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Covalent Modification of Schiff Base-Forming Proteins: *In Vitro* Evidence for Site Specificity and Behavioral Evidence for Production of Selective Hyposmia *in Vivo*[a]

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Chemical treatment of the olfactory epithelium can impair the sense of smell in two ways: production of a selective hyposmia or of general hyposmia. We have reported the former in tiger salamanders conditioned to respond to two odorants, cyclohexanone and dimethyldisulfide.[1-3] An example of the latter can be seen in the results of application of methylmercury hydroxide, CH_3HgOH (which rapidly attacks -SS- and -SH groups), directly to the receptor epithelium. As FIGURE 1A summarizes, responding to both odorants is impaired to an equal extent. By contrast, application of the sulfhydryl reagent iodoacetamide, ICH_2CONH_2 has, by itself, no effect on responding (FIG. 1B), nor does it enhance the effect of CH_3HgOH (FIG. 1C).

Our interest focuses on the blocker-fixer sequence, acetoacetic ester (CH_3COCH_2COOR) as blocker followed by sodium cyanoborohydride ($NaBH_3CN$) as fixer, which produces selective hyposmia to aldehydes and ketones, but does not affect responding to simple esters (e.g. ethyl butyrate), alcohols, or sulfides.[4,5] This two-step procedure is chemically specific for proteins with Schiff base-forming sites that bind electrically uncharged carbonyl compounds. *In vitro* experiments to probe this specificity were conducted with AAD (the bacterial enzyme acetoacetate decar-

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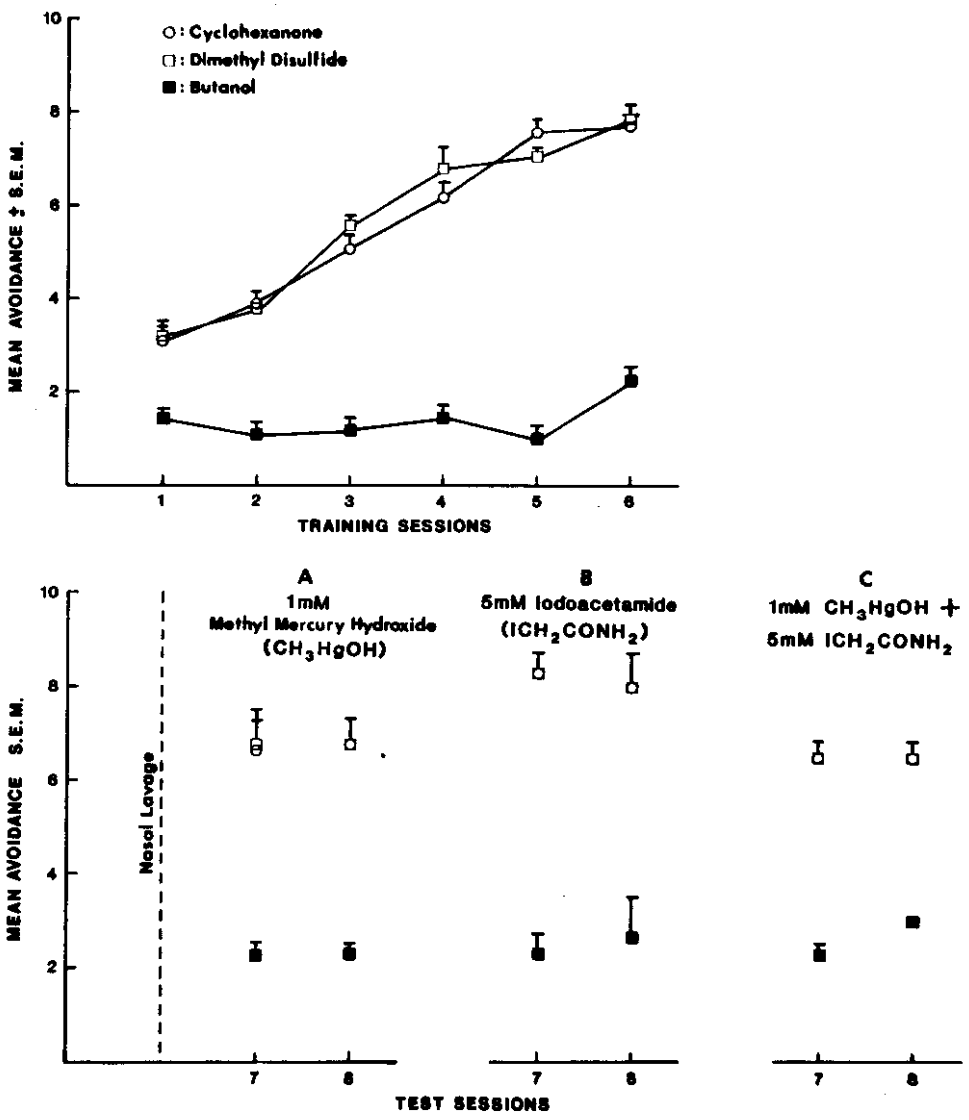

FIGURE 1. Effects of lavage with sulfhydryl reagents on olfactory discrimination by tiger salamanders. Top: acquisition of conditioned avoidance[1-3] to cyclohexanone and dimethyl sulfide (open symbols) with concurrent, unconditioned presentations of *n*-butanol (solid squares) ($n=11$). Bottom: mean performances for two postlavage test sessions for subgroups given bilateral lavage with **(A)** 100 μl methylmercury hydroxide ($n=3$); **(B)** 100 μl iodoacetamide ($n=3$); and **(C)** 100 μl methylmercury hydroxide followed by 100 μl iodoacetamide ($n=4$).

boxylase), a Schiff base-forming protein with a well-characterized active site. The following data provide evidence that a single active-site lysine undergoes covalent modification in this model system:

(1) Since all five hydrogens of CH_3COCH_2COOR exchange with solvent in the presence of AAD,[6] tritiated water can be used to incorporate radiolabel into the blocker and thence into the protein. A solution of 0.07 m*M* AAD and 3.4 m*M* ethyl 3-[^{14}C]acetoacetate (1 mCi/mmol) in tritiated water (0.3 Ci/ml) at pH 6 treated with 15 m*M* $NaBH_3CN$ for one hour incorporated nondialyzable label into AAD in a ratio

of $\frac{\text{dpm } ^3H}{\text{dpm } ^{14}C} = 5$ (after correction for a control in which $NaBH_3CN$ was omitted). In an experiment performed using *tert*-butyl acetoacetate, AAD incorporated comparable levels of ^3H: 0.056 μCi/mg protein from 3.2 m*M* $CH_3COCH_2COOC(CH_3)_3$ and 0.032 μCi/mg protein from 0.1 m*M* $CH_3COCH_2COOC(CH_3)_3$ [as opposed to 0.004 μCi/mg protein in a control experiment with 0.4 m*M* $CH_3COCH_2COOC(CH_3)_3$, in which $NaBH_3CN$ was omitted].

(2) Since acetic anhydride specifically blocks the active-site lysine of AAD,[7] acetylation can be used to prevent subsequent covalent modification by the blocker-fixer sequence, as shown to the left in FIGURE 2. AAD with >99% of its active sites acetylated shows a greatly reduced level of ^3H incorporation from 3.2 m*M* $CH_3COCH_2COOC(CH_3)_3$ plus $NaBH_3CN$ in tritiated water, 0.014 μCi/mg protein. AAD that was acetylated with 1-[^{14}C]acetic anhydride (13.8 μCi/mmol) and subsequently treated with 3.5 m*M* ethyl acetoacetate in tritiated water incorporated a ^3H-to-^{14}C ratio of 13 when $NaBH_3CN$ was omitted and a ^3H-to-^{14}C ratio of 14 under identical conditions when $NaBH_3CN$ was included. We draw two conclusions: (1) Incorporation of radiolabel into AAD does not depend on the identity of alkyl group R in the acetoacetic ester; (2) the blocker-fixer sequence specifically modifies the active-site lysine, as depicted by the reaction sequence to the right in FIGURE 2.

Intranasal lavage with 0.5 m*M* ethyl acetoacetate followed by 50 m*M* $NaBH_3CN$ produces a selective hyposmia not only in tiger salamanders, but in other species, as well. Results are tabulated below for cardiac conditioning of a starling *(Sturnus vulgaris)*. The bird was conditioned to respond to cyclohexanone and ethyl butyrate with an increase in heart-beat rate (with concurrent, unreinforced presentations of *n*-

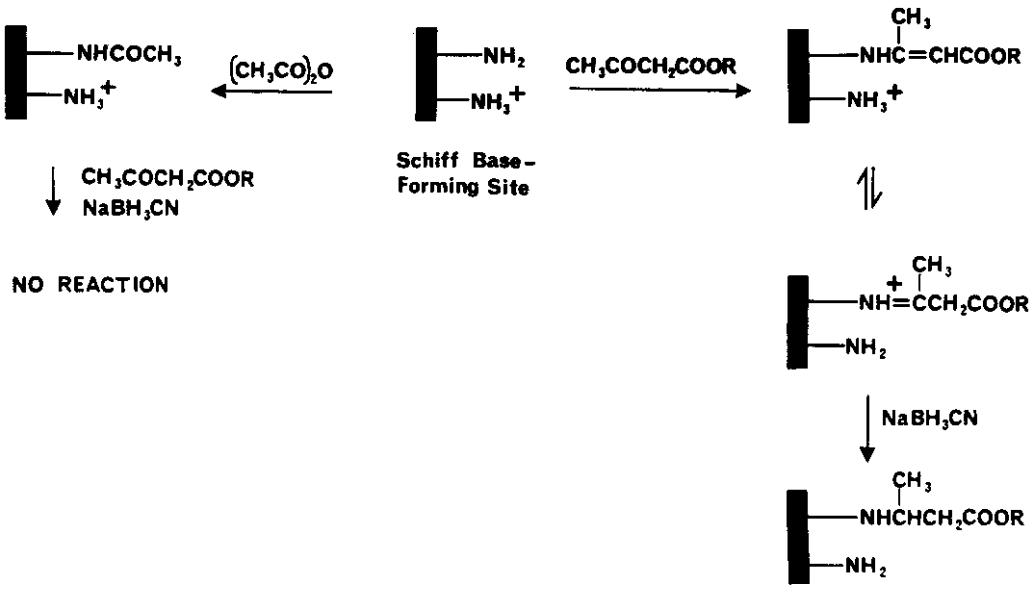

FIGURE 2. Site-selective covalent modification of a Schiff base-forming protein, as typified by the active site of acetoacetate decarboxylase (AAD), which contains two amine functions in close proximity. To the left, selective acetylation[7] blocks further modification by the blocker-fixer sequence of CH_3COCH_2COOR followed by $NaBH_3CN$. To the right, a mechanism for selective covalent modification of the active site via a blocker-fixer sequence.

TABLE 1. Two-Step Lavage Abolishes Significant Responding to Cyclohexanone

Odorant	Prelavage Prior	Prelavage During	Postlavage Prior	Postlavage During
Cyclohexanone	7.2 Hz[a]	8.3 Hz[a]	6.8 Hz[b]	7.0 Hz[b]
Ethyl butyrate	6.9 Hz[a]	7.7 Hz[a]	6.9 Hz[a]	8.0 Hz[a]

[a] Meets significance criterion $p < 0.001$.
[b] Fails to meet significance criterion $p < 0.15$.

butanol, to which the bird did not respond). Average heartbeat rates for 15 seconds before and for 15 seconds during odorant presentation are given (means of 10 trials). Paired *t*-tests of postlavage performance show that two-step lavage abolishes significant responding to cyclohexanone (TABLE 1).

Starlings recover intact olfactory function within one to two hours after lavage, much more quickly than do tiger salamanders (which take several days). This result is consistent with replacement of modified receptors via new protein synthesis, which, we have argued,[4,5] is required for recovery from the agonistic effect of irreversible covalent modification.

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