, where both blocker and fixer are removed from the reaction site. This situation can be simulated in vitro by dialyzing a reaction mixture while covalent modification is taking place. Kinetic analysis of this scheme, based on the assumption that removal obeys first-order

kinetics, with the same residence times (τ) for blocker and fixer, leads to eq 4. Once again, as in the derivation of eq 3, the dissociation constant K_X is taken to be the same for both P and SP.

Equation 4 is somewhat more complicated than its predecessors, but in the limit of large [X] ($\gamma \rightarrow \infty$), it reduces to the same functional form as eq 2 and 3. As in the case of BH_4^-, it is impossible to measure K_x for BH_3CN^- directly by inhibition studies, since AAD becomes irreversibly inactivated by borohydride reagents in the presence of substrate (although the enzyme is unaffected by treatment with borohydride alone).[2a]

$$\phi = 1 - \left[\frac{(1+\gamma)^{\beta}}{(1+\beta)^{\gamma}}\right]^{k_1\tau/(\gamma-\beta)}, \text{ where } \beta = \frac{S_0}{K_d} \text{ and } \gamma = \frac{X_0}{K_x} \quad (4)$$

An in vitro simulation was designed to test the validity of approximating eq 4 as $\phi = 1 - (1 + \beta)^m$. Sodium cyanoborohydride was added to a mixture of AAD with various concentrations of radiolabeled ethyl acetoacetate at pH 6 to give an initial concentration of $NaBH_3CN$ of 30 mM. The reaction mixtures were then set to dialyze against pH 6 buffer. A plot of ϕ as a function of initial blocker concentration gave a linear plot ($r^2 > 0.99$) of ln $(1 - \phi)$ vs. ln $(K_d/(K_d + S_0))$,[11] (just like the plots in Figure 1). The experimental value of the slope was 0.029. In the limit $\gamma \rightarrow \infty$, the exponent m is equal to $-k_1\tau$ (given the assumptions of equal K_x's and equal τ's). But since the limit and assumptions probably do not apply rigorously to the experimental conditions, the absolute value of m can be taken only as a crude estimate of $k_1\tau$. From measuring the rate of release of radioactivity from the dialysis bag, we estimate τ to be on the order of 1000 s. Therefore, the corresponding value of k_1 for conversion of the acetoacetic ester–AAD–BH_3CN ternary complex to an irreversible adduct is on the order of 10^{-5} s^{-1}. It is worth noting that if cyanoborohydride reduction had taken place via second-order attack of SP (as exemplified by Scheme I) rather than via first-order conversion of a ternary SPX complex, the extent of modification would have been expected to give a linear log–log plot of ln $[(1 - \phi)]$ vs. $[\ln (1 + \beta)]/\beta$. (This corresponds to eq 4 in the limit $K_x \gg X_0$). Experimentally, this alternative log–log plot exhibits substantial curvature (and a much poorer correlation coefficient, $r^2 = 0.96$). This warrants the inference that Scheme II represents an appropriate description of the conditions of the experiment with cyanoborohydride as the fixer.

Selectivity of labeling was assessed by examining polylysine and other proteins under the same conditions. In the presence of an initial concentration of 1 mM ethyl [3-^{14}C]acetoacetate without fixer, approximately one lysine in 10^4 of polylysine was labeled under dialysis conditions. When sodium cyanoborohydride was added to the reaction mixture to a concentration of 30 mM, the level of incorporated radioactivity corresponds to an increase of approximately 15%. In other experiments with BSA, bovine ribonuclease A(RNAseA), rabbit muscle aldolase, and phosphoglucose isomerase, no increase in incorporated radioactivity was detected when $NaBH_3CN$ was added to the reaction mixture prior to dialysis.

Behavioral Studies

Selective Deficits. Once an animal's nose has been treated with a covalent modification procedure, it is necessary to examine its sense of smell to find if any deficits are produced. In this work, we have chosen to use a behavioral assay as the most reliable way to determine whether specific deficits in the sense of smell ("selective hyposmia") result from direct treatment of the olfactory epithelium with a blocker–fixer sequence. (The term "anosmia" has been used previously to designate "odor blindness",[11,12,20] but "hyposmia" represents a more accurate description, since olfactory detection is impaired but not eliminated.) The technique for assessing selective hyposmias using trained tiger salamanders has been described elsewhere,[20] and the distinction between selective and general impairment has been described.[12] As we have previously reported, animals trained to avoid both cyclohexanone and dimethyl disulfide lose sensitivity to the ketone when lavage with blocker plus fixer is administered.[11] There are no effects from administration of blocker alone or of fixer alone at the same concentrations.

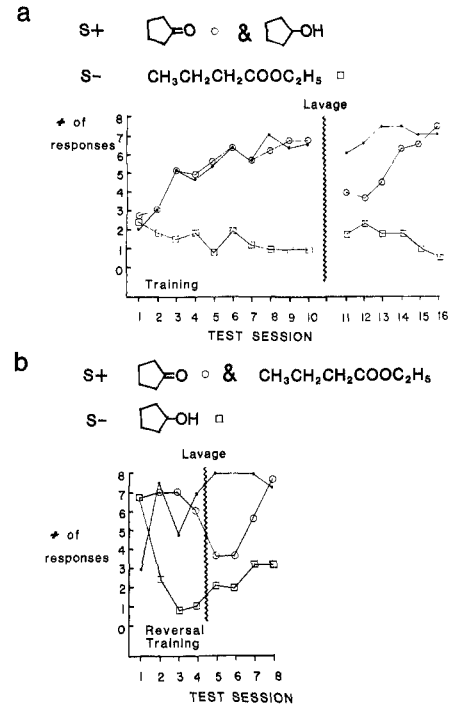


Figure 2. Selective hyposmias from two-step covalent modification of the olfactory epithelia of tiger salamanders (*Ambystoma tigrinum*). Error bars omitted for clarity. Each animal was trained to avoid both S+ odorants. Lavage was performed by irrigating both olfactory sacs with blocker (100 μL of 0.5 mM aqueous ethyl acetoacetate) followed by fixer (100 μL of 50 mM $NaBH_3CN$). Concurrent control experiments using lavage with blocker alone or fixer alone showed no effects resulting from lavage. (**a**) Four animals tested. Significant decrements in responding to cyclopentanone relative to cyclopentanol ($p < 0.01$ by t tests) observed for three sessions following lavage. (**b**) Three animals tested. Significant decrement in responding to cyclopentanone relative to ethyl butyrate ($p < 0.01$ by t tests) observed for two sessions following lavage.

Figure 2 summarizes a set of experiments for another group of odorants. Experimental animals were trained to avoid cyclopentanone and one other odorant, presented in a randomized order. These presentations were followed by negative reinforcement (bright light), and the subjects soon learned to avoid both odorant stimuli (designated as S+). Unreinforced presentations of a third odorant (S–) were interspersed among the reinforced presentations, and subjects did not learn to avoid the S– odorant.

For the first set of experiments, the S– odorant was ethyl butyrate. One of the S+ odorants was cyclopentanol (the other S+ was cyclopentanone). All subjects learned reliably to distinguish S+ from S– within a week (one training session per day), and performance did not change significantly between day 7 and day 10 of training, as Figure 2a depicts. On day 11, instead of a training session, the subjects were anesthetized, and lavage with blocker (0.5 mM ethyl acetoacetate) plus fixer (50 mM $NaBH_3CN$) was administered. On the next day, testing revealed that responding to cyclopentanone was significantly impaired, while responding to cyclopentanol was unaffected. Significant selective impairment ($p < 0.01$ by t tests) persisted for three sessions following lavage (days 12–14) before animals started to recover from the effects of lavage. Test sessions continued until all subjects exhibited the same level of performance as they had done immediately prior to lavage.

Once the subjects showed stable performance for two consecutive sessions, the roles of S– and one of the S+ stimuli were transposed. Figure 2b depicts the result of this reversal, where ethyl butyrate had become an S+ (along with cyclopentanone) and cyclopentanol an S–. All subjects learned to avoid ethyl butyrate just as well as cyclopentanone within four sessions. Right after the fourth session, animals were anesthetized, and lavage

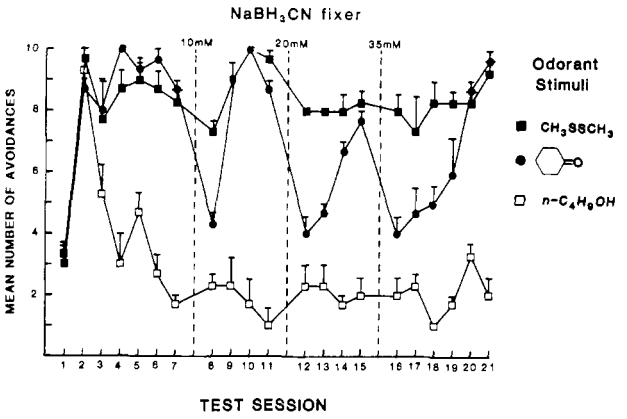


Figure 3. Effects of varying fixer concentration [BH_3CN^-] on selective hyposmia to ketones. Sessions 1–7 correspond to training the three tiger salamanders used in this study. Dashed lines represent lavages of both olfactory sacs with 100 μL of blocker (0.5 mM ethyl acetoacetate) followed by 100 μL of fixer. Error bars represent standard errors of means.

with blocker plus fixer was administered. Once again, responding to cyclopentanone was significantly impaired, while responding to the other S+ was unaffected. This selective decrement ($p < 0.01$ by t tests) persisted for two test sessions following lavage. Parallel control studies using blocker by itself or fixer by itself showed no significant effect on responding to any of the odorants.

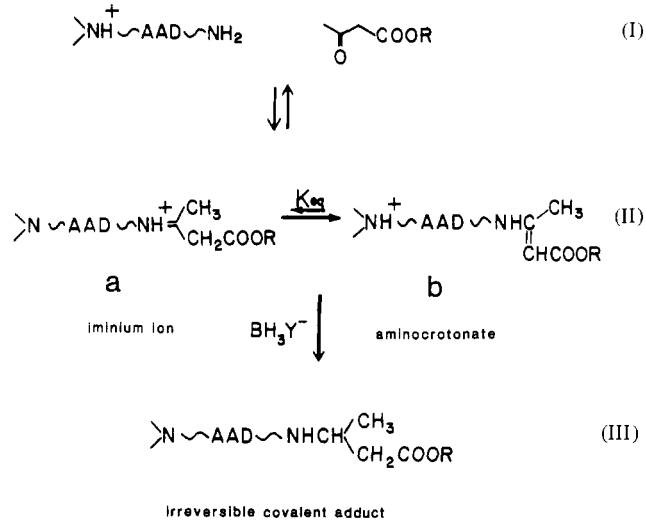
Dose-Response Relations. One might imagine that, with sodium cyanoborohydride as the fixer, some effect from free cyanide might be affecting the results. We therefore repeated the cyclohexanone–dimethyl disulfide experiments using sodium cyanide in place of sodium cyanoborohydride. Lavage with 0.5 mM ethyl acetoacetate (EAA) followed by 10 mM NaCN produced *general* hyposmia in a test session conducted as soon as the experimental animals had recovered from anesthesia (approximately 4 h after lavage) but no other effect. A repetition on the same subjects using 0.5 mM EAA followed by 20 mM NaCN produced a slightly more profound general hyposmia, but responding to the ketone-containing odorant was, if anything, less affected than responding to DMDS. This contrasts markedly with the selective hyposmia to cyclohexanone that we have previously reported to result from EAA followed by $NaBH_3CN$.[11]

Effects of varying the fixer concentration are summarized in Figure 3. Once again, a group of subjects was trained to avoid cyclohexanone and DMDS (with *n*-butanol as S–), and the first postlavage test sessions were performed as soon as possible after the animals had recovered from anesthesia (approximately 4 h after lavage). The first lavage was administered on day 8 by using EAA followed by 10 mM $NaBH_3CN$. Once all animals exhibited unimpaired performance for two consecutive sessions, a second lavage was administered by using EAA followed by 20 mM $NaBH_3CN$ (day 12). Once these animals had recovered, a third lavage using EAA followed by 35 mM $NaBH_3CN$ was performed. The duration of selective hyposmia increased as fixer concentration was raised. Comparison with the effects of lavage using EAA followed by 50 mM $NaBH_3CN$ (vide infra) supports the contention that these effects reflect the result of increasing fixer concentration rather than a cumulative effect over time.

Effects of lavage with constant fixer concentration (50 mM) but with a different blocker (EAA) concentration (0.2 vs. 0.5 mM) were examined. The duration of the selective hyposmia was only slightly affected. Statistical analysis (t tests) shows that significant hyposmia ($p < 0.01$) persists for 5 sessions after lavage by using 0.2 mM EAA and for 6 sessions after lavage by using 0.5 mM EAA. Profundity of the effect (the y axis) appears to be greater at the *lower* blocker concentration (vide infra). Thus, the evidence does not support the notion that raising blocker concentration increases the profundity of selective impairment.

Discussion

Reactions I–III depict a mechanism for covalent modification. Acetoacetic ester bound at the active site of AAD should exist predominantly as the aminocrotonate tautomer, structure **b**, and



the equilibrium constant K_{eq} should be much less than unity.[21] Close proximity of a protonated amino group has been inferred on the basis of the enzyme's stereochemistry,[16] as well as from other studies.[14,15] This may make it possible for borohydride to reduce the carbon–carbon double bond of **b**, although at a much slower rate than it attacks free acetoacetic ester.

Origins of specificity in this reaction sequence are apparent from the mechanism depicted. At physiological pH, ordinary lysine residues will be protonated, and reversible complexes are prevented from forming. For ε-amino groups whose basicity is depressed (e.g., the active site lysine of RNAseA), acetoacetic ester can readily form an iminium ion reversibly. However, the methylene protons are very acidic. The conjugate base is a vinylogous urethane, and, like other vinylogous amides, the unprotonated aminocrotonate ought to predominate at pH ≥ 6. Cyanoborohydride does not reduce electrically uncharged bonds; thus, a neutral aminocrotonate will be resistant to nucleophilic attack by BH_3Y^- (Y = CN).

The active site of AAD is unusual in that a proton acceptor (a prosthetic amine function) lies very near the active site lysine. The proximity of this prosthetic functionality is demonstrated by the stereoselectivity of deuterium exchange, as well as by the fact that the methyl as well as methylene hydrogens of ethyl acetoacetate rapidly with solvent in the presence of AAD.[22] This active site behaves very much like rigid diamines studied by Hine and co-workers,[23] where a specific three-dimensional arrangement leads to stereoselective catalysis. In the active site of AAD, the prosthetic amine function holds the proton near the aminocrotonate function, as represented by structure **b**. This either shifts the equilibrium in the direction of an iminium ion **a** (which can be reduced by cyanoborohydride) or delivers a proton to one end of the carbon–carbon double bond at the same time as BH_3Y^- delivers hydride to the other.

Labeling of polylysine is consistent with this portrait. A substantial level of incorporated radioactivity is observed when polylysine is treated with ethyl [3-^{14}C]acetoacetate alone. There is a high probability that not every ε-amino group in polymer of MW 40 000 is protonated, even at pH 6. These unprotonated nucleophiles can be acylated by the ester function, and the level of incorporation observed (corresponding to one acetoacetyl per 10 000 lysine residues) is consistent with that view. Against this high background, the level of additional labeling of polylysine when $NaBH_3CN$ is added appears to be small, but it is also consistent with the notion that two-step labeling can occur only when a Schiff base linkage to acetoacetic ester has a nearby protonated amine function to facilitate reduction by cyanoborohydride.

Although other enzymes have been labeled by reductive alkylation,[2,24,25] we find that only AAD is labeled by acetoacetic ester plus cyanoborohydride. Other Schiff base-forming enzymes require anionic substrates. We observe that rabbit muscle aldolase

is not measurably inhibited by ethyl acetoacetate up to 100 mM (nor by lithium acetoacetate). It is not surprising to find, therefore, that this enzyme is not labeled by two-step modification with ethyl [3-^{14}C]acetoacetate plus cyanoborohydride (although it is labeled by dihydroxyacetone phosphate plus borohydride[2b]). Neither is glucose-6-phosphate isomerase, another Schiff base-forming enzyme that requires an electrically charged substrate (even though the enzyme can be labeled by reductive alkylation with pyridoxal phosphate and borohydride[25]).

The specificity of labeling AAD stands as prima facie evidence that two-step labeling is specific for its active site. An additional piece of supporting evidence comes from the log–log plots exemplified by Figure 1. The relationship $\phi = 1 - (1 + \beta)^m$ is obeyed under four different sets of conditions when ϕ, the extent of modification, is measured as a function of $\beta = S_0/K_d$. Data from radioactive labeling agree with the results of enzyme activity assays at a higher value of S_0, although the experimental uncertainty of the assays of catalytic activity is very large. But the log–log plots give good fits only for values of β that correspond to $K_d = K_I$, where K_I is the inhibition constant measured for ethyl acetoacetate. This result serves to validate the quantitative expression $\phi = 1 - (1 + \beta)^m$ and also to substantiate the inference that covalent modification is taking place at the same sites where ethyl acetoacetate binds as a competitive inhibitor.

Derivations of the kinetic expressions are given in the Appendix section, and the underlying assumptions are detailed there. Among the pertinent assumptions are the requirement that fixer concentration, [X], is so high that temporal variations in [X] do not affect the extent of modification. Initial values of [X] were chosen to be more than an order of magnitude greater than S_0, so this supposition seems warranted. Yet if the values of [S] and [X] were to fall off exponentially with the same time constant τ (as they might in a dialysis experiment or an in vivo application), a different quantitative relation might have been expected, namely a linear plot of ϕ (which is $\approx -\ln(1 - \phi)$ at values of $\phi < 0.1$) vs. $\beta \ln(1 + \beta)$. Such a plot is not linear for our in vitro experiments, even for a reduction with cyanoborohydride conducted under dialysis conditions.

From these experimental results, we infer that two-step labeling of AAD proceeds via a ternary complex for ethyl acetoacetate as the blocker and either borohydride or cyanoborohydride as the fixer. The latter reagent is stable in aqueous solution above pH 3 and would appear to have a noncompetitive inhibition constant K_x that is much smaller than that for BH_4^-. Since tetraphenylborate anion has been reported as an inhibitor of AAD, it is not surprising to observe evidence that borohydrides can bind electrostatically at the enzyme active site, even though they are too reactive for direct examination of reversible inhibition kinetics. Moreover, since the noncompetitive inhibition constants vary widely among anions, it is also not surprising to find that cyanoborohydride binds much more tightly than borohydride. For purposes of comparison, we note that thiocyanate is reported to be a much better inhibitor than any of the halide anions and that the value of K_x for thiocyanate is 0.1 mM.[18] The differences between halide and thiocyanate may be a good model for the difference between BH_4^- and BH_3CN^-.

The quantitative relationship represented by eq 4 may very well pertain to in vivo labeling. Although it is based on the assumptions that K_x be the same for P and SP and that τ be the same for the blocker and fixer (and that their removal follow an exponential decay), eq 4 can be used to make semiquantitative predictions regarding a dose-response relation. We have previously discussed detection models to show that loose binding excitatory receptors ($K_d > 10^{-5}$ M) can exhibit sensitivity to very low ($<10^{-9}$ M) concentrations of odorant, as well as a dynamic range over 4 powers of 10.[26] We surmise that olfactory receptors may have dissociation constants in the micromolar range.[27] This means that β would be large when blocker concentrations are in the 0.1–1 mM range. On the other hand, the hydrophobicity of olfactory receptors would suggest that the dissociation constant for ions with the receptor, K_x, should be relatively large. In other words, γ ought to be small, even when fixer concentration is in the 10–50 mM range. In the limit where β is much larger than unity while γ is on the order of or smaller than unity, eq 4 becomes $1 - \phi \approx [\beta^{\gamma/\beta}/(1 + \gamma)]^{k_1\tau}$. Taking the logarithm of both sides, one gets for $\phi < 0.1$

$$\phi \approx k_1\tau[\ln(1 + \gamma) - (\gamma/\beta)\ln\beta] \quad (5)$$

How does the extent of modification vary with the initial concentrations of blocker and fixer? The sensitivity of ϕ to changes in β and γ may not be obvious from eq 5, so some plausible values can be inserted as examples. For $\gamma = 1$ and $\beta = 100$, increasing β by a factor of 2.5 leads to an increment of ϕ of 4%. Increasing γ by the same factor multiplies ϕ by 1.8. For $\gamma = 0.1$ and $\beta = 10$, the corresponding changes are 14% and 2.3, respectively. In other words, ϕ is much more sensitive to γ than to β in the limit of large β and small γ. This general result pertains to values of ϕ up to 0.9 just as it does to values < 0.1. It has not proved practical to confirm this expectation in vitro. However, the semiquantitative predictions of eq 5 have been probed in vivo.

We now summarize the implications of the chemical experiments regarding in vivo applications to the sense of smell:

(1) Two-step modification ought to block Schiff base-forming sites selectively. Receptors for ketones ought to be affected in preference to receptors for esters, alcohols, or sulfides.

(2) Application of blocker or fixer alone (at low concentrations) ought to have negligible effect.

(3) Use of a fixer (such as cyanide) that forms *reversible* covalent adducts with Schiff bases will have effects of much shorter duration than a fixer (such as cyanoborohydride) that forms irreversible covalent adducts.

(4) Alteration of the alkyl moiety of the acetoacetic ester ought not to affect its function as a blocker (assuming that K_d is not perturbed).

(5) Based on eq 5, the effect of changing blocker concentration ought to be small, while effects of changing fixer concentration should be large.

All these implications have been tested, and in every case the results from behavioral assay are consistent with the supposition that Schiff base-forming sites function in the olfactory detection of ketones:

(1) We have previously published data to show that olfactory detection of cyclohexanone is impaired by two-step labeling with ethyl acetoacetate followed by sodium cyanoborohydride.[11] Cyclohexanone has been reported to be different from many other odorants, in that the *spatial* distribution of electrically responsive receptors appears to be uniform over the surface of the olfactory epithelium of *A. tigrinum*, while the distribution of receptors for other odorants tends to be more localized.[4]

Figure 4 summarizes some of our results in experiments where olfactory detection of dimethyl disulfide was monitored concurrently (and was observed to be unaffected by the labeling procedure). The effects of lavage with 0.5 mM ethyl acetoacetate followed by 50 mM $NaBH_3CN$ are the same for animals trained and tested at two different odorant concentrations, 2% and 2.5% of vapor saturation. That these two concentrations are perceived differently can be gauged from a surgical section of the olfactory nerve, which leaves a reduced (but significant) level of discriminative responding at 2.5%, but which completely abolishes discrimination of S+ from S– at 2%.[11]

The data shown in Figure 2 also demonstrate the specificity of the decrement in responding to a ketone-containing odorant. Here, the unaffected odorants are an ester (ethyl butyrate), whose molecular dimensions should be very close to those of the blocker, and an alcohol (cyclopentanol), whose molecular shape is close to that of the ketone.

(2) In the experiments summarized in Figures 2 and 4, concurrent control experiments showed that lavage with 0.5 mM ethyl acetoacetate alone or 50 mM $NaBH_3CN$ alone had no significant effect on responding to olfactory cues. In the cyclohexanone experiments, we have reported that increasing the concentration of blocker by a factor of 100 causes a selective decrement in responding even when lavage with fixer is omitted.[20] This has been attributed to the action of endogenous nucleophiles, whose

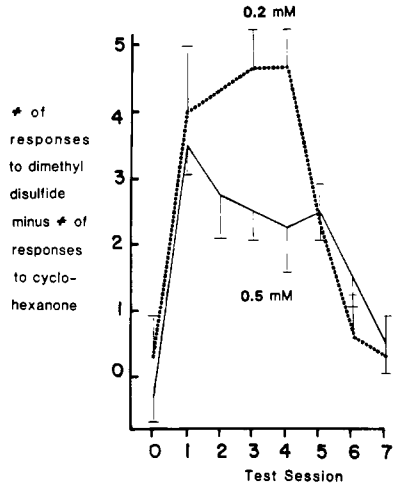


Figure 4. Selective hyposmia to ketones for two different blocker (ethyl acetoacetate) concentrations at constant fixer (50 mM $NaBH_3CN$) concentration. Degree of hyposmia is measured as the difference between avoidance responding to dimethyl disulfide and responding to cyclohexanone. Test session 0 corresponds to the last training session before lavage. Significant ($p < 0.01$ from t tests) selective hyposmia is observed for sessions 1–5 at 0.2 mM (three animals tested) and for sessions 1–6 at 0.5 mM (four animals tested). Subsequent test sessions do not show significant hyposmia even by the less stringent criterion of $p < 0.05$. Data for 0.5 mM plot taken from ref 11.

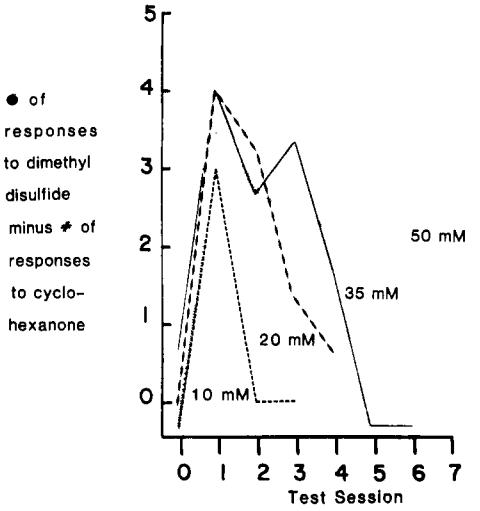


Figure 5. Selective hyposmia to ketones for four different fixer ($NaBH_3CN$) concentrations at constant blocker (0.5 mM ethyl acetoacetate) concentration. Data taken from Figure 3 and from ref 11. Degree of hyposmia is measured as the difference between avoidance responding to dimethyl disulfide and responding to cyclohexanone. Test session 0 corresponds to the last training session before lavage. Error bars omitted for clarity. A difference of > 2.0 corresponds to a significant ($p < 0.05$ from t tests) selective hyposmia.

effect at 0.5 mM acetoacetic ester is negligible but whose effect at much higher values of β become substantial[12] (as would be expected on the basis of eq 3).

(3) Two-step lavage using cyanide in place of cyanoborohydride does not produce selective hyposmia. Testing was performed at a shorter interval after lavage in these studies (4 h rather than 1 day), yet no selective hyposmia could be observed. General hyposmia for the first test session after cyanide lavage can be attributed to the reversible, systemic toxicity of cyanide without invoking any action at the receptor site.

(4) As we have previously reported, use of 2-(methylthio)ethyl acetoacetate as blocker in place of ethyl acetoacetate does not alter the results of our behavioral assays.[11] The hyposmia was restricted to cyclohexanone, and the profundity and duration of the effects were indistinguishable from the results of lavages using ethyl acetoacetate. No decrement of responding to dimethyl disulfide was observed, even though the new blocker contained a CH_2SCH_3 moiety.

(5) Figures 4 and 5 summarize effects of changing the blocker and fixer concentrations. Selective hyposmia is measured as the difference in responding to the two S+ odorants. As expected on the basis of eq 5, increasing the blocker concentration by a factor of 2.5 lengthens the duration of the effect only slightly. The *profundity* of the selective hyposmia appears to be greater at the *lower* blocker concentration. It is not clear that the variation in profundity is meaningful, as the two experiments were performed on two different sets of animals at two different times. In any event, there is no evidence to suggest that profundity increased with increasing blocker concentration.

Data for animals treated with 0.5 mM ethyl acetoacetate followed by varying fixer concentrations are summarized in Figure 5. The data for this figure are taken from Figures 3 and 4, and it is clear that there is a systematic increase in the duration of selective hyposmia (as measured by the difference between S+ responses) as the fixer concentration is increased. This result, too, is what would be expected on the basis of eq 5, for ϕ is much more senstive to γ than to β in the regime that we have proposed for two-step covalent modification of the olfactory epithelium.

The ability to detect ketone is impaired but not eliminated by chemical treatment. Out of 23 sessions shown in Figures 2–4 where significant ($p < 0.05$ by t tests) selective hyposmia was observed (comprising an average of 3.4 subjects per session), only five instances were observed where an animal failed to give more responses to ketone than to the S– odorant. We are forced to conclude that the conditions of two-step labeling do not obliterate all the pathways by which cyclopentanone and cyclohexanone are detected at 2% vapor saturation. By contrast, as we have reported elsewhere,[11] all ability to discriminate S+ from S– is lost when the olfactory nerves are surgically cut.

The inference that the nose detects ketones via Schiff base-forming sites offers a parsimonious interpretation of the results, but other classes of receptor must also be operating, too. The notion of redundancy in the olfactory code[29] is not inconsistent with the large body of electrophysiological data for salamanders.[4-6] Redundancy means that several different classes of receptor respond to a given stimulus. The most telling argument for such redundancy is that the *profundity* of selective hyposmia (the y axis of Figures 4 and 5) is not observed to increase as blocker or fixer is increased. If cyclohexanone could be discriminated only via a single class of receptors, the profundity of hyposmia would have increased as a consequence of increasing the extent of modification of those sites. Yet the only alteration of profundity was the increase observed when blocker concentration was *decreased*.

The apparent result of increasing fixer is to increase the *duration* of selective hyposmia rather than its profundity. Within the context of the models for olfactory detection discussed in the introduction to this paper, our results can be explained by the analogy to rod cells in the retina, Schiff base-forming receptor proteins that continually secrete neurotransmitter when the ligand is bound. Electrophysiological studies of the olfactory epithelia of salamanders show that after an initial burst of activity following presentation of an odorant pulse, continued stimulation causes continuous electrical output (although none of these *temporal* studies have been reported using continuous stimulation periods of time longer than several seconds). An olfactory receptor functioning in this fashion will contribute a background signal when the Schiff base-forming site is irreversibly covalently modified. Given a high enough background, detection of the ketone function is prevented, and other types of detectors have to bear the burden of discriminating S+ from S– stimuli. Increasing the extent of modification, ϕ, should not impair ketone detection any further, since information from the ketone receptor will already have been swamped by noise. But if regeneration of labeled sites via new protein synthesis takes place, then increasing ϕ ought to lengthen the time required to replace modified

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sites. In other words, when a sufficient number of sites are modified, the background signal from them interferes with the utilization of the unmodified sites. The greater the value of ϕ, the longer will be the time required for new protein synthesis to replace modified sites to a point where the noise level no longer interferes with the utilization of intact receptors.

Conclusion

We have presented here a selective two-step procedure for labeling Schiff base-forming proteins. A kinetic analysis of two-step covalent modification has been described, and experimental tests have been reported. The experimental results not only validate the analysis (as exemplified by Scheme II), but they also confirm that the site of covalent modification corresponds to the active site. Simulation of in vivo conditions in vitro substantiates the applicability of two-step labeling kinetics to living organisms. At least five specific predictions are made on the basis of experiments using AAD as a model. All these predictions are borne out with respect to olfaction by experiments using a behavioral assay with tiger salamanders.

One of the advantages of two-step modification is that control experiments are straightforward to design for in vivo studies. For the blocker–fixer sequence, omission of either the blocker or the fixer eliminates the selective hyposmia that is produced when they are applied in sequence. Many investigators have explored chemical treatment of the olfactory epithelium as an approach toward modifying receptor function.[30] In most of those experiments, the reactions have not been designed to produce selective deficits nor have the assays of function (which have usually been based on electrophysiology) been well suited to uncover selective hyposmias, even if they had happened to occur.

In the tiger salamander, our understanding of the mechanism for covalent modification, together with a germane chemical model (AAD) for which it is specific, makes it possible to extract meaningful information from studies of animal behavior. Moreover, the results to date give us confidence that it will be possible to identify and characterize olfactory receptor sites in this species.

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Appendix

Derivation of Equations 1–3. Assume that [S] is well approximated by $S_0e^{-t/\tau}$. Assume rapid equilibration of S + P $\rightleftarrows$ SP, so that [SP] = [S][P]/K_d, and rapid equilibration of X + P $\rightleftarrows$ PX as well as SP + X $\rightleftarrows$ SPX. The differential equation for Scheme II is then

$$\frac{\text{d[irreversible adduct]}}{dt} = k_1[\text{SPX}] \tag{A.1}$$

where

$$[\text{SPX}] = \frac{[\text{SP}][\text{X}]}{K_x} = \frac{[\text{S}][\text{P}][\text{X}]}{K_xK_d} \tag{A.2}$$

Since [P] is equal to P_0 − [irreversible adduct] − [SPX] − [SP], we get

$$\frac{\text{d[irreversible adduct]}}{dt} = \frac{k_1[\text{S}][\text{X}](P_0 - [\text{irreversible adduct}])}{(K_xK_d + K_d[\text{X}] + K_x[\text{S}] + [\text{S}][\text{X}])} \tag{A.3}$$

$$\frac{\text{d[irreversible adduct]}}{dt} = \frac{k_1[\text{S}][\text{X}](\text{P}_0 - [\text{irreversible adduct}])}{(K_x + [\text{X}])(K_d + [\text{S}])} \tag{A.4}$$

Substitute $S = S_0e^{-t/\tau}$ and multiply numerator and denominator by $e^{t/\tau}$.

$$\frac{\text{d[irreversible adduct]}}{dt} = \frac{k_1S_0[\text{X}](\text{P}_0 - [\text{irreversible adduct}])}{(K_x + [\text{X}])(K_de^{t/\tau} + S_0)} \tag{A.5}$$

By inspection, it is apparent that [irreversible adduct] = P_0 represents a particular solution of this inhomogeneous differential equation. Call $\alpha = S_0k_1[\text{X}]/K_d(K_x + [\text{X}])$ and $\beta = S_0/K_d$. For the general solution, consider the homogeneous differential equation, using Z in place of [irreversible adduct].

$$(e^{t/\tau} + \beta)Z' + \alpha Z = 0 \tag{A.6}$$

$$Z'/Z = \text{d}(\ln Z)/dt = -\alpha/(e^{t/\tau} + \beta) \tag{A.7}$$

Integrating both sides, we get

$$\ln Z = \alpha\tau \ln (1 + \beta e^{-t/\tau})/\beta + \text{constant} \tag{A.8}$$

Exponentiating, we get the general solution for the homogeneous part. Add to it the particular solution of the inhomogeneous part to get the general solution, using $\alpha/\beta = k_1[\text{X}]/(K_x + [\text{X}])$

$$[\text{irreversible adduct}] = \text{P}_0 + C(1 + \beta e^{-t/\tau})^{k_1\tau[\text{X}]/(K_x + [\text{X}])} \tag{A.9}$$

where C comes from the constant of integration.

Evaluate C by applying the initial condition that [irreversible adduct] = 0 at $t = 0$

$$\text{P}_0 + C(1 + \beta)^{k_1\tau[\text{X}]/(K_x+[\text{X}])} = 0 \text{ or } C = \frac{-\text{P}_0}{(1 + \beta)^{k_1\tau[\text{X}]/(K_x+[\text{X}])}} \tag{A.10}$$

$$C = \frac{-\text{P}_0}{(1 + \text{S}_0/K_d)^{k_1\tau[\text{X}]/(K_x + [\text{X}])}}$$

In the limit $t \rightarrow \infty$, we get eq 3. In the limit $K_x \gg$ X, letting $k_1/K_x = k_3$ and $\tau = 1/k_2[\text{X}]$, we get eq 1. Letting $t \rightarrow \infty$, get eq 2.

Derivation of Equation 4. Now consider removal by dialysis of S (initial concentration S_0) and X (initial concentration X_0) at equal rates, with residence time τ. The rate equation then becomes

$$\frac{\text{d[irreversible adduct]}}{dt} = \frac{k_1(S_0e^{-t/\tau})(X_0e^{-t/\tau})(\text{P}_0 - [\text{irreversible adduct}])}{(K_d + S_0e^{-t/\tau})(K_x + X_0e^{-t/\tau})} \tag{A.11}$$

Call $\beta = S_0/K_d$ and $\gamma = X_0/K_x$. Multiplying both sides, we get

$$(e^{t/\tau} + \beta)(e^{t/\tau} + \gamma)\frac{\text{d[irreversible adduct]}}{dt} + k_1\beta\gamma[\text{irreversible adduct}] = k_1\beta\gamma\text{P}_0 \tag{A.12}$$

for which, once more, [irreversible adduct] = P_0 is a particular solution. For the general solution, consider the homogeneous differential equation

$$(e^{t/\tau} + \beta)(e^{t/\tau} + \gamma)Z' + k_1\beta\gamma z = 0 \tag{A.13}$$

whence

$$\text{d}(\ln Z)/dt = \frac{-\beta\gamma k_1}{(e^{t/\tau} + \beta)(e^{t/\tau} + \gamma)} = \frac{\beta\gamma k_1}{\beta - \gamma}\left(\frac{1}{e^{t/\tau} + \beta} - \frac{1}{e^{t/\tau} + \gamma}\right) \tag{A.14}$$

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Integrating both sides, we get

$$\ln Z = \frac{k_1\tau}{\beta - \gamma} \ln \frac{(1 + \beta e^{-t/\tau})^\gamma}{(1 + \gamma e^{-t/\tau})^\beta} + \text{constant} \quad (A.15)$$

At $t = 0$

$$\ln Z = \frac{k_1\tau}{\beta - \gamma} \ln \frac{(1 + \beta)^\gamma}{(1 + \gamma)^\beta} + \text{constant} \quad (A.16)$$

Exponentiating, we get

$$Z_{t=0} = C\left[\frac{(1 + \beta)^\gamma}{(1 + \gamma)^\beta}\right]^{k_1\tau/(\beta-\gamma)} \quad (A.17)$$

Since, at $t = 0$, [irreversible adduct] $= 0 = P_0 + Z_{t=0}$, we get

$$C = -P_0\left[\frac{(1 + \gamma)^\beta}{(1 + \beta)^\gamma}\right]^{k_1\tau/(\beta-\gamma)} \quad (A.18)$$

At $t = \infty$, [irreversible adduct] $= P_0 + C$; hence,

$$[\text{irreversible adduct}]_{t=\infty} = P_0\left\{1 - \left[\frac{(1 + \gamma)^\beta}{(1 + \beta)^\gamma}\right]^{k_1\tau/(\beta-\gamma)}\right\} \quad (A.19)$$

Substituting back and setting $\phi = [\text{irreversible adduct}]_{t=\infty}/P_0$, get eq 4.

For the limit $K_x \gg X_0$, the denominator in eq A.11 becomes $K_x(K_d + S_0e^{-t/\tau})$. The corresponding homogeneous differential equation for second-order attack of SP is then

$$(e^{2t/\tau} + \beta e^{t/\tau})Z' + k_1\beta\gamma Z = 0 \quad (A.20)$$

whence the solution for the extent of modification becomes

$$\phi = 1 - e^{k_1\gamma\tau}(1 + \beta)^{k_1\beta\gamma\tau} \quad (A.21)$$

Registry No. $MeC(O)CH_2CO_2Et$, 141-97-9; $NaBH_4$, 16940-66-2; $NaBH_3CN$, 25895-60-7; acetoacetate decarboxylase, 9025-03-0; cyclopentanone, 120-92-3; cyclohexanone, 108-94-1.

Supplementary Material Available: Tables of individual session scores for Figure 2 and for control animals (2 pages). Ordering information is given on any current masthead page.

Biomimetic Models for Cysteine Proteases. 1. Intramolecular Imidazole Catalysis of Thiol Ester Solvolysis: A Model for the Deacylation Step

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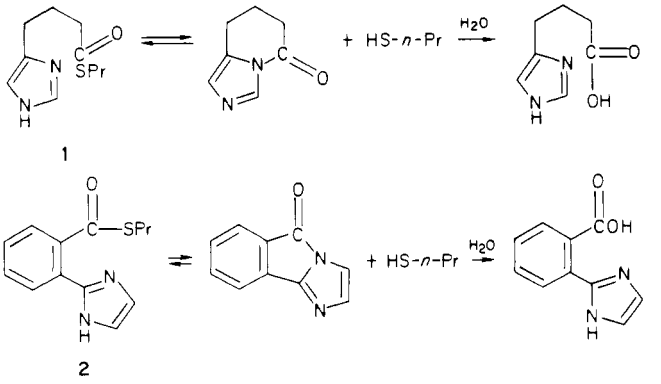
Abstract: The solvolysis of 2-[2-(acetylthio)phenyl]-4,5-dimethylimidazole (**3**) has been studied in ethanol–water solution as a model for the thiol ester intermediates occurring during the catalytic cycle of cysteine proteases. Additionally the solvolyses of *N*-acetyl-4,5-dimethyl-2-phenylimidazole (**4**) and benzenethiolacetate (**5**) were studied as reference compounds. At neutrality, a plateau in the pH–log k_{obsd} profile for solvolysis of **3** is seen due to the participation of imidazole. Evidence is given that the mechanism in this pH region is intramolecular general-base-catalyzed solvent attack on the thiol ester by imidazole. At pHs greater than 10, specific-base-catalyzed solvolysis of the thiol ester becomes predominant. There is no kinetic importance of an S-to-N acyl transfer during the course of solvolysis. The relevance of these results to the chemical mechanism of cysteine proteases is discussed.

I. Introduction

The catalytic cycle of the cysteine proteases, of which papain is the most widely studied example, is known to proceed through an intermediate thiol ester.[1] Present in the active site[2] of these enzymes is a histidylimidazole residue which presumably is catalytically involved with both the acylation and deacylation steps. The role of imidazole in the deacylation step has often been suggested[3] as that of a general base in assisting delivery of an attacking water molecule. Support for the involvement of imidazole comes mainly from the large solvent deuterium isotope effect measured for the enzymatic deacylation reaction[1d,e,4] and more recent spectrophotometric and NMR titration experiments of Shafer et al.[5] The only well-documented chemical precedent of this mechanism with a thiol ester is the intermolecular general-base-catalyzed hydrolysis of ethyl trifluorothioacetate.[6] It is

Scheme I



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