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Effects of General Anesthetics on the Bacterial Luciferase Enzyme from *Vibrio harveyi*: An Anesthetic Target Site with Differential Sensitivity†

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ABSTRACT: The effects of a diverse range of 36 general anesthetics and anesthetic-like compounds on a highly purified preparation of the bacterial luciferase enzyme from *Vibrio harveyi* have been investigated. Under conditions where the flavin site was saturated, almost all of the anesthetics inhibited the peak enzyme activity and slowed the rate of decay. However, a small number of the more polar agents only inhibited at high concentrations, while stimulating activity at lower concentrations. The inhibition was found to be competitive in nature, with the anesthetics acting by competing for the binding of the aldehyde substrate *n*-decanal. The anesthetic binding site on the enzyme could accommodate only a single molecule of a large anesthetic but more than one molecule of a small anesthetic, consistent with the site having circumscribed dimensions. The homologous series of *n*-alcohols and *n*-alkanes exhibited cutoffs in inhibitory potency, but these cutoffs occurred at very different chain lengths (about C_{10} for the *n*-alkanes and C_{15} for the *n*-alcohols), mimicking similar cutoffs observed for general anesthetic potencies in animals. Binding constants determined from peak height measurements showed that the inhibitor binding site was predominantly hydrophobic (with a mean $\Delta\Delta G^{\ominus}_{CH_2}$ of −5.0 kJ/mol), but fluctuations in the binding constants with chain length revealed regions in the binding site with polar characteristics. Binding constants to an intermediate form of the enzyme (intermediate II) were also determined, and these confirmed the principal features of the binding site deduced from the peak height measurements. The long-chain compounds, however, bound considerably tighter to the intermediate II form of the enzyme, and this was shown to account for the biphasic decay kinetics that were observed with these compounds. Overall, there was poor agreement between the EC_{50} concentrations for inhibiting the luciferase enzyme from *V. harveyi* and those which induce general anesthesia in animals, with bulky compounds being much less potent, and moderately long chain alcohols being much more potent, as luciferase inhibitors than as general anesthetics.

Many years ago it was noted that the presence of relatively low levels of general anesthetics inhibited the light emitted by cultures of various bioluminescent bacteria (Harvey, 1915; Taylor, 1934; Johnson et al., 1951). Subsequently, it was shown that a good correlation existed between the concentrations required to reduce light output from these bacteria and those required to induce general anesthesia in animals (Halsey & Smith, 1970; White & Dundas, 1970). The possible importance of these findings for understanding the molecular mechanisms that underlie general anesthesia is obvious and further enhanced when it is appreciated that the remarkable observation of the pressure reversal of general anesthesia in animals (Johnson & Flagler, 1950) was stimulated by the observation of similar effects in bioluminescent bacteria (Johnson et al., 1942). It is all the more surprising, therefore, that studies of the effects of general anesthetics on the bacterial luciferase enzyme itself have been so few and far between, despite considerable advances in understanding the kinetics and catalytic mechanism of the uninhibited enzyme [see Ziegler and Baldwin (1981) and Hastings et al. (1985) for reviews].

The few studies that have been reported over the last 2 decades have employed relatively impure preparations of the enzyme and have used only a handful of anesthetic agents (White et al., 1973; Adey et al., 1976; Middleton & Smith, 1976a,b; Banks & Peace, 1985). An added confusion is that different species of bacteria have been used in the various studies, and it is known that, at least in some cases, there can be marked species variations [see, for example, Hastings et al. (1969)]. While these studies have shown clearly that the bacterial luciferase enzyme is sensitive to at least some general anesthetics and that the predominant mode of inhibition is competitive in nature, the impression that is often given is that all luciferase enzymes are very sensitive to general anesthetics and, moreover, that the anesthetic response of the bacterium derives almost entirely from the effects of anesthetics on the

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luciferase enzyme itself. Although for some anesthetics, such as diethyl ether, this may indeed be true (Middleton & Smith, 1976a,b), there is clear evidence that for other agents, such as halothane, there is a significant difference between the sensitivity of the intact bacterium and the luciferase enzyme itself (Middleton, 1973; Adey et al., 1976). For those interested in the interactions of general anesthetic molecules with proteins, what has been lacking is a systematic study of the effects of a large and diverse range of anesthetics on a highly purified preparation of the bacterial luciferase enzyme. This is what we have attempted, and the results are reported in this paper.

Experimental Procedures

Purification of Luciferase. The luciferase enzyme used in this work was extracted and purified from *Vibrio harveyi* bacteria (strain MB20). The methods for the growth of the bacteria and the preliminary stages of purification (cell lysis, batch adsorption with DEAE-cellulose, and the ammonium sulfate cut) were essentially the same as those described by Hastings et al. (1978). Further purification of the enzyme was achieved with three chromatography columns (a DEAE-Sephacel ion-exchange column, an ACA 34 gel filtration column, and a Pharmacia FPLC ion-exchange column loaded with Mono Q monobeads). Sodium dodecyl sulfate electrophoresis showed that the final enzyme was greater than 90% pure. The purified enzyme, which consists of an $\alpha\beta$ heterodimer with subunit molecular weights of about 40 000 and 36 000 for the α and β subunits, respectively (Cohn et al., 1985; Johnston et al., 1986), was stored as a stock solution (about 1 mg/mL) in 50 mM potassium phosphate–0.1 mM DTT at pH 7.0 and kept at −20 °C until use. In the experiments designed to compare the anesthetic sensitivities of the *V. harveyi* and *Vibrio fischeri* luciferase enzymes, partially purified luciferase preparations were obtained from Sigma Chemical Co. and used without further purification.

Chemicals. *n*-Decanal (*n*-decyl aldehyde) was obtained from Sigma and FMN (riboflavin 5′-monophosphoric acid, sodium salt) from BDH. Palladium (10%) on activated charcoal was supplied by Aldrich. Propane and hydrogen gases were purchased from British Oxygen Corp. Methoxyflurane, enflurane, and isoflurane were obtained from Abbott; halothane was supplied by ICI and fluroxene by Ohio Medical Products. All other anesthetic agents (including the other *n*-alcohols and *n*-alkanes) were the purest grades available from Sigma, Aldrich, and BDH.

Assays of Luciferase Activity. All experimental procedures were performed at 24 ± 1.5 °C. Luciferase activity was measured with an assay method based on the rapid injection of reduced flavin to initiate the luciferase reaction. The reduced flavin solution was first prepared by bubbling H_2 through an FMN solution containing 10% palladium on activated charcoal. This was filtered and then rapidly injected from a gas-driven syringe into a buffered solution of luciferase and *n*-decanal, with or without anesthetic. After the rapid injection, any $FMNH_2$ that is not bound to luciferase is oxidized in under 1 s (Gibson & Hastings, 1962). Enzyme-bound flavin (intermediate I) reacts with oxygen to form the relatively long lived dihydro-4a-peroxyflavin intermediate II (Hastings et al., 1973; Vervoort et al., 1986). This latter intermediate binds *n*-decanal reversibly, and the resulting complex decays to give FMN, H_2O, *n*-decanoic acid, and blue-green light. The rapid oxidation of unbound flavin immediately after injection prevents turnover, so that the light output from the reaction, amplified with a photomultiplier tube (placed at the base of a glass reaction vial) and a current–voltage converter, rises rapidly to a peak and then decays slowly. Luciferase activity was taken as the peak light intensity (the maximum rate of photon emission); no attempt was made to measure absolute intensities. Final luciferase and flavin concentrations were typically 0.4 nM and 100 μM, respectively, in 50 mM potassium phosphate buffer at pH 7.0. Since activated charcoal adsorbs organic compounds, sufficient FMN was added to the reduction vessel to compensate for adsorption and sufficient time allowed for equilibration before the start of the experiment. The 100 μM level of flavin was chosen to saturate the $FMNH_2$ binding site on the luciferase enzyme (Meighen & Hastings, 1971). *n*-Decanal concentrations were varied between 0.25 and 1.7 μM in experiments performed to obtain double-reciprocal plots; for anesthetic dose–response determinations, the decanal concentration used was at, or close to, its K_m (0.85 μM, see below).

Anesthetics were added to assays either in buffered aqueous solutions or as concentrated ethanolic solutions. The final ethanol concentration never exceeded 1.5% of the EC_{50} concentration for inhibition of luciferase; nonetheless, the appropriate concentration of ethanol was present in the controls. Considerable care was taken with volatile anesthetics to minimize loss by evaporation. Solutions of volatile agents were prepared in capped vials shortly before each experiment and transferred to a 20-mL glass, gas-tight syringe. Appropriate volumes of the anesthetic solution were delivered to the reaction vials immediately prior to each assay. Aliquots of a halothane solution taken from this syringe over a 30-min period (the maximum period of use for a given syringe) and assayed spectrophotometrically showed no detectable loss of anesthetic.

Analysis of Anesthetic Dose–Response Data. Although it should be stressed that the bacterial luciferase reaction is complex and anesthetics could act at several points in the light and dark reaction pathways, our results (see below) suggest that a simple binding model can be used which is sufficient to account for our inhibition data. For a competitive inhibitor it is convenient (Franks & Lieb, 1984) to define a function $f(A)$, which is the factor by which the apparent Michaelis constant K_m^{app} changes with anesthetic concentration [A]; i.e., $K_m^{app} = f(A)K_m$, where K_m is the Michaelis constant. At a fixed substrate concentration [S], $f(A)$ is given by

$$f(A) = \frac{[S] + K_m}{K_m}\left(\frac{v_0}{v_i} - \frac{[S]}{[S] + K_m}\right) \quad (1)$$

where v_0/v_i is the ratio of control to inhibited luciferase activities at an anesthetic concentration [A].

In order to make a comparison between anesthetic concentrations needed to inhibit an enzyme and those required to induce general anesthesia in animals, it is essential to choose a standard substrate concentration in the enzyme measurements since, for a competitive inhibitor, the substrate levels affect the degree of enzyme inhibition. We have chosen to define an anesthetic EC_{50} concentration as the concentration of anesthetic required to half-inhibit the enzyme when the concentration of the competing substrate [S] is equal to its K_m (Franks & Lieb, 1984). This anesthetic EC_{50} concentration is simply related to the dissociation constant K_i in a way that depends upon the number of anesthetic molecules n involved in the inhibition.

If only a single molecule is involved in the inhibition, then it is easy to show that

$$f(A) = 1 + [A]/K_i \quad (2)$$

and

$$EC_{50} = 2K_i \quad (3)$$

where K_i is the inhibition constant (i.e., the enzyme–inhibitor equilibrium dissociation constant). To obtain a value for K_i and its associated standard error, $f(A)$ was plotted against [A] and a straight line fitted with the method of weighted least squares (Cleland, 1967). The weights used were derived by assuming a constant percentage error in the measurement of enzyme activity and are given by

$$w(A) = \frac{1}{\{f(A) + [S]/K_m\}^2} \quad (4)$$

K_i is then given by the ratio of the intercept on the y axis (c) and the slope (m) of the straight line (eq 2)

$$K_i = c/m \quad (5)$$

with a standard error given by

$$SE(K_i) = (1/m)[\text{Var}(c) - 2K_i \text{Cov}(c,m) + K_i^2 \text{Var}(m)]^{1/2} \quad (6)$$

where the variance and covariance terms in c and m are the diagonal and off-diagonal elements, respectively, of the variance–covariance matrix.

If two molecules are involved in the inhibition, then it can be shown (Franks & Lieb, 1984) that, if each molecule binds with the same inhibition constant K_i and if only one molecule is necessary to cause inhibition, $f(A)$ is given by

$$f(A) = (1 + [A]/K_i)^2 \quad (7)$$

and

$$EC_{50} = (\sqrt{3} - 1)K_i \quad (8)$$

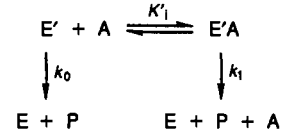
In this case, the square root of $f(A)$ was plotted against [A] and a straight line fitted with weights given by

$$w(A) = \frac{f(A)}{\{f(A) + [S]/K_m\}^2} \quad (9)$$

Thereafter, the procedure to obtain a value for K_i and its associated standard error was exactly as above. In most cases, K_i values were determined repeatedly and the means and their errors calculated; these data were then used to obtain values and standard errors for the anesthetic EC_{50} concentrations.

Measurement of the Lifetime of Intermediate II. Intermediate II, formed by mixing luciferase, $FMNH_2$, and O_2 in the absence of added aldehyde, decays spontaneously to enzyme, FMN, and H_2O_2 with a half-life of around 20 s at 20 °C (Hastings et al., 1985). The drop in bioluminescent activity of a sample of intermediate, monitored by mixing of successive aliquots with *n*-decanal, allows the rate of decay of intermediate II to be determined. The experimental protocol used to measure the lifetime of intermediate II was adapted from the method of Tu (1979); a 10-μL droplet of luciferase stock solution was placed at the bottom of a cuvette and 400 μL of buffered $FMNH_2$ (catalytically reduced as above) rapidly injected by hand to form intermediate II. This was immediately followed by the addition of either 1.6 mL of buffer (for controls) or a solution of anesthetic. Final luciferase and flavin concentrations were 0.55 nM and 100 μM, respectively, in the same phosphate buffer as above. (Flavin concentrations greater than 200 μM were observed to cause significant stabilization of the intermediate.) From each sample of intermediate, four 0.4-mL aliquots were taken and added, at intervals of approximately 1 min, to 4.6 mL of buffer and assayed for bioluminescent activity by rapid injection of 2.5 mL of a buffered 60 μM solution of *n*-decanal. (This dilution was chosen in order to minimize the inhibitory effects of the final concentration of the anesthetic on enzyme activity.) The decline in the peak intensity of successive aliquots, which reflects the decay of intermediate II, was found to be exponential and was retarded by the addition of anesthetics.

A scheme involving the dark reaction pathways, which was used to analyze the stabilizing effects reported here for anesthetic, has been previously proposed by Tu (1979):



The anesthetic (A) binds to intermediate II (E′) with a dissociation constant K_i' and forms a complex, E′A. k_0 and k_1 are the rate constants for the decay of E′ and E′A, respectively, where $k_0 \gg k_1$. The intermediate decays to yield the enzyme E and reaction products P (FMN and H_2O_2). This model, which assumes only that the on and off rates for the binding of A to E′ are faster than k_0, gives as the characteristic decay time τ for the decay of the total concentration of intermediate II (=[E′] + [E′A])

$$\tau = \frac{K_i' + [A]}{k_0 K_i' + k_1[A]} \quad (10)$$

In order to permit a simple linear regression analysis of dose–response stabilization data, we define the variable $\Delta\tau$:

$$\Delta\tau \equiv \tau - \tau_0 \quad (11)$$

where τ_0 ($\equiv 1/k_0$) is the characteristic decay time in the absence of inhibitor. If we also define $\tau_1 \equiv 1/k_1$, then from eqs 10 and 11 it follows that

$$\frac{1}{\Delta\tau} = \frac{(\tau_1/\tau_0)K_i'}{\tau_1 - \tau_0}\frac{1}{[A]} + \frac{1}{\tau_1 - \tau_0} \quad (12)$$

To obtain values for K_i' and τ_1, $1/\Delta\tau$ was plotted against $1/[A]$ and a straight line fitted with the method of weighted least squares (Cleland, 1967). The weights were $(\Delta\tau)^4/([SE(\tau)]^2 + [SE(\tau_0)]^2)$, where SE ($\tau$) and SE ($\tau_0$) are the standard errors in τ and τ_0, respectively. K_i' and τ_1 were obtained from the slope (m) and the intercept on the y axis (c) of the straight line (eq 12) as follows:

$$K_i' = m\tau_0/(1 + \tau_0 c) \quad (13)$$

and

$$\tau_1 = \tau_0 + 1/c \quad (14)$$

The standard errors in these parameters were obtained from the variance of τ_0 [Var (τ_0)] in control experiments and the variance and covariance terms of the regression variance–covariance matrix as follows:

$$SE(K_i') = (K_i'^2/m)[\text{Var}(\tau_0)/\tau_0^4 + \text{Var}(c) + \text{Var}(m)/K_i'^2 - 2\,\text{Cov}(c,m)/K_i']^{1/2} \quad (15)$$

and

$$SE(\tau_1) = [\text{Var}(\tau_0) + \text{Var}(c)/c^4]^{1/2} \quad (16)$$

Assay of NADH:FMN Oxidoreductase Activity. An NADH:FMN oxidoreductase enzyme, which catalyzes the reduction of FMN to $FMNH_2$ (Duane & Hastings, 1975), was present in the luciferase preparations supplied by Sigma. The activity of the oxidoreductase was assayed by allowing the $FMNH_2$ that it produces to participate in the luciferase-catalyzed luminescent reaction, under conditions where the inhibitory site on the luciferase enzyme was largely blocked by a high concentration of the *n*-decanal substrate. The reaction was initiated by the rapid injection of FMN into a

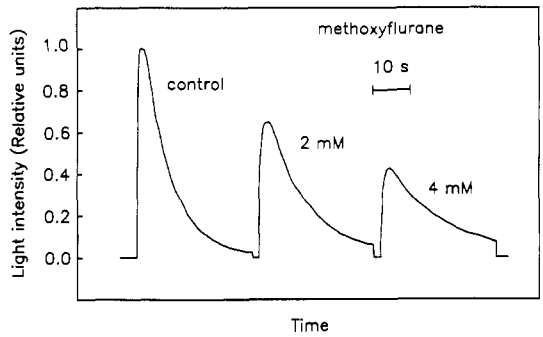


FIGURE 1: Time course of the light output from the $FMNH_2$-initiated luciferase reaction. The effect of increasing concentrations of methoxyflurane, as with most agents, is to decrease the peak height while also slowing the kinetics of the luminescence. Final concentration of *n*-decanal was 1.1 μM.

solution containing the luciferase/oxidoreductase mixture, *n*-decanal, and NADH (Boehringer Mannheim), with or without anesthetic. (NADH was added immediately prior to the reaction in order to minimize its degradation.) Final concentrations were 98 μM FMN, 4.25 μM *n*-decanal, and 0.2 mM NADH, in 50 mM potassium phosphate at pH 7.0. Following injection of FMN, the light intensity rose slowly to a maximum (after about 15 s) and then decayed slowly as the NADH was consumed, with a half-time of about 6 min. The maximum in the light intensity was taken as a measure of the activity of the oxidoreductase. The small reduction in light intensity due to direct effects on the luciferase enzyme was corrected for with the measured values of the *n*-decanal K_m and anesthetic K_i.

RESULTS

Anesthetic Inhibition of the Purified Luciferase Enzyme from V. harveyi. Under conditions where the flavin site was saturated, all of the anesthetics tested inhibited luciferase activity, provided the concentration of the *n*-decanal substrate was not large compared to its K_m. Almost all the anesthetics inhibited activity in a manner exemplified by the data for methoxyflurane, shown in Figure 1. In addition to the decrease in the peak light intensity with increasing anesthetic concentration, we also observed a slight increase in the time taken to reach the peak, together with a marked decrease in the rate of decay of the luminescence following the peak (see later). Similar effects were caused by reducing the *n*-decanal concentration, suggesting a competitive interaction with this substrate.

For a small number of agents, we observed a significant increase in luciferase activity at relatively low concentrations, although these agents also inhibited light output at higher concentrations. This enhancement in activity was accompanied by an *increased* rate of luminescent decay following the peak of intensity. Ethanol, *n*-propan-1-ol, and acetone exhibited this behavior, causing maximum stimulation of enzyme activity at 700, 200, and 100 mM, respectively. Similar behavior has been reported previously for these agents in a study of the luciferase enzyme from *V. fischeri* (Hastings et al., 1966). Inhibition by *n*-butan-1-ol was observed only at concentrations exceeding 30 mM; below this concentration butanol had no effect. This was probably because any stimulation of the enzyme activity by this agent was counteracted by its simultaneous inhibitory effect. Significantly, short-chain *n*-alkanes, such as propane, did not stimulate luciferase activity, suggesting that the excitation produced by ethanol, *n*-propan-1-ol, and acetone is mediated by interactions at small and relatively polar sites on the protein. However, chloroform

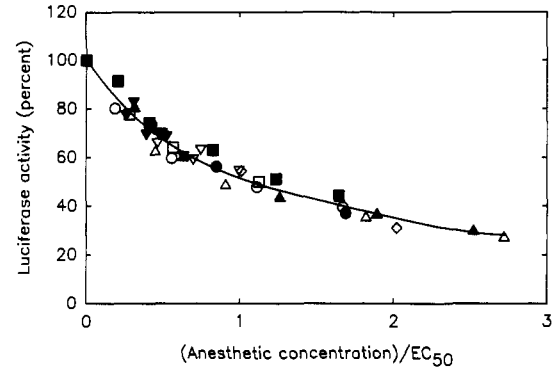


FIGURE 2: Normalized dose–response curve for luciferase activity (i.e., the peak intensity) in the presence of anesthetics is the same despite significant differences in the kinetics of the luminescent decay. The peak intensity is plotted against inhibitor concentration scaled as multiples of the EC_{50} concentration for a selection of anesthetics including short- and long-chain *n*-alcohols and *n*-alkanes: (▼) halothane; (▽) methoxyflurane; (◇) paraldehyde; (○) *n*-hexan-1-ol; (●) *n*-octan-1-ol; (△) *n*-decan-1-ol; (▲) *n*-dodecan-1-ol; (□) n-tetradcan-1-ol; (■) n-heptane. The final luciferase and *n*-decanal concentrations were 0.4 nM and 1.1 μM, respectively.

also produced a small degree of excitation (about 10% at 8.8 mM) before inhibiting activity at higher concentrations. Chloroform was unique among the anesthetics tested in that it also caused an extremely rapid spike of activity, which occurred considerably before the main maximum of light output, peaking in about 200 ms and rapidly decaying with a half-life of about 200 ms. These various excitatory effects could be due to the anesthetics acting at numerous points in the reaction pathways (Ziegler & Baldwin, 1981), and a detailed molecular interpretation is currently not possible.

For *n*-alcohols longer than *n*-decan-1-ol and *n*-alkanes longer than *n*-nonane, the decay kinetics changed significantly; the depression of the peak intensity was accompanied by a distinctly biphasic profile of luminescence decay (see inset to Figure 8). Increasing the concentration of these long-chain inhibitors was observed to accelerate the initial phase of decay while greatly retarding the final phase. (*n*-Undecan-1-ol and *n*-decane, the first members in this group, induced biphasic luminescence decay but did not significantly accelerate the initial phase of decay.) The long-chain alkanes *n*-tridecane and *n*-tetradecane did not measurably affect luciferase activity.

Reversibility of the effects of anesthetic binding was tested by exposing the luciferase enzyme to high anesthetic concentrations before dilution and comparing the results of these assays with those in which the enzyme had only been exposed to the dilute anesthetic levels. In all cases except one, the two assay methods gave identical results. The exception was ethanol, for which a small degree of irreversibility was noted at high concentrations ($\sim$1 M).

Figure 2 shows the normalized dose–response curve for the inhibition of peak light intensity. It can be seen that, despite the different effects on the kinetics of luminescent decay mentioned above (and discussed further below), a diverse range of anesthetics depressed the peak light intensity with an essentially identical normalized dose–response curve.

The mechanism of inhibition was determined by measuring luciferase activities over a range of *n*-decanal concentrations in the presence and absence of the anesthetics. From the control experiments, the K_m of *n*-decanal was determined to be 0.85 $\pm$ 0.08 μM (mean $\pm$ SE) [this compares with a K_m of 1.1 μM determined previously (Meighen & Mackenzie, 1973) under essentially the same conditions in 20 mM Bis-Tris]. Double-reciprocal plots were linear and showed that the inhibition was competitive in nature—most anesthetics had

Table I: EC_{50} Concentrations for Inhibition of Luciferase Enzyme from *V. harveyi* and Dissociation Constants for the Binding of Anesthetics to the Normal Form (K_i) and to the Intermediate II Form (K'_i) of the Enzyme[a]

agent	EC_{50}	K_i	K'_i	n
acetone (**1**)[b]	505 ± 78 mM			>2
ethanol (**2**)[b]	1.56 ± 0.22 M			>2
n-propan-1-ol (**3**)[b]	541 ± 108 mM			>2
n-butan-1-ol (**4**)[b]	79.1 ± 6.7 mM			2
urethane (**5**)	225 ± 53 mM			>2
diethyl ether (**6**)	24.0 ± 2.2 mM	32.8 ± 3.0 mM		2
butanone (**7**)	37.2 ± 1.3 mM	50.8 ± 1.9 mM		2
paraldehyde (**8**)	39.7 ± 1.2 mM	54.2 ± 1.7 mM		2
n-pentan-1-ol (**9**)	5.78 ± 0.04 mM	2.89 ± 0.02 mM		1
n-hexan-1-ol (**10**)	0.738 ± 0.026 mM	0.369 ± 0.013 mM	0.240 ± 0.030 mM	1
n-heptan-1-ol (**11**)	62.4 ± 11.2 μM	31.2 ± 5.6 μM	33.1 ± 1.9 μM	1
n-octan-1-ol (**12**)	8.88 ± 0.58 μM	4.44 ± 0.29 μM	4.07 ± 0.10 μM	1
n-nonan-1-ol (**13**)	7.90 ± 0.54 μM	3.95 ± 0.27 μM	4.20 ± 0.29 μM	1
n-decan-1-ol (**14**)	2.29 ± 0.16 μM	1.14 ± 0.08 μM	0.880 ± 0.042 μM	1
n-undecan-1-ol (**15**)	0.750 ± 0.048 μM	0.375 ± 0.024 μM	0.119 ± 0.010 μM	1
n-dodecan-1-ol (**16**)[c]	0.482 ± 0.048 μM	0.241 ± 0.024 μM	6.68 ± 1.17 nM	1
n-tridecan-1-ol (**17**)[c]	0.322 ± 0.034 μM	0.161 ± 0.017 μM	7.90 ± 0.39 nM	1
n-tetradecan-1-ol (**18**)[c]	0.356 ± 0.010 μM	0.178 ± 0.005 μM	7.69 ± 0.84 nM	1
n-pentadecan-1-ol (**19**)[c]	0.510 ± 0.020 μM	0.255 ± 0.020 μM	13.3 ± 1.6 nM	1
n-hexadecan-1-ol (**20**)[c]	0.564 ± 0.142 μM	0.282 ± 0.071 μM	37 ± 32 nM	1
propane (**21**)	5.9 ± 1.6 mM	8.1 ± 2.2 mM		2
n-pentane (**22**)	0.542 ± 0.040 mM	0.271 ± 0.020 mM		1
n-hexane (**23**)	61.6 ± 6.2 μM	30.8 ± 3.1 μM		1
n-heptane (**24**)	9.44 ± 0.10 μM	4.72 ± 0.05 μM		1
n-octane (**25**)	2.04 ± 0.06 μM	1.02 ± 0.03 μM		1
n-nonane (**26**)	0.608 ± 0.022 μM	0.304 ± 0.011 μM		1
n-decane (**27**)	0.246 ± 0.018 μM	0.123 ± 0.009 μM		1
n-undecane (**28**)[c]	0.122 ± 0.011 μM	61.0 ± 5.5 nM	1.44 ± 0.17 nM	1
n-dodecane (**29**)[c]	0.080 ± 0.034 μM	40 ± 17 nM		1
chloroform (**30**)[b]	21.2 ± 3.5 mM			2
halothane (**31**)	11.4 ± 1.4 mM	5.69 ± 0.72 mM		1
methoxyflurane (**32**)	4.28 ± 0.42 mM	2.14 ± 0.21 mM		1
fluroxene (**33**)	5.62 ± 0.12 mM	2.81 ± 0.06 mM		1
enflurane (**34**)	10.9 ± 1.3 mM	5.44 ± 0.64 mM		1
isoflurane (**35**)	13.2 ± 2.3 mM	6.58 ± 1.15 mM		1
benzyl alcohol (**36**)	13.5 ± 1.5 mM	6.75 ± 0.76 mM		1

[a] The errors are standard errors in the mean, and n is the number of molecules involved in the inhibition. [b] These agents stimulated activity at low concentrations. [c] These agents caused marked biphasic luminescence decay.

no statistically significant effect on V_{max}. These results are consistent with those of other laboratories (Adey et al., 1976; Middleton & Smith, 1976b; Banks & Peace, 1985). Notably, even long-chain agents, which produced biphasic luminescence decay, were found to be competitive inhibitors. Only for those compounds that were observed to cause excitation at low levels was V_{max} found to be increased significantly, but even in these cases the inhibition at higher concentrations appeared to be largely competitive in nature. The finding that the enzyme inhibition can be simply accounted for by the anesthetics competing for the binding of the aldehyde substrate suggests that, despite the overall complexity of the luciferase reaction pathways, a simple interpretation of the inhibition data in terms of a common mode of action is possible. This argument is supported by the data of Figure 2, which show an essentially identical dose–response curve for a diverse range of anesthetics. For these reasons we have interpreted our data using a simple binding model.

The number of anesthetic molecules (n) which are involved in the inhibition is reflected in the dependence of the function $f(A)$ upon anesthetic concentration [A] (see Experimental Procedures). If a single inhibitor molecule binds to the protein, then this function $f(A)$ increases linearly with [A] (eq 2). Most of the anesthetics tested showed this pattern of inhibition. This is exemplified by the data for fluroxene, shown in Figure 3a. For some relatively small anesthetics, such as ether and butanone, $f(A)$ increased parabolically such that a plot of $[f(A)]^{1/2}$ vs [A] was linear, consistent with eq 7, suggesting that two molecules were involved in the inhibition. Typical data showing $f(A)$ and $[f(A)]^{1/2}$ for butanone are plotted in Figure 3b. For small, but very weak, inhibitors, such as urethane, the $f(A)$ curve increases more rapidly (see Figure 3c) than that predicted by eq 7 (so that even $[f(A)]^{1/2}$ vs [A] is nonlinear), suggesting the presence of more than two inhibitory sites on the luciferase enzyme. Finally, Figure 3d shows the behavior of $f(A)$ and $[f(A)]^{1/2}$ for an anesthetic, *n*-propan-1-ol, that causes excitation at lower levels but inhibition at higher concentrations. From the steepness of the $f(A)$ curve at higher concentrations, it seems likely that, in addition to the site or sites underlying excitation, more than two inhibitory sites are also available for this agent.

As with firefly luciferase, the finding that multiple inhibitory binding sites are available only to small molecules suggests that these sites are contained within a single larger region, namely, a hydrophobic pocket (Franks & Lieb, 1984). A rigorous analysis of the $f(A)$ curves obtained for the inhibition of bacterial luciferase by ethanol, *n*-propan-1-ol, *n*-butan-1-ol, acetone, and chloroform is not possible because the curves are distorted by the excitatory effects observed at low concentrations; only the EC_{50} concentrations were determined for these agents. The inhibition due to propane was not sufficient for a determination of the order of the $f(A)$ plot; the K_i of this *n*-alkane was estimated by extrapolation, assuming that two propane molecules can bind to the enzyme. Values for EC_{50} concentrations and, where possible, the inhibition constants, K_i, are given in Table I for all the agents tested.

Because of their homology with the long-chain aliphatic substrate of luciferase (*n*-decanal), the *n*-alcohols and *n*-alkanes might be considered to be special cases. The inhibition constants, K_i, determined for *n*-alcohols and *n*-alkanes acting on

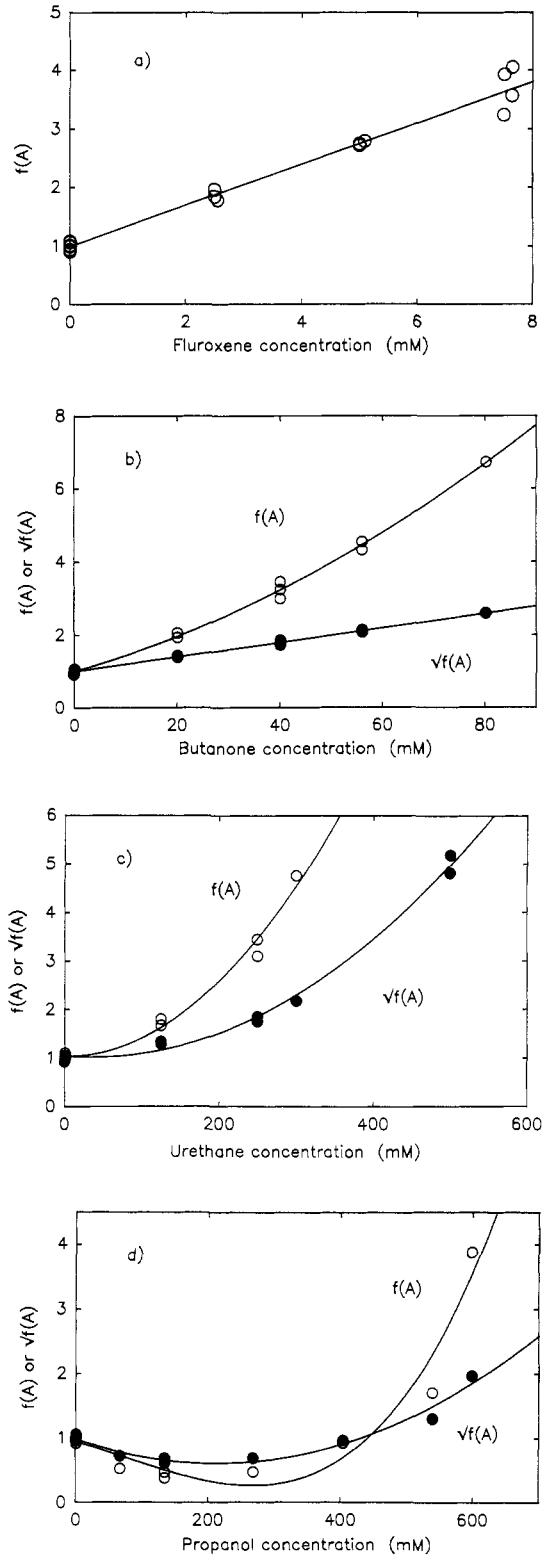


FIGURE 3: Inhibition of luciferase activity plotted as the function $f(A)$ or $[f(A)]^{1/2}$. Note that some data points are superimposed. (a) For anesthetics such as fluroxene, as with most agents, a plot of $f(A)$ vs [A] is linear, implying a single molecule binds to the enzyme. (b) For small compounds such as butanone, two molecules are involved in the inhibition and the plots of $f(A)$ vs [A] are parabolic. Consequently, a plot of $[f(A)]^{1/2}$ vs [A] is linear. (c) Very weak, polar inhibitors such as urethane show $f(A)$ curves that rise more steeply than the square of the inhibitor concentration, $[A]^2$. This implies the presence on the enzyme of more than two weak binding sites. (d) Some small polar compounds caused a significant increase in activity at lower levels before inhibiting at higher concentrations. The effects of such an agent, *n*-propan-1-ol, on plots of $f(A)$ and $[f(A)]^{1/2}$ vs [A] are complex and prevent a reliable assessment of the number of anesthetic molecules involved in the interactions with the enzyme.

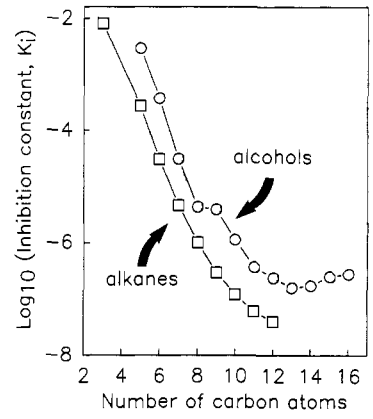


FIGURE 4: Inhibition constants K_i (in molar units) for the homologous series of *n*-alcohols (○) and *n*-alkanes (□) acting on luciferase. The K_i values and their associated standard errors are given in Table I.

bacterial luciferase are plotted in Figure 4 against the number of carbon atoms. For both homologous series, K_i decreases as the apolar methylene chain length increases, and for any chain length, the *n*-alcohol binds less tightly to the enzyme (i.e., with a higher K_i) than the corresponding *n*-alkane. Both of these features are indicative of the hydrophobic nature of the inhibitor binding site. Overall, the *n*-alcohol and *n*-alkane K_i curves follow roughly parallel courses, and both tend to level off after about C_{11}. *n*-Alcohols longer than *n*-undecan-1-ol and *n*-alkanes longer than *n*-undecane, even though they are more hydrophobic, do not bind significantly tighter to luciferase.

Stabilization of Intermediate II by n-Alcohols and n-Alkanes. Previous work has shown that intermediate II is stabilized by a variety of compounds (Baumstark et al., 1979; Tu, 1979). This provides an additional method for probing the interactions of *n*-alcohols and *n*-alkanes with luciferase. Stabilization was measured by following the decay in bioluminescent activity in aliquots of a sample of intermediate II prepared in a given concentration of *n*-alcohol or *n*-alkane. The progressive retardation of the decay of intermediate II by increasing concentrations of *n*-pentadecan-1-ol is illustrated in Figure 5a. For each inhibitor concentration, the first-order time constant (τ) was determined by the method of least squares. This time constant, characteristic of the intermediate lifetime, was found to have a dependence on inhibitor concentration, which is predicted by eq 10. *n*-Alcohols from *n*-hexan-1-ol to *n*-hexadecan-1-ol and one *n*-alkane, *n*-undecane, were tested and all found to behave in the same way.

The time constant in the absence of anesthetic, τ_0, was measured directly from the control data and found to be 16.1 ± 0.3 s (mean ± SE) at 25 °C. Plots of $1/\Delta\tau$ vs $1/[A]$ gave straight lines (see, e.g., Figure 5b), as predicted by eq 12, from which, knowing τ_0, values of K'_i and τ_1 and their associated standard errors were determined (see Experimental Procedures).

The time constant τ_1 for the decay of the intermediate II–alcohol complex (E′A) did not vary systematically with the *n*-alcohol chain length and did not reflect the variations reported by Tu (1979); an average value for τ_1 of 232 ± 39 s (mean ± SE) was calculated for the 11 *n*-alcohols studied. The variation of the dissociation constants K'_i determined for the stabilizing interaction of *n*-alcohols with intermediate II is given as a function of chain length in Figure 6. [Our values of K'_i agree reasonably well with those observed by Tu (1979) for C_8, C_{10}, C_{12}, and C_{14} *n*-alcohols, although Tu's K'_i values are, in general, somewhat higher—due, perhaps, to the presence in his assay mixture of bovine serum albumin, which

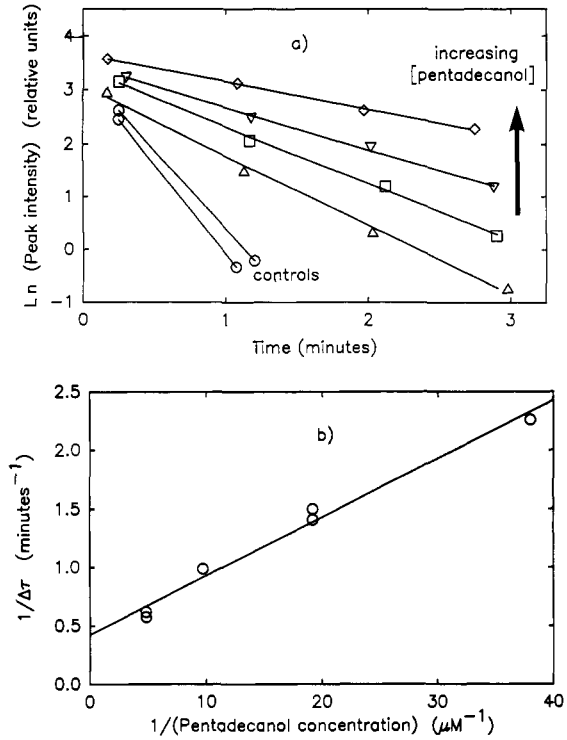


FIGURE 5: (a) Stabilization of intermediate II by *n*-pentadecan-1-ol. The data show the time-dependent decay in the luminescent activity of aliquots of intermediate II prepared in the presence of a range of *n*-pentadecan-1-ol concentrations: (○) control; (△) 26 nM; (□) 52 nM; (▽) 103 nM; (◇) 207 nM. Decay time constants (τ) were determined at each concentration from the reciprocal of the slope of the straight line fitted to the data by the method of least squares. (b) Plots of $1/\Delta\tau$ against $1/[A]$ gave straight lines. The gradient and intercept were determined with the method of weighted least squares and used to calculate K'_i and τ_1 and their associated standard errors (see Experimental Procedures for details).

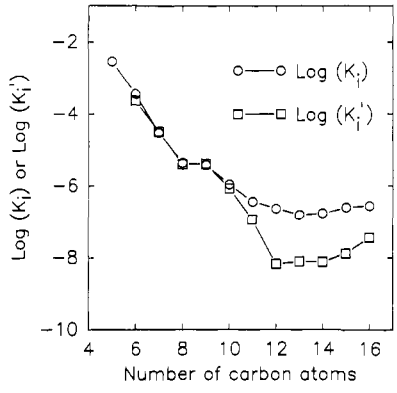


FIGURE 6: Dissociation constants (in molar units) determined from stabilization (K'_i) and inhibition (K_i) experiments for the homologous series of *n*-alcohols. K'_i (□); K_i (○). Values for K'_i and K_i and their associated standard errors are given in Table I.

might have bound some alcohol and thus reduced the free aqueous concentration.] For purposes of comparison, the corresponding dissociation constants, K_i, determined from the luciferase inhibition experiments are also plotted in Figure 6. For C_6 to C_{10} *n*-alcohols there is close agreement between K'_i and K_i values, even including the leveling out between *n*-octan-1-ol and *n*-nonan-1-ol. The surprising result shown in Figure 6 is that beyond C_{10} the K'_i and K_i curves diverge rapidly; between C_{10} and C_{12} the difference between K'_i and K_i expands to a factor of about 25. After C_{12} the K'_i curve levels off abruptly, so that the K'_i values for *n*-dodecan-1-ol, *n*-tridecan-1-ol, *n*-tetradecan-1-ol, and *n*-pentadecan-1-ol are all similar. This leveling out in K'_i values roughly mirrors that

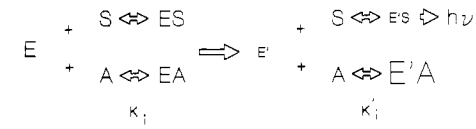


FIGURE 7: Schematic representation of the transition in the binding equilibrium of long-chain *n*-alcohols upon conversion of luciferase (E) to the luciferase–peroxyflavin complex, intermediate II (E'). S is the substrate *n*-decanal and A is a long-chain *n*-alcohol. Upon injection of $FMNH_2$, E converts to E', which is presumed to have a greater increase in affinity for A than for S. This shifts the equilibrium dramatically in favor of the complex E'A compared to E'S. This model accounts for the biphasic luminescence decay observed with long-chain *n*-alcohols and *n*-alkanes. (The relative sizes of the symbols for the enzyme and enzyme complexes are intended to represent schematically their relative concentrations.) See text for details.

seen in the K_i curve. These data show that for *n*-alcohols longer than *n*-decan-1-ol, intermediate II binds inhibitor molecules very much tighter than the initial form of the enzyme (i.e., $K'_i \ll K_i$).

The biphasic luminescence decay in luciferase assays inhibited by long-chain *n*-alcohols is probably a direct consequence of the ability of intermediate II to bind long-chain compounds so tightly. Consider an assay in which, prior to initiation, luciferase (E) in the reaction vial is equilibrated with a long-chain *n*-alcohol (A) and a subsaturating concentration of the substrate, *n*-decanal (S)—see LHS of Figure 7. Upon injection of $FMNH_2$ the luciferase in the vial is rapidly transformed into intermediate II. As a result, those enzyme molecules that have *n*-decanal bound proceed along the reaction pathway, causing a rapid rise in luminescence. At the same time, however, the conversion of luciferase to intermediate II (E') greatly enhances the affinity for the long-chain *n*-alcohol—an enhancement that is assumed to greatly exceed any increase in the affinity for the *n*-decanal substrate. The binding equilibrium is thus shifted heavily in favor of the intermediate II–alcohol complex, severely reducing the active pool of intermediate II–*n*-decanal complexes and therefore leading to a rapid drop in luminescence—the initial phase of luminescence decay. Soon thereafter a new equilibrium is established, determined by the new *n*-decanal and *n*-alcohol dissociation constants (RHS of Figure 7). In inhibition experiments, the *n*-alcohol concentrations used ranged typically from 1–6 times the value of K_i, which, for the long-chain alcohols, corresponds to about 25–150 times K'_i. Since the *n*-decanal concentration was normally close to the K_m, the new equilibrium is dominated almost entirely by the intermediate II–alcohol complex, and the reaction rate is limited by the release of intermediate II molecules from sequestration within intermediate II–alcohol complexes. This gives rise to the slow, second phase of luminescence decay.

This model naturally accounts for the finding that increasing the concentration of long-chain *n*-alcohols increased the rate constant of the initial phase of luminescence decay, since the higher the *n*-alcohol concentration, the higher will be the rate of sequestration of intermediate II molecules into complexes with the *n*-alcohol. Two simple and testable predictions also derive from this model. First, only monophasic decay should be seen following the injection of *n*-decanal into a vial containing intermediate II in equilibrium with a long-chain *n*-alcohol. In this case, since conversion of luciferase to the high-affinity form (intermediate II) is complete before initiation of the luminescence reaction, one would not expect to observe the initial fast rise and fall of luminescence which is associated with the shift in binding affinities that accompanies this conversion in $FMNH_2$-initiated reactions. Second, one would also predict that the decay of luminescence in *n*-deca-

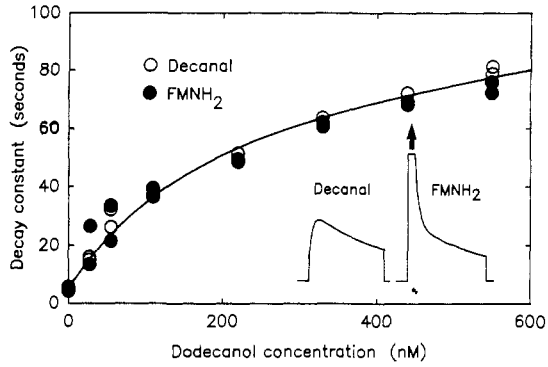


FIGURE 8: The time constant of the final phase of the $FMNH_2$-initiated luciferase reaction (●) varies in exactly the same way with *n*-dodecan-1-ol concentration as the decay constant measured for *n*-decanal-initiated reactions (○). Note that some data points are superimposed. The inset shows a comparison of the time dependence of the light output from luciferase reactions initiated by $FMNH_2$ and *n*-decanal in the presence of a final *n*-dodecan-1-ol concentration of 0.55 μM. The experimental conditions, except for the order of addition of substrates, were identical for the two assays. Solutions containing 2.56 μM *n*-decanal and 329 μM $FMNH_2$ were used in both cases. $FMNH_2$ initiation: 2.5 mL of $FMNH_2$ solution was injected into a vial containing 2.5 mL of *n*-decanal solution, 2.5 mL of a solution of *n*-dodecan-1-ol, and 10 μL of luciferase stock solution. *n*-Decanal initiation: 2.5 mL of *n*-decanal solution was injected into a solution of intermediate II prepared by injection of 2.5 mL of $FMNH_2$ solution into 2.5 mL of the *n*-dodecan-1-ol solution in the presence of 10 μL of luciferase stock solution. Final concentrations in 50 mM phosphate buffer at pH 7.0 were 0.2 nM luciferase, 0.85 μM *n*-decanal, and 110 μM $FMNH_2$. Note that, in the inset, the trace from the *n*-decanal-initiated reaction has been reduced 10-fold in the vertical direction compared to that for the $FMNH_2$-initiated assay in order to aid the comparison. Note also that the peak of the $FMNH_2$-initiated assay is off-scale.

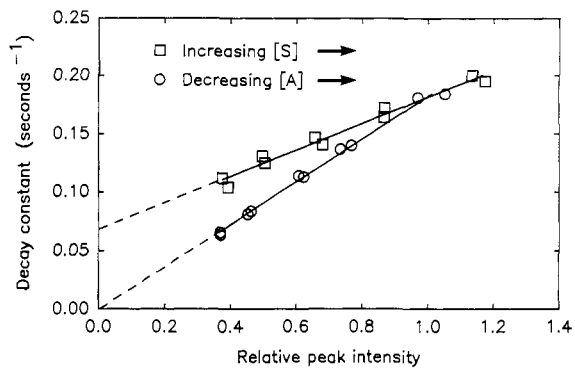


FIGURE 9: Relationship between the peak intensity and the luminescence decay constant as either the substrate level ([S]) or inhibitor concentration ([A]) (in this case *n*-heptan-1-ol) is changed. The relative peak intensities have been normalized so that a *n*-decanal concentration of 1.1 μM at 25 °C gives unit peak intensity. (□) Effect of increasing the *n*-decanal concentration from 0.25 to 1.67 μM; (○) effect of reducing the *n*-heptan-1-ol concentration from 0.14 to 0 mM in the presence of 1.1 μM *n*-decanal. Notice that, as [S] is reduced and as [A] is increased, the observed decay constants extrapolate to values close to those observed independently from stabilization experiments for k_0 ($=1/\tau_0 = 0.062\ s^{-1}$) and k_1 ($=1/\tau_1 = 0.004\ s^{-1}$), respectively (see text). Note that some data points are superimposed.

nal-initiated reactions should proceed with the same rate constant as the final phase of the biphasic luminescence decay observed in the $FMNH_2$-initiated reaction (provided the concentrations of enzyme, substrates, and inhibitor were the same in both assays). These predictions were tested experimentally for *n*-dodecan-1-ol. The inset to Figure 8 shows that, compared to the characteristically biphasic profile observed with the flavin-initiated reaction, the *n*-decanal-initiated light output has a monophasic decay. Figure 8 shows, also as predicted, that the late phase of the flavin-initiated reaction decays at the same rate as the luminescence following *n*-decanal injection. These observations can be taken as supporting evidence that the intermediate II form of the luciferase enzyme has a much higher affinity for long-chain *n*-alcohols than the initial form of the enzyme.

The dissociation constant determined for *n*-undecane from stabilization experiments ($K'_i = 1.4 \pm 0.2$ nM; mean ± SE) was found, like that of *n*-dodecan-1-ol, *n*-tridecan-1-ol, *n*-tetradecan-1-ol, and *n*-pentadecan-1-ol, to be very much lower than the K_i (61 ± 6 nM), indicating that the hypothesis developed above to account for the biphasic luminescence decay induced by long-chain *n*-alcohols may also be applied to long-chain *n*-alkanes. This suggests that the hydroxyl group on *n*-alcohol molecules plays no special role in the stabilization of intermediate II; the increased affinity of intermediate II for long-chain compounds primarily reflects an increased affinity for the hydrophobic methylene chain.

Two final points arising from the anesthetic stabilization of intermediate II are worth mentioning. The first relates to the exact values of K_i for long-chain *n*-alcohols and *n*-alkanes. In the flavin-initiated assays, which were used to measure inhibition by long-chain compounds, the observed peak intensity (luciferase activity) was determined by the equilibrium between luciferase and its complexes with *n*-decanal and the inhibitor prior to initiation and by the rate, following conversion to intermediate II, at which the equilibrium shifted in favor of the intermediate II–inhibitor complex. This latter process renders the peak intensity somewhat less than it would otherwise be; thus, the measured K_i values are actually lower than the true luciferase–inhibitor dissociation constant for long-chain compounds, although it is not possible at present to determine the magnitude of this discrepancy.

Second, most of the anesthetics slowed the luminescence decay roughly in proportion to the reduction in peak height. This is shown quantitatively in Figure 9 for *n*-heptan-1-ol. As the peak intensity tends to zero due to increasing inhibitor concentrations, the luminescence decay constant appears to approach zero as well. Since the inhibition is competitive in nature, i.e., the inhibitors can be regarded as acting simply to reduce the effective substrate concentration [by a factor of $K_i/(K_i + [A])$], it might be expected that the decay constant would also tend to zero if the substrate concentration was reduced. This, however, does not occur (see Figure 9). As the substrate concentration, and hence the peak intensity, approaches zero, the luminescence decay constant drops to only about 40% of its value at a *n*-decanal concentration equal to the K_m. This behavior can, however, be attributed to the ability of inhibitors to stabilize intermediate II. Since the in vitro luciferase reaction does not permit enzyme turnover, any route on the reaction pathway which leads to nonproductive decay of intermediate II contributes to the observed rate of luminescence decay. At very low substrate concentrations, most of the intermediate II formed following initiation is not complexed with a substrate molecule. Under these conditions, the spontaneous decay of intermediate II is probably the major dark pathway. Consistent with this reasoning, Figure 9 shows that as the substrate concentration approaches zero the luminescence decay constant may be extrapolated to 0.068 s^{-1} which is comparable to the observed decay constant for the spontaneous decay of intermediate II ($1/\tau_0 = k_0 = 3.73\ min^{-1} = 0.062\ s^{-1}$). In contrast, at very high *n*-alcohol or *n*-alkane concentrations, the vast majority of intermediate II is stabilized in a complex with an inhibitor molecule; the rate of luminescence decay is thus controlled by the rate of nonproductive decay of this complex, which has a decay constant

equal to $k_1 = 1/\tau_1$ (= 0.26 min^{-1} = 0.004 s^{-1}). Figure 9 shows that the estimated luminescence decay constant at high inhibitor concentrations (i.e., as peak intensity → 0) approaches this value to within experimental error.

Comparison between V. harveyi and V. fischeri Luciferase Enzymes. In order to aid a comparison between our results on the inhibition of the *V. harveyi* enzyme and those of some previous workers (Adey et al., 1976; Middleton & Smith, 1976a,b), who studied the effects of anesthetics on the luciferase enzyme from *V. fischeri* bacteria, we compared the effects of two very different anesthetic agents (halothane and *n*-decan-1-ol) on the two enzymes. For these studies, so that a fair comparison could be made, we used the partially purified luciferase preparations that were available commercially. We first determined the Michaelis constants, K_m, for both enzymes when *n*-decanal was used as the aldehyde substrate at saturating levels of reduced flavin (99 μM). The values (mean ± SE) for the two enzymes were very significantly different: 0.67 ± 0.04 μM for *V. harveyi* and 5.6 ± 1.8 μM for *V. fischeri*. (The K_m value determined for the commercial, partially purified *V. harveyi* enzyme, differed only slightly from the value determined above for our very pure enzyme.) Interestingly, this factor of roughly 8 between the K_m values for the two enzymes is almost exactly reflected in the inhibition constants (K_i) for *n*-decan-1-ol, which were found to be 1.22 ± 0.05 and 9.16 ± 0.49 μM for *V. harveyi* and *V. fischeri*, respectively.

This striking difference between the enzymes in the inhibition constants for *n*-decan-1-ol, however, is not reflected in the binding of halothane, which is structurally very dissimilar to the *n*-decanal substrate. The K_i values for halothane were determined to be 7.02 ± 0.82 mM for the *V. harveyi* enzyme and 8.53 ± 0.57 mM for the *V. fischeri* enzyme. We did find, however, that while for the *V. harveyi* enzyme only a single halothane molecule could bind, for the *V. fischeri* enzyme two molecules of halothane were involved in the inhibition. This would be expected if the inhibitor binding site is larger for the *V. fischeri* enzyme than for the *V. harveyi* enzyme.

Effects of Halothane on the NADH:FMN Oxidoreductase Activity from V. harveyi. Since, in the intact bacterium, the reduced flavin utilized by the luciferase enzyme is generated by NADH:FMN oxidoreductase (Hastings et al., 1985), we tested the effects of a single anesthetic, halothane, on the activity of the oxidoreductase enzyme(s) and found that the activity is clearly diminished, with an EC_{50} concentration of about 5 mM.

Discussion

Anesthetic Binding to the Luciferase Enzyme from V. harveyi. All the general anesthetics tested inhibited the light output from the luciferase enzyme, although some small and polar compounds, such as ethanol, were also observed (at lower concentrations) to increase enzyme activity, presumably by binding to small and polar regions on the protein. Since the flavin site was saturated, the inhibition could be attributed, in almost all cases, to the anesthetics competing for the binding of the aliphatic aldehyde substrate *n*-decanal. The hydrophobic nature of this substrate is obvious, and there is also some direct evidence that its binding site on the enzyme is fairly apolar (Nicoli & Hastings, 1974; Merritt & Baldwin, 1980). It seems reasonable, therefore, to suppose that most, if not all, of the anesthetics act competitively at the substrate binding site itself, rather than indirectly via an allosteric conformational change. For a few agents that bind very weakly indeed (in the sense of having large EC_{50} values), more than two inhibitory sites are clearly available, as judged by the steepness of the $f(A)$ curves (see, for example, Figure 3c). It seems likely that at least some of these additional binding sites are not within the aldehyde binding pocket but elsewhere on the protein and that part of the inhibition might not be competitive in nature.

Although a diverse range of anesthetic compounds can bind to the luciferase enzyme and inhibit activity, some agents are far more effective inhibitors than others. This is not, however, obvious if EC_{50} concentrations alone are considered. This is because EC_{50} values are expressed in terms of aqueous concentrations and what is being compared are the *relative* affinities between protein and water, and different agents are capable of very different interactions with water. A clearer picture emerges if the affinities are expressed in terms of ratios between the EC_{50} concentrations and saturated aqueous concentrations (C_{sat}). For compounds with low solubilities this ratio approximates the thermodynamic activity and can be considered to be an "effective concentration" (Brink & Posternak, 1948). Ferguson (1939) and Brink and Posternak (1948) showed that, for most general anesthetics, this ratio is about 0.02 when EC_{50} concentrations for producing general anesthesia are considered. For inhibiting the bacterial luciferase enzyme, on the other hand, it turns out that the ratio EC_{50}/C_{sat} varies greatly from agent to agent. For example, for the aliphatic *n*-alcohols between *n*-pentan-1-ol and *n*-decan-1-ol, this ratio varies between about 0.002 and 0.02, while for large and bulky compounds like halothane, chloroform, enflurane, and isoflurane the values range from about 0.3 to 0.9. Thus, when looked at in this way, the bacterial luciferase enzyme can be seen to bind molecules that resemble its natural substrate (a long-chain aldehyde) much tighter than large and bulky compounds, which are effectively excluded from tight binding interactions.

Surprisingly, however, while aliphatic *n*-alcohols bind to the protein very tightly, the same is not true for *n*-alkanes if thermodynamic activities are considered. On average, the *n*-alkanes do have K_i values about 10 times smaller than those of the corresponding *n*-alcohol, yet their solubilities in water are lower by a factor of about 1000 (reflecting the lack of the polar hydroxyl group). Consequently, the ratios of EC_{50}/C_{sat} for the *n*-alkanes between *n*-pentane and *n*-octane vary between 0.34 and 1.1, suggesting very weak binding to the protein when referred to the pure compounds. The difference between the *n*-alcohols and *n*-alkanes obviously reflects interactions that are possible between the protein and the polar alcohol hydroxyl group. This can also be seen if the EC_{50} concentrations are expressed in terms of partial pressures (calculated from Ostwald solubility coefficients), since in the gas phase we can reasonably assume that intermolecular interactions are negligible. When this is done, it turns out that the *n*-alcohols are several orders of magnitude *more* potent than *n*-alkanes rather than apparently less potent when aqueous concentrations are considered. For example, the partial pressure of *n*-octan-1-ol required to half-inhibit the luciferase enzyme is about 2×10^{-7} atm compared to a value of 6.4×10^{-3} atm of *n*-octane—a difference of over 30 000-fold! Thus, although the luciferase binding site has an overall hydrophobic nature, it appears also to contain one or more distinctly polar regions.

Additional details about the anesthetic binding site can be deduced from the variations in the binding constants K_i and K'_i with *n*-alcohol and *n*-alkane chain lengths (see Figures 4 and 6). First, the binding constants for both homologous series tend to level out after about C_{11}. A similar leveling out has been previously observed for the binding of these compounds to another protein, firefly luciferase (Franks & Lieb, 1985). This leveling out is most easily understood in terms of the

inhibitors binding to a site on the protein of circumscribed dimensions. Above a certain chain length (about C_{11} or C_{12}), the binding pocket is essentially "full" so that any additional methylene groups are forced into water and therefore do not contribute significantly to binding. Second, the decrease in the binding constant with chain length, particularly for the lower members in the series, is remarkably steep. From the slopes of the curves, the apparent free energy of binding of the methylene groups ($\Delta\Delta G^{\ominus}_{CH_2}$) can be calculated. The average value for the *n*-alkanes and *n*-alcohols between C_5 and C_8 is −5.0 kJ mol^{-1} per CH_2 group. The magnitude of this value exceeds even that for partitioning of *n*-alcohols between the completely apolar solvent *n*-hexadecane and water (Aveyard & Mitchell, 1969; Franks & Lieb, 1986), suggesting a particularly intimate, as well as hydrophobic, interaction between the inhibitors and the protein. Third, the pronounced "kink" in the *n*-alcohol binding curve between C_8 and C_9 (and the corresponding, but much smaller, change in slope in the *n*-alkane binding curve) suggests that, at some point along the length of a predominantly hydrophobic binding site, there exists a discrete polar region. (It may be that this feature is much more pronounced for the *n*-alcohols compared to the *n*-alkanes because the polar hydroxyl group on the *n*-alcohol chain constrains the position of the inhibitor, whereas the *n*-alkane molecule might be more free to bind in slightly different positions.)

Furthermore, the finding that the intermediate II form of the enzyme can bind long-chain compounds very much tighter than the normal form of the enzyme provides a simple explanation for the different kinetics of luminescence that were observed with the long-chain inhibitors. The physical basis of this enhanced affinity is unclear. Holzman and Baldwin (1981) showed that the affinity of the luciferase from *V. harveyi* for 2,2-diphenylpropylamine, which competes for the aldehyde substrate, was increased in the presence of $FMNH_2$, and these workers have argued (Holzman & Baldwin, 1981, 1982) that this was a consequence of a conformational change in the enzyme. Alternatively, because of the close proximity of the aldehyde and flavin binding sites on the enzyme (Meighen & Mackenzie, 1973; Nicoli et al., 1974; Cousineau & Meighen, 1976; Tu & Henkin, 1983; Fried & Tu, 1984), a direct interaction between the flavin and inhibitor molecules cannot easily be ruled out.

Comparison between V. harveyi and V. fischeri Luciferase Enzymes. It is well established that the luciferase enzymes from these two species of bacteria differ in many important respects, despite a considerable degree of sequence homology (Hastings et al., 1969; Baldwin et al., 1979; Holzman & Baldwin, 1980). It is perhaps not surprising, therefore, that the interactions between general anesthetics and the enzymes are also found to differ substantially. We restricted our comparison to two contrasting anesthetics, one (*n*-decan-1-ol) that resembles the aldehyde substrate and one (halothane) that does not. *n*-Decan-1-ol was found to bind very much tighter to the enzyme from *V. harveyi* compared to that from *V. fischeri*, with affinities that closely reflected the different values of the aldehyde K_m. (This amounts to strong independent evidence that the inhibition is at the aldehyde site itself rather than via an allosteric interaction.) Halothane, however, bound roughly equally well to the two enzymes, although the *V. fischeri* enzyme could accommodate two halothane molecules in the binding site rather than just one, as is the case for the *V. harveyi* enzyme. An interesting consequence of this is that, despite the roughly equal binding constants, the enzyme from *V. fischeri* is rendered considerably more sensitive to halothane

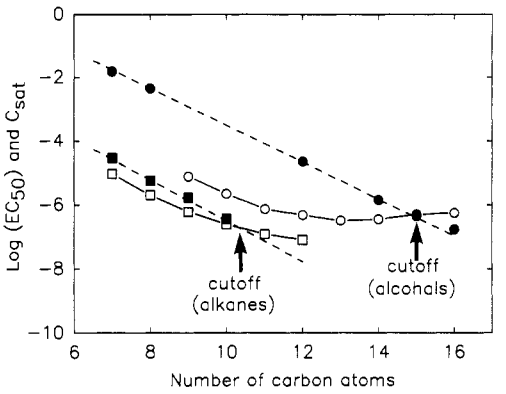


FIGURE 10: Cutoff effect. EC_{50} values (molar units) for luciferase inhibition by long-chain *n*-alcohols (○) and *n*-alkanes (□) are plotted against the number of carbon atoms in the chain. The corresponding saturated aqueous molar concentrations, C_{sat}, are plotted for the *n*-alcohols (●) and *n*-alkanes (■) with least-squares fits drawn as dashed lines. The EC_{50} and C_{sat} lines intersect at very different positions for the two homologous series. This trend is also observed in the general anesthetic potencies for animals, where the cutoff occurs at quite different chain lengths for the two series (see text for details). The solubility data is taken from the compilation of Bell (1973).

inhibition, since for a single molecule of a competitive inhibitor the $EC_{50} = 2K_i$ (eq 3) while for two molecules of a competitive inhibitor the $EC_{50} = 0.732K_i$ (eq 8). It should be stressed, however, that both luciferase enzymes are still remarkably insensitive to inhibition by halothane, requiring concentrations that are 20–50 times higher to half-inhibit activity than those needed to induce general anesthesia in animals (Kita et al., 1981). [The greater sensitivity of intact bacteria (Middleton, 1973) compared to the isolated enzyme (Adey et al., 1976) may be due, at least in part, to inhibition of oxidoreductase enzymes (see Results).]

Comparison between EC_{50} Concentrations for Inhibiting Luciferase Enzymes and for Inducing General Anesthesia. Although there is no general consensus as to the molecular nature of general anesthetic target sites (Franks & Lieb, 1982, 1987; Miller, 1985; Dluzewski et al., 1983; Richards, 1980), there is growing evidence that these sites are protein molecules (Richards, 1980; Franks & Lieb, 1984, 1985, 1986). Assuming, for the purposes of this discussion, that this is the case, what inferences can be drawn from our data regarding molecular mechanisms of general anesthesia? One aspect of anesthetic potency that is peculiar to homologous series is the so-called "cutoff" effect. This is the well-known observation that as one ascends an homologous series (the best studied being the *n*-alcohols) potency increases with chain length only up to a certain point, after which it disappears. For general anesthesia, the point at which this occurs depends upon the homologous series. For the *n*-alcohols, the cutoff occurs between C_{12} and C_{14} (Meyer & Hemmi, 1935; Pringle et al., 1981; Alifimoff et al., 1989). For the *n*-alkanes, the cutoff is significantly earlier, between C_6 and C_{10}, although the exact point seems to be species dependent (Meyer & Hemmi, 1935; Gary-Bobo & Lindenberg, 1952; Mullins, 1954, 1971).

A very similar pattern emerges when one looks at the effects of these compounds on the luciferase enzyme. Figure 10 shows a plot of the EC_{50} concentration (the concentration needed to half-inhibit the enzyme when the concentration of the *n*-decanal substrate is equal to its K_m) against chain length for the long-chain *n*-alkanes and *n*-alcohols. The cutoffs occur with the enzyme when the EC_{50} concentration exceeds the maximum possible aqueous concentration, shown as dashed lines. As with animals, the cutoff for the *n*-alkanes comes significantly earlier (at about C_{10}) than that for the *n*-alcohols (at

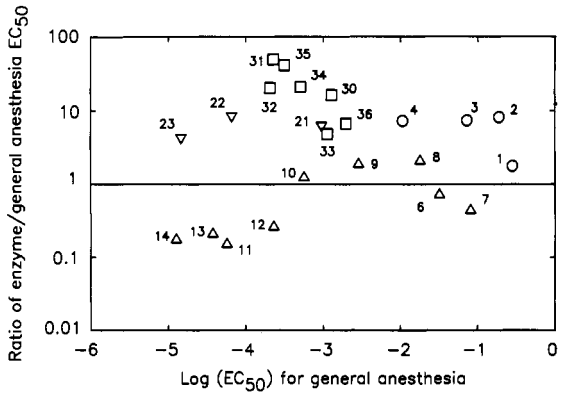


FIGURE 11: Correlation between EC_{50} concentrations for inducing general anesthesia and inhibiting the bacterial luciferase enzyme from *V. harveyi* is poor. The ratio of the enzyme EC_{50} to general anesthesia EC_{50} is plotted against the logarithm (to the base 10) of the general anesthesia EC_{50}. The different symbols refer to different groups of anesthetics: (○) polar agents that cause some enzyme excitation at lower levels, (△) agents with some polar component which only cause inhibition, (▽) apolar *n*-alkanes, and (□) large and bulky agents. The anesthetics are numbered as in Table I. The six largest *n*-alcohols and *n*-alkanes are excluded from this comparison because they are close to or beyond the region of cutoff (Franks & Lieb, 1985) so that a fair comparison cannot be made. EC_{50} concentrations for general anesthesia were taken from the following sources: compounds **1**, **6–8**, and **30**, Table V of Brink and Posternak (1948); compounds **2–4** and **9–14**, Table II of Alifimoff et al. (1989); compounds **31**, **32**, and **36**, Kita et al. (1981). For compound **21**, the gaseous concentration given in Table I of Mullins (1954) and for compounds **22** and **23** the gaseous concentrations given in Table XI of Brink and Posternak (1948) were converted to aqueous concentrations by use of Henry's law constants at 37 °C, calculated with the data in Table IV of Abraham (1982). For compounds **33–35**, the gaseous partial pressures given by Steward et al. (1973) were converted to aqueous concentrations with the Ostwald solubility coefficients at 37 °C given by Allott et al. (1973).

about C_{15}). The cutoffs for the enzyme are a consequence of the leveling out in the binding curves (see Figure 4), which is in turn due to the inhibitors binding to a pocket of circumscribed dimensions. Attempts have been made to account for the cutoff effect in terms of either lipid solubility (Pringle et al., 1981) or slight changes in lipid chain disordering (Miller et al., 1989). Partitioning of long-chain compounds into lipid bilayers, however, continues to increase well after their biological activity has disappeared, with no hint of a cutoff (Franks & Lieb, 1986; Requena & Haydon, 1985). Moreover, lipid bilayer disordering at general anesthetic EC_{50} concentrations is so small that it is unlikely to be physiologically relevant (Richards, 1980; Franks & Lieb, 1978, 1982, 1987). The fact that binding to protein pockets provides such an economical explanation of cutoff effects is strong support for the view (Richards, 1980; LaBella, 1981; Franks & Lieb, 1978, 1982, 1987) that general anesthetics exert their primary effects on protein molecules rather than on lipid bilayers.

Why, though, do the *n*-alkanes cut off before the *n*-alcohols? For the luciferase enzyme, the reason appears to be that the protein binding site has a polar as well as a hydrophobic component. As a result of the polar component, the *n*-alkanes bind only about 10 times tighter to the enzyme than do the *n*-alcohols, compared to the factor of about 10000 which might be expected if the binding site were as hydrophobic as *n*-hexadecane (Lieb & Stein, 1986). However, a much larger factor (of the order of 1000) separates the aqueous solubilities of the *n*-alcohols and *n*-alkanes (see the dashed lines in Figure 10). Consequently, the alkane EC_{50} concentrations are a much higher fraction of their aqueous solubilities than are the alcohol EC_{50} concentrations, so that when the two EC_{50} curves level out, the alkane cutoff occurs much earlier than the alcohol cutoff.

How do the EC_{50} concentrations for inhibiting the activity of the purified luciferase enzyme from *V. harveyi* compare with those needed to induce general anesthesia? A comparison is made in Figure 11, where the ratio between the enzyme and animal EC_{50} concentrations is plotted against the animal EC_{50} concentration. Overall, it is obvious that the agreement is very poor. Leaving aside those agents for which the enzyme EC_{50} values are distorted by excitation, it can clearly be seen that the majority of the anesthetics are rather less potent as inhibitors of the luciferase enzyme than as general anesthetics. The most striking exceptions are the moderately long chain alcohols (C_7 to C_{10}), which closely resemble the aldehyde substrate (*n*-decanal). It is likely that the large and bulky anesthetic agents (such as halothane and isoflurane) are very poor inhibitors of the bacterial luciferase enzyme because of steric factors. This is suggested by the fact that benzyl alcohol binds relatively weakly to the enzyme while *n*-hexan-1-ol binds relatively strongly, yet both are alcohols having very similar general anesthetic potencies. Moreover if the bulky compounds are roughly graded in terms of "cross-sectional area" (crudely defined as the molar volume divided by the longest molecular dimension), there is a reasonable correlation (not shown) between this parameter and the EC_{50} ratios plotted in Figure 11. These considerations suggest that the anesthetic target sites in animals have, on average, at least one critical dimension that is larger than the anesthetic binding site on the bacterial luciferase enzyme.

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