THE INTERACTIONS OF GENERAL ANAESTHETICS WITH A BACTERIAL LUCIFERASE ENZYME

A thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

by

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October 1988

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ABSTRACT

The anaesthetic sensitivity of a highly purified luciferase enzyme from *Vibrio harveyi* luminous bacteria has been assessed in detail and compared with the corresponding pharmacological profile of whole animals. Of the fifty–four different anaesthetics tested, almost all were found to inhibit bacterial luciferase by preventing the binding of the long–chain aldehyde substrate. Differences in the details of the kinetics of reactions inhibited by different groups of anaesthetics were observed; these were investigated and accounted for. Significantly, the potency of *n*–alcohols and *n*–alkanes as luciferase inhibitors increases with chain–length but then, quite suddenly, disappears. This "cut–off" behaviour, which was attributed to the finite dimensions of the luciferase pocket, mimics the cut–off in anaesthetic potencies of these agents, strongly suggesting that the target sites underlying general anaesthesia are protein pockets.

Notably, bulky halogenated agents are much less potent as luciferase inhibitors than as anaesthetics, indicating the comparative narrowness of the enzyme pocket. To test this hypothesis, the potencies, as inhibitors of luciferase, of cycloalcohols and *n*–alcohols – compounds which, though chemically similar, are structurally distinct – were compared. As predicted, the bulkier cycloalcohols are much less effective, even when their greater aqueous solubility (measured using a novel experimental technique) is taken into account. Analysis of alcohol and alkane data showed that the distribution of polar and apolar regions in the luciferase pocket, as well as its geometry, distinguishes it from the target sites in animals.

Surprisingly, the anaesthetic sensitivity of *V. harveyi* luciferase was found to be quite distinct from that of *V. fischeri* luciferase; moreover the NADH:FMN oxidoreductase of *V. harveyi* was shown to be inhibited by halothane. The bearing of these results on previously published findings is discussed.

Measurement of the stabilisation of the luciferase–peroxyflavin complex by *n*–alcohols confirmed the luciferase cut–off effect and revealed that this reaction intermediate has a much higher affinity for long–chain compounds than the enzyme on its own. This difference in affinities accounts for observed differences in the kinetics of reactions inhibited by short– and long–chain alcohols and alkanes.

Acknowledgements

This thesis and this thesis writer owe a considerable debt of thanks to a great many people. I am particularly grateful to Nick Franks, my supervisor, and to Bill Lieb for their skilful guidance and example over the last three years. Thanks are also due to my fellow Ph.D. student, Guy Moss, who proved his patience and resourcefulness in numberless discussions. I am further and sincerely indebted to the Biophysics Group for technical and not so technical support. My thanks go also to the physics workshop who built the apparatus and to Tony Cass, Sarah Jones and David Manuel at the Centre for Biotechnology who calmly bore the problems generated by a physicist in a biochemistry laboratory. To all at Tooting Bec, ta very much! Lastly, I want to thank my Mum and Dad; only I know the full value of their contribution to this work.

S.C.

October 1988.

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CHAPTER 1

WHY STUDY THE INTERACTIONS OF GENERAL ANAESTHETICS WITH A BACTERIAL LUCIFERASE ENZYME?

1.1 Introduction: Failure and Success

In 1663, while investigating the efficacy of alcohol (or "spirit of wine") as a preservative of biological material, Robert Boyle recorded that:

"we have for curiosity sake, with this Spirit, preserv'd from further stinking, a portion of Fish, so stale, that it shin'd very vividly in the dark."

This shining was undoubtedly due to the presence of luminous bacteria thriving on the rotting fish and would most likely have been extinguished upon immersion in alcohol. However, the celebrated seventeenth century scientist does not mention this in his report and seems, somewhat uncharacteristically, to have missed an important discovery: the depressant effect of an anaesthetic on bacterial bioluminescence! Commenting on Boyle's experiment in his history of luminescence, E. N. Harvey makes the curious remark that "it is unfortunate that alcohol did not also preserve the luminescence of the fish" (Harvey, 1957). In my view, this is not the least bit unfortunate, as I hope the contents of this thesis will show.

Harvey's disappointment with the probable outcome of Boyle's experiment did not prevent him making the first direct study of the effects of several anaesthetic agents on bacterial bioluminescence (Harvey, 1915). He observed that ether, chloroform and a number of alcohol compounds all reversibly depressed this bioluminescence and described the effect as "the apparent anaesthesia of a solution" (Harvey, 1917). With this work began the detailed quantitative investigation of the inhibition of bacterial bioluminescence, and of bacterial luciferase (the enzyme ultimately responsible for it), as possible models of the action of general anaesthetics on the central nervous system. In this introductory chapter I wish to trace the path

of investigation from Harvey's early work to the identification of bacterial luciferase as a current focus of interest. Initially however, in order to establish precisely why a study of the effects of anaesthetics on a bacterial enzyme might be considered useful, I shall review the most popular recent theories of the molecular mechanisms thought to underlie general anaesthesia.

1.2 Physicochemical and Thermodynamic Approaches to Understanding Anaesthesia

Every day, in hospitals all over the world, thousands of patients are rendered unconscious by anaesthetic drugs. Yet despite such widespread use, the molecular mechanism of these agents is unknown. One can define general anaesthesia as drug–induced, reversible loss of consciousness but this definition suffers from our lack of understanding of the structures and processes in the central nervous system which maintain consciousness. On the operating table anaesthesia is usually gauged by the lack of purposeful response of a patient to a jab of the surgeon's scalpel. In the case of laboratory animals, the loss of the righting reflex is often used to determine anaesthesia. The crudity of these methods reflects the crudity of our understanding of the transition from consciousness to unconsciousness.

1.2(a) Solvent Models of the Anaesthetic Target

In general, although anaesthetic molecules are chemically inert and relatively apolar (or hydrophobic), the range of compounds which can induce anaesthesia shows tremendous structural diversity. Thus the monatomic gas, xenon, is an anaesthetic, as are *n*–decanol, a long–chain alcohol, and alfaxalone, a steroid compound. Despite this diversity, a remarkable discovery made at the turn of the century led many people to the view that anaesthetics have a common mode of action – the unitary hypothesis of anaesthesia. H. Meyer and E. Overton found that anaesthetic potency [usually defined as the inverse of the dose (ED_{50}), whether it be a partial pressure or an aqueous concentration, which anaesthetises 50% of a population under test] correlates with solubility in olive oil (Meyer, 1899; Meyer, 1901; Overton, 1901). Thus the more potent an anaesthetic, the more soluble it is in the relatively hydrophobic environment of olive oil. This was the first major breakthrough in the understanding of anaesthesia. The fact that potency could be related to a simple solubility parameter implied that no covalent bonds were involved in anaesthesia – consistent with the inertness of anaesthetic compounds. It seemed to imply that

anaesthesia might be induced merely by the physical presence of anaesthetic molecules at the target site. This notion, that the mode of action of general anaesthetics is mechanical, rather than chemical, is common to most of the current theories of anaesthesia.

The success of the Meyer–Overton correlation also points out the relatively hydrophobic nature of the site of anaesthetic action. If the site is hydrophobic, then is is easy to see why the most apolar agents are also the most potent. This is because the aqueous concentration (ED_{50}) required to achieve a critical anaesthetic concentration in a hydrophobic site falls as the apolarity or hydrophobicity of the agent increases; hence the potency ($\equiv 1/ED_{50}$) is observed to increase. In recent years, evidence has been advanced to suggest that *n*–octanol is a better solvent model of the site than olive oil. (Franks and Lieb, 1978). The amphiphilic (polar and apolar) nature of octanol serves to emphasise that the site of anaesthetic action is itself amphiphilic and should not be regarded as purely apolar.

The correlation of anaesthetic potency of a broad range of agents with solubility in simple solvents such as olive oil or octanol suggests a common mode and perhaps even a common site of action for these agents. However, the correlation has yet to be extended to include many anaesthetics which are given intravenously *e.g.* steroid agents, ketamine, propanidid because the doses required for an anaesthetic equilibrium are unknown. It remains to be seen whether such compounds may be retained within the framework of a unitary hypothesis of anaesthetic action. It is also important to realise that the Meyer–Overton correlation fails to account for the sudden loss of anaesthetic potency by the longest members of homologous series. For example, the *n*–alcohols are anaesthetics up to dodecanol. Tridecanol is partially effective but tetradecanol has no anaesthetic activity whatsoever (Pringle *et al.*, 1981). Similar cut–off behaviour has been reported for alkanes, perfluoroalkanes and barbiturates (Janoff and Miller, 1982). However, there is no suggestion of a corresponding cut–off in the solubilities of these agents in olive oil or octanol. Initially, it was pointed out that the apparent loss of potency might be due to the extreme length of time taken by such agents to reach an effective concentration at the anaesthetic target (Meyer and Hemmi, 1935). More recently however, the cut–off has been viewed as an important clue to the nature of the site of action and will therefore be treated in some detail in section 1.3.

The presumed hydrophobic nature of the general anaesthetic target has focussed most attention on two possible physiological sites in the central nervous system. These are the lipid bilayer of neuronal membranes and hydrophobic clefts or pockets

in proteins crucial to normal neuronal activity. In section 1.3 I shall consider the various theories of anaesthetic action which have arisen from studies of model lipid and protein systems. Firstly, however, I wish to discuss the insights into the anaesthetic target that may be gained by observing how anaesthetic potency varies with temperature and pressure.

1.2(b) Antagonism of Anaesthesia by Increasing Temperature

In principle, since anaesthesia is a physicochemical interaction, the enthaply of this interaction may be determined by measuring the variation of the required anaesthetic dose as a function of temperature. A number of workers have undertaken such studies and generally it is found that anaesthetic potency (measured in the gas phase) falls as body temperature rises (Cherkin and Catchpool, 1964; Eger *et al.*, 1965; Regan and Eger, 1967; Janoff and Miller, 1982). For example, raising the temperature of dogs from 28 to 38 °C almost doubles the partial pressure of halothane which is required to induce anaesthesia (Eger *et al.*, 1965). The enthalpy change, ΔH, calculated for halothane and various other inhalational agents is similar to the values of ΔH determined for partitioning of these agents into olive oil (from the gas phase), suggesting that the variation of anaesthetic potency may be more or less accounted for by the change in solubility at the site of action (Franks and Lieb, 1982). Unfortunately this finding cannot distinguish between lipid and protein sites of action.

However, it may be naive to attempt to relate temperature–anaesthetic data for whole animals to the enthalpy of a molecular interaction. Shifts from normal body temperature, especially in warm–blooded animals, may well interfere with attempts to take a measure of anaesthetic potency which is comparable to that taken at normal body temperature. Regan and Eger (1967), for example, observed a linear decline in ED_{50} values for dogs (expressed as partial pressures) as body temperature was lowered. By extrapolation, they estimated that the anaesthetic requirement would fall to zero somewhere between 18 and 21 °C and deduced that cold itself may well have an anaesthetic effect. This observation emphasises the problems that may be associated with a simplified analysis of temperature–anaesthetic potency relationships.

In spite of these reservations, a final point is worth noting. It will be seen in section 1.3, below, that most lipid theories of anaesthestic action predict an enhancement of anaesthetic potency by increasing temperature – quite contrary to

the trend dictated by the experimental evidence. In contrast, it has been argued that a protein binding site provides a simple explanation for the loss of potency as the temperature rises: heat simply weakens the association of the anaesthetic with the protein target, releasing it into the gas phase (Franks and Lieb, 1982). However, this latter theory has yet to be tested experimentally even on a model protein system.

1.2(c) Antagonism of Anaesthesia by Increasing Pressure

The discovery that high pressures, of around 200 atmospheres, cause anaesthetised tadpoles to regain swimming activity (Johnson and Flagler, 1950) was followed by similar demonstrations of pressure reversal with newts (Lever *et al.*, 1971), mice (Halsey and Wardley−Smith, 1975; Miller and Wilson, 1978) and rats (Halsey *et al.*, 1978). Typically, pressures in the region of 100−200 atmospheres, applied hydrostatically or with helium, are required to fully reverse the effect of an ED_{50} dose of inhalational agents including halothane, enflurane, ether, nitrous oxide and cyclopropane (Halsey and Wardley−Smith, 1975). In addition, the ability of pressure to reverse the anaesthesia induced by intravenously administered agents, such as the barbiturate, thiopentone, and the steroid anaesthetic, althesin, has also been demonstrated (Lever *et al.*, 1971; Halsey *et al.*, 1978).

In simple terms, the mechanical action of pressure at constant temperature is to reduce volume. The phenomenon of pressure reversal has therefore led some workers to suggest that the interaction of an anaesthetic molecule with its target may result in a volume increase which can be directly opposed by pressure (Miller *et al.*, 1973; Halsey *et al.*, 1978). Miller and his coworkers (1973) observed that increasing the pressure caused the same linear percentage increase in the required anaesthetising partial pressures of nitrogen, nitrous oxide, carbon tetrafluoride and sulphur hexafluoride acting on newts. They postulated the critical volume hypothesis which asserts that "anaesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical amount by the absorption of an inert substance." Attempts to test this hypothesis with anaesthetics which are very different from the rather narrow range of agents on which it was founded have proved difficult. Using mice, a study of several intravenously administered drugs (urethane, α−chloralose and phenobarbital) showed that these agents are antagonised twice as much by pressure as gaseous agents (Miller and Wilson, 1978). These authors were obliged to conclude that, unless their result was due to artefacts arising from the more complicated pharmacokinetics of intravenous agents, either a smaller critical volume

was required for anaesthesia by these anaesthetics or they acted at a separate site with greater compressibility than the site of action of gaseous agents. Both of these explanations require modifications of the critical volume hypothesis.

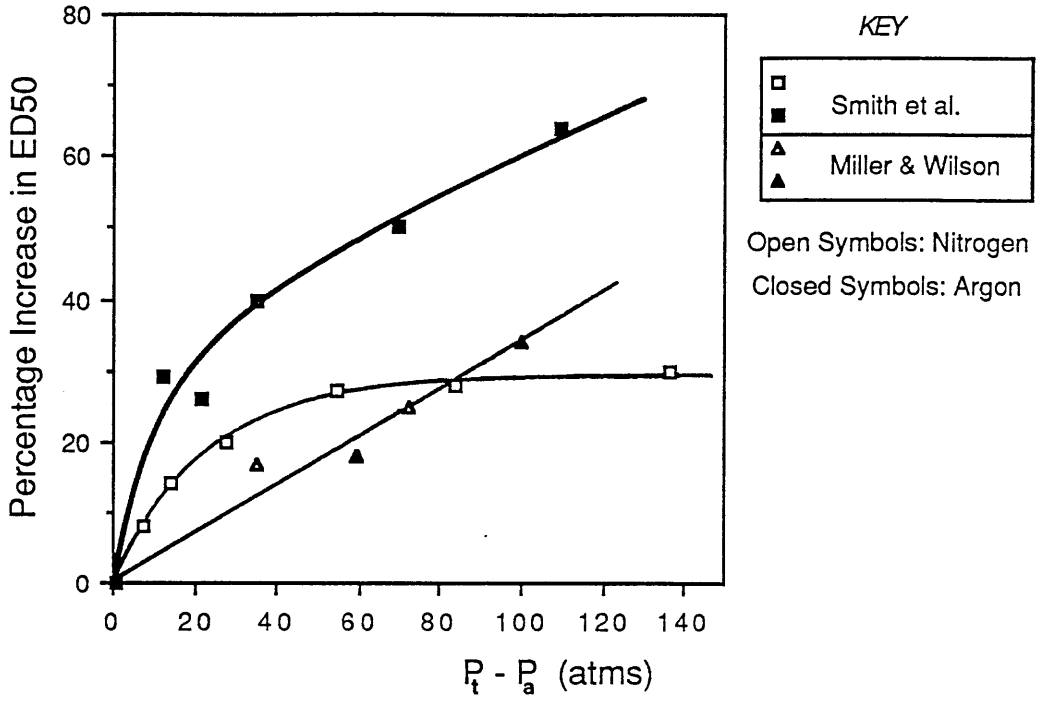

Figure 1.1: Differing reports of the antagonism by pressure of anaesthesia in mice. The data show the percentage increase (relative to controls at 1 atm) in the anaesthetic doses of nitrogen and argon required to keep 50% of a population of mice anaesthetised as the pressure is increased. $P_t - P_a$ is the difference between the total pressure of anaesthetic and helium, (P_t), and the anaesthetic partial pressure, (P_a). The data of Smith et al. (1979) [squares] are quite different from the observations of Miller and Wilson (1978) [triangles].

Further opposition to this theory has arisen because some of the original data cited in support of it have not been reproduced elsewhere. In contrast to the observations of Miller and his colleagues (Miller *et al.*, 1973; Miller and Wilson, 1978), who found that pressure caused a uniform linear percentage increase in the ED_{50} values of gaseous agents (including argon and nitrogen) acting on newts and mice, Smith *et al.* (1979) observed non−linear and disparate increases in the ED_{50} partial pressures of nitrogen and argon determined with mice. This discrepancy is shown in Figure 1.1. Thus, although pressure reversal is well established as a phenomenon, this significant discrepancy in the quantitative details of the effects of pressure on anaesthesia has yet to be resolved.

The anaesthetising aqueous concentrations of intravenous agents such as methohexitone, althesin and ketamine have also been observed to rise disparately and non-linearly with increasing pressure (Halsey *et al.*, 1978). It seems unlikely that this diversity is related to the complicated pharmacokinetics of intravenously administered agents since differing degrees of pressure reversal were also observed with tadpoles exposed to aqueous solutions of methohexitone and althesin (Halsey *et al.*, 1986). These results and the results obtained by Smith *et al.* (1979) with gaseous agents have led to the formulation of the multi-site expansion hypothesis, an extension of the critical volume hypothesis which proposes that anaesthesia is caused "by the expansion of more than one molecular site with different molecular properties" (Halsey *et al.*, 1978, 1986). This theory embraces the diversity of pressure-anaesthetic interactions.

However, a considerable amount of work remains to be done before specific clues about the nature of the anaesthetic target can be derived from pressure reversal studies. So far, such studies have failed to discriminate between lipid and protein sites of action, a state of affairs which is due to the complexity of pressure-anaesthetic interactions. For example, the formulation of the multi-site expansion hypothesis includes the possibility that pressure, rather that acting directly on the anaesthetic target, may stimulate secondary sites leading ultimately to an antagonism of anaesthetic effects (Halsey *et al.*, 1978); this idea has recently been emphasised by Wann and Macdonald (1988). In addition, side-effects of pressure may frustrate efforts to use this variable to probe the site of anaesthetic action in quantitative detail. Tremors, hyperexcitability and changes in the electro-encephalogram (EEG) are among the effects produced by pressure alone, even at levels lower than the 150 atmospheres associated with reversal of anaesthesia (Miller and Miller, 1975; Halsey, 1982). The ability of certain anaesthetics to lower the threshold at which pressure induces convulsions (normally around 100 atmospheres in mice), while others raise it, further complicates analysis, though to an unknown extent (Halsey, 1982). Thus, although pressure represents an intriguing and potentially valuable tool for the investigation of anaesthetic mechanisms, that value has yet to be fully realised.

1.3 Molecular Models of General Anaesthetic Action

It is most often claimed that anaesthetics act at neuronal membranes, the

structures responsible for the transmission of electrical and chemical signals within the brain. The presence of anaesthetics is held to disrupt, either directly or indirectly, the function of proteins essential to normal neuronal activity but the mechanism by which anaesthetics precipitate this protein dysfunction is as yet, undetermined. Recent reviews have concentrated on lipid bilayers and hydrophobic protein pockets as the most likely primary sites of anaesthetic action (Richards, 1980; Dluzewski *et al.*, 1983; Franks and Lieb, 1982; Miller, 1985). In this section I wish to discuss the lipid and protein theories which have received most attention. Because of limitations of space, this discussion is necessarily selective. My main aim is to provide a context for the work on anaesthetic–bacterial luciferase interactions which is described in this thesis.

1.3(a) Is the Anaesthetic Target the Lipid Bilayer in Neuronal Membranes?

Lipid theories of anaesthetic action commonly propose that anaesthetic molecules dissolve in the lipid bilayer of neuronal membranes and perturb its structure. This disruption of the lipid environment of intrinsic membrane proteins is postulated to cause some of them to malfunction, leading to anaesthesia. The most obvious attraction of these theories is that they provide a simple explanation for the correlation between anaesthetic potency and solubility in the lipid medium of olive oil. Although lipid bilayers have a well defined structure, unlike olive oil, the solubilities of twenty–one agents (including *n*–alcohols, volatile compounds *and* barbiturates) in a phosphatidylcholine bilayer have been successfully correlated with their anaesthetic potencies (Janoff *et al.*, 1981). The lipid portion of real membranes differs considerably in composition from pure phosphatidylcholine, containing (among other things) 30 to 50 mol% cholesterol, but experimental evidance obtained with phospholipid–cholesterol bilayers suggests that this seems unlikely to upset the overall correlation (Miller, 1985). Lipid theories of anaesthetic action also have the advantage of being conceptually attractive: it is easy to imagine how the fluid bilayer might accomodate a structurally diverse range of agents.

In the 1960s the advent of artificial lipid bilayers provided an opportunity to study the effects of anaesthetics on bilayer structure in detail. Many perturbations have been observed and the most interesting theories which have arisen from these findings are described below.

Permeabilisation Theories: Lipids in solution spontaneously form into liposomes, spherular bilayer shells. One of the earliest investigations of the effects of

anaesthetics on lipid bilayers demonstrated that clinical doses of ether, chloroform and *n*−alcohols enhanced the cation efflux from liposomes by about 30% (Bangham *et al.*, 1965). Halothane and pentobarbital also increase cation permeability significantly at clinical levels (Pang *et al.*, 1979). Interestingly, certain steroid anaesthetics have been shown to produce similar increases in permeability, whereas non−anaesthtic steroids have little or no effect (Connor *et al.*, 1974). The relatively large effect of anaesthetics have been attributed to their ability to increase the freedom of movement of lipid molecules at the water interface (Johnson and Bangham, 1969; Pang and Miller, 1978). Additionally, it was observed that pressures in the region of 150 atmospheres are required to reverse the effect of clinical concentrations of ether, butanol and nitrogen (Johnson and Miller, 1970). This model thus accounts simply for the pressure reversal of anaesthesia. It seems unlikely that enhancement of cation permeability from whole cells is relevant to anaesthesia since basal biomembrane permeabilities are much greater that that of simple liposomes. However, evidence has been presented to show that anaesthetics cause catecholamine neurotransmitters to leak from their synaptic vesicles by enhancing the proton efflux from these vesicles (Bangham and Mason, 1980; Barchfield and Deamer, 1985). The proton efflux causes the vesicle pH to rise so that the catacholamines revert from the charged to the uncharged form. The uncharged neurotransmitters thus pass across the bilayer and impair the signalling capacity of the synapse. Several anaesthetics have been shown to deplete the dopamine content of rat brain vesicles. However, clinical concentrations caused only a slow, partial release − 20% in 25 minutes (Bangham and Mason, 1980). This may be too small an effect to be clinically relevant, but further investigation of this hypothesis may prove interesting.

Expansion Theories: The solution of anaesthetic molecules in the lipid bilayers of neuronal membranes inevitably causes the bilayer to expand; this expansion, it has been proposed, may perturb lipid−protein interactions so as to abolish the proper functioning of certain membrane proteins. The proponents of the critical volume hypothesis (Miller *et al.*, 1973) and the multi−site expansion hypothesis (Halsey *et al.*, 1978), discussed in section 1.2(c), have cited lipid bilayers as possible sites of the expansion that they deduce from pressure reversal studies. However, it is important to point out that bilayer expansion may differ for different agents. Measurements of the drop in capacitance of lipid bilayers formed using black film techniques and equilibrated with saturated solutions of *n*−alkanes were interpreted as a demonstration of the thickening effect of these agents (Haydon *et al.*, 1977). In contrast, no thickening effect of similar bilayers was observed with low levels of benzyl alcohol (Reyes and Latorre, 1979). This discrepancy may be attributed to

the fact that alkanes partition preferentially into the hydrocarbon core of bilayers whereas the polar hydroxyl group of benzyl alcohol anchors it close to the lipid/water interface. Thus the direction of membrane expansion depends to an extent on the nature of the anaesthetic molecule and theories which attribute protein dysfunction to anaesthetic-induced expansion must explain why similar membrane concentration of anaesthetics which expand the bilayer in different ways, produce the same endpoint: anaesthesia. An attempt to rationalise the inhibition of the sodium channel in squid giant axons in terms of the differential distribution and effects of alkanes and alcohols has been made by Haydon and Urban (1983). However, in general, this aspect of expansion theories has received little attention.

In any case, most of the data on membrane expansion shows that clinical doses produce very small effects. X-ray diffraction studies revealed no significant thickening of phospholipid-cholesterol bilayers at up to eleven times the anaesthetising concentrations of halothane and chloroform (Franks and Lieb, 1978). Volume increases, which have been studied with red blood cell membranes and artificial cholesterol-containing bilayers, are typically in the range 0.1-0.2% for surgical levels of anaesthetics (Kita *et al.*, 1981; Franks and Lieb, 1981; Bull *et al.*, 1982). As an indication of the small magnitude of this increase, it may be noted that a temperature rise of around 2°C produces a similar volume expansion (Rand and Pangborn, 1973; Melchior *et al.*, 1980). It is thus also apparent that expansion hypotheses predict an enhancement of anaesthesia with rising temperatures, contradicting the experimental evidence obtained with whole animals.

Disordering Theories: Although cell membranes are well defined structures they are thought to be "fluid" in the sense that there is lateral diffusion of lipid and protein molecules within the bilayer. In the hydrocarbon region, the mixture of saturated and unsaturated acyl chains is normally in a disordered, liquid-like state (Lehninger, 1982). Disordering theories postulate that anaesthetics act by further fluidising or disordering the lipid bilayer in nerve cell membranes, thereby interfering with the active conformation of some membrane proteins. An impressively diverse selection of agents, including halothane, chloroform, butanol (Boggs *et al.*, 1976) and urethane, α-chloralose, phenobarbital and ketamine (Pang *et al.*, 1980) have been shown to disorder lipid-cholesterol bilayers. In addition certain anaesthetic steroid compounds (*e.g.* alfaxalone) were observed to disorder bilayers, whereas non-anaesthetic steroids (*e.g.* betaxalone) had little or no effect (Lawrence and Gill, 1975). However, evidence for significant disordering of bilayer structure by clinical levels of anaesthetics is controversial. Those studies which claim an effect have extrapolated from the disorder measured at supraclinical concentrations (Mastrangelo

et al., 1978; Pang *et al.*, 1980). Other workers, using low concentrations found no discernable disordering (Boggs *et al.*, 1976; Lieb *et al.*, 1981). In the latter investigation, no effect was detected at up to five times the anaesthetising concentrations of halothane or chloroform using a technique sensitive to temperature variations of $1-2^{\circ}C$ (Lieb *et al.*, 1981).

Other evidence from studies of model bilayer systems is difficult to reconcile with the theory that anaesthetics act by fluidising the lipid portion of neuronal membranes. For example, butanol and dodecanol respectively disorder and order lipid−cholesterol bilayers although both are anaesthetics (Richards *et al.*, 1978). It has been pointed out that bilayer composition (*e.g.* cholesterol and ganglioside content) can have a large effect on the disordering efficacy of anaesthetics (Pang and Miller, 1980; Harris and Groh, 1985) and this may eventually explain the discrepant results for butanol and dodecanol. However, temperature poses a further problem for disordering theories. Pang *et al.* (1980) calculated that a $0.35^{\circ}C$ rise would apparently mimic the fluidising effect of an anaesthetic dose. One possible reason to explain why anaesthesia is not brought about by such a small temperature rise, Pang and his colleagues have argued, is that anaesthetic effects may be concentrated in particular regions of the membrane, producing large local effects on proteins, whereas the fluidisation attributed to temperature is dispersed globally so that local effects are small. Convincing experimental evidence in support of this reasoning may have to wait for a comprehensive investigation of the anaesthetic distribution in real membranes. In any case, the decline in anaesthetic potency with rising temperature is not explained by disordering theories; moreover, only tens of atmospheres of pressure seem to be needed to reverse anaesthetic induced disorder, a further quantitative difficulty for these hypotheses (Boggs *et al.*, 1976).

Phase Transition Theories: Anaesthetics depress the temperature, T_m, at which a pure phospholipid bilayer shifts from the gel to the liquid crystalline state (Hill, 1974; Mountcastle *et al.*, 1978). Several workers have therefore suggested that unconsciousness may be induced when anaesthetics depress T_m below normal body temperature. The advantage of such a hypothesis is that it shows how the normally small perturbation of the bilayer by anaesthetics might be amplified (Janoff and Miller, 1982). However, experiments with pure lipid bilayers indicate that anaesthetising concentrations of inhalational agents and *n*−alcohols depress T_m by only $0.3-0.6^{\circ}C$. These model systems therefore seem too simple to account for anaesthesia data. Normal body temperature, even in warm blooded animals, fluctuates by more than a degree without inducing unconsciousness (Wright, 1949). As with disordering theories, phase transition hypotheses also predict increasing

potency with increasing temperature – contrary to anaesthesia data. In addition, pressure reversal of the depression of T_m has consistently been observed at much less than the 150 atmospheres required to remove anaesthesia (Mouncastle *et al.*, 1978; Trudell *et al.*, 1975). The equipotent anaesthetics *cis*– and *trans*–tetradecenol respectively lower and raise T_m of phosphatidylcholine bilayers (Pringle and Miller, 1978); interestingly, later work had suggested that this discrepancy may be abolished if the choline head group on the lipid is replaced by ethanolamine (Firestone *et al.*, 1987). This result indicates how bilayer composition can influence phase–transition effects. Although the gel–liquid crystalline phase transition in pure lipid bilayers does not seem to provide a plausible anaesthetic mechanism, the study of more sophisticated artificial bilayers, containing mixtures of lipid types, or of real membranes may give a clearer indication of the possible importance of these theories to an understanding of general anaesthesia.

Summary: Lipid perturbation hypotheses provide a simple framework to account for general anaesthesia. They can explain the Meyer–Overton correlation and the structural diversity of anaesthetic molecules. Inhalational agents, *n*–alcohols, *n*–alkanes, steroids and barbiturates have all been shown to perturb the structural and dynamic properties of lipid bilayers. Experimental evidence also indicates that lipid perturbation theories go some way to accounting for the ineffectiveness of certain non–anaesthetic steroids.

At clinical concentrations, the perturbations caused by anaesthetics are small and one has to posit that there is a strong coupling between the perturbation and the disruption of membrane function. Studies of the anaesthetic enhancement of cation permeability suggest one possible example of strong coupling (Pang *et al.*, 1979). Some efforts have been made to demonstrate the functional link between lipid environment and protein function (*e.g.* Johannson *et al.*, 1981) but, to date, there is little evidence to suggest that the perturbation associated with clinical levels of anaesthetics are sufficient to disrupt membrane protein activity.

In general, lipid perturbation hypotheses cannot yet properly account for temperature or pressure reversal of anaesthesia – except possibly in the case of permeabilisation theories. Additionally, it has not been possible to explain the cut–off effect – the loss of anaesthetic potency by the longest members of homologous series – with lipid bilayer models. An early report that the membrane solubilities of *n*–alcohols cut off at the same point as anaesthetic potency for that series (Pringle *et al.*, 1981) proved to be incorrect (Franks and Lieb, 1986). However, as has already pointed out elsewhere (Miller, 1985), real neuronal

membranes are considerably more sophisticated than artificial bilayers in composition and, hence, also in their properties. Interest in lipid bilayer theories is now shifting more towards the study of intact biomembranes but, due to the complexity of such systems, significant advances have yet to be made.

1.3(b) Is the Anaesthetic Target a Protein?

Much of the interest in the hypothesis that anaesthetics act by direct interaction with sensitive functional proteins in the central nervous system stems from the disenchantment of some workers with lipid theories of anaesthetic action (Richards *et al.*, 1978; LaBella, 1981; Franks and Lieb, 1982). Theoretically, protein theories have a number of inherent attractions. The selective action of anaesthetics, depressing some central nervous functions while leaving others relatively unimpaired, might reasonably derive from the sensitivity and insensitivity of different proteins. Moreover, simple molecular mechanisms relating anaesthetic binding to protein malfunction are instantly conceivable. Anaesthetic molecules may block the binding of an endogenous ligand either directly, by occupying its binding site, or indirectly through an allosteric interaction. Alternatively, anaesthetics, when they bind, may lock a protein into an active or an inactive conformation which induces unconsciousness. Lastly, in contrast to lipid theories, this approach to anaesthesia admits the possibility that cytosolic proteins may be affected by anaesthetic agents.

Theoretical attractions are, of course, no substitute for experimental evidence and while there is no shortage of reports concerning anaesthetic–protein interactions, only a few of these are of significant interest to the phenomenon of anaesthesia. Unfortunately, much of the experimental evidence with which one might test protein theories is fragmentary; most studies of the effects of anaesthetics on proteins have involved only two or three agents. For example, haemoglobin, one of the most intensively studied of all proteins, is reported to bind the anaesthetic, xenon, in a number of preformed cavities without significantly affecting the structure of the molecule (Schoenborn, 1965). The binding of halothane to haemoglobin presumably does not cause much structural perturbation either, since the affinity of the protein for oxygen is unaffected (Weiskopf *et al.*, 1971). In contrast, halothane is known to competitively inhibit adenylate kinase; x–ray diffraction analysis of the inhibited complex has located the halothane molecule at the hydrophobic binding site of the adenine moiety of the adenosine 5'–monophosphate (AMP) substrate (Sachsenheimer *et al.*, 1977). Other studies have investigated the anaesthetic sensitivity of pepsin (Tang, 1965), chymotrypsin (Miles *et al.*, 1962) and acetylcholinesterases (Braswell

and Kitz, 1977) using a variety of agents including ether, methoxyflurane, ethanol and chloroform. However, a convincing correlation between protein sensitivity and anaesthetic potency failed to emerge.

Two proteins of more interest, which have been shown to be structurally perturbed by about a dozen agents in a manner which correlates broadly with their anaesthetic potencies, are bovine serum albumin (BSA) and β-lactoglobulin (Balasubramanian and Wetlaufer, 1966). These proteins have been investigated in some detail and a useful amount of information about the likely nature of anaesthetic-protein interactions has been obtained.

Certain anaesthetic *n*-alkanes and *n*-alcohols are known to bind to BSA and β-lactoglobulin at concentrations which induce anaesthesia. This binding seems to involve interactions with a large number of low affinity sites which are located in the hydrophobic regions at the interfaces between the subunit-like domains of the protein (Wishnia and Pinder, 1964; Ray *et al.*, 1966). A study of the binding of a range of organic compounds (some of which are known to have anaesthetic activity) revealed that these also bound at these interfaces, with affinities which correlated with their octanol-water partition coefficients (Helmer *et al.*, 1968). Interestingly, a similar result was obtained for organic compounds binding to subunit interfaces on bovine haemoglobin (Kiehs *et al.*, 1966). These findings indicate that, despite their relative rigidity, protein sites can display solvent-like properties and may be able to account for the solvent correlations associated with anaesthesia (see section 1.2(a)). In contrast to BSA and bovine haemoglobin, β-lactoglobulin does not admit anaesthetic molecules to its interfacial regions but rather has a high affinity hydrophobic binding pocket on each of its two monomers. These sites are identical; both of them bind two molecules of butane with equal affinity, two molecules of pentane with unequal affinity but only one molecule of iodobutane (Wishnia and Pinder, 1966). A subsequent investigation noted that neopentane ($C(CH_3)_4$), an agent which has the same molar volume as pentane ($H(CH_2)_5H$) but is more globular in its proportions, binds exceedingly weakly (100 times weaker than pentane) to the β-lactoglobulin site (Wishnia, 1969). The site therefore appears to have a well defined shape with which neopentane cannot make good binding contacts. This behaviour provides, on a small scale, an explanation of the inability of larger members of homologous series (*e.g.* alkanes and alcohols) to cause anaesthesia. BSA and β-lactoglobulin thus represent two distinct and interesting cases of anaesthetic-protein interactions.

One class of protein that has received more detailed attention are the

light−emitting luciferase enzymes; these are naturally of particular interest to the subject matter of this thesis. The research effort that has been devoted to the study of the effects of general anaesthetics on bacterial luciferase will be considered separately in the following section, as a preface to the experimental work described in this report. Before that, I want to consider the most recent results obtained with firefly luciferase, a completely different protein, which have sparked a new interest in anaesthetc−protein interactions.

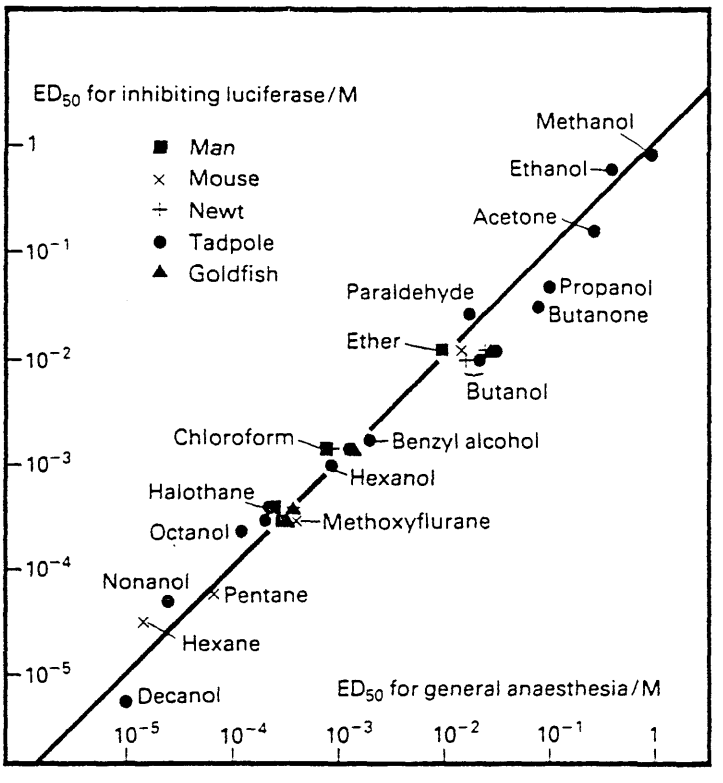

Figure 1.2: The anaesthetic ED_{50} concentrations required to inhibit firefly luciferase are essentially identical to those which induce general anaesthesia. Reproduced by kind permission of the authors from Franks, N. P. and Lieb, W. R. (1985) Chemistry in Britain 10, 919−921.

In 1973 the luminescence produced by the addition of ATP to the cell−free extract from firefly tails was found to be inhibited by clinical concentrations of methoxyflurane, halothane, chloroform, enflurane and fluroxene (Ueda and Kamaya, 1973), a result which was interpreted in terms of anaesthetics binding to a hydrophobic pocket on the luciferase protein. This conclusion was confirmed and

the understanding of anaesthetic−firefly luciferase interactions extended by a subsequent study which used a highly purified preparation of the protein. Franks and Lieb (1984) demonstrated that firefly luciferase can be inhibited 50% by eighteen different anaesthetics at concentrations which are very close to those necessary for anaesthesia. This striking correlation (Figure 1.2) contains agents as diverse as ethanol, benzyl alcohol, hexane, paraldehyde and halothane and spans a potency range of five orders of magnitude. Anaesthetics were shown to compete with the aromatic luciferin substrate [4,5−dihydro−2−(6−hydroxy−2−benzothiazoyl)−4−thiazole carboxylic acid] for binding to the enzyme − apparently by occupying the hydrophobic substrate pocket. Like BSA and bovine haemoglobin, firefly luciferase seems to possess solvent−like properties which can account for the Meyer−Overton correlation. Moreover, Franks and Lieb (1985) observed that alkanes longer than hexane and alcohols longer than pentadecanol were ineffective luciferase inhibitors, thus mimicking the anaesthetic cut−off, which occurs at decane for alkanes in mice (Mullins, 1971) and at dodecanol for alcohols in tadpoles (Meyer and Hemmi, 1935). Further discussion of the molecular details of the cut−off effect will be held over until Chapter 5 where experimental results with bacterial luciferase which are pertinent to this phenomenon will be discussed.

So far, firefly luciferase appears to be quite exceptional among proteins. It supplies a plausible model to explain both the Meyer−Overton correlation and the cut−off effect. Consequently, it is possible to speculate that there is a reasonable chance of finding an anaesthetic binding pocket similar to the one on this luciferase on one or more of the proteins at work in the central nervous system. Nonetheless, a number of important questions concerning the plausibility of protein sites of anaesthetic action remain to be answered. More detailed investigation of the effects of temperature and pressure on anaesthetic−protein interactions will be necessary to test whether temperature and pressure reversal of anaesthesia can be modelled with a protein target, although this has been demonstrated theoretically (Franks and Lieb, 1982). In addition, the interactions of more complicated anaesthetics such as barbiturates, steroids and ketamine with protein targets have yet to be subjected to the same degree of scrutiny as the interactions of these agents with lipid bilayers. Some studies have been made in this direction. Keane and Biziere (1987), for example, have recently reviewed the evidence concerning the stimulation of γ−aminobutyric acid (GABA) receptors by a range of anaesthetics, including steroids and barbiturates, and suggested a causal link between these effects and general anaesthesia. However, the GABA receptor complex is a membrane−bound protein and it is not yet clear whether anaesthetics act on it directly or via membrane perturbations.

Despite such reservations, it is clearly worthwhile to pursue the investigation of the interaction of anaesthetics with proteins and, to this end, the present study of bacterial luciferase was undertaken. The reasons for selecting bacterial luciferase as worthy of interest to the field of anaesthesia research are presented in the next section.

1.4 The Inhibition of Bacterial Bioluminescence and Bacterial Luciferases by General Anaesthetics

Since Harvey's first report in 1915, the range of anaesthetics known to depress bacterial bioluminescence has been extended considerably. Aliphatic and aromatic alcohols, carbamates and a wide variety of inhalational anaesthetics have all been shown to reduce the light output from a number of species of luminous bacteria (Taylor, 1943; Johnson *et al.*, 1951; Halsey and Smith, 1970; White and Dundas, 1970; Middleton, 1973). Using as much of this data as possible I have plotted the correlation between the anaesthetic concentrations (ED_{50}) which depress luminescence by 50% and those required to induce anaesthesia in 50% of a population of whole animals (Figure 1.3 – next page). It is clear from this figure that the relative sensitivities of bacterial bioluminescence and whole animals to general anaesthetics are very similar (although, in absolute terms, the bioluminescence is slightly less sensitive). This impressive correlation has fostered the idea that valuable anaesthesia research may be performed on a simple bacterial cell – an idea which is supported by the results of experiments to probe the effect of temperature and pressure on the anaesthetic inhibition of bacterial bioluminescence.

Temperature Effects: Firstly, studies with bioluminescent bacteria in the absence of any inhibitor show that each species has a characteristic optimum temperature; deviations from this optimum lead inevitably to a reduction in the light output (Johnson *et al.*, 1945). This result is a useful reminder of the perturbation of physiological systems due to temperature alone. Obviously, in the case of whole animals, any general depression due to temperature alone cannot be assessed or compensated for: a dog, for example, is either conscious or unconscious and it is not possible to observe in-between states. The determination of animal ED_{50} values as a function of temperature suffers accordingly. However, in studies using luminous bacteria, the ED_{50} at a given temperature is the anaesthetic dose which depresses the light output by 50% relative to a control sample *at the same*

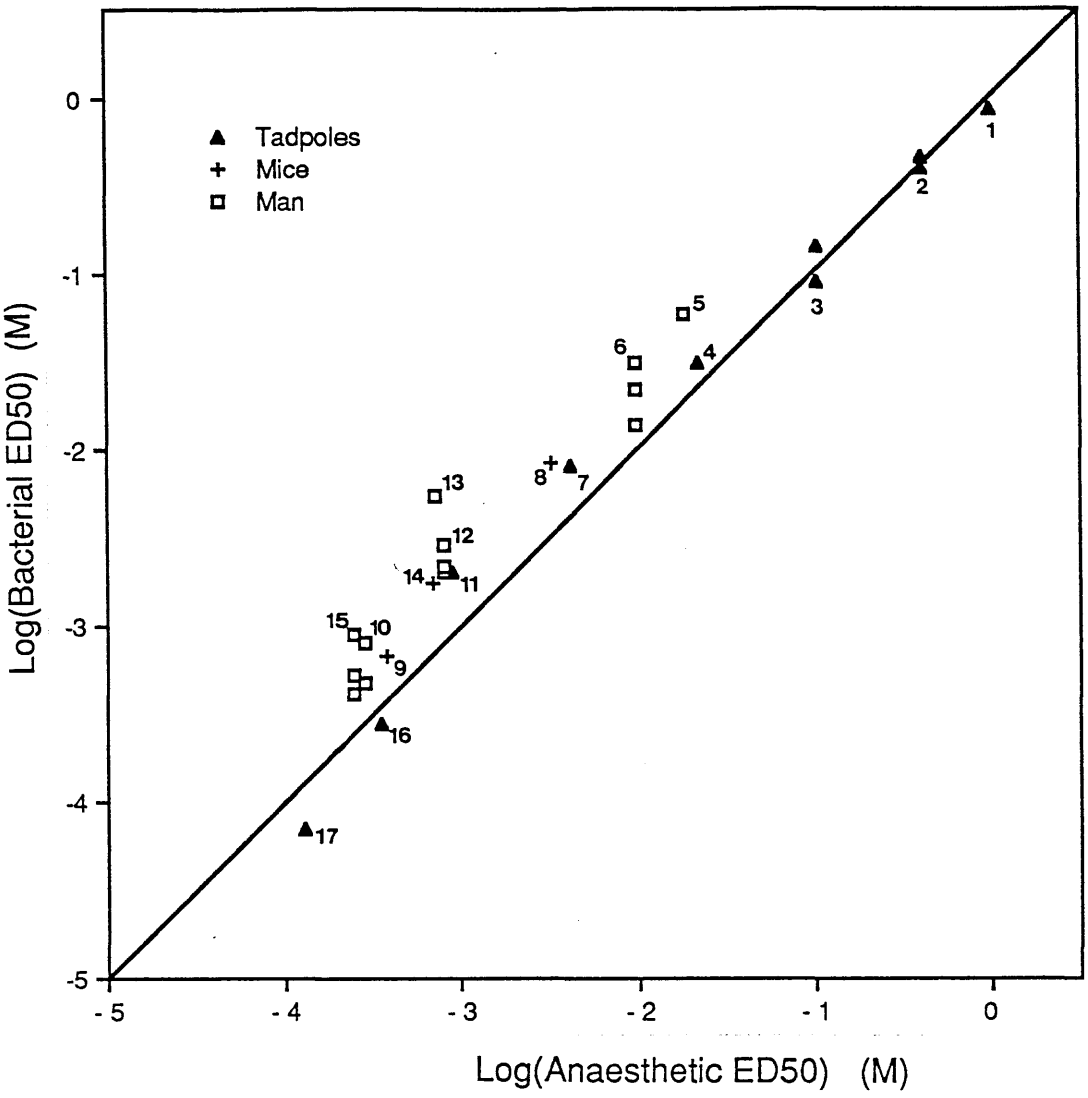

Figure 1.3: *The anaesthetic sensitivity of whole animals is mimicked by bacterial bioluminescence. Over a 10,000−fold range of concentrations, animal and bioluminescence* ED_{50} *values are proportional, although bioluminescence is somewhat less sensitive to anaesthetics in absolute terms. The line is a line of identity. Anaesthetics are labeled as follows: 1, methanol; 2, ethanol; 3, propanol; 4, butanol; 5, nitrous oxide; 6, diethyl ether; 7, pentanol; 8, chlorodifluoromethane (*$CHClF_2$*); 9, trichloroethylene; 10, methoxyflurane; 11, hexanol; 12, chloroform; 13, cyclopropane; 14, dichlorodifluoromethane (*CCl_2F_2*); 15, halothane; 16, heptanol; 17, octanol. Sources of data: (I) Bacterial bioluminescence. Taylor (1934), Bacillus fischeri: compounds 1−4, 7, 11, 16, 17.* ED_{50} *values were estimated by interpolation of Taylor's graphical data since his "critical narcotic concentrations" were effectively the concentration required for 90% inhibition. Halsey and Smith (1970), Photobacterium phosphoreum, T =* $21^{\circ}C$*: compounds 5, 6, 8, 12, 14, 15. White and Dundas (1970), Photobacterium phosphoreum, T =* $25^{\circ}C$*: compounds 6, 9, 10, 12, 13, 15. Middleton (1973), Vibrio fischeri, T =* $28^{\circ}C$*: compounds 2, 3, 6, 10, 12, 15. (II) General Anaesthesia. Steward et al. (1973), Man, T =* $37^{\circ}C$*:*

compounds 5, 6, 12, 13, 15. Miller and Smith (1973), Mouse, T = 37°C: compounds 6, 8, 10, 14, 15. Vernon (1913), Tadpole, T = 18°C: compounds 1–4, 7, 11, 16, 17. Firestone et al. (1986), Man, T = 37°C: compound 13. Rat, T = 37°C: compound 9.

Doses quoted as partial pressures P_{50} (atm) were converted into aqueous concentrations, ED_{50} (M), using the formula, derived in Appendix 1:

$$ED_{50}\ (M) = \frac{\lambda_T P_{50}(atm)}{RT} \qquad (1.1)$$

where λ_T is the water/gas partition coefficient (Ostwald solubility coefficient) expressed as a ratio of molar concentrations at temperature T (Kelvin). R is the universal gas constant (0.08206 lit atm deg^{-1} mol^{-1}). λ_T values were calculated from the Ostwald solubility coefficient and its temperature coefficient (k, percent per degree) at 310K with the equation:

$$\lambda_T = \lambda_{310}\left[1 + \frac{k}{100}\right]^{(310-T)} \qquad (1.2)$$

This equation may be applied over the temperature range 15–40°C (Allot et al., 1973). Temperature coefficients for CCl_2F_2 and $CHClF_2$ were not available in the literature. However, it was noted that the temperature coefficients of five other chlorinated and fluorinated agents (chloroform, haltohane, methoxyflurane, fluroxene and trichloroethylene (Allot et al., 1973)) are very similar and average at −3.95 ± 0.16 (s.d.) percent per degree. This average value was therefore used to calculate Ostwald solubility coefficients for CCl_2F_2 and $CHClF_2$ at temperatures below 37°C

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temperature. In this way, one can correct for the effects of temperature on the light output. This measure of anaesthetic potency has been observed to fall with rising temperature for ethanol and urethane (Johnson *et al.*, 1945) and for halothane, chloroform and methyoxflurane (Flook *et al.*, 1974). These results reveal an interesting parallel with whole animal data (bearing in mind the qualitative difference in the ED_{50} measurement for the two systems) and indicate the possible relevance of luminous bacteria to anaesthetic studies. However the potency of diethyl ether (in the gas phase) as an inhibitor of bacterial bioluminescence was actually enhanced, slightly, by temperature – opposite to the results of Cherkin and

Catchpool (1964) with goldfish. Furthermore, the enthalpies of binding calculated with bacteria differ significantly from whole animal data (Flook *et al.*, 1974). These discrepancies may possibly be due to the qualitative difference, mentioned above, in the methods for the determination of temperature effects on anaesthetic and inhibitory potencies. In order to make a fairer comparison between luminous bacteria and whole animals in this regard, it may be advisable to measure the bacterial ED_{50} at a given temperature, T, as the dose which reduces the light output to a level which is 50% of a control sample at the optimum temperature, T_0. If the results of such a comparison prove comparable to whole animal data, this would warrant a much more detailed study of luminous bacteria (and of bacterial luciferase and other sensitive proteins) since the effects of temperature alone can be excluded from such systems, permitting a simpler analysis of temperature−anaesthetic interactions.

Pressure Effects: Perhaps the neatest demonstration of the importance of luminous bacteria, and hence of bacterial luciferase, to the study of general anaesthesia is the fact that the ability of pressure to antagonise or reverse anaesthesia was predicted and then discovered as a result of observations on the pressure reversal of anaesthetic inhibition of bacterial bioluminescence (Johnson *et al.*, 1942). Interestingly, luminous bacteria may be of some help in elucidating the difficulties with pressure reversal data (mentioned in section 1.2(c)). More than thirty years ago, pressure was observed to have different effects on the inhibition of bacterial bioluminescence by the homologous series of *n*−alkyl carbamates ($NH_2COO(CH_2)_nH$). Pressure antagonised the inhibition due to methyl, ethyl and propyl carbamate but had little or no effect on the inhibition by butyl and amyl carbamate and actually contributed to the depression of light output caused by hexyl and octyl carbamate (Johnson *et al.*, 1951). Strikingly, this result was reproduced, though in much less detail, with tadpole anaesthesia. Pressure reversed the effect of a 100 mM dose of ethyl carbamate (urethane) but had no effect on tadpoles anaesthetised by 1 mM amyl carbamate (Johnson and Flagler, 1951). To a certain extent, this result thus resembles the disparate pressure reversal effects of different agents observed by Halsey *et. al.* (1978, 1986). Although, somewhat surprisingly, the notion of a progressive change in the susceptibility of the members of a homologous series to pressure reversal has received little attention to date, it seems possible that more detailed investigation (with tadpoles and luminous bacteria) may prove fruitful.

Why is bacterial bioluminescence sensitive to anaesthetic inhibition? Using bacteria

of the species *Bacillus fischeri*, Taylor (1934) demonstrated that the depression of bioluminescence by alcohol and carbamate anaesthetics is not linked to some overall depression of cell activity – as judged by the rate of respiration.. If anything, Taylor found, cell respiration was either unaffected or slightly stimulated by anaesthetics. This result, which has been confirmed more recently with *Photobacterium phosphoreum* (Halsey and Smith, 1970), naturally directed attention towards the luminescent apparatus of the cell as the most likely target of anaesthetic action. However, direct evidence for this had to wait for the first successful preparation of active cell–free extracts from luminous bacteria (Strehler, 1953). Strehler and Johnson (1954) subsequently demonstrated that such extracts were sensitive to inhibition by urethane and ethanol and that this inhibition was anatgonised by pressure. Unfortunately, their study only reported inhibition and pressure effects for a single concentration of each inhibitor and for a single high pressure (approximately 400 atmospheres) – and these findings have yet to be pursued in other laboratories.

Further progress towards understanding the details of anaesthetic effects on the light emitting process was made in the 1960s. By this time the basic luciferase–catalysed reaction mechanism was well established:

$$\text{Reduced Flavin} + O_2 + \text{Aldehyde} \xrightarrow{\text{Luciferase}} \text{Oxidised Flavin} + H_2O + \text{Carboxylic Acid} + \text{Photon}$$

This reaction will be considered in greater detail in Chapter 2. For the time being it is sufficient to say that reduced flavin ($FMNH_2$) and oxygen bind rapidly to the enzyme forming a luciferase–peroxyflavin complex which reacts with a long–chain aldehyde to produce light. Hastings *et al.* (1966) observed the inhibition of this reaction *in vitro* by the anaesthetic alcohol, *n*–decanol, and concluded that decanol binds reversibly to the luciferase–peroxyflavin complex, thereby preventing the light emitting reaction with the aldehyde. This was the first clue that anaesthetics might inhibit bioluminescence, at least partially, by competing with the aldehyde substrate for binding to luciferase. Consistent with this idea is the finding that ether, though it depresses bioluminescence, does not otherwise distort the emission spectrum (Halsey and Smith, 1970).

An elegant series of experiments with whole bacteria indicated that halothane might also inhibit luciferase competitively. The light output from a solution of bacteria is quickly extinguished when the solution is bubbled with nitrogen.

Following the readmission of oxygen, luminescence rises rapidly to a peak and then declines to a steady state level. White *et al.* (1973), using *Photobacterium phosphoreum*, noted that when one observes a series of such flashes, by switching alternately between nitrogen and oxygen, the flash height is dependent on the period of anoxia (nitrogen bubbling). They observed that, at 10°C, at least 2 minutes anoxia was required to obtain a maximal flash height and deduced, not unreasonably, that this reflected the time required for the build-up of the luciferase-$FMNH_2$ complex following each flash. In the presence of 1.0% halothane the time required for this build-up, and hence for the maximal flash height was unaffected – although the flash height itself was reduced by about 65%. This is consistent with a lack of any effect of the anaesthetic on the binding of $FMNH_2$ to the enzyme. In further experiments with *Vibrio harveyi* bacteria, formerly designated MAV (Baldwin *et al.*, 1975), White and his colleagues observed the effect of pulsing an aldehyde-saturated vapour over the bacterial solution. Each pulse stimulated the luminescence, but if the time between pulses was less than 4 minutes (at 22°C) the stimulation was reduced. This was attributed to the finite time required for the accumulation of the luciferase-peroxyflavin complex following each pulse – (this complex reacts with aldehyde to produce light). 3.5% halothane had no effect on the rate of accumulation. Nor did it affect the intensity of the stimulated luminescence. However, if the aldehyde vapour was diluted so that the stimulated intensity was half maximal, in the absence of the anaesthetic, then 3% halothane was observed to cause a significant (25%) depression. These results were interpreted as a reflection of the fact that halothane does not interfere with the interaction between oxygen and the luciferase-$FMNH_2$ complex. The depression of light output that was observed, was antagonised by high levels of aldehyde – affirming the notion of competitive inhibition.

Following this demonstration that the only effect of an anaesthetic on luciferase *in vivo* seems to be targeted at the aldehyde binding site, other studies with partially purified luciferase from *Vibrio fischeri* bacteria have shown that ether (Middleton and Smith, 1976) and methoxyflurane (Adey *et al.*, 1976) also inhibit the enzyme by preventing binding of the aldehyde substrate. More recently, a report of competitive inhibition of the same preparation of luciferase by steroid compounds has been published (Banks and Peace, 1985). While inhibition was clearly demonstrated for some agents, the results could not be correlated with anaesthetic potencies. This is perhaps not surprising, since steroids are larger and structurally more complicated than the long-chain aldehyde substrate of luciferase.

That paper by Banks and Peace is in fact the only study of

anaesthetic−bacterial luciferase interactions to have appeared in the last twelve years. This is probably a reflection of the general lack of interest in protein theories of anaesthesia at a time when many scientists were searching for and finding interesting examples of anaesthetic−induced lipid bilayer perturbations. While the exact nature of the anaesthetic target remains open to question, the recent results obtained with firefly luciferase (Franks and Lieb, 1984) have prompted a reassessment of the likelihood that the physiological anaesthetic target is a protein. On the basis of the evidence discussed in this section, bacterial luciferase clearly represents a promising opportunity to probe anaesthetic−protein interactions in greater depth.

To date, firefly luciferase is the only protein which has been shown to be sensitive to a relatively wide range of anaesthetics. The obvious question is: what makes this particular protein susceptible to anaesthetic inhibition in a way that so many other proteins have proved not to be? The experimental evidence suggests that anaesthetics act by binding to the hydrophobic pocket on firefly luciferase which is normaly occupied by the aromatic luciferin substrate. In a similar manner, a small number of anaesthesics have been shown to bind to the "luciferin" pocket on bacterial luciferase, only in this case the "luciferin" is a saturated, aliphatic aldehyde. The binding site on bacterial luciferase which accomodates anaesthetics is therefore likely to be quite different from the pocket on firefly luciferase. The primary aim of this project was to assemble a broad pharmacological profile of the anaesthetic interactions with bacterial luciferase. Such a profile, it was hoped, would reveal any significant differences between the two luciferases and consequently provide new insights into the properties which render a protein binding site sensitive to anaesthetics; in this way, clues to the molecular mechanism of general anaesthesia might also be obtained.

CHAPTER 2

INTRODUCTION TO BACTERIAL LUCIFERASE ENZYMES

Bacterial luciferase is the enzyme which catalyses the light−emitting reaction in bioluminescent bacteria. Over the past thirty−five years, following the first crude extraction of this enzyme from the bacteria (Strehler, 1953), a sustained research effort has been devoted to elucidating its structure and mechanism. Although differences between luciferases from different species of bacteria have been identified, *in vitro* the enzyme catalyses the same light−emitting oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long−chain *n*−aldehyde (RCHO):

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} FMN + H_2O + RCOOH + h\nu$$

(Ziegler and Baldwin, 1981). Since molecular oxygen is divided between the long−chain carboxylic acid and the H_2O produced in the reaction, luciferase has been identified as a monooxygenase (Suzuki *et al.*, 1983). The other reaction products are oxidized flavin (FMN) and a photon ($h\nu$), usually in the blue−green region of the spectrum. The quantum efficiency (photons per reaction) is estimated at 0.1.

Considerable progress has been made towards elucidation of the structure and mechanism of bacterial luciferase. Two recent reviews provide comprehensive summaries of the extent of this progress (Ziegler and Baldwin, 1981; Hastings *et al.*, 1985). In this chapter I shall concentrate principally on the main features of luciferase structure and the reaction that it catalyses which are relevant to the study of the interactions of general anaesthetics with the enzyme.

2.1 Species Variation and Nomenclature

Any discussion of the literature concerning bacterial luciferase must contend with

the fact that studies of the enzyme have not concentrated systematically on the luciferase from a single species. Thus, various aspects of the luciferase structure and function have been elucidated with a number of different species. As will be pointed out in the discussions below, properties observed with one species may not always be generalised to all luciferases. Further confusion arises because a number of species have undergone name changes over the years. The luciferase purified and studied in the experiments described in this thesis is of the species *Vibrio harveyi*. Previously this species has been designated as *Benecka harveyi* (Hastings *et al.*, 1985) and before that simply as MAV (Baldwin *et al.*, 1975). *Vibrio fischeri* was previously named *Photobacterium fischeri* (Hastings *et al.*, 1985) and, prior to that, *Achromobacter fischeri* (Hastings *et al.*, 1969). Other species mentioned here are *Photobacterium phosphoreum* and *Photobacterium leiognathi*, neither of which has suffered a name-change.

2.2 Solubility

Since the primary aim of this thesis is to extend our understanding of the nature of direct anaesthetic-protein interactions, it is important to establish the absence of any contaminating membrane lipid in the purified *V. harveyi* luciferase which was used in experiments. In general, bacterial luciferases are regarded as cytosolic rather than membrane-bound proteins (Ziegler and Baldwin, 1981; Hastings *et al.*, 1985), the main evidence for this being their high aqueous solubility; Vervoort *et al.* (1986a), for example, were able to prepare a 3 mM solution of *V. harveyi* luciferase for their NMR studies. By way of contrast, Balakrishnan and Langerman (1977) found that a substantial proportion (80%) of the luciferase from *Ph. leiognathi* bacteria was only released from the cell debris (during purification) when the debris was treated with sodium deoxycholate; they inferred from this that carbohydrate groups on the luciferase linked it, probably extrinsicaly, to the cell membrane. However, the same study showed that *V. harveyi* luciferase is not glycosylated; presumably therefore, it forms no covalent attachement with the membrane (Balakrishnan and Langerman, 1977). A separate investigation, which confirms this finding, revealed that although a number of membrane-bound polypeptides are associated with the expression of the luminescent apparatus in *V. harveyi* cells, none of these is the luciferase enzyme (Ne'eman *et al.*, 1977).

2.3 Reaction Mechanism

The main features of the proposed luciferase reaction scheme (Holzman and Baldwin, 1983; Kurfürst *et al.*, 1984) are shown in Figure 2.1 and discussed below.

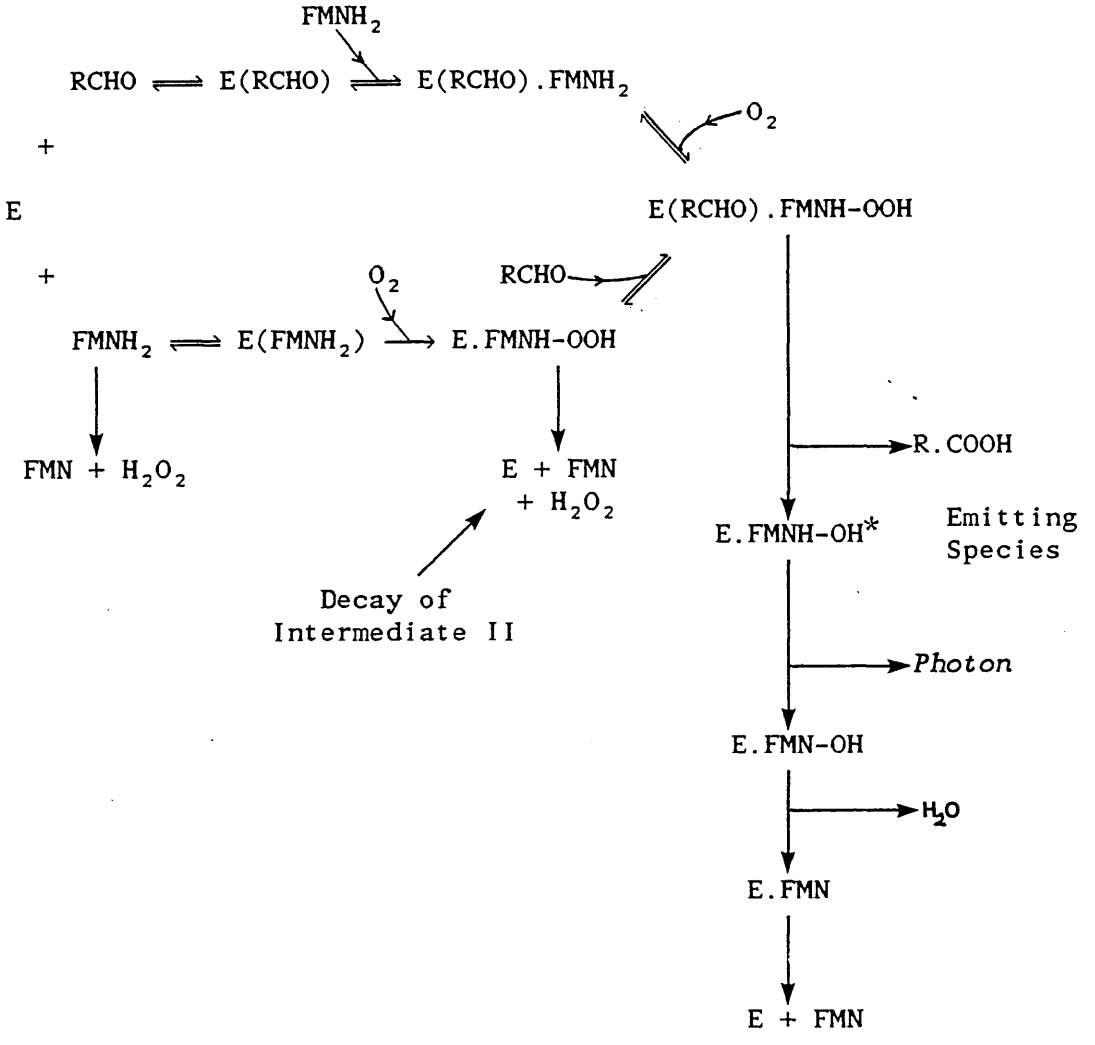

Figure 2.1: Main features of the proposed bacterial luciferase reaction scheme. For simplicily, dark side–reactions other than the decay of E.FMNH–OOH, which may occur on the pathway from E(RCHO)FMNH–OOH have not been shown.

Random Binding of Substrates: Only recently has it been suggested that the reduced flavin or the long–chain aldehyde substrates can bind independently of the other to luciferase. Previously, most workers had assumed that $FMNH_2$ bound first, and then oxygen, before the aldehyde site became available for occupation. However,

Holzman and Baldwin (1981) have shown that, in the absence of reduced flavin, the addition of *n*−decanal releases *V. harveyi* luciferase bound to an immobilised inhibitor, 2,2−diphenylpropyamine (DΦPA), which is known to compete with the aldehyde substrate for binding to the enzyme. Other experiments with the same luciferase, which provide further evidence in support of the ability of the aldehyde to bind to luciferase before $FMNH_2$, revealed that inhibition by aldehyde substrates occurs if they are allowed to equilibrate with luciferase prior to initiation of the luminescent reaction by injection of $FMNH_2$ (Holzman and Baldwin, 1983). The simplest explanation of the inhibition kinetics for this process, according to these authors, is that an inactive complex is formed when a *second* aldehyde molecule binds to an enzyme−aldehyde intermediate intermediate. Nevertheless, although it seems clear that random binding of luciferase substrates occurs, it remains to be verified that the luciferase−aldehyde complex is an active or a dead−end intermediate.

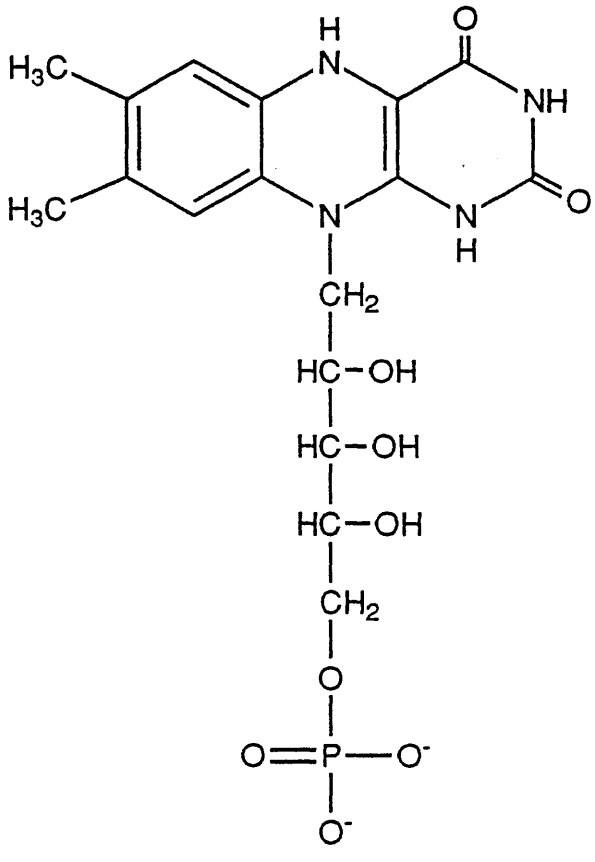

Figure 2.2: The structure of reduced flavin mononucleotide.

Flavin Binding: Strehler *et al.* (1954) showed that reduced flavin mononucleotide (Figure 2.2) is a necessary substrate in the luminescent reaction. For *V. fischeri* luciferase, the second order rate constant for the reaction of this compound with luciferase (to form $E-FMNH_2$, also known as intermediate I) is estimated to be

extremely fast: $2x10^8$ $M^{-1}s^{-1}$ (Hastings and Gibson, 1963). Later work showed that reduced flavin binds tightly to *V. harveyi* luciferase, with a dissociation constant for the E−$FMNH_2$ complex of around 0.8 μM (Meighen and Hastings, 1971). Interestingly, Holzman and Baldwin (1981) observed that the affinity of *V. harveyi* luciferase for the immobilised inhibitor, DΦPA, was enhanced by the addition of $FMNH_2$ and suggested that $FMNH_2$ induces a conformational change in luciferase, or rather, stabilises a particular conformer of the protein molecule. This conclusion is supported by later experimental work which, in particular, implicated the phosphate moiety on the ribityl side−chain of the flavin as crucial to the conformational shift (Holzman and Baldwin, 1982, 1983). A more detailed discussion of this feature is included in section 6.2(e) where the postulated ability of $FMNH_2$ to induce a conformational change is used to explain the effects that long−chain alcohols and alkanes were observed to have on the luciferase reaction.

Under normal conditions (20% oxygen, room temperature), $FMNH_2$ autoxidises, apparently autocatalytically, with a half−life of the order of 0.1 seconds (Gibson and Hastings, 1962). As a result, *in vitro* assays of the luciferase reaction do not usually permit sustained turnover of the enzyme [unless some means of replenishing the $FMNH_2$ concentration, such as the presence of an NADH−coupled flavin reductase, is available − see section 6.3(b)]. This feature distinguishes luciferase assays from most other enzyme reactions. Initiation of the luciferase reaction produces a biphasic luminescent output comprising a sharp initial rise in intensity, which peaks in about a second, followed by an exponential decay with a characteristic decay time of around 2−20 seconds, depending on species (Ziegler and Baldwin, 1981). This decay, which far outlasts the lifetime of unbound $FMNH_2$, reflects the presence of a long−lived intermediate in the reaction pathway (see below). Although luciferase reactions are often performed in the presence of a high initial concentration of $FMNH_2$ (typically 50−100 μM), the formation of a similarly high concentration of FMN in the seconds following initiation has no detectable effect on the reaction since the oxidised product binds very weakly to luciferase (*V. harveyi*), with a dissociation constant of 0.4 mM at 24°C (Baldwin *et al.*, 1975).

Reaction with Oxygen: Intermediate I has a very high affinity for oxygen. Using *V. fischeri* luciferase, Hasting and Gibson (1963) estimated that the second order rate constant for the reaction of oxygen with intermediate I is around $2x10^8$ $M^{-1}s^{-1}$. This reaction results in the formation of intermediate II which, on the basis of fluorometric measurements and chemical reasoning, was postulated to be a luciferase−4a−hydroperoxyflavin complex (E−FMNH−OOH in Figure 2.1) (Hastings *et al.*, 1973). Vervoort and his coworkers (1986a) have confirmed this identity in a

recent NMR study. Intermediate II is quite stable, possessing a lifetime of about 20 seconds at 20°C, breaking down to yield FMN and hydrogen peroxide, H_2O_2 (Hastings *et al.*, 1985). Many workers have studied this intermediate closely. Becvar and his colleagues (1978), for example, found that its lifetime is extended by low temperatures, high phosphate and high ionic strength. Tu (1979), on the other hand, measured the stabilisation of the intermediate caused by a number of aliphatic hydrocarbon compounds including *n*−alcohols and *n*−carboxylic acids. Such studies proved to be of value during the course of the work described in this thesis. Experiments based on Tu's method helped to elucidate the interpretation of several aspects of the results of experiments to investigate inhibition by anaesthetics [Sections 6.2(d)−(f)].

Reaction with Aldehyde: A long−chain n−aldehyde was identified as a necessary factor in the luminescent reaction by Strehler and Cormier (1954). *N*−aldehydes (*e.g.* *n*−decanal, Figure 2.3) react with intermediate II, reversibly at first (Shannon *et al.*, 1978; Baumstark *et al.*, 1979), and then via a series of irreversible steps, which are not yet fully understood (Shannon *et al.*, 1978) leading to the formation of the emitting species and finally the production of light. Although luciferases are not terribly specific for *n*−aldehydes of a particular chain length, there is some species variation in this regard. Thus *V. harveyi* luciferase has been shown to catalyse reactions involving aldehydes from C_4 to C_{14}, with *n*−decanal being the optimal substrate (*i.e.* the aldehyde which produces the greatest peak intensity when added to the reaction as a saturated aqueous solution) (Hastings *et al.*, 1969). The K_m for decanal on this enzyme has been determined as 1.1 μM (Holzman and Baldwin, 1983). In contrast, *V. fischeri* luciferase can catalyse the oxidation of aldehydes from C_6 to C_{20}, tetradecanal having the optimal chain−length (Hastings *et al.*, 1963).

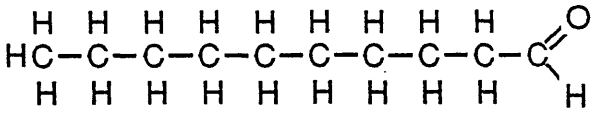

Figure 2.3: The structure of n−decanal.

The Emitting Species: The nature of the molecular species which ultimately emits the photon from the reaction continues to be the subject of debate. Kurfürst *et al.*

(1984, 1986) have suggested that, over a number of steps, a peroxyhemiacetal complex, formed by the reaction of intermediate II with the aldehyde substrate, breaks down releasing the carboxylic acid and producing a luciferase−bound 4a,5−dihydroflavin 4a−hydroxide in the excited state (E.FMNH−OH*). The step immediately prior to the formation of the emitter is held to be rate limiting, under conditions of saturating aldehyde concentrations (Shannon *et al.*, 1978). This hydroxide intermediate seems to be a strong candidate for the role of emitting species. Kurfürst and his colleagues have isolated and identified a luciferase−bound flavin 4a−hydroxide as a reaction product and found that not only does it have a fluorescence emission spectrum (λ_{max} = 485 nm) which is very similar to the bioluminescence spectrum, but also that it decays spontaneously to yield H_2O and luciferase−bound FMN, the observed final products of the reaction (Kurfuerst *et al.*, 1987). There is some dissent from this view of the emission process. Matheson and Lee (1983) have proposed a complicated alternative scheme in which two as yet unidentified intermediate species (formed sequentially from the reaction of aldehyde with intermediate II), along with other "minor fluorophores present in the luciferase preparation" are responsible for the light output. However, this scheme appears to be untenable for *V. harveyi* since it has not proved possible to identify more than a single emitting species (Lee *et al.*, 1988).

In other bioluminescent bacteria, observations that the colour of the light output *in vivo* does not match that produced *in vitro* have indeed led to the discovery of protein−bound fluorophores, distinct from the luciferase−bound flavin intermediate, which appear to act as acceptors (and emitters) of the energy released in the luciferase−catalysed reaction. Species known to contain such complexes include *Ph. phosphoreum* and a yellow strain of *V. fischeri* (see Hastings *et al.* (1985) for a review). Lastly, it may be noted that even in the absence of such acceptor/emitter protein complexes, the colour of the light emitted is found to be species dependent. For example, the emission maximum for *V. harveyi* is 492 nm but occurs at 496 nm for *V. fischeri* (Hastings *et al.*, 1969), probably reflecting small differences in the hydrophobic natures of the active sites on the two enzymes.

2.4 Luciferase Structure

Bacterial luciferase exists and functions as an $\alpha\beta$ heterodimer. The amino acid sequences of the α and β monomers have now been determined for *V. harveyi* luciferase (α, Cohn *et al.*, 1985; β, Johnston *et al.*, 1986) providing accurate values

of the molecular weights as 40,108 and 36,349 Daltons respectively. Despite recent efforts (Swanson *et al.*, 1985), the three−dimensional structure of bacterial luciferase remains unknown. Nonetheless, an amount of structural information has been gathered from biochemical experiments.

Chemical Modification Studies on *V. harveyi* Luciferase: One of the first studies to use chemical modifying agents found that *n*−ethylmaleimide inactivated luciferase by covalently attaching to a reactive cysteinyl residue on the α subunit (Nicoli *et al.*, 1974). Finding that the aldehyde substrate protected against this inactivation and that the modified enzyme had no detectable affinity for the reduced flavin substrate, Nicoli and her coworkers deduced that the active site is located on the α subunit. This conclusion is supported by the demonstration that the affinity labeling probe, 2−bromo−[1−^{14}C]−decanal, which is highly homologous with the aldehyde substrate and most likely binds in the aldehyde pocket on luciferase, also modified a cysteinyl residue (possibly the same one) on the α subunit (Fried and Tu, 1984). Cousineau and Meighen (1976) located a histidyl residue also on the α subunit close to the active site by showing that modification of this residue by ethoxyformic anhydride was antagonised by dodecanal and $FMNH_2$.

The observation that 2,4−dinitrofluorobenzene inactivates luciferase by a specific modification of either the α or the β subunit (Welches and Baldwin, 1980) provided the first indication that the active site on the α subunit is close to the $\alpha\beta$ subunit interface. The protection against this inactivation afforded by aldehyde substrates showed that, from its location at the active site, the modifier was within reach of either subunit. As with previous studies, no affinity for $FMNH_2$ was detected in the modified enzyme. A more or less identical result was obtained using the photo−activated labeling probe, 1−diazo−2−oxoundecane which, like the brominated probe mentioned above, shares structural homology with the aldehyde substrate and competes with it for binding to the enzyme (Tu and Henkin, 1983).

Chemical modification studies have also been used to probe the apolar nature of the active site. Nicoli and Hastings (1974) found that the apparent second order rate constant for inactivation of luciferase by members of the homologous series of *n*−alkylmaleimides increased dramatically with increasing chain−length. From their data, the free energy of binding per methylene group may be estimated as 2.1 kJ mol^{-1} at 25°C, which suggests a relatively hydrophobic environment at the active site − (in comparison, partitioning of methylene groups from water to hexadecane contributes a free energy of binding of around −3.51 kJ mol^{-1} at 20°C. (Aveyard and Mitchell, 1969)). Merritt and Baldwin (1980) studied the electron spin

resonance spectra of spin−labeled maleimides covalently bound to the active site and estimated that it was about as apolar as 2−propanol. In section 5.3, evidence will be presented to suggest that the aldehyde binding pocket within the active site is somewhat more hydrophobic than the active site appears to be as a whole on the basis of the above results.

Subunit Complementation Studies: The ability to reversibly denature luciferase into its constituent α and β subunits and renature hybrid luciferase dimers consisting of α and β subunits from different sources has been exploited by a number of researchers to further elucidate the structure and catalytic mechanism of the enzyme. An investigation of a large number of mutant strains of *V. harveyi* luciferases showed that those mutants with altered luminescent reaction kinetics invariably possessed "lesions" (defects) in the α subunit. (Lesions were located by denaturing the mutant dimer, $\alpha_m\beta_m$, and testing which of the hybrids renatured with wild−type subunits, $\alpha\beta_m$ or $\alpha_m\beta$, possessed wild−type kinetic characteristics) (Cline and Hastings, 1972). This finding suggests that the active centre is located on the α subunit and is therefore consistent with the results of chemical modification studies. Cline and Hastings also observed that those mutants which were more sensitive than the wild−type to denaturation by temperature had lesions in either subunit and that lesions in β appeared to hamper renaturation of hybrids. This led them to suggest that the β subunit is at least required to stabilise the proper conformation of the active site − (the α subunit has no catalytic activity in isolation). A later investigation with *V. harveyi* mutants identified a further possible role for the β subunit by showing that a mutant enzyme with a lower affinity for $FMNH_2$ than the wild−type had a lesion on the β subunit (Anderson *et al.*, 1980). Confirmation of this role was provided by Meighen and Bartlet (1980) in a study of the properties of a hybrid formed from the α subunit of *V. harveyi* and the β subunit of *Ph. phosphoreum*; the hybrid had the flavin affinity of the *Ph. phosphoreum* wild−type. This study also found that the kinetic parameters of the other steps in the reaction (aldehyde binding and specificity and the rate of luminescence decay) were determined by the α subunit. These results were repeated in experiments with hybrids formed using *Ph. leiognathi*, *Ph. phosphoreum* and *V. fischeri* luciferases (Ruby and Hastings, 1980). Interestingly, although the α subunits of *V. harveyi* (Meighen and Bartlet, 1980) and *V. fischeri* (Ruby and Hastings, 1980) can both form active hybrids with the β subunit of *Ph. phosphoreum*, they do not form active hybrids with each others' β subunits (Hastings *et al.*, 1969), an indication of the structural similarities and differences between these different species.

CHAPTER 3

MATERIALS AND METHODS

The principal aims of the experiments described in this thesis were to observe and interpret the interactions of general anaesthetics with highly purified bacterial luciferase. To this end a number of different experimental techniques were employed. In the first instance the concentrations at which a wide range of general anaesthetics inhibit the luciferase reaction by 50% (ED_{50}'s) were measured. The solubilites and anaesthetising concentrations of a novel homologous series of anaesthetics, the cycloalcohols, were also determined. Additionally, the ability of certain agents (principally long-chain alcohols) to stabilise the lifetime of the luciferase-peroxyflavin complex, intermediate II on the luciferase reaction pathway, was investigated. This chapter describes the preparation of purified bacterial luciferase and contains the details of all the solutions, apparatus and techniques which were employed in this project.

3.1 Extraction and Purification of Luciferase from *Vibrio harveyi* Bacteria

Vibrio harveyi bacteria (strain MB20) were grown and the luciferase from them extracted and purified using the facilities available at the Centre for Biotechnology at Imperial College. I am very much indebted to Dr. Tony Cass and his staff for their generous assistance at each stage of the procedure. The protocols for growth, extraction and purification follow the method of Hastings *et al.* (1978) quite closely.

Growth of Cells: Bacteria from an agar stab were streaked onto agar-NaCl complete on a single petri dish. "NaCl complete", the growing medium, was prepared with 7 g of $Na_2HPO_4.7H_2O$, 1 g of KH_2PO_4, 0.5 g of $(NH_4)_2HPO_4$, 0.1 g of $MgSO_4$, 30 g of NaCl, 5 g of tryptone, 3 g of yeast extract and 2 ml of glycerol, per litre of distilled water. After growing overnight at 30°C (the temperature used for all incubations), 6-12 bright colonies of the bacteria were selected and streaked onto separate plates for a further overnight incubation. The

six brightest of these colonies were used to inoculate six shaker flasks, each containing 250 ml of the growing medium. The cells were again grown overnight, this time with vigorous aeration. Following this incubation, 0.5 ml of cells from the two brightest flasks were added to two larger flasks containing 1 litre of medium; these were incubated and aerated for four hours and then added to 400 litres of the growing medium in the large fermentor. The careful selection of bright colonies at each stage maximized the yield of luciferase. The luminescence and cell density of bacteria in the fermentor were monitored every half−hour and the cells finally harvested at the peak of luminescence, which ocurred at the transition between the end of the logarithmic growth phase and the beginning of the stationary (or death) phase. In order to minimize the degradation of luciferase which accompanies the onset of the stationary phase, the cells were harvested quickly by chilled, continuous flow centrifugation and the resulting cell paste stored in 500 g blocks at $-20^{\circ}C$.

Cell Lysis: A 500 g block of cells was carefully broken into small pieces. The pieces were placed in a 2 litre beaker and the cells thawed by putting the beaker into a warm water bath; 10 mg of DN−ase was added to the thawing cells to chop up DNA polymers. The resulting creamy brown paste was mixed with about 100 ml of a cold 3% NaCl solution forming a thin cell suspension. In order to break open the cells, the suspension was added slowly to 7 litres of lysis buffer [5 mM phosphate, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM DL−dithio−threitol (DTT), pH 7.0] in the cold room at $4^{\circ}C$. EDTA inhibits some protease activity by chelating metal ions essential to them while DTT maintains luciferase activity by keeping sulphydryl groups reduced. Continuous stirring helped to ensure good lysis. At intervals, in order to monitor the release of luciferase from the bacterial cells a small sample of the mixture was withdrawn, centrifuged and assayed for luminescent activity in both the supernatant and the re−suspended pellet. (The assay method is described at the end of this section). Lysis was taken to be complete when 90% of the activity was found in the supernatant. At this point the whole suspension was centrifuged, using 500 ml pots, at 10,000 rpm and $4^{\circ}C$ for 30 minutes. The supernatant from each pot was then pooled in a clean beaker in the cold room and an aliquot from the pool assayed to obtain an initial measure of its luciferase activity.

Adsorption onto DEAE−Cellulose: The protein in the pooled supernatant was removed from solution by adsorbing it on to cellulose. The cellulose, 200 g of preswollen Whatman microgranular DE−52, was washed thoroughly before use by vacuum filtration on a Büchner funnel using a muslin cloth as a support. Eight litres of water, 4 litres of 0.25 M NaOH and another 8 litres of water were washed

successively through the cellulose. It was finally converted to the phosphate form by further washes with 2 litres of 0.2 M H_3PO_4, 8 litres of water, 200 ml of 1 M phosphate at pH 7.0 and lastly another 2 litres of water. Before addition of the cellulose, the pH of the supernatant was raised to pH 6.8 (if necessary) by adding a few grammes of Tris powder. With continuous stirring 200 ml of settled cellulose was added slowly to the supernatant; the pH was held at 6.8 by further addition of Tris. Uptake of luciferase causes the activity of the supernatant to drop. When 90% of the activity had disappeared from the supernatant, adsorption of protein onto the cellulose was taken to be complete.

The supernatant/cellulose suspension was poured into another Büchner funnel, also covered with a muslin cloth, and the supernatant drawn off by vacuum filtration. The vacuum was released and the "cake" of cellulose, with the proteins bound, allowed to soak in 500ml of distilled water for 5 minutes before the vacuum was again applied to remove the liquid. Following a further 5 minute wash in distilled water, 500 ml of a low ionic strength buffer (0.15 M phosphate, pH 7.0) was added to remove weakly bound proteins such as the flavin reductase. About 10% of the luciferase dissociated into solution at this stage. To remove luciferase, the cellulose was repeatedly soaked in fresh 500 ml volumes of 0.35 M phosphate, pH 7.0. Every fraction collected in this process (including the distilled water and 0.15 M phosphate washes) was assayed for luciferase activity. Five to ten washes in 0.35 M phosphate were usually sufficient to elute all of the luciferase. Fractions with greater than 10% of the peak activity were pooled and the pool assayed to obtain an overall measure of activity; typically, the volume of protein solution at this stage was 1.2 litres. This entire process was performed at room temperature with washing solutions at 4°C.

The Ammonium Sulphate Cut: Ammonium sulphate was added to the pooled washes to give a 40% solution (242 g/litre). This solution was stirred for an hour in the cold room and then centrifuged to remove any precipitate. The supernatant was retained and its ammonium sulphate concentration boosted to 75%. The precipitation of luciferase was monitored by assaying centrifuged aliquots of the solution; when the activity had dropped to 10% of its original value, the suspension was centrifuged and the pellets from this final centrifugation resuspended in a minimum volume (about 30 ml) of 0.15 M phosphate buffer, pH 7.0, containing 0.1 mM DTT. This solution was dialysed for 36 hours at 4°C against 4 litres of the same buffer (changed twice) to remove ammonium sulphate. Following dialysis the volume of the protein solution was about 120 ml.

Column Chromatography: Purification of the dialysis product was performed on three columns in succession. The first, a DEAE−Sephacel ion−exchange column (Pharmacia; 50 mm diameter x 30 cm length; 400 ml bed volume) was equilibrated in the cold room at 4°C with 0.15 M Phosphate, 1 mM EDTA, 0.1 mM DTT, pH 7.0 at a flow rate of 200 ml/hr. The sample was applied and eluted at the same rate with a linear phosphate gradient from 0.15 to 0.5 M phosphate, pH 7.0. The fractions were assayed for luciferase activity and absorbance at 280 nm to determine specific activity. Those fractions with greater than 10% of the peak specific activity were pooled (total volume ≃ 300 ml) and concentrated to a volume of 20 ml in preparation for loading onto the ACA 34 Gel Filtration column (LKB; 50 mm diameter x 1 m length; 1800 ml bed volume). This column separates proteins on the basis of molecular weight. The protein was simply eluted at 80 ml/hr with the equilibration buffer (0.15 M phosphate, 1 mM EDTA, 0.1 mM DTT, pH 7.0) at 4°C. As with the previous column, fractions were asayed for specific activity and the best ones pooled. This product was concentrated to 10 mg/ml; purity at this stage was typically 40−60%.

In the final purification step the luciferase was run on an FPLC ion−exchange column (Pharmacia HR 5/5; 5 mm diameter x 5 cm length; 1 ml bed volume) loaded with Pharmacia Mono Q Monobeads. Normally a 2 ml sample of 10 mg/ml product from the previous column was purified. Before application to the column, the enzyme buffer was changed to that which is used for column equilibration (25 mM Tris, 0.1 mM DTT, pH 8.0). This was achieved with repeated cycles of concentration and dilution (with the equilibration buffer) of the protein solution using an Amicon ultrafiltration cell. The buffer solutions used to produce a salt gradient were (A) 25 mM Tris and (B) 25 mM Tris with 0.75 M NaCl. Both solutions contained 0.1 mM DTT and were titrated with HCl to pH 8.0. The enzyme was applied to the column, which was equilibrated at 60% of A and 40% of B, in 500 µl loads and eluted by raising the proportion of B linearly to 55% over 20 minutes. The fractions were assayed for specific activity and selected ones examined on an SDS gel (See Figure 3.1 over the page for an example). The best fractions from each 500 µl loading were pooled giving a final product containing in excess of 90% luciferase. The buffer was changed to 50 mM phosphate, 0.1 mM DTT, pH 7.0 using the Amicon in the same way as before. This final luciferase solution was concentrated to 1 mg/ml and stored for future use in small aliquots (0.5 ml) in the freezer at −20°C.

Note on Luciferase Purity: All of the experiments reported in this thesis (except some preliminary work with dithionite assays − see section 4.2) were performed

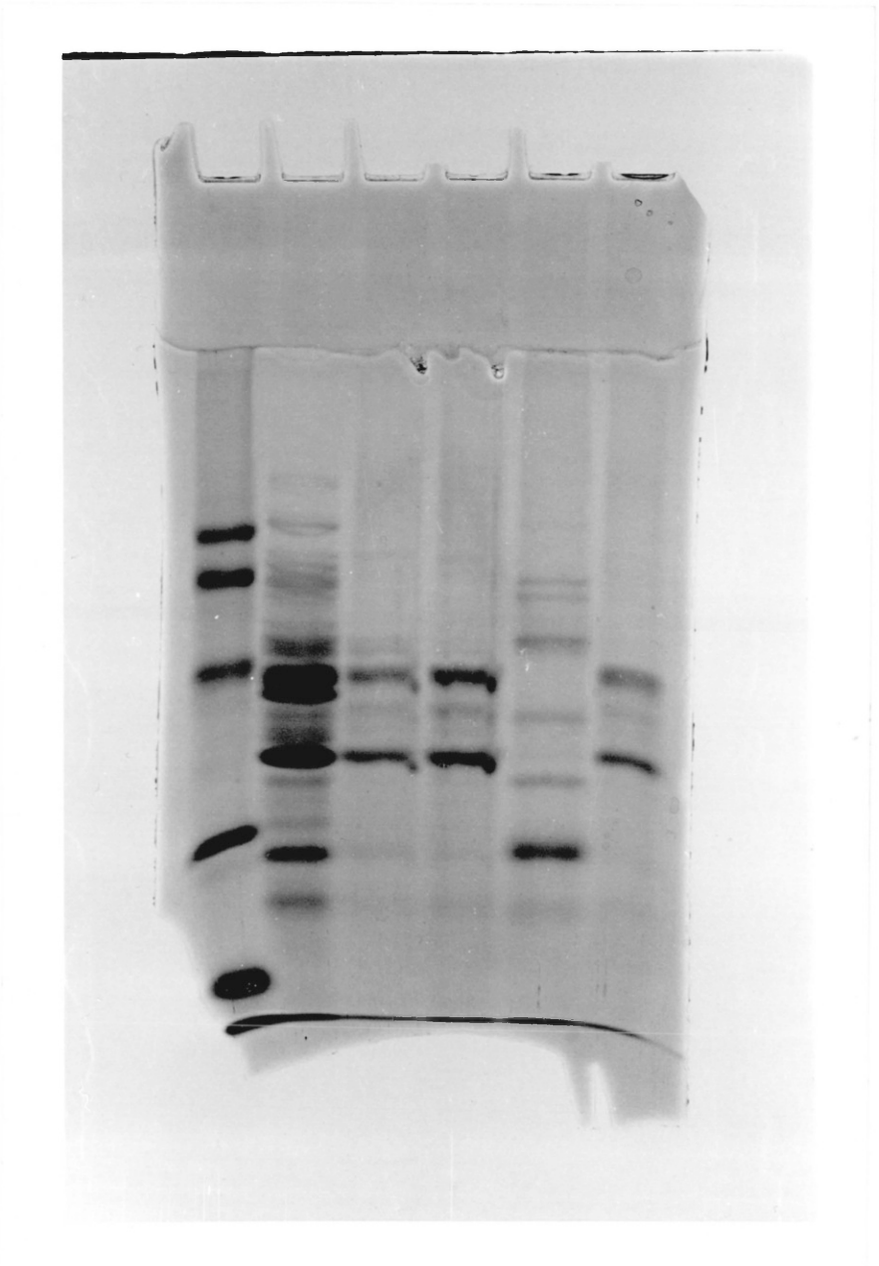

Figure 3.1: Photograph of 10% SDS polyacrylamide gel showing bacterial luciferase purity at the end of the purification process. Tracks are identifiable as follows: (1) Molecular weight markers; (2) Starting material for FPLC column; (3), (4), and (6) Pools of fractions with the highest specific luciferase activity from three separate loadings of starting material on the FPLC column – α and β identify the luciferase subunits; (5) Typical composition of a fraction from the second maximum in the protein eluted from the column – note that this fraction contains a negligible amount of luciferase.

using luciferase from two separate preparations. Although there was no discernable difference in the purity of the luciferase obtained on each of these two occasions, the decay of luminescence in the reaction using the product from the second purification was about 40% faster for a given aldehyde concentration. The origin of this effect is unknown but it was not observed to cause any difference in the interactions of either the decanal substrate or general anaesthetics with luciferase: the Michaelis constant for decanal and the ED_{50} concentrations of anaesthetics were the same (within experimental error) for the two luciferase samples.

Assaying for Luciferase Activity: During the enzyme preparation these assays were performed using the LKB photometer in the Centre for Biotechnology. 10 μl of enzyme solution, of appropriate dilution, was added to a plastic cuvette, followed by 10 μl of 10 mM FMN in 50 mM phosphate buffer, pH 7.0. These two droplets were mixed by the addition of 470 μl of assay buffer (50 mM phosphate, pH 7.0 with 0.2% BSA). The FMN was reduced by injecting 5 μl of sodium dithionite solution (15 mg/ml kept under nitrogen), the cuvette then placed in the photometer and the reaction initiated by the rapid, manual injection of 500 μl of 10 mM *n*−decanal emulsion (prepared by sonication) in 50 mM phosphate, pH 7.0. Activity was taken as the peak intensity of the resulting light emission.

3.2 Preparation and Handling of Solutions

The vast majority of experiments involved the measurement of the effect of general anaesthetics on some aspect of the function of bacterial luciferase. This section details the preparation of substrate and anaesthetic solutions used in luciferase work. The solutions which were used in determinations of the solubility and anaesthetizing concentrations of cycloalcohols are described along with the methods for these experiments in section 3.4 of this chapter.

3.2(a) Reagents for the Luciferase Reaction

All reagents were of the highest purity commercially available. Phosphate salts, sodium dithionite and FMN (riboflavin 5'monophosphoric acid, monosodium salt) were obtained from BDH; EDTA, DTT and *n*−decanal from Sigma and 10% palladium on activated charcoal from Aldrich.

Buffer: A 50 mM phosphate buffer at pH 7.0 was used throughout. The buffer was prepared on the day of the experiment from two 1 M stock solutions of K_2HPO_4 and KH_2PO_4 at pH 10 and 4 respectively. Microbial growth was prevented by the high and low pH of these solutions and by storing them in the fridge at 4°C for no longer than two weeks. On the day of an experiment the buffer solution was typically prepared by mixing 44 ml of 1 M K_2HPO_4, 36 ml of 1 M KH_2PO_4 and 1520 ml of de−ionized water. This gave a 50 mM solution of pH slightly greater than 7; the solution was titrated to pH 7.0 with several drops of 6 M HCl

Luciferase: As described in the last section, luciferase was stored in 50 mM Phosphate, 0.1 mM DTT, pH 7.0, at −20°C. The concentration of luciferase in this stock solution was approximately 1 mg/ml. (This corresponds to a concentration of 13 μM, since the molecular weight of *Vibrio harveyi* luciferase is 76,457 Da). For use in experiments the stock was thawed on ice over several hours and a small aliquot diluted into buffer at 0°C. This solution was kept on ice for the entire duration of the experiment. In most experiments a 10 μl aliquot of this diluted solution was withdrawn by micropipette for each assay. The final luciferase concentration was usually about 0.4 nanomolar.

Decanal: Decanal solutions were made up on the day of an experiment in order to minimise problems arising from oxidation of the aldehyde group. Due to the very low solubility of decanal (the saturating concentration in water ≃ 140 μM) it was first dissolved in ethanol and then, just before the start of the experiment, a 20 μl aliquot of this ethanolic solution was diluted in 250 ml of buffer. The buffer was stirred continuously to facilitate the rapid dispersion and solution of the aldehyde. The concentrations of decanal solutions prepared in this way ranged from 6 to 60 μM, well below saturation. The decanal solution also contained 1.3 mM ethanol which is much lower than an ethanol concentration which was observed to have a detectable effect on the luciferase reaction. Nonetheless in experiments where the amount of aldehyde was varied, the concentration of ethanol was adjusted so that it remained constant. In order to do this, 20 μl of neat ethanol was added to 250 ml of buffer to give the same ethanol concentration that was present in the decanal solution. Reaction vials were then prepared by adding x ml of decanal solution to (V−x) ml of the buffer with added ethanol (where x is a variable, and V a constant, volume). Therefore the total volume of buffer which contained ethanol was fixed at $x + (V-x) = V$. Typically, V was no more than 2.5 ml out of a total final volume of 7.51 ml; as a result, the maximum ethanol concentration present was only 0.4 mM.

Flavin: The reduced flavin required for the reaction was prepared from a solution of oxidized flavin (FMN). This is readily soluble in buffer but must be protected from photochemical degradation (usually by covering the solution with foil or black plastic). In several initial experiments FMN was chemically reduced using sodium dithionite ($Na_2S_2O_4$); however dithionite was observed to affect the luciferase reaction (See Chapter 4 for details) and a different method, the reduction by H_2 gas on a palladium catalyst, was adopted.

Reduction by Hydrogen: The flavin was reduced by bubbling hydrogen gas through a solution of FMN containing a palladium catalyst. The most convenient form of the catalyst available was as activated charcoal powder containing 10% palladium. Preliminary experiments showed that 15 mg of the charcoal powder was sufficient for the reduction of 100 ml of 400 μM FMN which had hydrogen, regulated at 4 p.s.i., bubbling gently through it. Reduction was complete in about 30 minutes. The presence of activated charcoal inevitably depleted the flavin concentration and, under the above conditions, 40% of the flavin was absorbed into the charcoal. Nine-tenths of this absorbtion occurred within 40 minutes; thereafter the flavin concentration in the reduction flask declined at a rate of 9-12 μM per hour. A 40% excess of flavin was therefore used in preparation of $FMNH_2$ solutions by this method. The concentration of flavin was measured spectrophotometrically [ϵ_{450} = 12,220 M^{-1} cm^{-1} (Whitby, 1953)] at the beginning and end of each experiment and never varied by more than a few percent. The flavin concentration of a given experiment was taken as the mid-point of the initial and final concentrations. The average flavin concentration of seventy experiments where a concentration of 100 μM was desired, was calculated to be 103 ± 9.6 μM (standard deviation).

Reduction by Dithionite: Two methods of flavin reduction by sodium dithionite were tried before dithionite was rejected as a reducing agent for the purposes of the luciferase experiments described in this thesis. In the first method luciferase assays were initiated by rapid injection of a solution of oxidized flavin into a vial containing luciferase, *n*-decanal and a small volume (usually 50 μl) of a sodium dithionite solution to give a final dithionite concentration (in 7.51 ml of reactants) of 640 μM, sufficient to reduce 100 μM FMN, the normal working level. Since dithionite is susceptible to oxidation, its solution was prepared by adding dithionite powder to buffer which had been perfused with oxygen-free nitrogen for an hour. The solution was kept under nitrogen for the duration of the experiment.

In the second method the reaction was initiated with $FMNH_2$ which had been reduced in advance with dithionite. In a manner quite similar to the method

described above, the $FMNH_2$ solution was prepared by adding dithionite to a flask of FMN solution which had been perfused with oxygen-free nitrogen for an hour and was subsequently kept under nitrogen for the whole experiment. In this case the nitrogen also served to keep the flavin in the reduced state.

Even with the precaution of keeping solutions containing dithionite under nitrogen, both methods suffered from oxidation of the reducing agent. The half life was observed to be in the region of two hours. For all experiments therefore, sufficient dithionite was added so that its concentration after 90 minutes (the typical length of an experiment) was still adequate for complete flavin reduction.

3.2(b) Anaesthetic Solutions

In all, fifty-four agents were studied for their effect on bacterial luciferase. They mostly fall into several broad categories: *n*-alcohols, *n*-alkanes, α,ω-*n*-alkyldiols, cycloalcohols, halogenated hydrocarbons, and barbiturates. For all luciferase experiments buffer solutions of anaesthetics were prepared. Since the agents used in this study came in gaseous, liquid and solid forms and since their aqueous solubilities spanned ten orders of magnitude, a variety of strategies was required to dissolve them in buffer.

The agents were obtained from a variety of sources and over half were at least 99% pure. All the volatile inhalational anaesthetics were obtained from medical suppliers. The agents and their sources are listed below.

BDH: *n*-alcohols from propanol to decanol; dodecanol; hexadecanol; *n*-alkanes from pentane to decane; chloroform; butanone; paraldehyde. ALDRICH: pentadecanol; cyclododecanol; α,ω-*n*-alkyldiols from 1,4-butanediol to 1,10-decanediol except 1,7-heptanediol; 1,12-dodecanediol; 1,14-tetradecanediol; 1,16-hexadecanediol; benzyl alcohol; adamantanol. SIGMA: undecanol; tridecanol, tetradecanol; hexadecanol; n-alkanes from undecane to tetradecane; urethane; barbital (sodium salt); pentabarbital. LANCASTER SYNTHESIS: cycloalcohols from cyclohexanol to cyclooctanol; 1,7-heptanediol. ABBOTT: methoxyflurane (Penthrane); isoflurane (Forane); enflurane (Ethrane). I.C.I.: halothane (Fluothane). OHIO MEDICAL PRODUCTS: fluroxene (Fluoromar). B.O.C.: propane. FISONS: diethyl ether. MAY & BAKER: acetone. K & K LABORATORIES: cyclodecanol. JAMES BURROUGHS: ethanol.

Soluble Agents: Those agents with solubilities greater than 1 mM were dissolved directly into buffer. In most of these cases gentle stirring was sufficient for complete solvation; (*n*−alcohols from ethanol to nonanol, α,ω−*n*−alkyldiols from butanediol to nonanediol, diethyl ether, acetone, butanone, paraldehyde, benzyl alcohol, urethane and barbital were prepared in this way). Evaporation of volatile agents in this group (*e.g.* diethyl ether, butanone) was minimised by making up their solutions in stoppered volumetric flasks. As a matter of routine, all other solutions were prepared in beakers which were then covered with cling−film. Due to the viscosity of liquid cycloalcohols (cyclohexanol to cyclooctanol inclusive) these were added to buffer which had been warmed to 40°C. The alcohol was dissolved by stirring and the solution then allowed to cool slowly to room temperature.

Volatile Agents: Buffer solutions of halothane, chloroform, methoxyflurane, isoflurane, enflurane and fluroxene were prepared very carefully in order to prevent loss by evaporation. An hour before the start of an experiment a small volume of anaesthetic was injected under the surface of 21 ml of buffer in a scintillation vial. This volume of buffer filled the vial almost completely thus minimising the loss of anaesthetic from solution due to partitioning between air and buffer in the vial. The amount of anaesthetic delivered was measured by weighing the vial before and after injection. The top of the vial was covered with foil, capped and the mixture vortexed for several minutes to disperse the anaesthetic into solution. For use in the experiment the solution was transferred into a 20 ml gas−tight glass syringe fitted with a hypodermic needle. The cap of the vial was removed and the foil carefully pierced with the syringe needle to allow loading with minimum evaporation. Air bubbles were quickly expelled from the syringe before it was clamped to a calibrated syringe microburet (Micrometric Instrument Co. (Ohio); Model No. SB2). In a preliminary test, samples of a halothane solution were taken from the syringe over a half−hour period and the concentration of halothane determined spectrophotometrically at 245 nm: no detectable loss of halothane occurred. In contrast, a 33% drop in the concentration of a halothane solution in an open vial was observed by the same method after the same period. The syringe−microburet combination therefore enabled precise delivery of volatile anaesthetic solutions the reaction vial; delivery was timed for 15−20 seconds prior to initiation of luciferase reactions. In several experiments a saturated solution of halothane in buffer was prepared by a very similar method. In these cases double the amount of halothane calculated to be necessary for saturation of the 21 ml of buffer was added to the vials. The mixture was vortexed, allowed to equilibrate overnight, vortexed again and then centrifuged at 1000 rpm for 30 minutes at a temperature of 24°C before being loaded into the microburet syringe.

Alkane Solutions: Due to the exceptionally low solubilities of *n*−alkanes, saturated solutions of these agents were prepared. Neat alkane (100 μl pentane, 30 μl hexane, 20 μl heptane and 10 μl of each of the higher alkanes) was added to 21 ml of buffer in clean scintillation vials, shaken vigorously and equilibrated overnight on a rotator at 4 rpm. It was found necessary to centrifuge the solutions of pentane, hexane and heptane (in sealed centrifuge tubes at 10,000 rpm for 15 minutes at 24°C) in order to remove excess alkane from the body of the solution. Since these three alkanes are also quite volatile, they were handled in exactly the same way as the volatile anaesthetics mentioned above, that is, with the syringe microburet. In order to test whether trace impurities in the the alkanes had any effect on the results of luciferase inhibition experiments, a different method of preparation of solutions of alkanes from octane to tetradecane was also used. Ethanolic solutions of each alkane were prepared such that the addition of 10 μl of this solution to the 5.01 ml of reactants (luciferase and decanal) delivered only five times the amount of alkane required to saturate the solution. 10 μl of ethanol was added to the controls in these experiments giving a final ethanol concentration, after injection of 2.5 ml of $FMNH_2$, of 23 mM. This concentration, which is 1.5% of the concentration required to half inhibit the luciferase reaction under these experimental conditions, was observed to cause a general 10% increase in activity. It is not known whether the mechanism whereby ethanol increases activity is interferes with competitive inhibition. However, in no case was a significant difference observed in the inhibition of luciferase by alkane solutions prepared by either method. The method of ethanolic solutions was not used to test for the effects on inhibition of trace impurities in solutions of shorter alkanes because of their volatility. Propane solutions were prepared by bubbling propane gas at a rate of 0.25 $cm^3\ s^{-1}$ through continuously stirred buffer at atmospheric pressure for 24 hours. This method has previously been shown to give a solution of propane equilibrated with 1 atmosphere of the gas (Franks and Lieb, 1985). The concentrations of alkane solutions were calculated from aqueous solubility data summarized by Bell (1973).

Other Agents of Low Solubility: Two approaches were adopted, both involving ethanolic solutions. In most cases 20 μl of an ethanolic solution of the agent was dissolved in 100 ml of buffer. For experiments where the amount of this anaesthetic solution was varied, the ethanol concentration was held constant by the same method as for *n*−decanal solutions [see section 3.2(a)]. The maximum final ethanol concentration never exceeded 2.0 mM when these types of solution were used (including the contribution of ethanol in the decanal solution, when present). The agents handled in this way were: *n*−alcohols from octanol to hexadecanol,

cyclodecanol, cyclododecanol, $\alpha,\omega-n-$alkyldiols from nonanediol to hexadecanediol and adamantanol.

The inhibition caused by pentobarbital was determined by adding 10−25 μl of an ethanolic solution of these agents directly to the 5.01 ml of luciferase and decanal. As long as the concentration of the anaesthetic was kept below its aqueous solubility there was no difficulty in dispersing the ethanolic solution into buffer. The final ethanol concentrations ranged from 23 to 57 mM and control reactions with these concentrations of ethanol were also recorded. Such controls had peak intensities which were from 10 to 25% higher than those recorded in the absence of ethanol.

3.3 The Rapid−Mixing Apparatus

The apparatus used to initiate and record the luciferase reaction is shown in the photograph in Figure 3.2 and presented schematically in Figure 3.3. It was used to initiate the reaction by injecting either of the two substrates, $FMNH_2$ or $n-$decanal into a vial containing luciferase and the other substrate. This section describes the basic set−up of the apparatus and provides an account of its performance. Additional modifications required by the two different methods of initiation will be detailed in section 3.4, where these methods are described.

3.3(a) Description of the Apparatus

The apparatus has two principal components: a gas−driven syringe assembly, designed for prompt, reproducible delivery of the initiating substrate solution, and a photomultiplier unit which records the light output through the base of a glass reaction vial. The injection mechanism is supported on a stainless−steel frame which is composed of two vertical struts spanned by horizontal cross−spars. The barrel of a 5 ml glass injection syringe (Chance, luer−lock) is clamped between the middle two cross−spars above a midget air cylinder (Schraeder Bellows, Part No.40400), itself bolted to the lowest spar. The syringe plunger rests on a small platform screwed to the top of the air cylinder rod. The air cylinder is supplied, via a levered midget disc valve (Schraeder Bellows Type 3/2, Part No.B3213H), with nitrogen regulated at 16−17 p.s.i.. The disc valve, which is fixed to the base of the apparatus, is opened by throwing the lever; the nitrogen pressure immediately

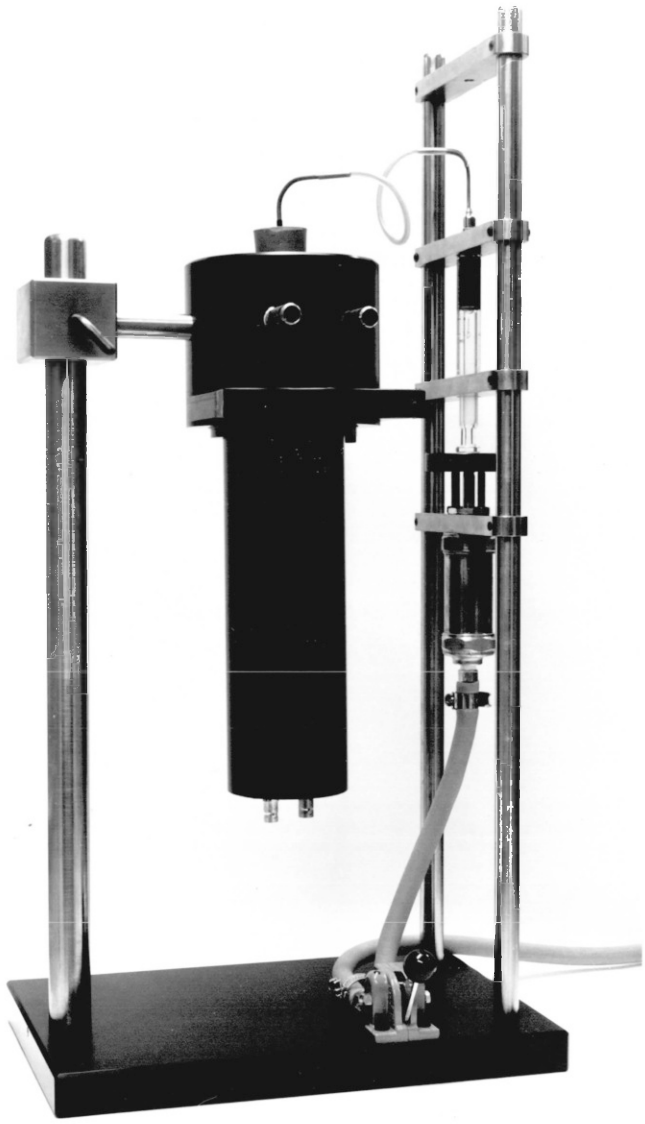

Figure 3.2: Photograph of the basic set-up of the rapid-mixing apparatus. [This set-up was used for aldehyde-initiated assays - see section 3.4(b)]. The rubber collar normally in place around the neck of the syringe plunger is not shown.

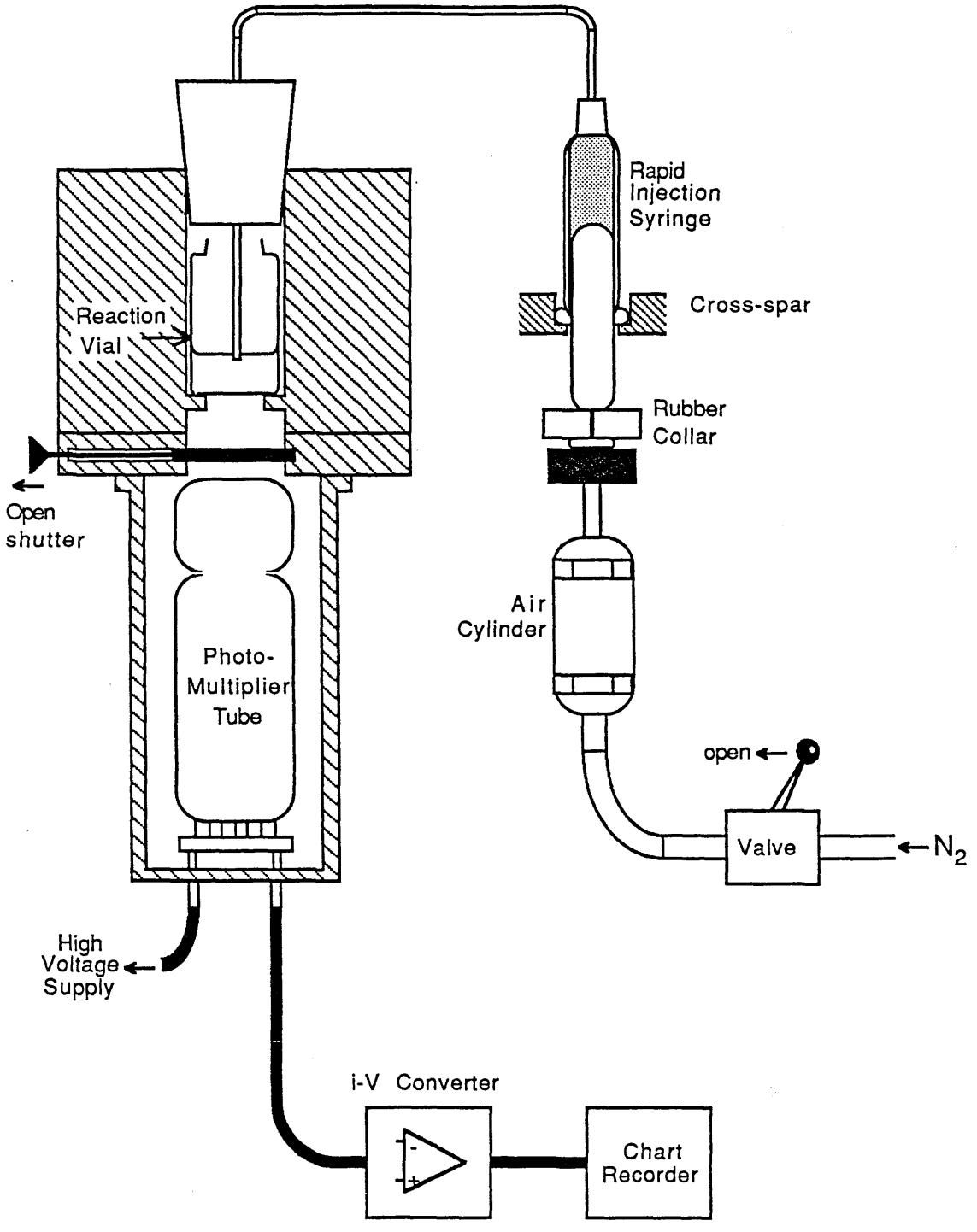

Figure 3.3: Schematic diagram of the basic set–up of the rapid–mixing apparatus – (as used in aldehyde initiated assays).

pushes the cylinder rod and plunger upwards, expelling the contents of the syringe, via a delivery tube, into the reaction vial. Once the reaction has reached completion, closure of the disc valve shuts off the nitrogen pressure and simultaneously allows the gas to exhaust from the air cylinder. A spring returns the rod (and platform) to its original resting position.

The distance of travel of the platform from this resting position to the point of fullest extension determines the volume of solution driven from the syringe. This distance can be adjusted by altering the heights of two screws which are bolted to the lowest cross-spar and which press against the underside of the platform in its rest position. For all of the work described in this thesis the delivered volume was set at 2.5 ml. Since the plunger of the syringe is not fixed to the platform on which it rests, it was necessary to prevent it overshooting upwards once the cylinder rod (and platform) has come to an abrupt halt - at the point of fullest extension. To do this a close-fitting, removable rubber collar was placed around the narrow neck of the plunger so that it sat on the rim formed where the neck meets the plunger base. The height of the cross-spar supporting the syringe barrel was then altered such that when the rod was fully extended (having expelled 2.5 ml from the syringe), the top of the collar exactly fitted the gap between the rim of the plunger base and the underside of this cross-spar. This prevents any further upward travel of the plunger. The collar also acts as an effective shock absorber preventing any damage to the syringe plunger or barrel that might result from the very abrupt movements of the cylinder rod.

The contents of the syringe are fed to the reaction vial in a delivery tube made with sections of metal and plastic tubing. A broad stainless-steel needle (diameter = 2 mm), bent through a gentle 90 degree turn, is luer-locked to the top of the syringe. This needle is connected to an L-shaped stainless-steel tube by a 340 mm length of clear, flexible plastic tubing. One arm of the second steel tube passes through a rubber bung which provides a light-tight seal over the compartment housing the reaction vial. Starting at the end joined to this second steel tube, a 150 mm length of the clear plastic tubing is wrapped in black plastic to ensure complete light-tightness of the vial compartment. The bung also acts as a firm support (by friction) for the end of the delivery tube; the ability to position this end of the tube in the same place for each reaction is a necessary condition for consistent mixing.

The reaction vial compartment sits on top of the light-recording unit of the apparatus. This compartment, which is cut out of the middle of a cylindrical

head–block, was tailored to be a good fit for Scintran scintillation vials (base diameter = 25 mm, height = 56 mm) thus minimizing any lateral variation in the position of the vials which might distort the distribution of light emitted towards the photomultiplier tube. Its temperature may be regulated by passing temperature–controlled water through the head–block. This enables luciferase reactions to be observed at a broad range of temperatures; however no such experiments were undertaken in this study – the head–block was simply allowed to equilibrate at room temperature, which varied in the range 22.5 to 25.5 °C for the work described in this thesis.

In its compartment, the reaction vial sits on a narrow rim just above a manually operated shutter; immediately beneath is the photomultiplier housing. The photomultiplier tube (Thorn EMI Type 9558B) plugs into a support assembly which is fixed to the base of the housing. This assembly enables the vertical position of the tube to be adjusted so that its window is as close to the shutter and reaction vial as possible – in order to maximize the collection of light. A high–voltage coaxial socket on the underside of the housing passes the operating d.c. voltage to the tube (supplied by a Brandenburg photomultiplier supply, model 475R). An adjacent BNC connector feeds the current from the tube's dynode resistor chain to the amplifier. Amplification is performed with a simple one–stage current–voltage converter with three gain settings (relative approximate gains: 0.5, 1 and 10; see

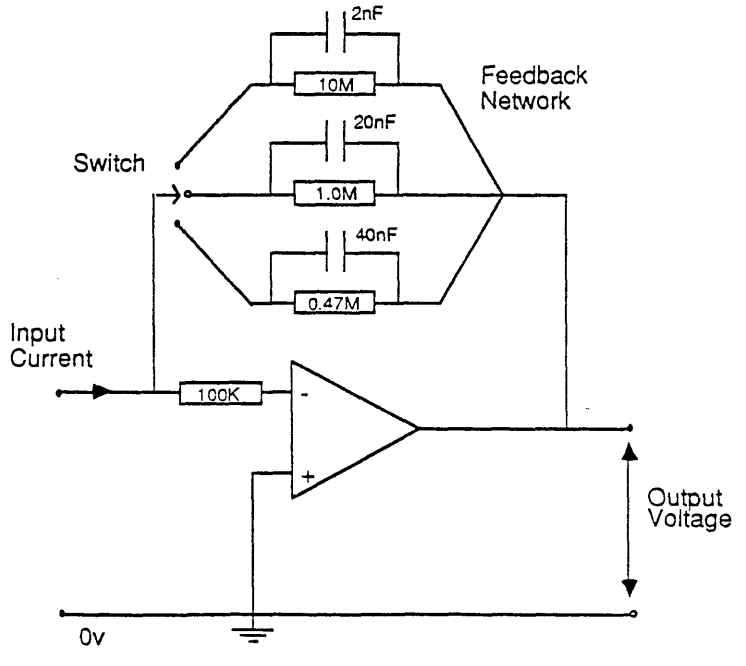

Figure 3.4: Circuit diagram of the current–voltage converter used to amplify photomultiplier signals.

Figure 3.4 for a circuit diagram) which simultaneously filters the signal to remove extraneous noise. The amplified signal is fed finally to a chart recorder.

3.3(b) Performance of the Apparatus

Upon rapid initiation, the intensity of light emitted from the luciferase reaction rises to a peak in about 1 sec and thereafter decays quite slowly with a half−life greater than 2.5 seconds under most conditions. For the peak intensity to be a meaningful measurement of the rate of a given reaction (a subject that I will return to in the next chapter) the apparatus has to fulfil two main performance criteria: (i) mixing of the reactants must be complete in less than a second and (ii) the recorded signal must be directly proportional to the light output of the reaction. The performance of the system with regard to these two criteria is described below.

Mixing of the Reactants: Good mixing will be achieved if excessive sloshing or splashing of the reactants does not occur as a result of the rapid injection of the initiating slug of substrate solution. The parameters which affect the quality of mixing are: the nitrogen pressure used to drive the injection of substrate solution, the length of delivery tubing between the syringe and reaction vial, the position of

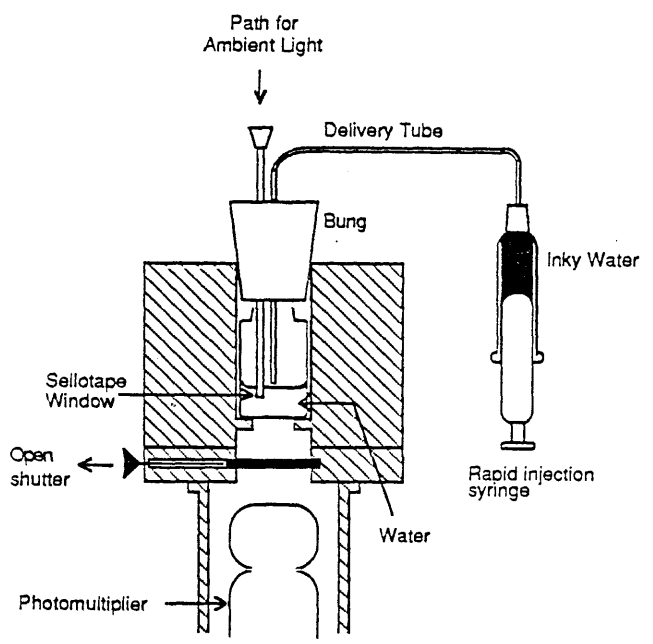

Figure 3.5: Schematic diagram of the experimental set−up used to test the speed of mixing of the injected slug (inky water) with the contents of the vial (clear water). Notice that the cross−spars supporting the syringe and the rubber collar are not shown.

the tip of the delivery tube relative to the surface of the reactants in the vial and the relative volumes (or masses) of the injected slug and the vial contents. These parameters were optimized by observing the change in opacity of water in the reaction vial as a slug of inky water was injected. The increase in opacity which occurred upon mixing was observed by using the photomultiplier to record the level of light transmitted through the vial contents (FIgure 3.5). A path for ambient light was created by inserting a narrow metal tube through the bung which seals the top of the vial compartment so that it just penetrated the surface of the water in the vial. Splashing of water into this tube, which would have distorted the measurement of transmitted light, was prevented by fixing a Sellotape window over the end of the tube. The injection of the slug of inky water caused the transmitted intensity to fall from a high to a low level and the ambient light and ink solution were adjusted so that this gave a difference signal of about 20mV. Due to the speed of changes in the transmitted intensity following injection, the signal was captured on a digital storage oscilloscope and then plotted out onto a chart recorder. An example of an early observation of mixing using this technique is given in figure 3.6(a). The large peak in transmission between injection an equilibration was associated with a violent

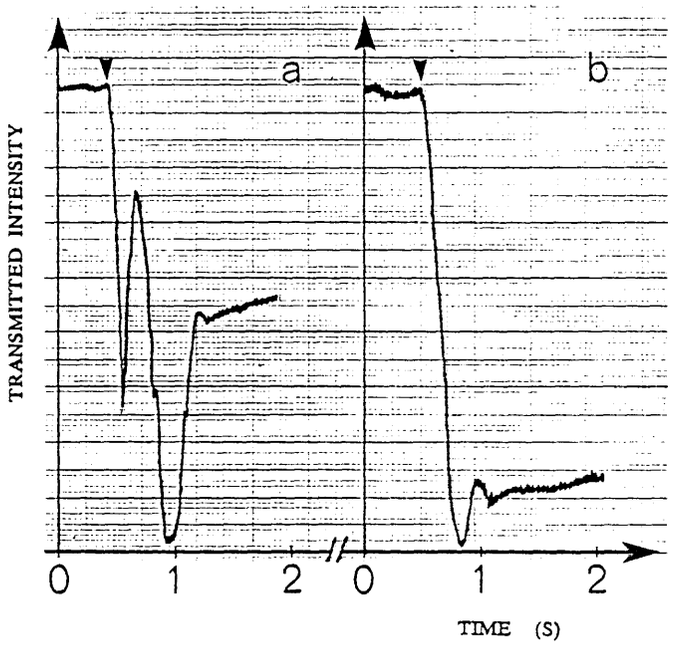

Figure 3.6: Typical output from the experiment to measure mixing speed. The transmitted intensity falls from a high to a low level following the injection of the slug of inky water. Injection is indicated by the arrowheads. (a) An early result. The large peak represents a large "bounce" in the vial contents. (b) A result obtained after tuning. The difference in the stabilised level of transmitted intensity following injection is due to the different concentrations of ink used.

"bounce" of the mixture as the force of injection caused it to leap up the sides of the vial; (this was observed visually). By adjusting the various parameters mentioned above it was possible to tune this "bounce" out of the mixing process. The trace corresponding to optimized mixing is given in Figure 3.6(b).

The optimum values of the relevant parameters are:

* Nitrogen injection pressure = 16 p.s.i.
* Delivery tube length = 30 cm
* Volume of injection slug = 2.5 ml
* Volume of reactants in vial = 5.0 ml
* Position of delivery tube tip: just under the surface of the reactants

For optimum mixing the time between injection of the slug and equilibration at the lower level of transmission, t_{eq}, was observed to be 0.4 secs. However complete mixing is very probably achieved sooner than this because t_{eq} is extended by the time taken for air bubbles to rise out of the ink−water mixture following injection. The actual mixing time is therefore difficult to determine but I estimate that it may be as low as 0.1−0.2 seconds − well under the minimum time to peak observed for any luciferase reaction.

Once the parameters which determine mixing were set, the actual volume delivered by the injection was determined by injecting pure water into 5.0 ml of the same and measuring the increase in mass of water in the vial. The average (± standard deviation) of five injections performed at 24°C was 2.48 ± 0.02 g; this corresponds to an injected volume of 2.49 ± 0.02 ml.

Amplification of the Light Output: A proper measurement of the light output from a reaction depends on linear amplification by both the photomultiplier and the current−voltage converter. The current−voltage converter was tested to check that the gain of each setting was independent of the input current and frequency over a useful range. In Figure 3.7 the input−output relationship, up to saturation of the output voltage, is given for amplification of a d.c. input current on each gain setting. The slopes, calculated by the method of least squares are 0.471 ± 0.001, 0.998 ± 0.001 and 9.70 ± 0.04 V/μA.

Under most of the experimental conditions used in this project the light output from luciferase reactions reached a peak in not less than 1 second, corresponding to a fundamental frequency of around 0.5 Hz. The fastest observed signal peaked in

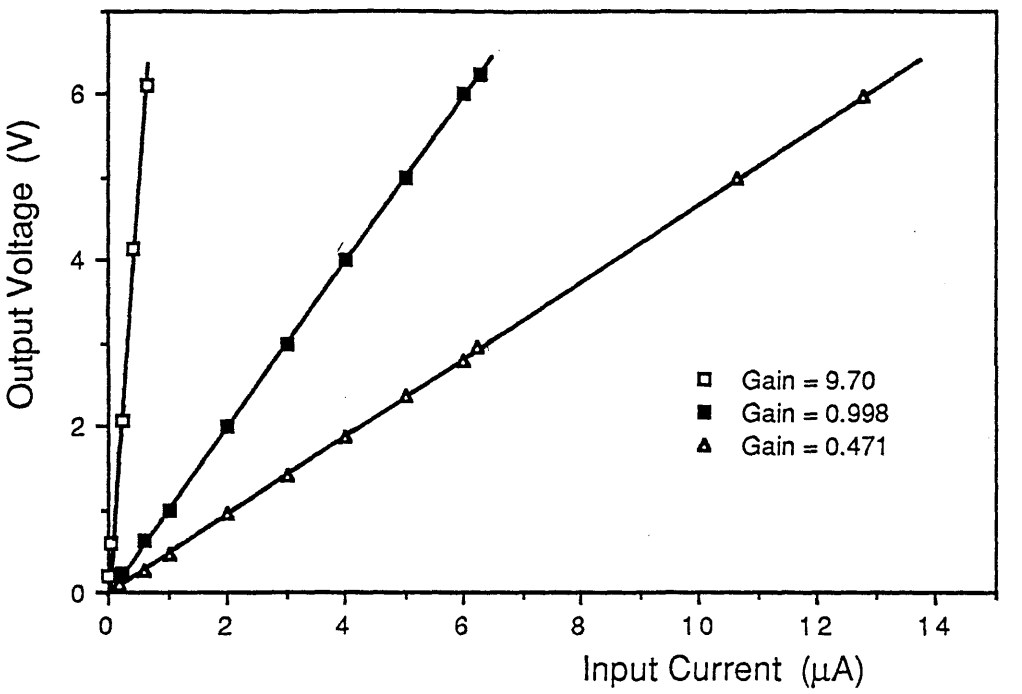

Figure 3.7: The d.c. input–output relationship of the current–voltage converter. The constant gradients indicate that the gain on all three settings is independent of the magnutude of the input d.c. current.

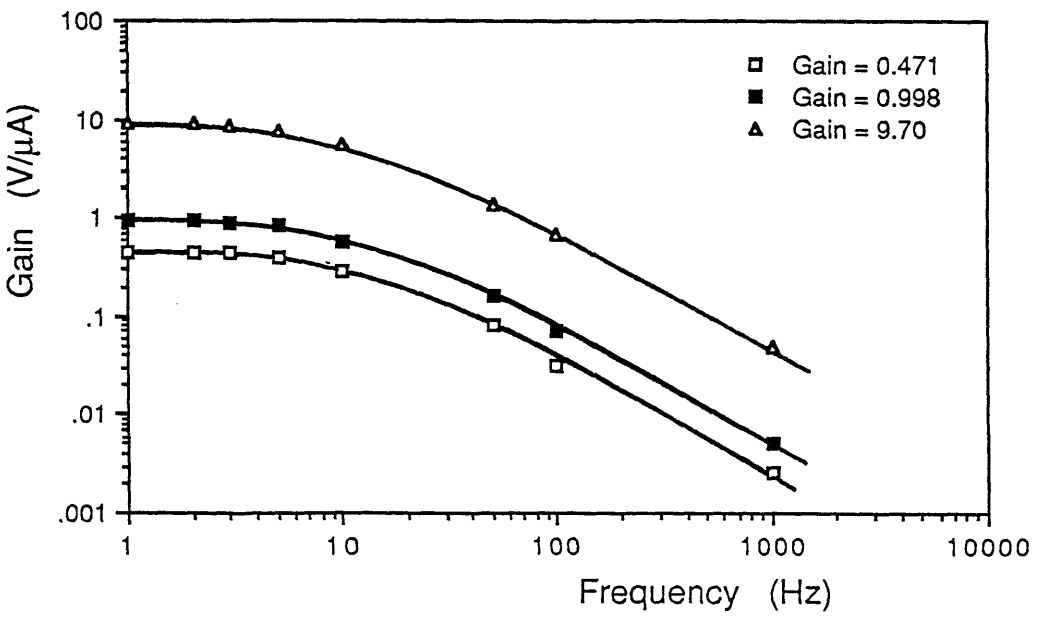

Figure 3.8: The frequency response of the current–voltage converter (to a sinusoidal input current).

0.4 seconds (1.25 Hz). The frequency response of the i−V converter is shown in Figure 3.8. Within experimental error (≃ 0.5%), there is no loss of gain up to 1 Hz; at 2 Hz the loss of gain is around 1%. Therefore the fastest recorded signal in my work is subject to a slight systematic error; however this deviation is negligible compared to the other errors (*e.g.* in pipetting) associated with each assay. In order to record signals faster than 1.25 Hz the converter would, of course, need to be modified.

Once it had been established that the i−V converter was working properly, it was used to test the photomultiplier. With the i−V converter on its lowest gain setting, a range of concentrations of luciferase was assayed for activity. The concentration range was extended such that the highest concentration gave a peak intensity close to the maximum output level of the converter. In this way the amplification of light levels all the way up to the maximum observable with this equipment was tested. Figure 3.9 shows that, within experimental errors, light amplification by the photomultiplier is satisfactorily linear.

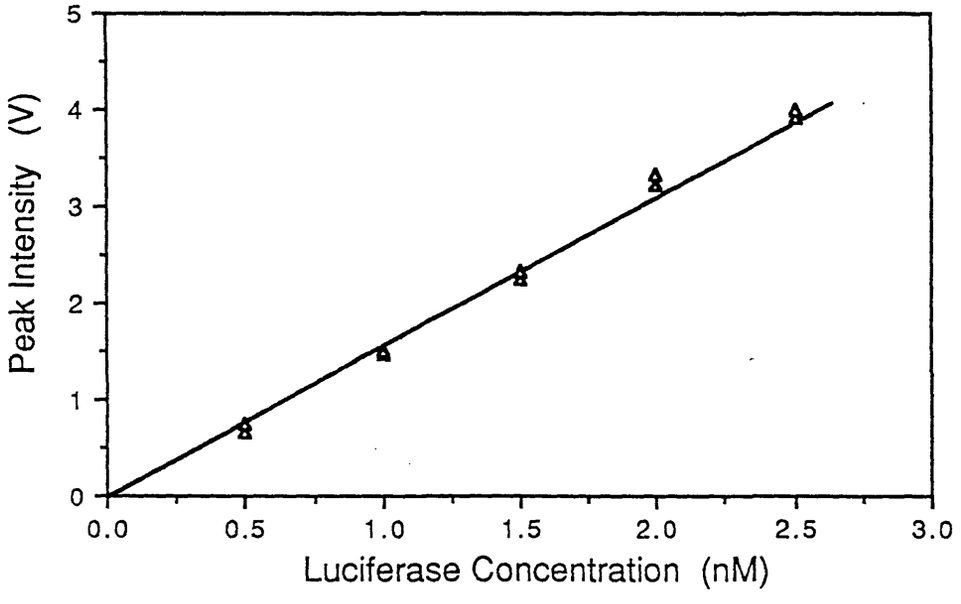

Figure 3.9: The input−output relationship of the photomultiplier tube. The data show the peak intensity recorded from luciferase assays at a range of enzyme concentrations. The assays were performed by mixing 2.5 ml of $FMNH_2$ with 5.01 of luciferase and decanal solutions. Final concentrations (in 7.51 ml): decanal, 0.85 μM; $FMNH_2$, 101 μM. $T = 25.0^{\circ}C$.

3.4 Experimental Methods

Various experimental techniques were used to investigate the inhibition of the luciferase−catalysed reaction by anaesthetics, the stabilisation of the luciferase−peroxyflavin complex by particular agents and to measure the solubilities and anaesthetizing concentrations of a novel homologous series of anaesthetics, the cycloalcohols. These techniques are described in detail in this section.

3.4(a) Measurement of Luciferase Inhibition − (The $FMNH_2$−Initiation Method)

Previous work with partially−purified luciferase has shown that a number of anaesthetics inhibit the luciferase−catalyzed reaction by competing with the long−chain aldehyde substrate for binding to the enzyme (see section 1.4). It was therefore desirable to allow the anaesthetic and the aldehyde substrate to equilibrate with luciferase before initiation of the reaction, which was achieved by the rapid injection of reduced flavin. This is the $FMNH_2$−initiation method.

The modifications to the basic apparatus required by $FMNH_2$−initiation depend on the method chosen for the preparation of reduced flavin ($FMNH_2$) from oxidized flavin (FMN). Most experiments were performed (for reasons which will be discussed later) using flavin which had been reduced by hydrogen on a palladium catalyst. This method of reduction presented two technical obstacles. Firstly, the form of the palladium catalyst used was 10% palladium on activated charcoal powder. This powder had to be removed by filtration from the $FMNH_2$ solution before it could be used to initiate the reaction. Secondly, flavin reduced by hydrogen is not buffered against autoxidation. Therefore the $FMNH_2$ solution had to be transferred to the rapid injection syringe without exposure to air. The set−up designed to overcome these obstacles is shown schematically in Figure 3.10. The flavin solution and charcoal powder were held in a 500ml Quick−fit conical flask sealed at the top with a rubber Suba−seal bung. Three hypodermic needles inserted through this bung provided entry and exit ports for hydrogen gas and a port for the removal of the reduction product, $FMNH_2$. A length of capillary tubing fitted to the hydrogen entry needle fed the gas to the bottom of the solution. The narrow diameter of this tubing produced fine bubbles which were dispersed through the solution by continuous stirring with a magnetic flea before reaching the surface. This double strategy speeded up the process of reduction. The magnetic stirrer, which was positioned close the photomultiplier, was switched off just before the start of the experiment in order to avoid interference on the photomultiplier

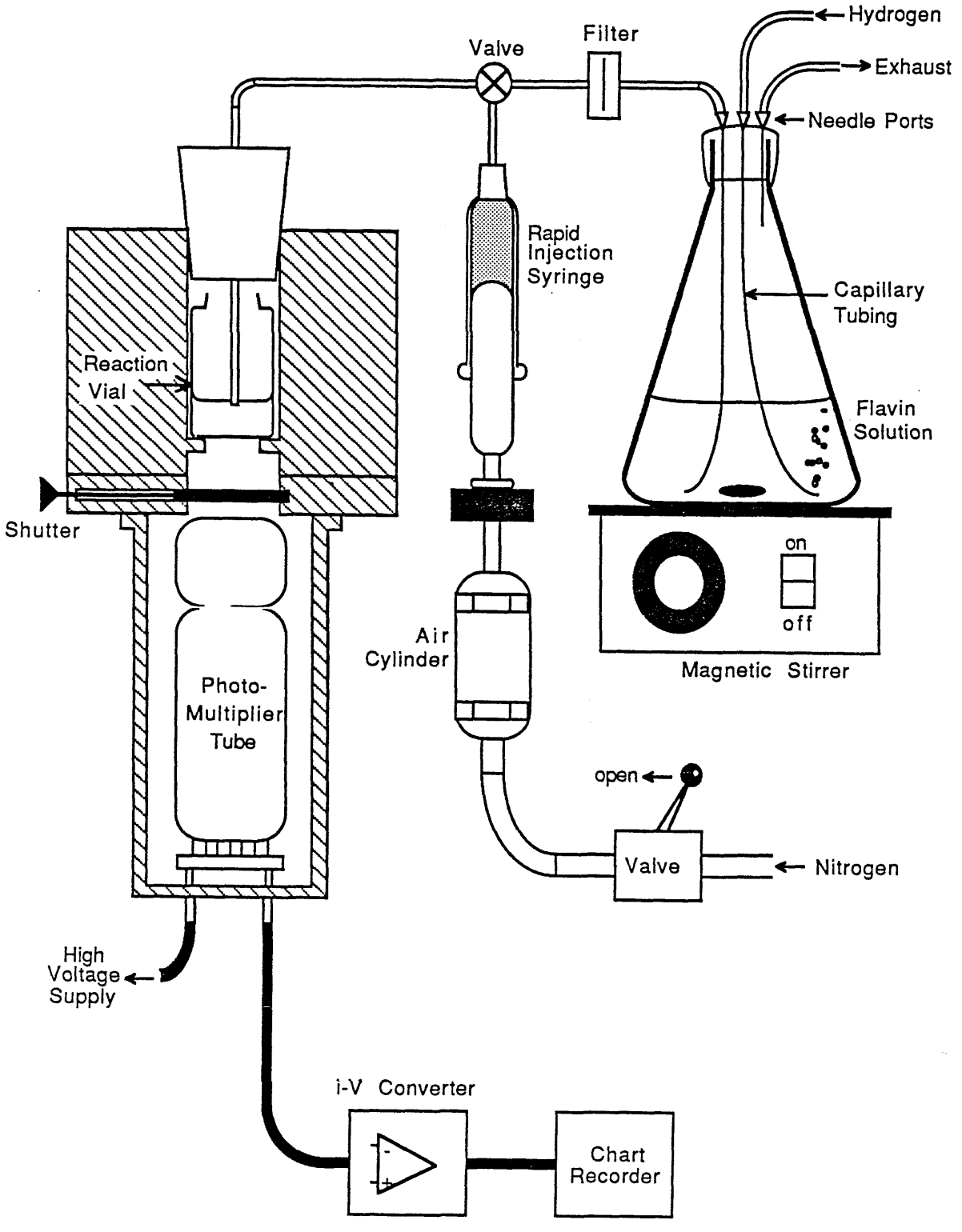

Figure 3.10: Schematic diagram of the experimental set-up of the rapid-mixing apparatus used for $FMNH_2$-initiated luciferase assays. Note that the rubber collar, which would have been in place around the neck of the plunger, is not shown.

signal. Since flavin is degraded by exposure to light, the conical flask was covered with a black plastic bag. It was not necessary to protect any of the supply tubing in this way because $FMNH_2$ was present in these sections of the apparatus for only a very short time before initiation of each reaction.

A nylon, luer-locking, two-way valve fitted to the top of the rapid injection syringe provided access to the $FMNH_2$ solution along a gas-tight pathway. With the valve open to this pathway the action of pulling down the syringe plunger loaded the syringe. The $FMNH_2$ solution travelled up a length of capillary tubing, through the needle port and into a Nuclepore filter unit. A reusable polycarbonate filter with a 3μm pore size removed all the charcoal particles without severely restricting the flow of solution. From the filter the solution passed through a short length of plastic tubing, which acted simply as a spacer between the injection assembly and the reducing flask, and into the syringe. Once the syringe had been loaded the valve was set to close off the $FMNH_2$ loading pathway, simultaneously opening the way to the delivery tube. The presence of this valve required the optimum nitrogen pressure for injection to be increased from 16 to 17 p.s.i..

The reduction of FMN to $FMNH_2$ was begun an hour before the start of an experiment. Immediately before each assay a clean scintillation vial was loaded with decanal, anaesthetic and buffer solutions to a volume of 5.00 ml. In order to ensure that both the rapid-injection syringe and the delivery tube which connects the syringe to the reaction vial were properly loaded prior to each assay, fresh $FMNH_2$ was flushed through them to a 'waste' beaker by loading the syringe, expelling this flavin to waste and then reloading. 10 μl of luciferase solution (kept on ice) was then pipetted into the vial and mixed with the anaesthetic and aldehyde by brief agitation of the vial. The enzyme concentration was always chosen to be very much less than that of either the aldehyde or the anaesthetic so that their initial concentrations were not significantly depleted in the reaction. Finally the vial was inserted into the apparatus and the reaction initiated with an injection of 2.5 ml of $FMNH_2$, giving a final volume of 7.51 ml. The signal was recorded on the chart recorder for long enough to take a measure of the peak intensity and, if necessary, the rate of decay of luminescence.

Several preliminary $FMNH_2$-initiation experiments were carried out using flavin that had been chemically reduced with sodium dithionite, the second of the dithionite reduction methods described in section 3.2(a). This method of preparation employed almost exactly the same apparatus as for reduction by hydrogen. The FMN solution in the flask had oxygen-free nitrogen bubbled through it (instead of hydrogen) and,

in the place of palladium, the chemical reducing agent sodium dithionite was added to the solution. Since the dithionite dissolves completely, the Nuclepore filter unit was removed. In all other aspects the set−up and method were identical to those described above.

3.4(b) Measurement of the Stabilisation of the Luciferase−Peroxyflavin Complex − (The Aldehyde Initiation Method)

The luciferase−peroxyflavin complex, conventionally known on the reaction pathway as intermediate II, is formed by mixing $FMNH_2$ and luciferase in the presence of oxygen. Subsequent addition of a long−chain aldehyde produces a relatively high yield luminescence as the aldehyde reacts with the intermediate. However in the absence of added aldehyde the intermediate decays without the production of light to give oxidized flavin (FMN) and hydrogen peroxide (H_2O_2). The reaction scheme for this latter process is:

$$E + FMNH_2 \rightleftharpoons E\text{-}FMNH_2 + O_2 \longrightarrow E\text{-}FMNH\text{-}OOH \longrightarrow E + FMN + H_2O_2$$

(where E represents luciferase and E−FMNH−OOH the peroxyflavin complex). Since $FMNH_2$ autoxidizes very quickly, the rapid formation of intermediate is followed by a relatively slow exponential decay. Experiments were performed to measure the rate of decay of intermediate II in the presence and absence of particular agents. These experiments, which are described below, involved the aldehyde initiation method.

No modifications to the basic rapid−injection apparatus were required for observation of luciferase reactions initiated by injection of decanal. The syringe was loaded by sucking decanal solution up the delivery tube from a beaker of solution. $FMNH_2$ was prepared by the hydrogen reduction method using the same apparatus as for the $FMNH_2$ initiation method only in this case the nylon valve, instead of connecting the flavin solution to the rapid injection syringe, provided a resealable port for the extraction of reduced flavin (See Figure 3.11). A plastic, gas−tight syringe was plugged into the valve port and filled with freshly reduced flavin. With the valve closed the syringe was removed, fitted with a hypodermic syringe needle and used to deliver $FMNH_2$ to the cuvette in which the intermediate II was prepared. The needle enabled the flavin to be injected as a fine jet for thorough mixing with luciferase.

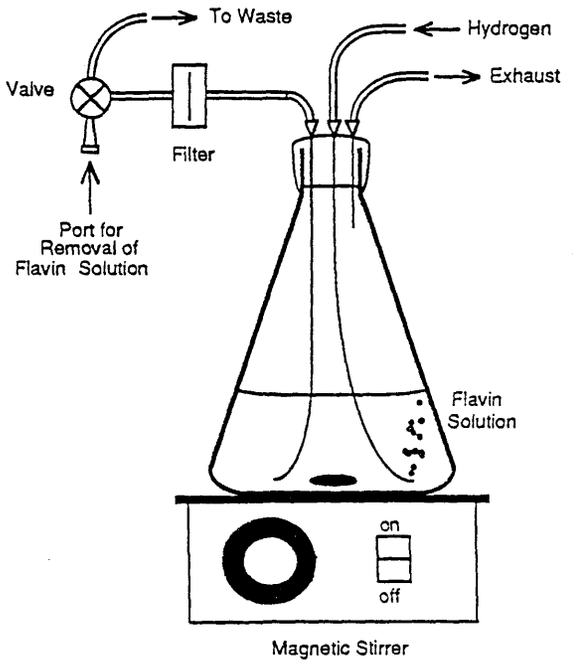

Figure 3.11: Apparatus for preparation of catalytically reduced $FMNH_2$ used in stabilisation experiments.

It is known that some long-chain alcohols stabilise the lifetime of the luciferase-peroxyflavin intermediate. In order to study the stabilizing effects of a wider range of agents, a protocol was developed, based on the method of Tu (1979), for the determination of this lifetime. When a solution of the luciferase-peroxyflavin intermediate is injected with aldehyde, the peak intensity of the resulting light emission is proportional to the concentration of intermediate at the time of injection. Therefore in order to measure the lifetime of the intermediate, it is sufficient to observe the decay in the peak intensity of assays of aliquots of a sample taken at a number of time intervals. The concentrations of aldehyde and anaesthetic present will of course affect the peak intensity but if these are constant for a given sample of intermediate II, the observed decay rate is unaffected. Since the decay of the intermediate is exponential, the gradient of a plot of the logarithm of the peak intensity against time yields the decay constant. Fuller details of the theory behind the method are given along with the results in section 6.2(a).

Samples of the intermediate complex were prepared in the following manner: a 10μl drop of luciferase was placed in the bottom of a plastic cuvette and 0.4 ml of $FMNH_2$ forcibly injected (as described above) for good mixing. A small volume (up

to 1.6 ml) of the anaesthetic solution was added and the total volume of the intermediate sample made up to 2.01 ml with phosphate buffer. At recorded times a 0.4 ml aliquot (typically) was withdrawn fron this mixture and diluted in buffer in a glass reaction vial to give a total volume of 5.00 ml. The vial was placed quickly in the apparatus and assayed for activity by injecting 2.5 ml of *n*−decanal. Further decay of the intermediate inevitably occurred in the time interval, Δt, between dilution of the aliquot in buffer and initiation of the luminescent reaction. (Except for controls, the rate of this decay was faster than in the ~~the~~ original sample because of dilution of the stabilising agent.) Therefore in order to ensure that the proportion of intermediate which decayed in this interval was the same for all aliquots of a given sample, Δt was held constant (at 10 ± 1 seconds).

3.4(c) Solubility Measurements

A simple method was developed for measurement of the aqueous solubilities (C_{sat}) of compounds which can inhibit bacterial luciferase. This was used to determine aqueous solubilities of cycloalcohols for which reliable data was not found in the literature. Using the $FMNH_2$−initiated assay, dose−response data were taken for a range of dilutions of a saturated and a concentrated standard solution of a given agent. From these data sets it was possible to make an accurate determination of the volume of the saturated solution (in 7.51 ml total) and the concentration of the standard solution which inhibited the reaction by 50%. These quantities, V_{50}(ml) and the ED_{50}(M) respectively, are related to C_{sat} by the equation:

$$\frac{V_{50}}{7.51} \times C_{sat} = ED_{50} \qquad (3.1)$$

which can be rearranged to give:

$$C_{sat} = ED_{50} \times \frac{7.51}{V_{50}} \qquad (3.2)$$

Saturated solutions of cycloalcohols were prepared by adding double the amount of cycloalcohol necessary for saturation (estimated from preliminary experiments) to 20 ml of de−ionized water in each of two clean scintillation vials. Over a two hour period, on the day of the experiment, the vials were vortexed vigorously, one for 1½ minutes and one for 3 minutes, at half hour intervals. No difference in the

inhibition by these two solutions was observed, indicating that saturation of the aqueous phase had been achieved in both. The solutions of cyclohexanol, cycloheptanol, and cyclooctanol, which are quite viscous at room temperature, were heated to about 40°C to aid equilibration. Solutions of cyclodecanol and cyclododecanol, which are respectively greasy and solid at room temperature, were heated to beyond their melting points (40 and 75°C respectively) to facilitate dispersion into solution. Following equilibration, the cycloalcohol solutions (except saturated cyclodecanol) were centrifuged at 20,000 rpm for 30 minutes at a temperature of 24°C to separate undissolved cycloalcohol. The saturated solution was loaded into clean vials for use in the experiment. Cyclodecanol, perhaps because of its rather greasy nature at room temperature, could not be separated in this way. Instead the excess cyclodecanol was removed using a 0.2 μm Sartorius Minisart filter. In order to test for loss of cyclodecanol from solution due to adsorption onto the filter, the first 3 ml of each solution was filtered and discarded and the remaining 17 ml then filtered as consecutive lots of 9 and 8 ml into two clean vials. Significant loss of cyclodecanol would have resulted in different degrees of inhibition by the separate filtrates. No such difference was observed.

In assays of the inhibition due to saturated solutions, reaction vials were loaded with 1.0 ml of 6.4 μM decanal in 250 mM phosphate buffer. A volume of the saturated solution was then added and the total volume made up to 5.00 ml with deionized water giving a buffer concentration of 50 mM. The 250 mM phosphate buffer was prepared from the two 1 M stock solutions (K_2HPO_4 and KH_2PO_4) such that dilution to 50 mM gave a pH of 7.0. 10 μl of luciferase was injected and the reaction initiated, as described in section 3.4(a), by the rapid injection of $FMNH_2$. Concentrated standard solutions, which were made up in de-ionized water, were treated in exactly the same way. Errors due to day to day variations in substrate concentrations were avoided by assaying both the standard and saturated solutions in the same experiment using the same flavin and aldehyde solutions.

3.4(d) Tadpole Experiments

In order to assess the relevance of the effect of cycloalcohols on bacterial luciferase to general anaesthesia, it was necessary to determine whether these agents act as anaesthetics. Experiments were performed on 1-2 week old *Xenopus laevis* (average length = 9 mm) at a temperature of 23 ± 1 °C. The anaesthetic end-point was defined as the lack of a sustained swimming response to a gentle prod with a smooth glass rod. Concentrated solutions of cycloalcohols were prepared

in tap water. Due to the viscosity of cyclohexanol, cycloheptanol, cyclooctanol, and cyclodecanol (which was noted earlier), these agents were added to tap water which had been warmed to 40−60°C; the water was kept at this temperature until the alcohol had been stirred completely into solution. (In the cases of cycloheptanol and cyclodecanol, trace amounts of the alcohol appeared to be undissolved after two hours of continuous stirring. These were removed with a 0.4μm polycarbonate filter. The amount of material removed by filtration was estimated to have a negligible effect on the final concentration). The solutions were allowed to cool to room temperature before use. Cyclododecanol, as well as being a solid at room temperature, is the least soluble of the cycloalcohols. It was therefore decided that the most accurate way to prepare a concentrated solution was to make up a saturated solution. Excess cyclododecanol was added to 1 litre of water in a conical flask heated to 90°C (15°C above its melting point). The mixture was stirred on heat for an hour and shaken vigouously at ten minute intervals − similar treatment to that used to make up saturated solutions for solubility measurements. The saturated solution was cooled to room temperaure and filtered through Whatman Grade 1 filter paper and a Nuclepore 0.4 μm polycarbonate filter to remove undissolved particles of cyclododecanol.

Eight tadpoles were placed in each of 6 beakers containing 300 ml of a dilution of the concentrated cycloalcohol solution. This number of tadploes in this volume of water was estimated, by a crude calculation, not to cause substantial depletion of the aqueous concentration of anaesthetic at equilibrium: the average dry weight of the tadpoles was determined to be 5 mg. Half of this was assumed to be fatty tissue likely to absorb the anaesthetic. Partitioning data for the cycloalcohols were not available but it was noted that the product of solubility (Bell, 1973) and membrane/buffer partition coefficient (for erythrocyte membranes (Roth and Seeman, 1972)) for n−hexanol, n−octanol and n−decanol lies in the narrow range 0.4−0.8. Assuming the value of this product for the cycloalcohols to be 0.6, the molar partition coefficient of cyclododecanol was estimated at 2000. From this estimate the depletion of an aqueous concentration of cyclododecanol by 8 tadpoloes in 300 ml was calculated to be approximately 10%. Depletion would have been less for the other cycloalcohols, since their partition coefficients would most likely have been smaller.

The tapdoles at each particular concentration were assayed for swimming responses and steady−state anaesthesia determined when the number of tadpoles responding became constant. Equilibration was complete in 20−60 minutes. The number of anaesthetized tadpoles at equilibration was recorded and the tadpoles

transferred quickly to recovery beakers filled with tap water at 23°C. In these beakers the tadpoles soon recovered completely. In one experiment a single tadpole did not recover and was excluded from the data analysis. The concentration required to anaesthetize half of the tadpoles (ED_{50}) was determined by the method of Waud (1972). No correction was made for depletion of the aqueous concentration by uptake into the tadpoles because of the small size of the estimated depletion and the crudity of the calculation to determine it.

CHAPTER 4

CHARACTERISATION OF THE *IN VITRO* LUCIFERASE–CATALYSED REACTION AND ITS RESPONSE TO GENERAL ANAESTHETICS

The *in vitro* luciferase reaction used in this study differs from most other enzyme–catalysed reactions because luciferase is prevented from turning over. Consequently, the intensity of the light output following initiation of the reaction rapidly reaches a peak and then decays. Typical examples of the luminescent output which were recorded in the presence of a number of different concentrations of the substrate, *n*–decanal, and of an inhibitor, diethyl ether, are shown in Figures 4.1 and 4.2. Clearly the peak intensity, the time taken to reach this peak and the rate of decay following it all vary. It does not seem possible to identify a steady

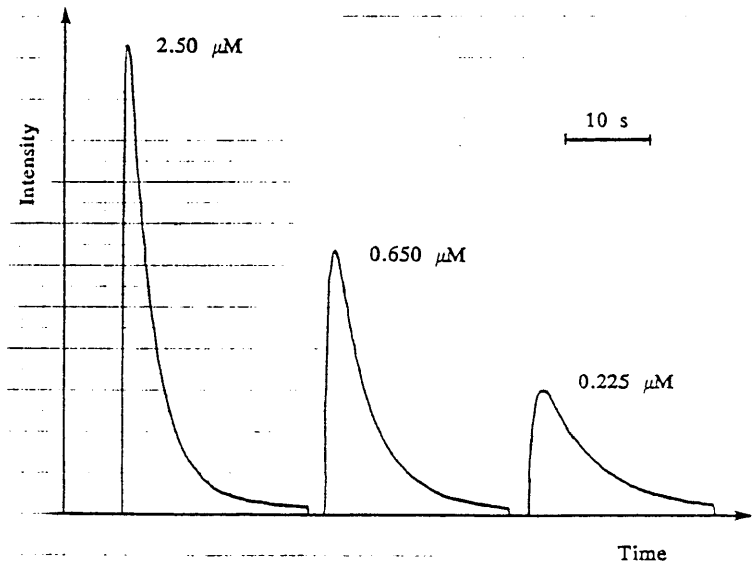

Figure 4.1: The effect of the decanal concentration on the time–dependence of the luminescent output of the in vitro luciferase reaction initiated by injection of $FMNH_2$. *The profiles were traced from chart recorder output. Final concentrations: decanal, 0.225–2.50 μM;* $FMNH_2$, *117 μM; luciferase, 1.7 nM. T = 25.3°C.*

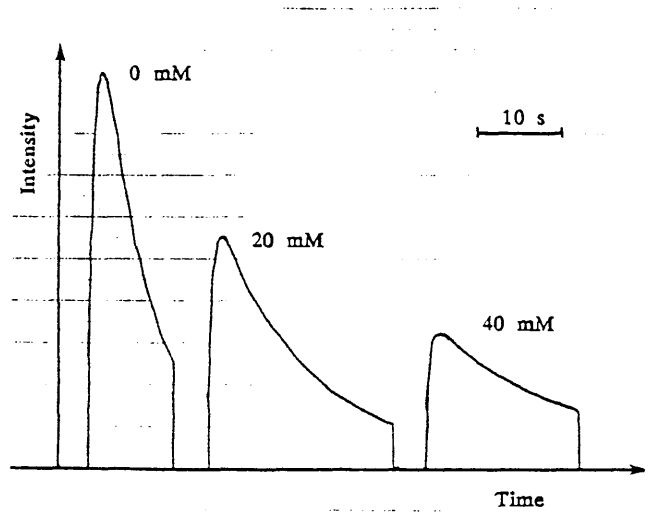

Figure 4.2: The effect of a general anaesthetic, diethyl ether, on the time–dependence of the luminescent output from the in vitro luciferase reaction initiated with $FMNH_2$. The profiles were traced from chart recorder output. Final concentrations: diethyl ether, 0–40 mM; decanal, 1.1 μM; $FMNH_2$, 107 μM; luciferase, 1 nM. T = 25.1°C.

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state rate of catalysis, which is the usual measure of reaction velocity; however it can be argued that the peak intensity is an equivalent measurement of the rate. The argument for using the peak intensity is presented in the first part of this chapter. This is followed by a detailed and quantitative description of the effects on the $FMNH_2$–initiated luciferase reaction of both the flavin and aldehyde substrates and of a wide range of general anaesthetics. Finally, a method for determining the anaesthetic dissosciation constants for luciferase is developed.

4.1 Measurement of Luciferase Reaction Rates

Clearly it is valuable to be able to interpret observations of changes in the rate of the luciferase–catalysed reaction in terms of a molecular mechanism of interaction. Conventionally the interactions of substrates and inhibitors with enzymes are modelled according to the Michaelis–Menten reaction scheme, which is usually presented as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E \qquad (4.1)$$

where E, S, ES and P are the free enzyme, free substrate, enzyme–substrate complex and reaction products respectively and k_1, k_{-1} and k_2 are the rate constants. It is possible to show that, at the steady state condition ($d[ES]/dt = 0$) the rate (v) of the reaction is given by:

$$v = \frac{k_2[E]_0[S]}{[S] + K_m} \qquad (4.2)$$

where [S] is the substrate concentration, K_m the Michaelis constant [$\equiv (k_{-1}+k_2)/k_1$], and $[E]_0$ the total enzyme concentration. This derivation assumes that the enzyme turns over (so that $[E]_0 = [E] + [ES]$) and that a steady state can be attained and maintained before significant depletion of the substrate has occurred. (This is achieved by having $[S] \gg [E]_0$).

However *in vitro* luciferase reactions do not appear to fulfil either of these assumptions. Since $FMNH_2$ is autoxidized very quickly, each luciferase molecule catalyses just one reaction before the loss of reduced flavin brings further catalysis to a halt. Consequently, in the absence of enzyme turnover, the injection of $FMNH_2$ into a vial containing luciferase, decanal and dissolved oxygen leads rapidly to the formation of the luciferase–peroxyflavin complex, intermediate II, which reacts with *n*–decanal to produce light (Ziegler and Baldwin, 1981 – see section 2.3). (A portion of the intermediate decays spontaneously to give oxidized flavin and hydrogen peroxide but no photon). Since there is no way of replenishing the pool of intermediate II which is formed at the start of the reaction, the reaction rate quickly reaches a maximum and thereafter decays as the intermediate is used up. Correspondingly the intensity of the light output, which is a direct measure of the reaction rate (being the rate of photon production), rises rapidly to a peak and then decays. Thus, under these circumstances, there is no steady state.

(The absence of a steady state was not a problem in the analysis of the rates of those luciferase reactions which were initiated with decanal in order to take a measure of the enzyme (or intermediate) concentration. In experiments of this type [which are described in section 3.4(b)] the substrate and inhibitor concentrations were held constant so that the profiles of the time–dependence of intensity (*i.e.* reaction rate) differed only by a scale factor which was directly proportional to the enzyme concentration. The peak intensity, being the easiest parameter to measure

accurately, was taken as a measure of the enzyme concentration.)

In experiments designed to measure the inhibition of the luciferase reaction by anaesthetics, which were performed by injecting $FMNH_2$ into a vial containing luciferase and variable amounts of decanal and anaesthetic, the problem of identifying a meaningful measure of rate is exacerbated by the fact that there are significant changes in all the parameters which describe the light output of the reaction (see Figures 4.1 and 4.2). It is conventional simply to take the peak intensity, which is the maximum rate of photon emission (and hence the maximum rate for the reaction), as *the* rate of the reaction under conditions of varying substrate and inhibitor concentrations. Previous studies of the inhibition of similar *in vitro* luciferase reactions (using luciferase from *Vibrio fischeri* bacteria) by methoxyflurane (Adey *et al.*, 1976) and diethyl ether (Middleton and Smith, 1976) used the peak intensity and have shown that it obeys the Michaelis–Menten equation. In this way the competitive nature of the inhibition by these agents was demonstrated. However since the assumptions necessary for a proper Michaelis–Menten analysis are not fulfilled in luciferase reactions, it is not completely clear whether the results of such an analysis have their conventional meanings. For example, does the K_m for decanal derived from this treatment of luciferase data correspond to the K_m parameter as defined for other enzymes which behave in exactly the way that the Michaelis–Menten analysis demands? It is possible to answer this question by considering the problem in a little more detail.

For luciferase reactions in the presence of saturating concentrations of reduced flavin and the absence of any inhibitor, the Michaelis–Menten reaction scheme may be represented as:

$$E' + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E'S \xrightarrow{k_2} E_r + P + \text{photon} \qquad (4.3)$$

where E', intermediate II, has taken the place of E. E_r is "redundant" enzyme which, having participated in the reaction, either productively or non–productively, is rendered useless by the disappearance of $FMNH_2$; S is the decanal substrate and P includes the products of oxidation. The rate of the reaction, v, is given by:

$$v = k_2[E'S] \qquad (4.4)$$

and the rate equation for [E'S] is:

$$\frac{d}{dt}[E'S] = k_1[E'][S] - k_{-1}[E'S] - k_2[E'S] \qquad (4.5)$$

When the rate reaches a maximum, which occurs at $t = t_{pk}$:

$$\frac{dv}{dt} = 0 = k_2\left[\frac{d}{dt}[E'S]\right]_{t=t_{pk}} \qquad (4.6)$$

Therefore:

$$\left[\frac{d}{dt}[E'S]\right]_{t=t_{pk}} = k_1[E']_{pk}[S]_{pk} - k_{-1}[E'S]_{pk} - k_2[E'S]_{pk} = 0 \qquad (4.7)$$

for the concentrations $[E']_{pk}$, $[S]_{pk}$ and $[E'S]_{pk}$ at $t = t_{pk}$, which gives:

$$\frac{k_{-1} + k_2}{k_1} = \frac{[E']_{pk}[S]_{pk}}{[E'S]_{pk}} \qquad (4.8)$$

If the decanal concentration greatly exceeds that of luciferase, there is a negligible depletion of this substrate and $[S]_{pk}$ can be set equal to the initial substrate concentration, [S]. Since the Michaelis constant is defined as $K_m \equiv (k_{-1}+k_2)/k_1$, then

$$K_m = \frac{[E']_{pk}[S]}{[E'S]_{pk}} \qquad (4.9)$$

The concentrations $[E']_{pk}$ and $[E'S]_{pk}$ at $t = t_{pk}$ can be related to the total luciferase concentration, $[E]_0$, by the equation:

$$[E]_0 = [E']_{pk} + [E'S]_{pk} + [E_r]_{pk} \qquad (4.10)$$

where $[E_r]_{pk}$ is the concentration of enzyme which has, effectively, been used up in the time to peak. This equation can be used to substitute for $[E']_{pk}$ in equation (4.9). Re-arranging (4.9):

$$[E'S]_{pk} = \frac{([E]_0-[E_r]_{pk})[S]}{[S] + K_m} \qquad (4.11)$$

Hence:

$$v_{pk} = k_2[E'S]_{pk} = \frac{V_{max}[S]}{[S] + K_m} \qquad (4.12)$$

where $V_{max} = k_2([E]_0 - [E_r]_{pk})$ is the maximum theoretical rate (which is approached as $[S] \rightarrow \infty$). V_{max} should not be confused with the maximum rate of a reaction, *i.e.* the peak intensity. Normally the inverse of equation (4.12) is used in the analysis of enzyme kinetics:

$$\frac{1}{v_{pk}} = \frac{1}{[S]} \cdot \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \qquad (4.13)$$

Thus a plot of the reciprocal of the peak intensity as a function of the reciprocal of the decanal substrate concentration will give a straight line if $([E]_0 - [E_r]_{pk})$ is constant. It will be shown in section 4.3 that such a plot is indeed linear, as has already been demonstrated by other workers (Adey *et al.*, 1976; Middleton and Smith, 1976) . Therefore peak intensity data can be used to determine K_m and V_{max}. (K_m is minus the inverse of the intercept of the line on the abscissa; V_{max} is simply the inverse of the intercept on the ordinate). A more direct test of the constancy of $([E]_0 - [E_r]_{pk})$ would remove any remaining uncertainty as to the meaning of the K_m calculated by fitting data to equation (4.13). Unfortunately this is not possible because there is no way of determining $[E_r]_{pk}$.

4.2 Digression: The Effects of Sodium Dithionite on the Luciferase Reaction

In experiments to measure the inhibition of the luciferase-catalysed reaction by general anaesthetics the reaction was initiated with an injection of $FMNH_2$, which had been reduced with hydrogen on a palladium catalyst, into a vial containing luciferase and *n*-decanal. The characteristic behaviour of this version of the *in vitro* reaction and the effects of general anaesthetics on it will be discussed in section 4.3. Before that however, I want to digress briefly to the reasons for rejecting sodium dithionite, a commonly used chemical reducing agent, as a means of preparing $FMNH_2$ for inhibition experiments. Although this discussion is not directly relevant to the results presented in this thesis, it is of some interest. Despite the fact that dithionite is widely used as a reducing agent, there is no reference in the literature to most of the effects that sodium dithionite was observed, in this study, to have on the luciferase reaction.

The method for reducing FMN to $FMNH_2$ by sodium dithionite was originally chosen because it seemed to provide a safe and convenient way to prepare this substrate. However it soon became apparent that the presence of dithionite modified the kinetics of the luciferase reaction. Two protocols for initiating luciferase

reactions involving dithionite−reduced $FMNH_2$ were considered before both were ultimately rejected. The effects observed with the two methods differed considerably and are discussed below.

FMN−Initiation: Luciferase reactions were initiated by injecting oxidized flavin into a vial containing luciferase, *n*−decanal and sodium dithionite, the flavin being reduced upon injection (section 3.2(a)). The problems associated with this method are evident in Figure 4.3, where double reciprocal Lineveaver−Burk plots of the peak intensity and decanal concentration at 10, 30, 100 and 200 μM flavin are presented together. Clearly, Michaelis−Menten kinetics are not observed at 10 and 30 μM flavin. The behaviour of the reaction at these flavin concentrations is very

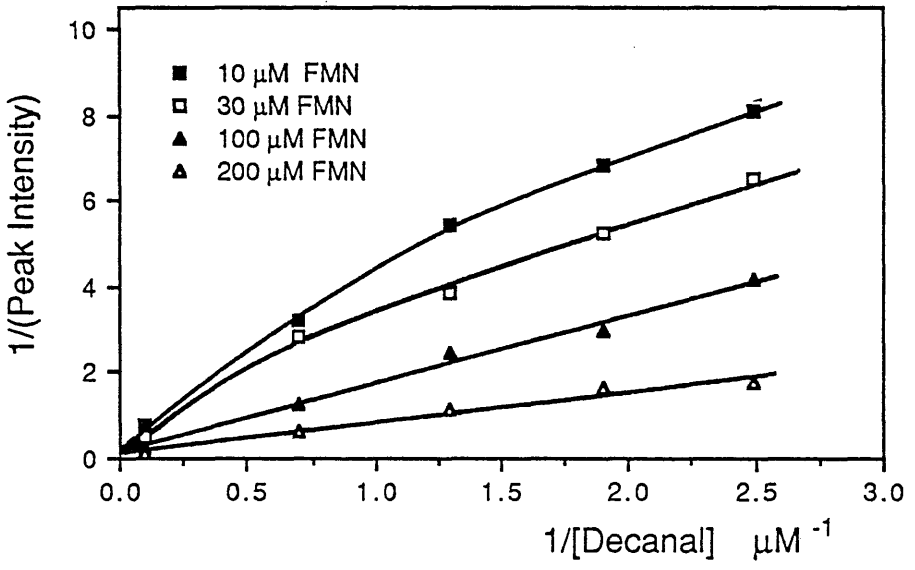

Figure 4.3: Lineweaver−Burk plot of the maximum rate (peak intensity) and decanal concentration for FMN−initiated luciferase reactions. Final concentrations (in 7.51 ml total): decanal, 0.4−10 μM; FMN, 10, 30, 100 and 200 μM; dithionite, 0.74 mM; luciferase, 4 nM. T = 23−25°C.

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difficult to interpret. If the data obtained at high concentrations of decanal (> 1 μM) are considered in isolation, the system actually appears to behave quite well and the same estimate of the decanal K_m (approximately 14 μM) is observed at each flavin concentration. In contrast, if the data at low decanal concentrations are treated separately, the decanal K_m appears to increase with increasing flavin concentrations (see Table 4.1). This latter observation suggests that flavin, in some way, may antagonise the interaction of decanal with luciferase. However, it is not possible to justify rigorously any estimate of the K_m from curved double reciprocal

plots and the real reason for the curvature shown in Figure 4.3 remains unknown. Further investigation of this complex behaviour was not carried out since the primary concern was to find an experimental protocol which was devoid of such artefacts.

Table 4.1: The dependence on the flavin concentration of the K_m of decanal determined using the FMN−initiation method at low decanal concentrations (< 1 μM). The K_m values were calculated as the inverse of the intercepts on the abscissa of the line fitted to data points obtained at decanal concentrations below 1 μM.

[Flavin] μM	Decanal K_m μM
10	0.83 ± 0.05
30	1.7 ± 0.3
100	14 ± 2
200	13 ± 4

Another problem with FMN−initiation was that the light output in the absence of decanal was a substantial fraction (5%) of the luminescence evoked by a decanal concentration equal to its K_m, *i.e.* the half maximal rate, (at 100 μM flavin). Since it is not known whether the luminescence pathway which does not require added decanal is affected by the presence of this substrate, it was not possible to correct for this light output. This left a further question mark over the results of FMN−initiated assays.

$FMNH_2$−Initiation: The next simplest protocol was to initiate the reaction by injecting flavin which had already been reduced by dithionite into a vial containing just luciferase and *n*−decanal. Unlike the FMN−initiation method, this protocol had been used previously by other workers (*e.g.* Hastings *et. al.*, 1978). Initially the method appeared very promising: it gave none of the confusing behaviour that had been observed with FMN−initiation. Michaelis−Menten kinetics were observed at 10 and 100 μM flavin. The double reciprocal plots in Figure 4.4 gave K_m values for *n*−decanal of 2.4 ± 0.2 and 1.8 ± 0.3 μM respectively for these flavin concentrations; there thus appeared to be little or no effect of the flavin concentration on the interaction of *n*−decanal with luciferase under these experimental conditions. Additionally, the light output in the absence of decanal was a negligible fraction (0.3−0.6%) of the half−maximal rate (at [decanal] = K_m).

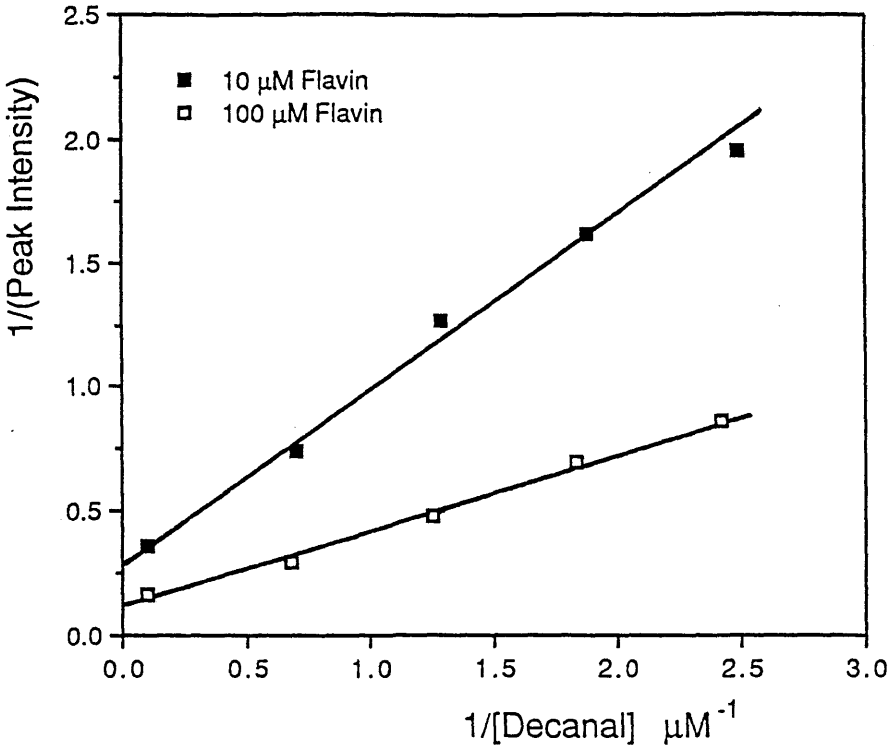

Figure 4.4: Lineweaver–Burk plot of the maximum rate and decanal concentration from luciferase reactions carried out with the $FMNH_2$–initiation method, using flavin which was reduced by dithionite. Final concentrations (in 7.51 ml): decanal, 0.4–10 μM; $FMNH_2$, 10, 100 μM; dithionite, 0.27 mM; luciferase, 2.7 nM. T = 23–24°C.

However, despite this initial promise, further experiments revealed that there were still problems associated with the presence of sodium dithionite. The most important of these, since it led to the rejection of this method, was the effect of dithionite on the maximum initial reaction rate. Figure 4.5 shows the dependence of the rate on dithionite concentrations up to 0.90 mM. Beyond a minimum level required for proper reduction of the flavin substrate (approximately equal to the sum of the oxygen and flavin concentrations in the sample to be reduced), the light output appears to vary in a complex manner with the concentration of added dithionite. Between 0.24 and 0.48 mM dithionite there is a slow decline in peak intensity; this is followed by a slight rise as the concentration is increased to 0.60 mM and thereafter the peak intensity declines again in a weakly concentration dependent manner. Although this increase in the peak intensity at 0.60 mM is quite small, it does appear to be significant. Figure 4.6, which shows the time–dependence of the light output of the reaction for several dithionite concentrations, reveals that it is associated with a distinctive luminescence profile.

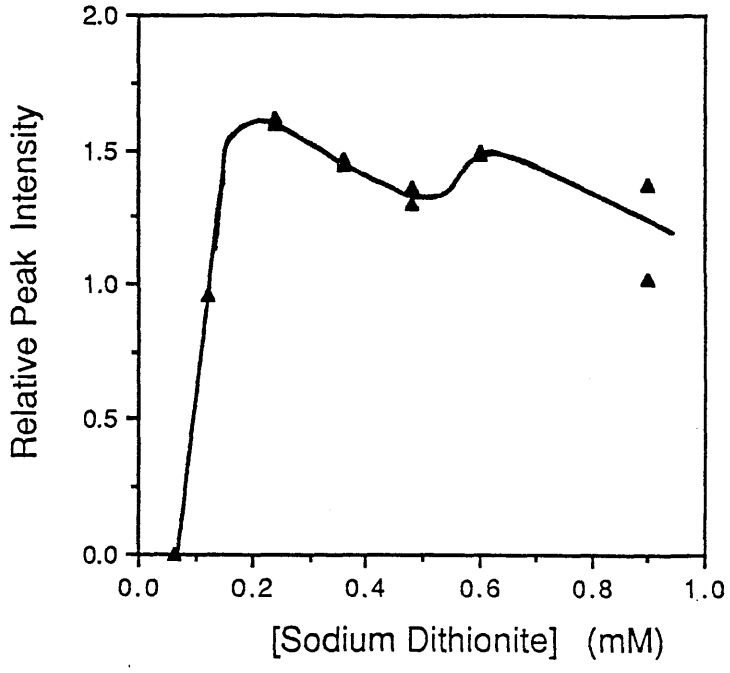

Figure 4.5: The effect of the dithionite concentration on the maximum rate (peak intensity) of the luciferase reaction. Reactions were initiated by injection of dithionite–reduced $FMNH_2$. Final concentrations (in 7.51 ml): decanal, 9.9 μM; $FMNH_2$, 100 μM; luciferase, 1.7 nM. T = 24.0°C.

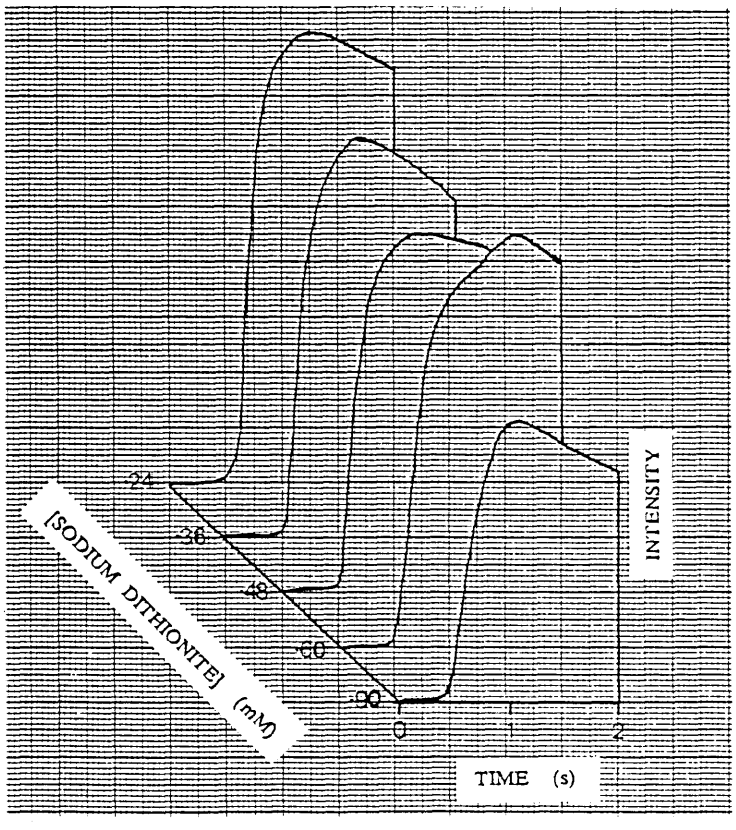

Figure 4.6: The variation with dithionite concentration of the time–dependence of the light output of the luciferase reaction. Conditions are as for Figure 4.5. Notice the distinctive profile at 0.60 mM dithionite.

FMN and sodium dithionite (or its oxidation products) are known to catalyse the oxidation of $FMNH_2$ (Gibson & Hastings, 1962; Meighen & Hastings, 1971). However it is difficult to see how this would account for the complex behaviour observed here.

The dependence of the luciferase reaction on the dithionite concentration suggested that the presence of dithionite would affect luciferase–anaesthetic interactions. To investigate this possibility, dose–response data were collected for *n*–butanol [at 100 μM flavin and 1.8 μM decanal (= K_m)] for two dithionite concentrations. The concentrations of butanol required to reduce the peak intenstiy by 50% under these conditions at 0.41 and 1.00 mM dithionite were 133 and 78 mM respectively. Clearly dithionite has a big effect. In principle it might have been possible to quantify anaesthetic inhibition for a fixed dithionite concentration; in practice however, dithionite levels declined quite quickly through autoxidation. Under experimental conditions, where the dithionite solution was kept under oxygen–free nitrogen which had been "scrubbed" with a separate dithionite solution, the half–life of the reducing agent was observed to be only 126 minutes. Therefore this second dithionite reduction method had to be discarded for inhibition experiments.

One final feature of luciferase reactions carried out in the presence of dithionite deserves mention. The decay of luminescence in reactions initiated by dithionite–reduced $FMNH_2$ was observed to have two distinct exponential phases. What is more, the decay constants of these two phases, 0.191 and 0.055 s^{-1} respectively at 24°C, were independent of the decanal concentration present (in 100 μM flavin and 0.27 mM dithionite). Sufficient data was not recorded to test whether the luminescent decay of FMN initiated assays was also biphasic; however the decay rate of such assays, up to 9 seconds after the peak, did not change significantly from 0.189 s^{-1} at 24°C over a decanal concentration range from 0.4 to 10 μM. (The final flavin concentration was also 100 μM in this experiment although the dithionite concentration was much higher at 0.74 mM). Later in the chapter it will be shown that for luciferase reactions which were initiated with $FMNH_2$ that had been reduced catalytically (by hydrogen on palladium), and therefore in the absence of dithionite, the decay rate of luminescence was observed to decrease with decreasing decanal concentration. This marked contrast serves to highlight the dramatic influence that dithionite appears to be exerting on the bacterial luciferase reaction.

4.3 The *in vitro* Luciferase Reaction using Flavin Reduced by Hydrogen

Since sodium dithionite was shown to interfere with the luciferase reaction, the flavin substrate for all subsequent experiments was reduced instead with hydrogen on a palladium catalyst. The evidence presented in this section demonstrates that reduced flavin solutions prepared in this way have no adverse effects on the luciferase reaction. This evidence is effectively a characterisation of the luciferase assay which was used in all inhibition experiments.

As I have already noted, a number of studies on the inhibition of luciferase from *Vibrio fischeri* bacteria had suggested that anaesthetics compete with the long-chain aldehyde substrate for binding to the enzyme (Hastings *et al.*, 1966; Adey *et al.*, 1976; Middleton and Smith, 1976); it was therefore important to examine the interaction of this substrate with luciferase. Figure 4.1 illustrates the variation with decanal concentration of the time-dependence of the light output from the reaction. The final flavin concentration in this experiment was 117 μM. If the concentration of decanal is lowered, the peak and the decay rate of luminescence both fall, while the time taken to reach the peak increases. The dependence of the maximum rate (peak intensity) of the reaction on the decanal concentration is presented as a Lineweaver-Burk double reciprocal plot in Figure 4.7. It is clear that the Michaelis-Menten equation can account satisfactorily for the data. As was explained in section 4.1, this permits a proper determination of the K_m of decanal. The average of eight such determinations yielded the value 0.85 ± 0.08 μM for this parameter. This compares well with a value of 1.1 μM obtained by Holzman and Baldwin (1983) for *V. harveyi* luciferase at 22-25°C in 20 mM Bis-Tris buffer.

[A final comment on the effect of sodium dithionite may now be made. Using flavin reduced by dithionite, the $FMNH_2$-initiation method yielded 1.8 ± 0.3 μM as the value of the K_m of decanal (for a final flavin concentration of 100 μM). This is more than twice the value obtained with catalytically reduced flavin. Additionally, substrate inhibition by decanal was observed to occur at a lower concentration (10 μM) in the absence of dithionite. Together these results suggest that sodium dithionite in some way impedes the interaction of decanal with luciferase. The use of dithionite in the study of luciferase reactions has to be limited by these considerations. It should not affect the results of assays to determine luciferase activity where the substrate concentrations are fixed; however in other applications due attention should be paid to the possible modifying effects of this reducing agent.]

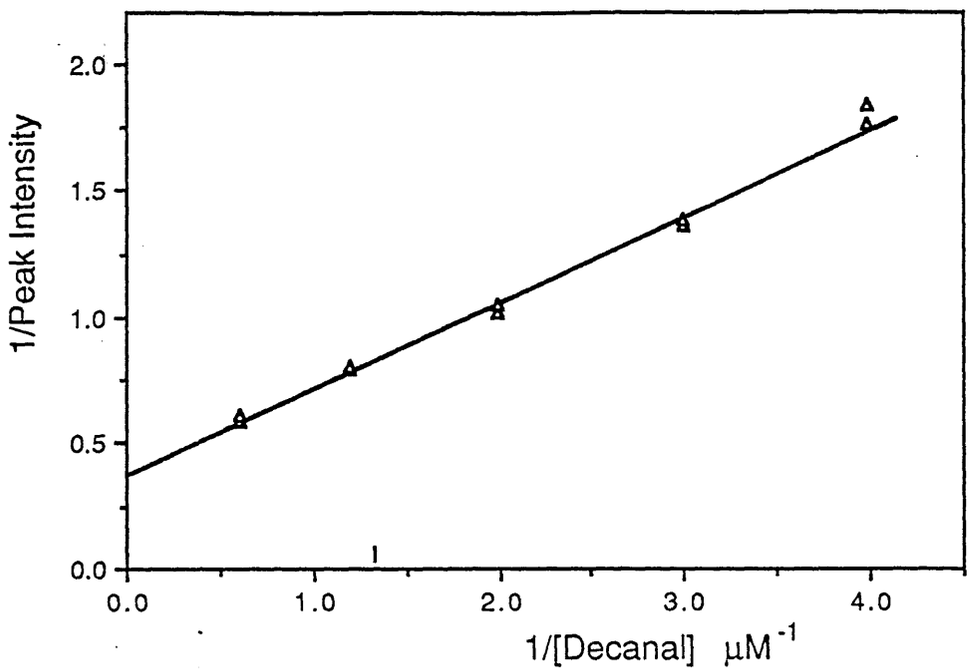

Figure 4.7: Lineweaver–Burk plot of the maximum rate and decanal concentration for luciferase reactions initiated by injection of $FMNH_2$ which had been reduced with hydrogen on a palladium catalyst. Final concentrations (in 7.51 ml): decanal, 0.25–1.7 μM; $FMNH_2$, 96 μM; luciferase, 1.2 nM. T = 23.3°C.

In the presence of a very low final concentration of $FMNH_2$ (2.8 μM), there was only a slight change in the K_m of decanal. The data from this experiment, plotted in Figure 4.8, gave a K_m of 0.62 ± 0.08 μM, close to the result determined at 96 μM flavin. For all subsequent inhibition experiments, which were carried out in final flavin concentrations ranging from 90 to 110 μM, it therefore seems highly unlikely that the K_m of decanal would have deviated significantly from 0.85 μM. The interaction of $FMNH_2$ itself with luciferase was also investigated. However the K_m of this substrate was not measured using catalytically reduced flavin because of the technical difficulty in diluting a flavin solution prepared in this way without causing oxidation. A determination, by the aldehyde initiation method, using flavin that had been reduced by dithionite, which buffers against oxidation, yielded a result of 0.34 ± 0.11 μM. With the same experimental method, but in the presence of 0.1% BSA, Meighen and Hastings (1971) obtained a value of 0.8 μM. Although the influence of dithionite upon the reaction may well have affected both of these measurements, it is likely that the K_m for $FMNH_2$ in the absence of dithionite would be of a similar order of magnitude. In any case, there seems little doubt that the high concentrations of $FMNH_2$ which were used in all inhibition

experiments would have been more than sufficient to saturate flavin binding to luciferase.

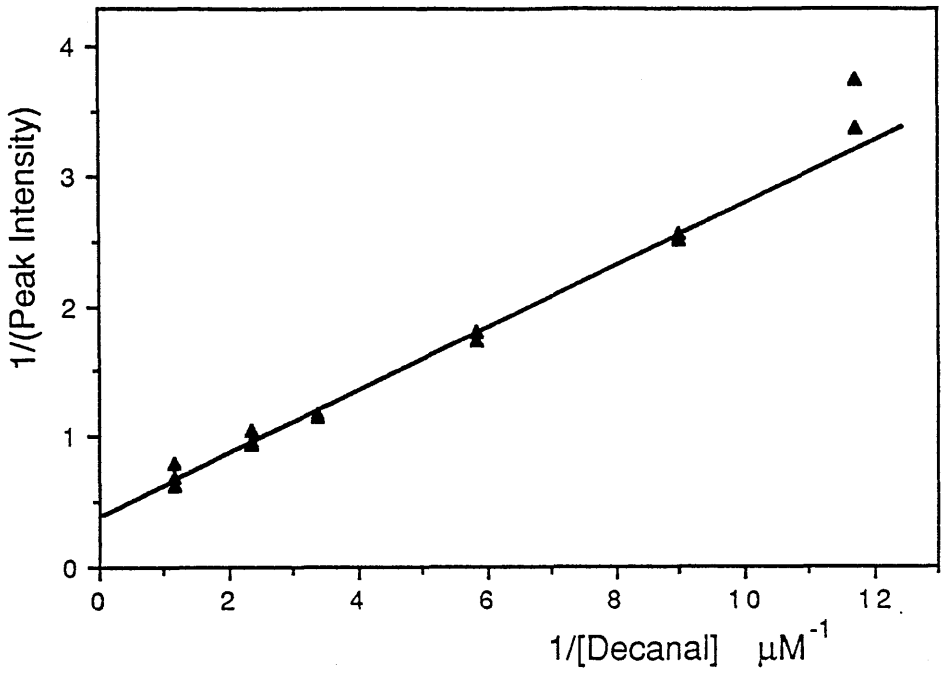

Figure 4.8: Lineweaver–Burk plot of the maximum rate and decanal concentration for luciferase reactions initiated by injection of a low concentration of $FMNH_2$. Final concentrations (in 7.51 ml): decanal, 0.09–0.85 μM; $FMNH_2$, 2.8 μM; luciferase, 0.6 nM. T = 25.4°C.

The influence of the decanal concentration on the decay of luminescence is shown in semi–logarithmic plots of the time–dependence of the light output in Figure 4.9. The decay constant, calculated from the slopes of such plots, increases in a hyperbolic fashion as the decanal concentration is raised (Figure 4.10). A double reciprocal plot of decay constant and decanal concentration is therefore linear (Figure 4.11) and predicts a maximum decay constant of 0.225 s^{-1} (at 23.3°C) for very high levels of the aldehyde substrate. [Note that the enzyme sample which gave faster luminescence decay (section 3.1) had an apparent maximum decay constant of 0.325 s^{-1} at 25.1°C].

In the absence of added decanal the peak intensity of the light output was only 1% of that observed in the presence of 0.85 μM decanal (= K_m). Given that the combined random errors associatied with each assay exceeded 1%, it was not necessary to attempt to correct for this extra luminescence (even if it could have

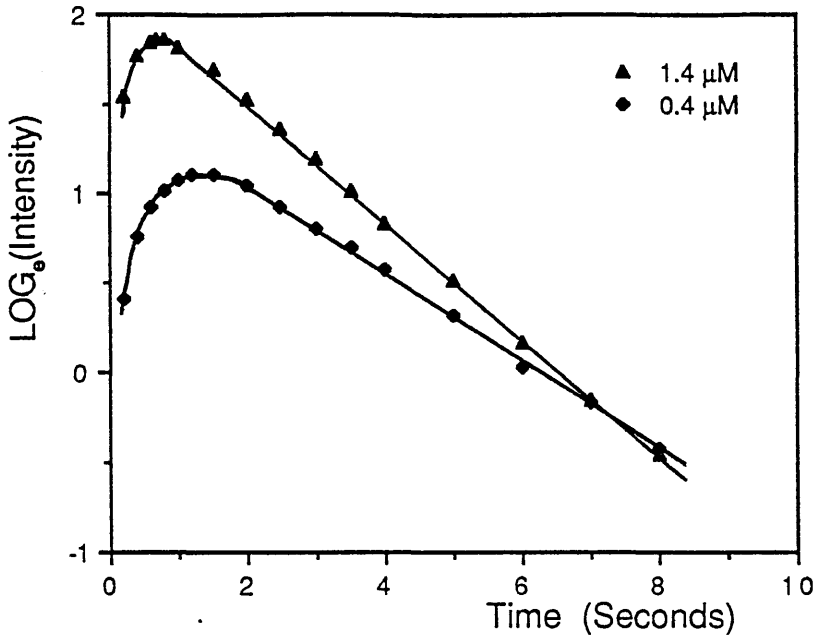

Figure 4.9: The decay of luminescence from the $FMNH_2$–initiated luciferase reaction is expontential. The data show the linear decline in the natural logarithm of intensity as a function of time. The rate constant of this decay (the luminescence decay constant) falls as the decanal concentration is reduced. Final concentrations: $FMNH_2$, 107 μM; luciferase, 0.4 nM. T = 20.0°C.

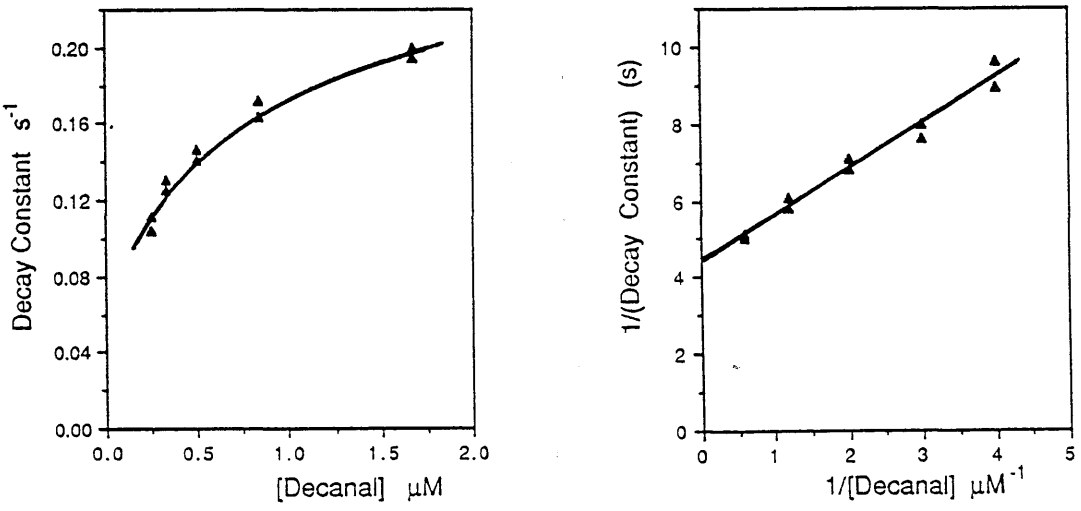

Figure 4.10 (LEFT): The dependence of the luminescence decay constant on decanal concentration in $FMNH_2$–initiated assays. Conditions were as in Fig. 4.7.
Figure 4.11 (RIGHT): Double reciprocal plot of the data in Figure 4.10 showing the linear relationship between the inverse of the luminescence decay constant and the inverse of the decanal concentration.

been shown to persist with decanal present). Additionally, the presence of a small amount of hydrogen in the flavin solution was estimated not to have any effect on the luciferase reaction. The flavin was prepared using hydrogen regulated at 4 p.s.i. above atmospheric pressure (which corresponds to 1.27 atmospheres of hydrogen). As an indication of the improbability of any effect on the luciferase protein of this level of hydrogen, it is known to require 130 atms of the gas to anaesthetise tadpoles (Miller & Smith, 1973).

4.4 The Effects of General Anaesthetics

The anaesthetic sensitivity of bacterial luciferase was investigated, in the first instance, by observing and analysing the effects of a wide range of general anaesthetics on the luminescent reaction catalysed by the enzyme. In these experiments the reaction was initiated with an injection of $FMNH_2$ into a vial containing luciferase, decanal and the anaesthetic agent under test [section 3.4(a)]. A small number of other agents which, in contrast, are known not to induce anaesthesia, were also examined for an effect on the luciferase enzyme. In this way

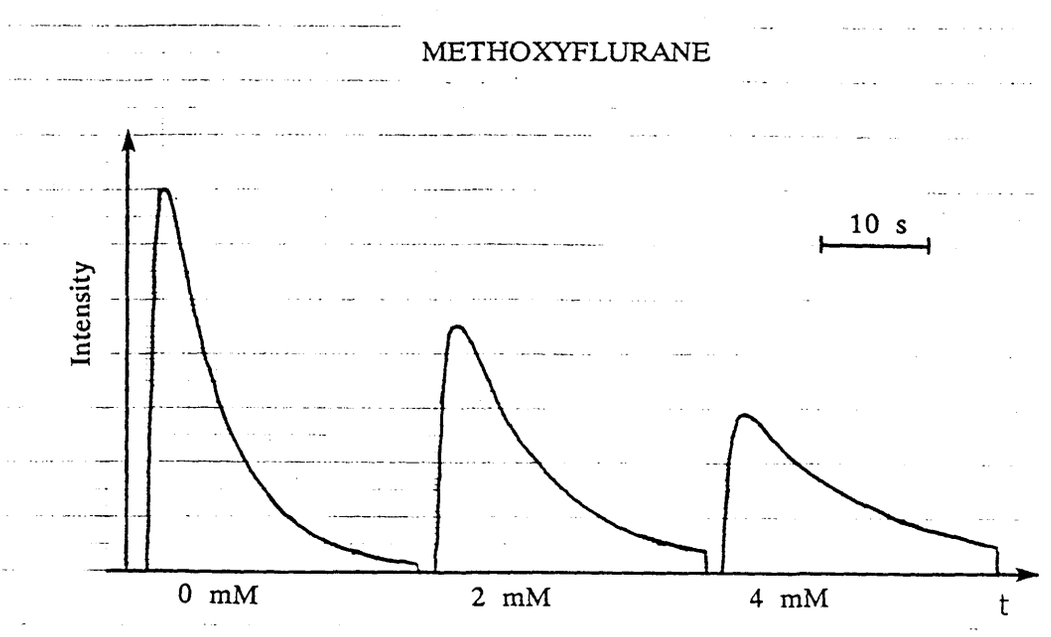

Figure 4.12: The effect of methoxyflurane on the time-dependence of the luminescent output of the $FMNH_2$-initiated luciferase reaction. The peak intensity is reduced, the time taken to reach the peak increased and the luminescence decay rate retarded. Final concentrations: decanal, 1.1 μM; $FMNH_2$, 96 μM; luciferase, 0.4 nM. $T = 23.8^{\circ}C$.

a full pharmacological profile was obtained so that a detailed comparison could be made with anaesthesia data for whole animals. This section contains a general description of the various effects that anaesthetics were observed to have on the luciferase catalysed reaction.

The light output from the reaction was sensitive to almost all of the agents tested. In a substantial majority of these cases the effect on the light output was identical to that shown in Figure 4.12 for methoxyflurane, a well known general anaesthetic. It is apparent that an increase in the concentration of methoxyflurane has a similar (but not quite identical – see below) effect as a reduction in the decanal concentration in that the peak and decay rate of luminescence are both reduced, while the time to peak is extended. This is indicative of the competitive nature of the inhibition. Dose–response data for methoxyflurane, halothane, *n*–heptanol and paraldehyde are presented in Figures 4.13(a)–(d). If this data is replotted with the anaesthetic concentrations scaled as multiples of the concentration (ED_{50}) of each which was required to depress the maximum rate (peak intensity) by 50%, it becomes clear that the dose response behaviour is exactly the same for these diverse agents (Figure 4.14). The inhibition by these agents was shown to be reversible because assays in which luciferase was exposed to a high concentration of anaesthetic before dilution to a lower concentration gave the same results as when the enzyme had been exposed only to the lower dose.

A plot of the logarithm of intensity of the light output as a function of time for the methoxyflurane data of Figure 4.12 reveals that the decay of luminescence is exponential in the presence of this anaesthetic (Figure 4.15). The first order decay constants calculated from this data, as well as from data for halothante, *n*–heptanol and paraldehyde, follow dose–response curves which look similar to those plotted for the fall in the initial maximum rate of the reaction (Figures 4.16(a)–(d)). Indeed it appears that, for these anaesthetics, the maximum initial rates are directly proportional to the associated decay constants for a broad range of concentrations (Figure 4.17). This apparent proportionality is a defining characteristic of the inhibition of bacterial luciferase for the majority of anaesthetics which were investigated in this study. (See tabulated data at the end of this chapter for a full list of the agents which produce this characteristic effect). At this point it may be noted that the relationship between the reduction in the peak intensity and the concomitant retardation of the decay of luminescence is not quite the same for addition of anaesthetic and reduction of the substrate concentration. Figure 4.17 shows that, as the peak intensity is reduced by increasing the amount of anaesthetic present, the luminescence decay constant appears to approach zero; in contrast, if

the peak intensity is reduced to zero by reducing the substrate concentration (Figure 4.10), the decay constant extrapolates to a finite, non−zero value. An explanation for this difference in behaviour will be given in section 6.2.

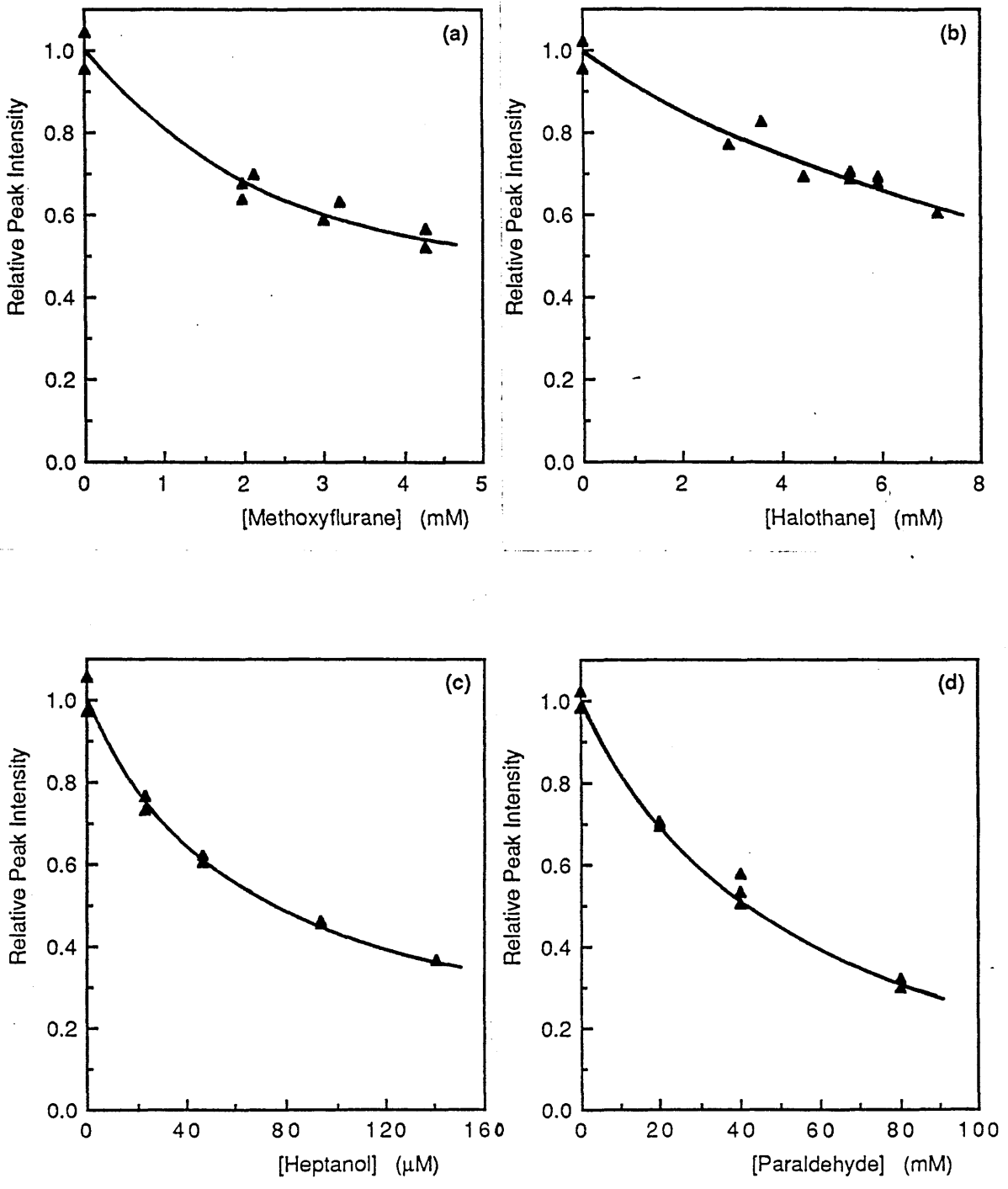

Figure 4.13(a)−(d): The inhibition of the $FMNH_2$−initiated luciferase reaction by general anaesthetics. The data show the dose−response of the reduction of the maximum rate (peak intensity) by (a) methoxyflurane, (b) halothane, (c) heptanol and (d) paraldehyde. Final concentrations (in 7.51 ml): decanal, 1.1 μM; $FMNH_2$, 94−103 μM; luciferase, 0.4 nM. T = 22.5−25.0°C.

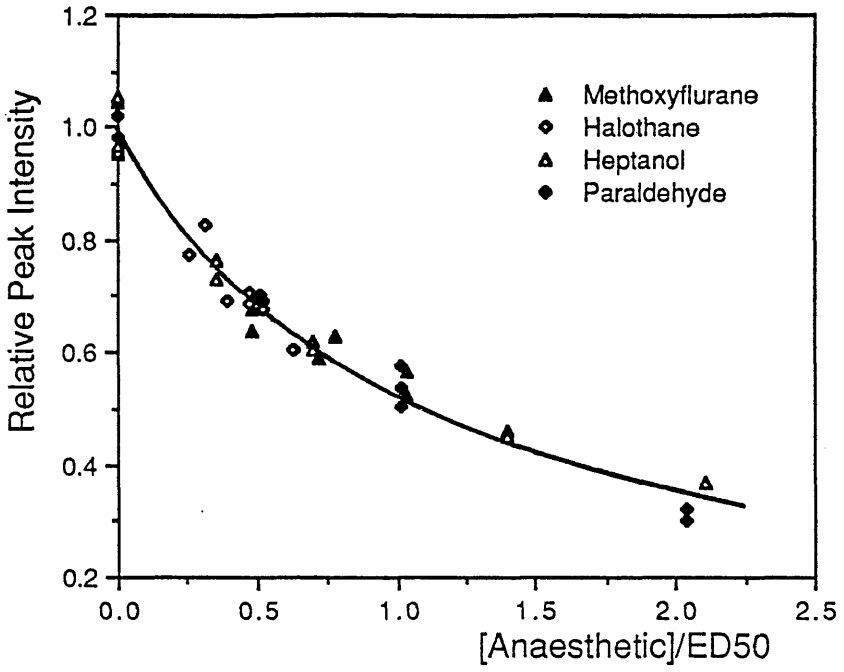

Figure 4.14: The dose–response behaviour of the maximum rate of the luciferase reaction is identical for methoxyflurane, halothane, heptanol and paraldehyde. Anaesthetic concentrations are given as multiples of the ED_{50} of each agent. Conditions are as in Figure 4.13.

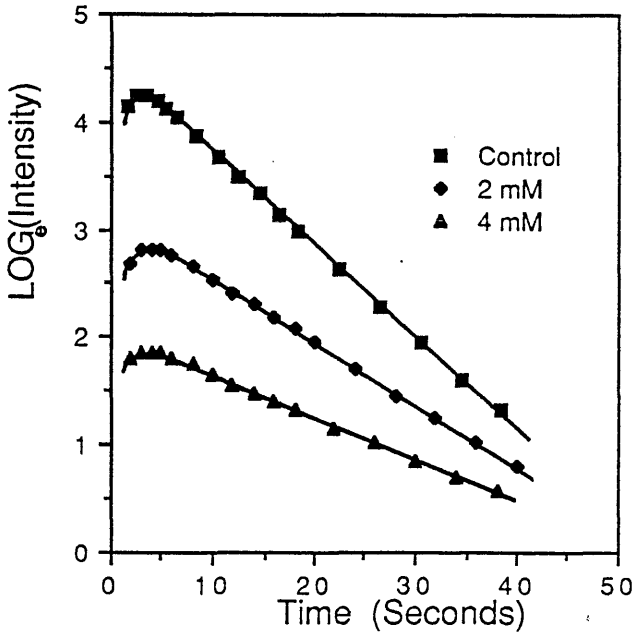

Figure 4.15: Methoxyflurane retards the exponential decay of the luminescent output of the $FMNH_2$–initiated reaction. Conditions are as in Figure 4.12.

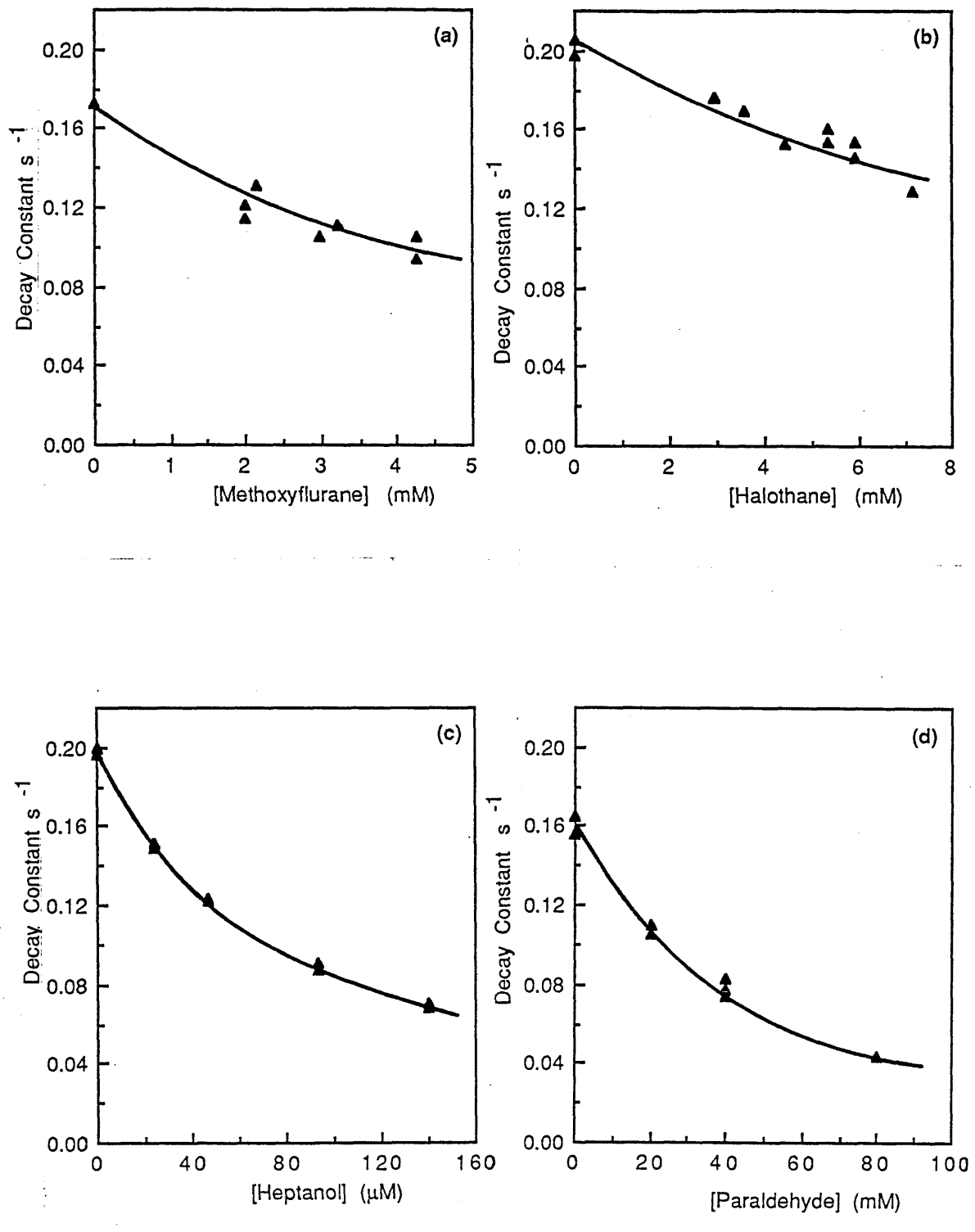

Figure 4.16(a)–(d): The dose–dependence of the reduction of the luminescence decay constant by (a) methoxyflurane, (b) halothane, (c) heptanol and (d) paraldehyde. This dose–response behaviour parallels the dependence of the maximum rate on the anaesthetic concentration – see Figure 4.13. Conditions are as in Figure 4.13.

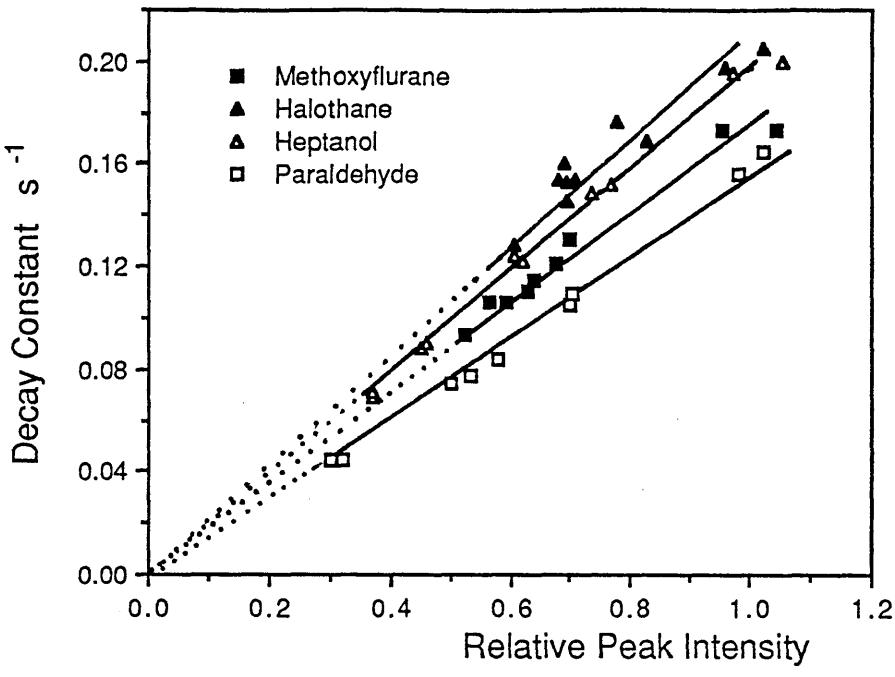

Figure 4.17: As suggested by comparison of Figure 4.13 and 4.16, the depression of the maximum rate of the luciferase reaction by methoxyflurane, halothane, heptanol and paraldehyde is apparently proportional to the concomitant reduction in the luminescence decay constant. Most anaesthetics also give this behaviour – see Table 4.2. The fact that the data points do not all fall on the same line results from the differences in temperature in the four experiments represented here. The highest temperature gives the steepest gradient: halothane, 25.2°C; heptanol, 25.0°C; methoxyflurane, 23.8°C; paraldehyde, 22.5°C.

Anaesthetics which Excite: Apart from the main group of anaesthetics described above, a small number of agents were observed to increase the peak intensity of the luminescent reaction at low concentrations. Ethanol, propanol, acetone and chloroform fall into this category; the dose–response curves for these agents show that varying degrees of excitation are produced (See Figures 4.18(a)–(d)). At sufficiently high concentrations inhibition was eventually observed. The reversibility of this inhibition was tested with the method described above; only the effect of ethanol was not completely reversed by dilution.

The dose–response behaviour of the inhibition caused by ethanol is very steep. This can be attributed to the fact that inhibition by ethanol is not fully reversible, an unsurprising result given the high concentrations used in the experiment. The peak intensity of the light output in the presence of an inhibitory concentration of

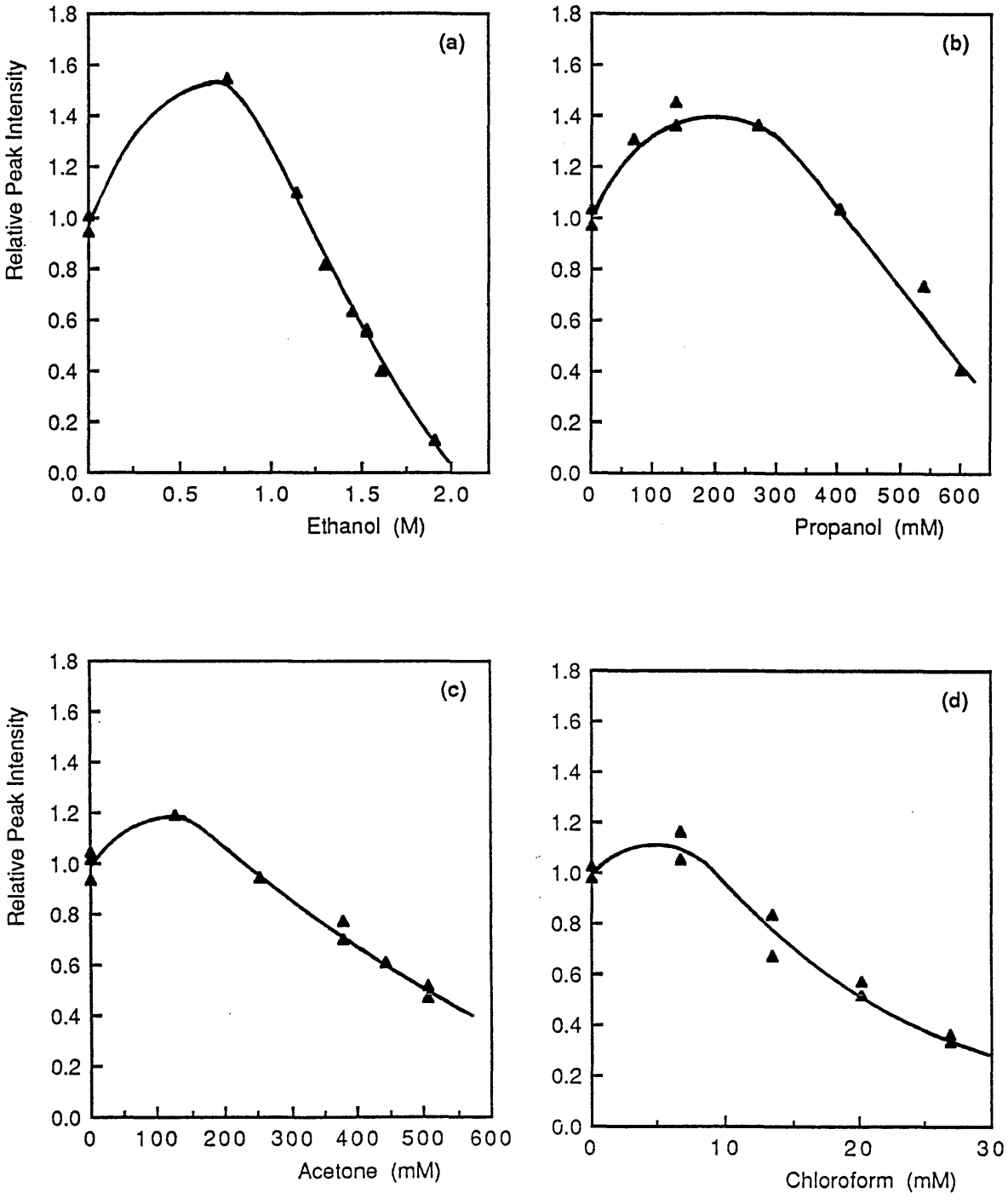

Figure 4.18(a)–(d): Stimulation of the maximum rate (peak intensity) of the $FMNH_2$–initiated luciferase reaction by low concentrations of (a) ethanol, (b) propanol, (c) acetone and (d) chloroform. Notice the variation in the maximum degree of stimulation. Final concentrations (in 7.51 ml): decanal, 0.85 μM; $FMNH_2$, 93–97 μM; luciferase, 0.13 nM. T = 24.0–25.9°C.

this alcohol was observed to decline if the time allowed for the incubation of luciferase, decanal and ethanol before initiation was increased. It is estimated that for a concentration of ethanol which reduces the peak intensity by 50% (with a decanal concentration of 0.85 μM), about 15% of that inhibition is not reversible. This result is unaffected by raising the concentration of decanal to 4.43 μM – indicating that the irreversible inhibition produced is due to ethanol acting at sites other than the aldehyde binding pocket. Figure 4.19 shows that the excitation due to ethanol is associated with a corresponding rise in the decay constant of luminescence. At higher concentrations, for which inhibition is observed, the decay constant falls below that of the control. However it does not fall as sharply as the peak intensity, probably because the irreversible component of the inhibition by ethanol does not greatly affect the process of decay.

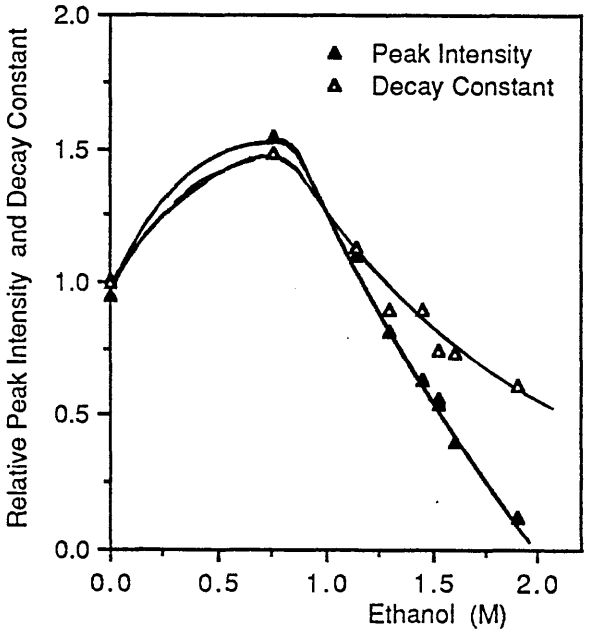

Figure 4.19: Comparison of the effects of ethanol on the maximum rate and the luminescence decay constant of $FMNH_2$–initiated reactions. Control values are normalised to unity. Conditions are as in Figure 4.18.

Chloroform is unique among the anaesthetics studied in that it causes a very fast spike in intensity prior to the normal peak. This feature is most prominent at high doses of chloroform. It is too fast to be measured quantitatively with the amplification equipment used in these experiments, however the spike is clearly visible in the traces shown in Figures 4.20(a) and (b). No account of this

behaviour was taken in the analysis of dose–response data – the normal, slower peak intensity was used as the measure of the maximum rate.

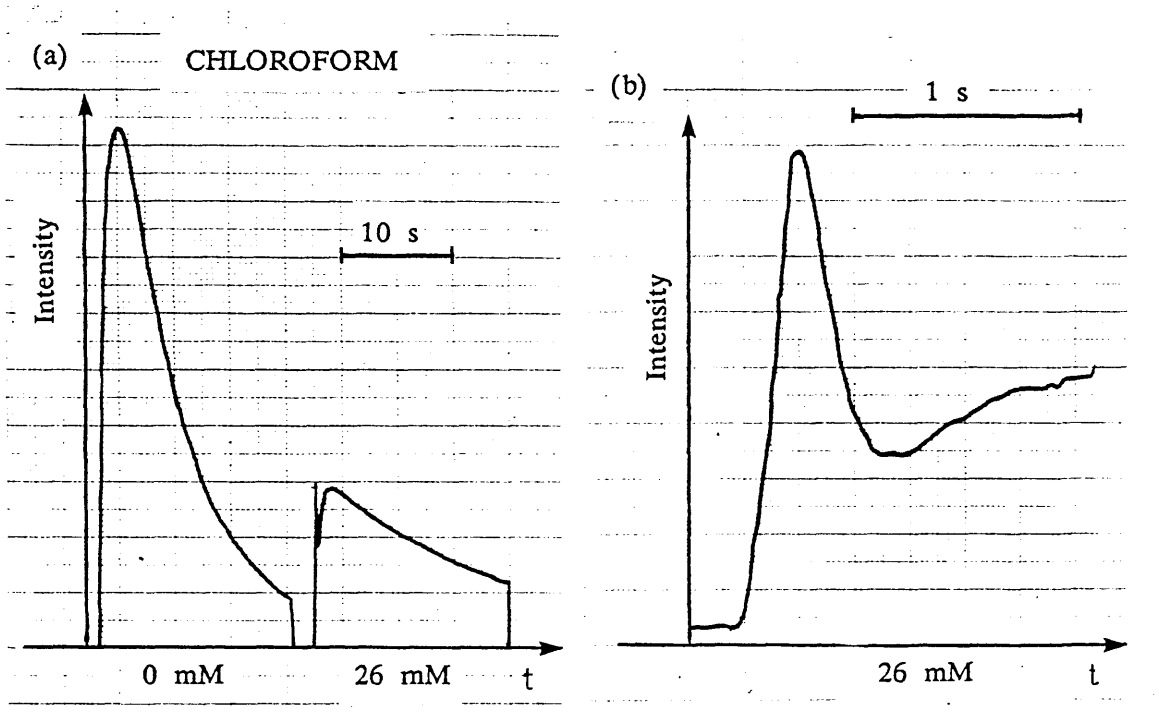

Figure 4.20: The effect of chloroform on the time–dependence of the luminescence output from the $FMNH_2$–initiated luciferase reaction. The profiles were traced from chart recorder output. Notice in (a) the fast spike prior to the main peak in intensity. This feature is shown magnified in (b). This latter signal was recorded on a digital storage oscilloscope and plotted on a chart recorder. [Note that the main peak in intensity shown in (a) is not present in (b)]. Final concentrations: decanal, 1.1 μM, $FMNH_2$, 96 μM, luciferase, 0.4 nM. $T = 22.5^{\circ}C$.

Lastly it should be noted that although butanol does not increase the maximum rate at low concentrations, it does not cause a net reduction either, until its concentration exceeds 40 mM (Figure 4.21).

Inhibition by Long–Chain Alcohols and Alkanes: *N*–alcohols from pentanol to decanol and *n*–alkanes from propane to nonane depress the peak intensity and retard the decay of the light output in exactly the same manner as most other anaesthetics (see above). However undecanol and decane, although they reduce the peak intensity, caused the light output following the peak to decay in a biphasic manner. In the presence of larger alcohols and alkanes, the biphasic nature of the

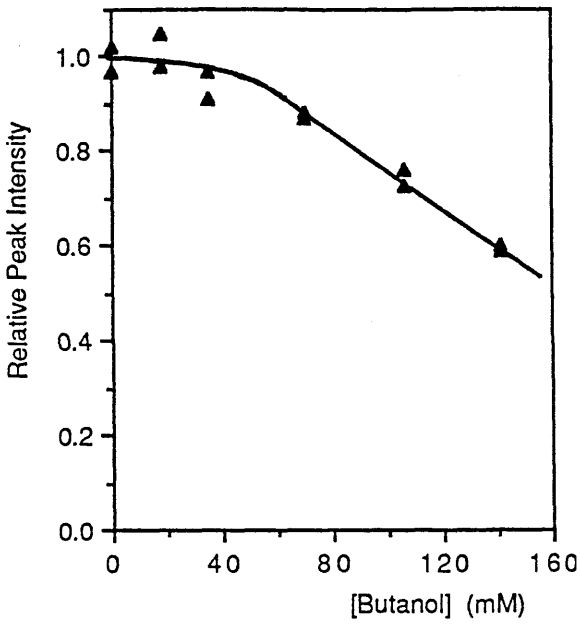

Figure 4.21: The depression of the maximum rate of the $FMNH_2$-initiated luciferase reaction by butanol. Notice that butanol has no effect at low concentrations. Final concentrations: decanal, 0.85 μM; $FMNH_2$, 107 μM; luciferase, 0.4 nM. T = 23.8°C.

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decay of luminescence is even more pronounced. These effects can be seen in examples of the light output profiles obtained with roughly equipotent doses (judged by their effect on the peak intensity) of decanol, undecanol and dodecanol (Figure 4.22). (The parallel transition for alkanes occurs for nonane, decane and undecane). The dependence of the luminescence decay constant on the concentration of decanol, given in Figure 4.23(a) follows the same dose–response curves as those shown in Figures 4.16(a)–(d) for methoxyflurane, halothane, *n*–heptanol and paraldehyde. Curiously, the decay constant of the initial fast phase induced by undecanol is independent of its concentration [Figure 4.23(b)] In contrast, the initial phase of decay is accelerated by increasing the concentration of dodecanol [Figure 4.23(c)]. For both undecanol and dodecanol, the final decay phase is retarded in a dose–dependent manner. At concentrations of these agents which reduce the maximum inital rate by 50% (ED_{50}) dodecanol retards this phase more than undecanol which, in turn, retards the decay more than decanol (Figure 4.24).

Decane acts identically to undecanol. Tridecanol, tetradecanol, undecane and dodecane all act in exactly the same way as dodecanol; all long–chain agents inhibit reversibly. Pentadecanol produces a similar type of inhibition although the

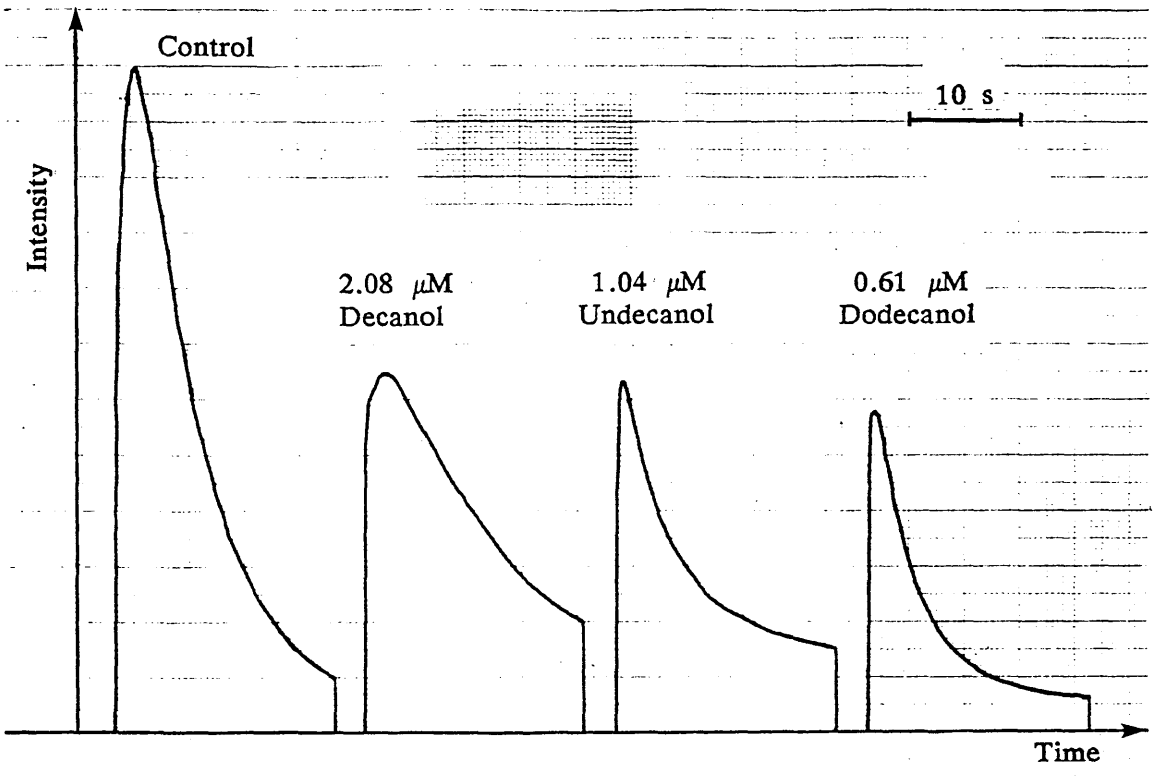


Figure 4.22: The effects of approximately ED_{50} doses of decanol, undecanol and dodecanol on the time-dependence of the luminescent output of the $FMNH_2$-initiated luciferase reaction. Notice the biphasic nature of the decay in the presence of undecanol and dodecanol. Final concentrations: decanal, 1.1 μM; $FMNH_2$, 88 μM, luciferase, 0.4 nM. T = 22.6°C.

acceleration and retardation of the two phases of decay are not as marked as observed with doses of dodecanol which cause the same drop in peak intensity. The inhibition by hexadecanol, even at concentrations close to saturation, is too slight for any quantitative assessment of the decay constants to be made. Tridecane and tetradecane were observed not to inhibit the reaction at all.

Although undecanol and dodecanol (and other long-chain alkanes and alcohols) induce a very different process of luminescence decay than that observed in the presence of decanol, the depression of the maximum initial rate of the reaction by these agents follows the same dose-response curve. (See Figure 4.25 where the peak intensity has been plotted as a function of multiples of the ED_{50}'s of the alcohols from decanol to tetradecanol). This suggests that, despite the very obvious differences in the overall effects of these agents on the reaction, they seem to share a similar mode of action. In order to investigate this seemingly contradictory

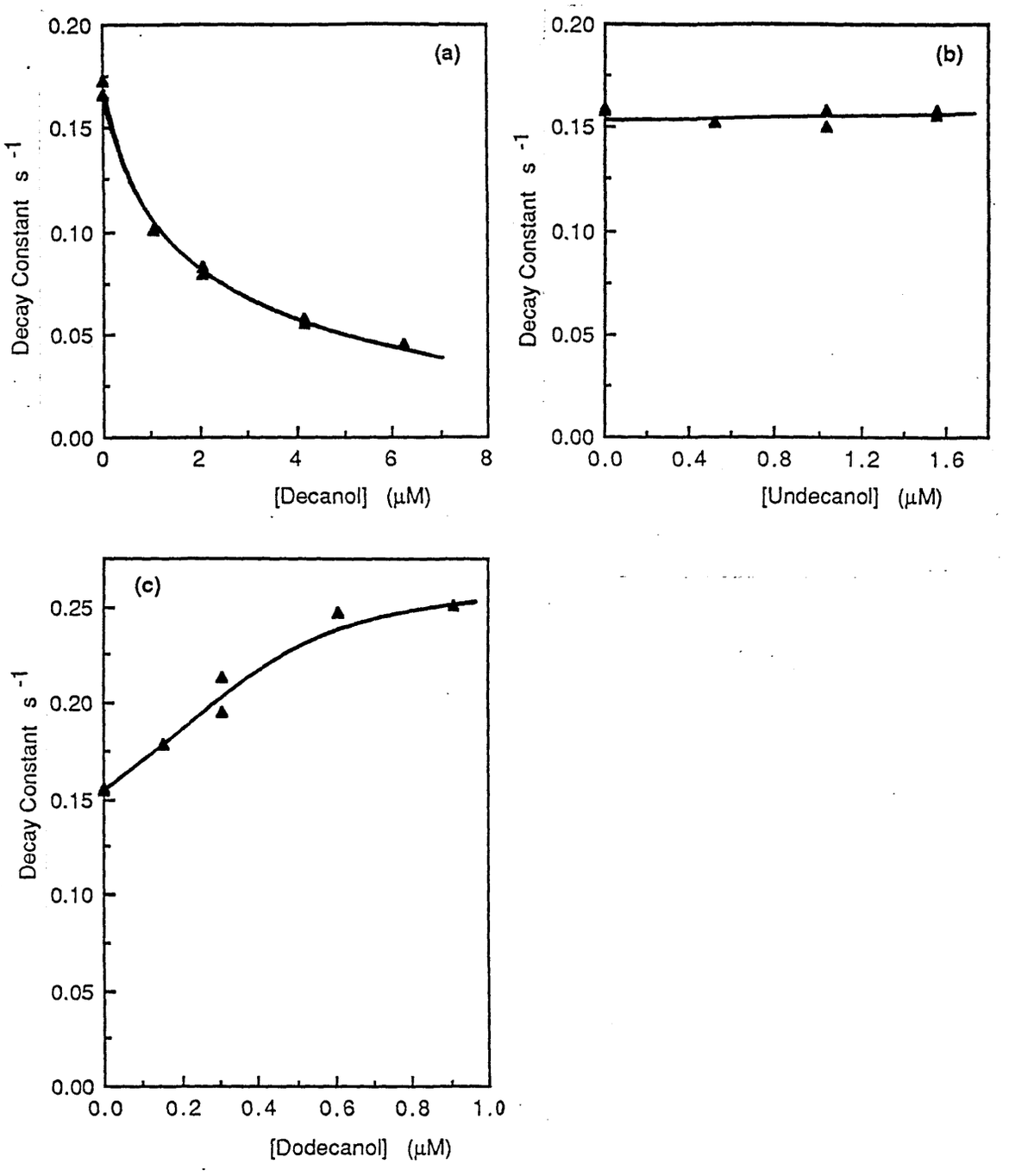


Figure 4.23(a)–(c): The dose–dependence of the decay constants for the early phase of the decay of luminescence in the presence of long–chain alcohols. (a) decanol reduces the decay constant – [remember that the luminescence decay in the presence of decanol is <u>not</u> biphasic. The decay constants of the early and late phases are identical]. (b) The decay constant of the initial phase of decay in the presence of undecanol is independent of the concentration. (c) In the presence of dodecanol, the decay constant of the initial phase is increased by raising the concentration. Conditions are as in Figure 4.22.

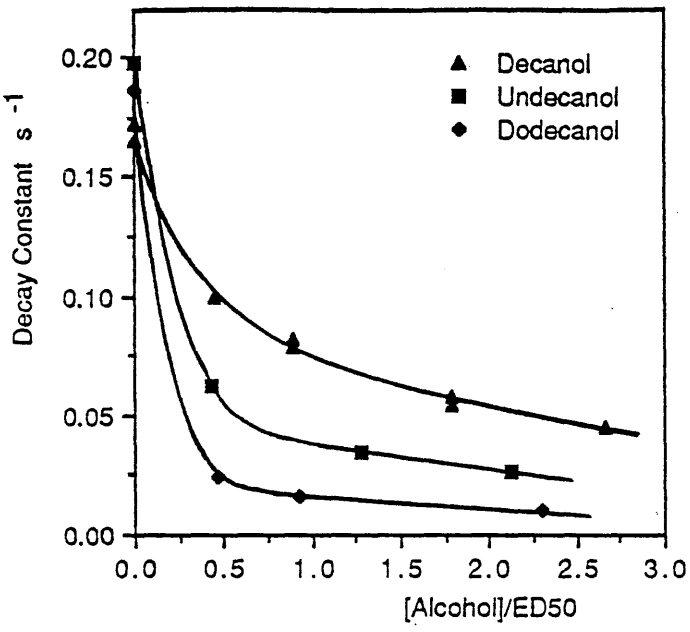


Figure 4.24: Comparison of the reduction in the luminescence decay constant due to decanol with the dose dependence of the decay constants for the final phase of decay observed in the presence of undecanol and dodecanol. Alcohol concentrations are scaled as multiples of the ED_{50} concentration of each agent. Final concentrations: decanal, 1.1 μM; $FMNH_2$, 88–107 μM; luciferase, 0.4 nM. T = 22.6°C (decanol); 24.3°C (undecanol, dodecanol).

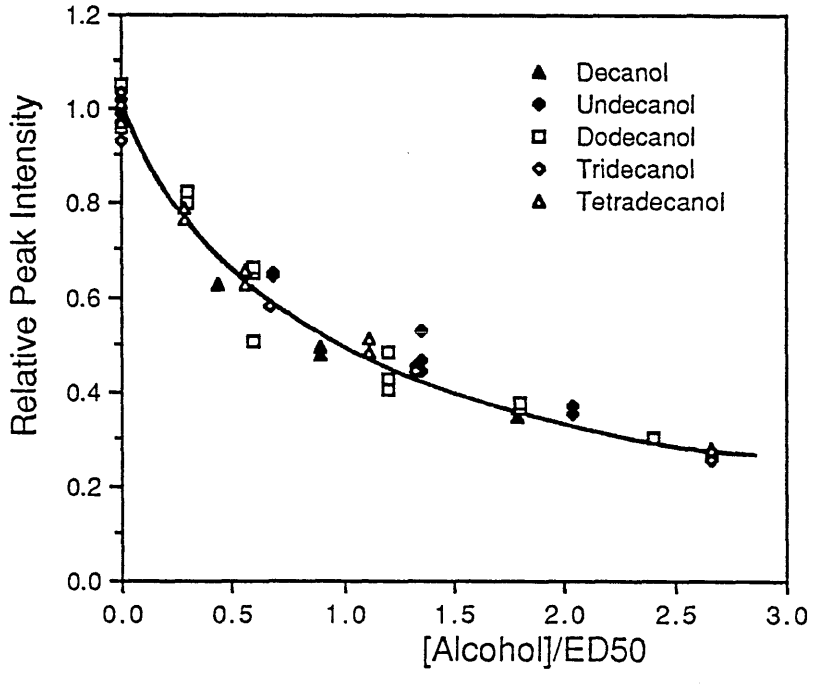


Figure 4.25: The dose–response behaviour of the maximum rate of the luciferase reaction in the presence of long–chain alcohols is the same, whether or not biphasic luminescence decay is observed. Final concentrations: decanal, 1.1 μM; $FMNH_2$, 88–107 μM; luciferase, 0.4 nM. T = 22.6–24.3°C.

observation, experiments were performed to determine the mechanism of inhibition; these are discussed in the next section.

4.5 The Mechanism of Inhibition

The analysis in section 4.1 and the experimental results in section 4.3 demonstrated that the reaction catalysed by bacterial luciferase obeys the Michaelis−Menten equation. It is therefore possible to determine experimental values of the K_m of decanal and V_{max}. By observing changes in these parameters due to general anaesthetics, deductions about the mechanism of inhibition can be made. Experiments were performed to measure the variation in $1/v_{pk}$ as a function of 1/[S] in the presence of fixed concentrations of anaesthetics which were representative examples of the three classes of luciferase inhibitor that were identified in the previous section.

In that section it was noted that an increase in the concentration of an anaesthetic such as methoxyflurane had a similar effect on the light output of the reaction as a decrease in the decanal concentration. The competitive nature of the inhibition by such agents, which is suggested by this result, is confirmed by the data presented in Figure 4.26(a). Identical results were obtained for diethyl ether, octane and decanol (See Figures 4.26(b)−(d)). The maximum theoretical reaction rate (V_{max}) is not affected by these agents. The inhibition that they cause is simply due to an increase in the apparent K_m of decanal. This means that, in the presence of such agents, it requires a greater aqueous concentration of decanal to attain the half maximal rate (since for [S] = K_m, $v_{pk} = \frac{1}{2}V_{max}$). Such a result would be obtained if the anaesthetic simply reduces the aqueous concentration of the aldehyde substrate directly, by reacting with it to produce an inactive product. This seems highly unlikely, however, in view of the fact that competitive inhibition was observed for a diverse group of agents, none of which is very reactive. A second, more plausible mechanism is that binding of the anaesthetic molecule to luciferase prevents decanal participating in the luminescent reaction. Conceivably, there are two mechanisms by which this might occur: either the anaesthetic could bind to the aldehyde binding site and thus physically block decanal from binding there itself, or alternatively, the anaesthetic may bind to a separate site causing an allosteric interaction which either blocks the aldehyde site or prevents the aldehyde, once bound, from any further interaction with the flavin substrate. Although it is not possible to distinguish between these binding models, the active site on *Vibrio*

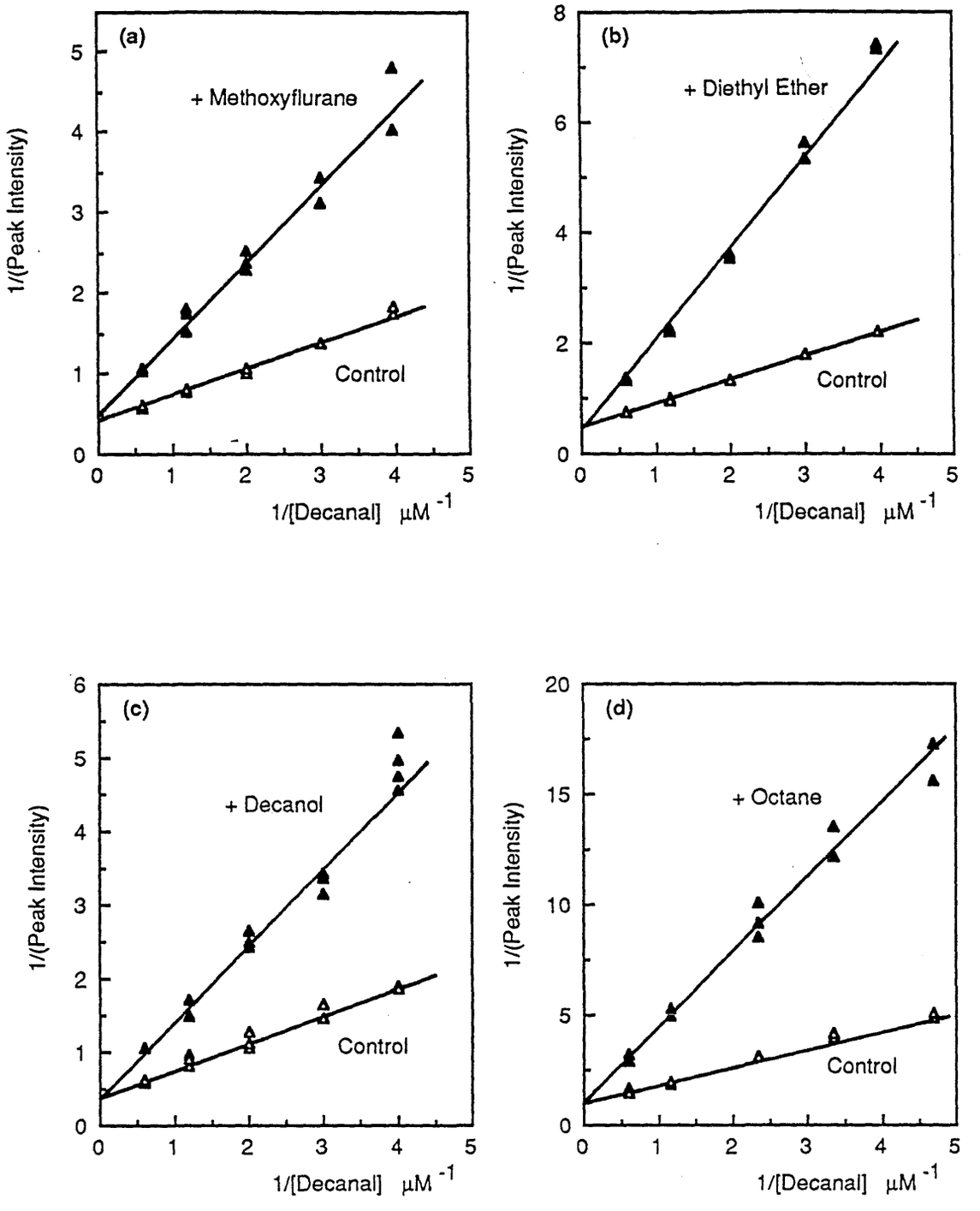


Figure 4.26(a)–(d): Lineweaver–Burk plots showing purely competitive inhibition of the $FMNH_2$–initiated luciferase reaction by (a) 4.9 mM methoxyflurane, (b) 35 mM diethyl ether, (c) 2.2 μM n–decanol and (d) 4.0 μM n–octane. Final concentrations (in 7.51 ml): decanal, 0.21–1.7 μM; $FMNH_2$, 95–97 μM; luciferase, 0.4 nM. T = 23.1–24.9°C.

harveyi luciferase is considered to be hydrophobic (Nicoli and Hastings, 1974; Meighen and Bartlet, 1980), a property which is possessed by the anaesthetic target(s) in animals, and it seems most likely that anaesthetics are binding there, rather than at some secondary site.

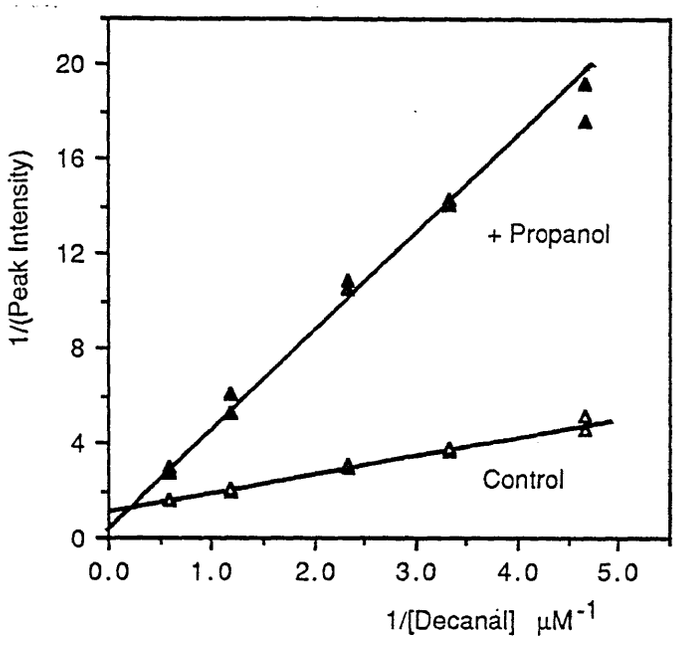


Figure 4.27: Lineweaver–Burk plot showing inhibition of the $FMNH_2$–initiated luciferase reaction by propanol. Note that the lines do not meet on the ordinate. Final concentrations: decanal, 0.21–1.7 μM; $FMNH_2$, 97 μM; luciferase, 0.4 nM. $T = 24.9^{\circ}C$.

The inhibitory mechanism of propanol, an agent which increases the peak intensity at low concentrations, was also investigated. The double reciprocal plot (Figure 4.27) indicates that the inhibition is competitive in nature, since the apparent K_m of decanal is increased. The inhibition by propanol can therefore be removed by raising the decanal concentration. However, propanol also increases V_{max}, an effect which is probably responsible for the observed excitation and which cannot be antagonised by an increase in the levels of decanal. Consequently propanol appears also to be acting at a second site on luciferase from where it exerts its effect on V_{max}. Since $V_{max} = k_2([E]_0 - [E_r]_{pk})$ it is possible that propanol may be increasing the rate constant k_2 in equation 4.3 (which actually represents a combination of the rate constants for several steps on the reaction pathway). A

similar mechanism probably underlies the excitation caused by ethanol, acetone and chloroform. The fact that the reaction rate is unaffected by butanol at concentrations less than 40 mM, may result from a net cancellation of inhibitory and excitatory effects.

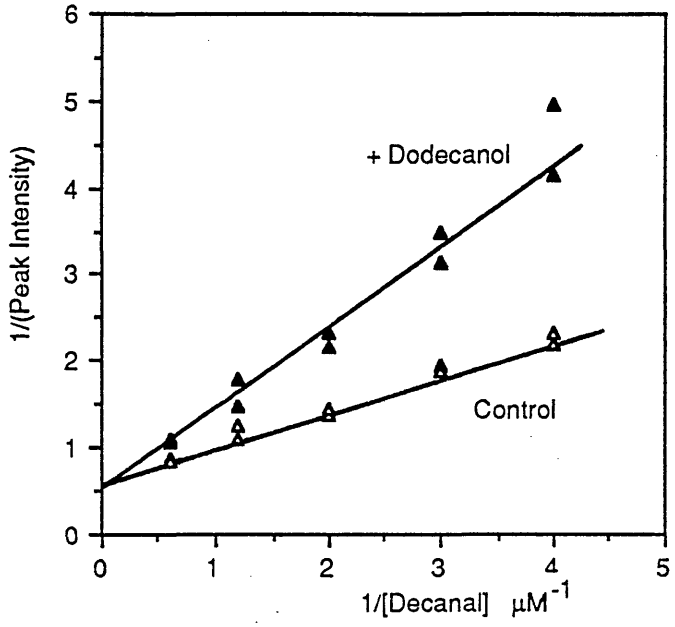


Figure 4.28: Lineweaver–Burk plot showing purely competitive inhibition of the $FMNH_2$–initiated reaction by dodecanol. Final concentrations: decanal, 0.25–1.7 μM; $FMNH_2$, 113 μM; luciferase, 0.4 nM. T = 22.0°C.

As discussed in the previous section, long–chain alkanes and alcohols, although they depress the maximum initial rate, produce a very different luminescence decay profile than that observed with most other anaesthetics. Nonetheless, it is clear from Figure 4.28 that dodecanol, an example of this kind of agent, acts as a purely competitive inhibitor since it has no significant effect on V_{max}. On the one hand it is hardly surprising that dodecanol binds at the aldehyde site, given the close homology between it (and other long–chain agents which act in the same way) and the long–chain aldehyde substrate. This accounts for the fact that the dose–response behaviour for this alcohol is the same as that for other agents, even though it induces a very different type of luminescence decay. On the other hand, this finding gives no clue as to why dodecanol, and agents like it, should induce a biphasic rather than monophasic decay of luminescence. A fuller discussion of this particular question is given in section 6.2.

4.6 Quantitative Analysis of Inhibition

On the basis of the knowledge that the mechanism of inhibition of bacterial luciferase is competitive, it is possible to determine quantitatively the affinity of anaesthetic molecules for their binding site on the enzyme. Competitive inhibition of the luciferase catalysed reaction may be represented by the scheme:

$$\begin{array}{l} E' + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E'S \xrightarrow{k_2} E_r + \text{photon} + P \\ + \\ A \\ \Big\| K_i \\ E'A \end{array} \qquad (4.14)$$

E', S, E'S, E_r and P have the same meanings as in section 4.1. E'A is the inactive complex formed when the anaesthetic molecule, A, associates with the luciferase peroxyflavin intermediate. The anaesthetic dissociation constant, K_i, is defined as:

$$K_i = \frac{[E'][A]}{[E'A]} \qquad (4.15)$$

Following the same derivation as in section 4.1, it is possible to show that the maximum rate (peak intensity), v_i, in the presence of an anaesthetic concentration [A] is:

$$v_i = k_2[E'S] = \frac{V_{max}[S]}{[S] + K_m\{1 + [A]/K_i\}} \qquad (4.16)$$

where $V_{max} = k_2([E]_0 - [E_r]_{pk})$, as before. In the absence of any anaesthetic, the maximum rate of the control is:

$$v_0 = k_2[E'S] = \frac{V_{max}[S]}{[S] + K_m} \qquad (4.17)$$

The ratio v_0/v_i is therefore:

$$\frac{v_0}{v_i} = \frac{[S] + K_m(1 + [A]/K_i)}{[S] + K_m} \qquad (4.18)$$

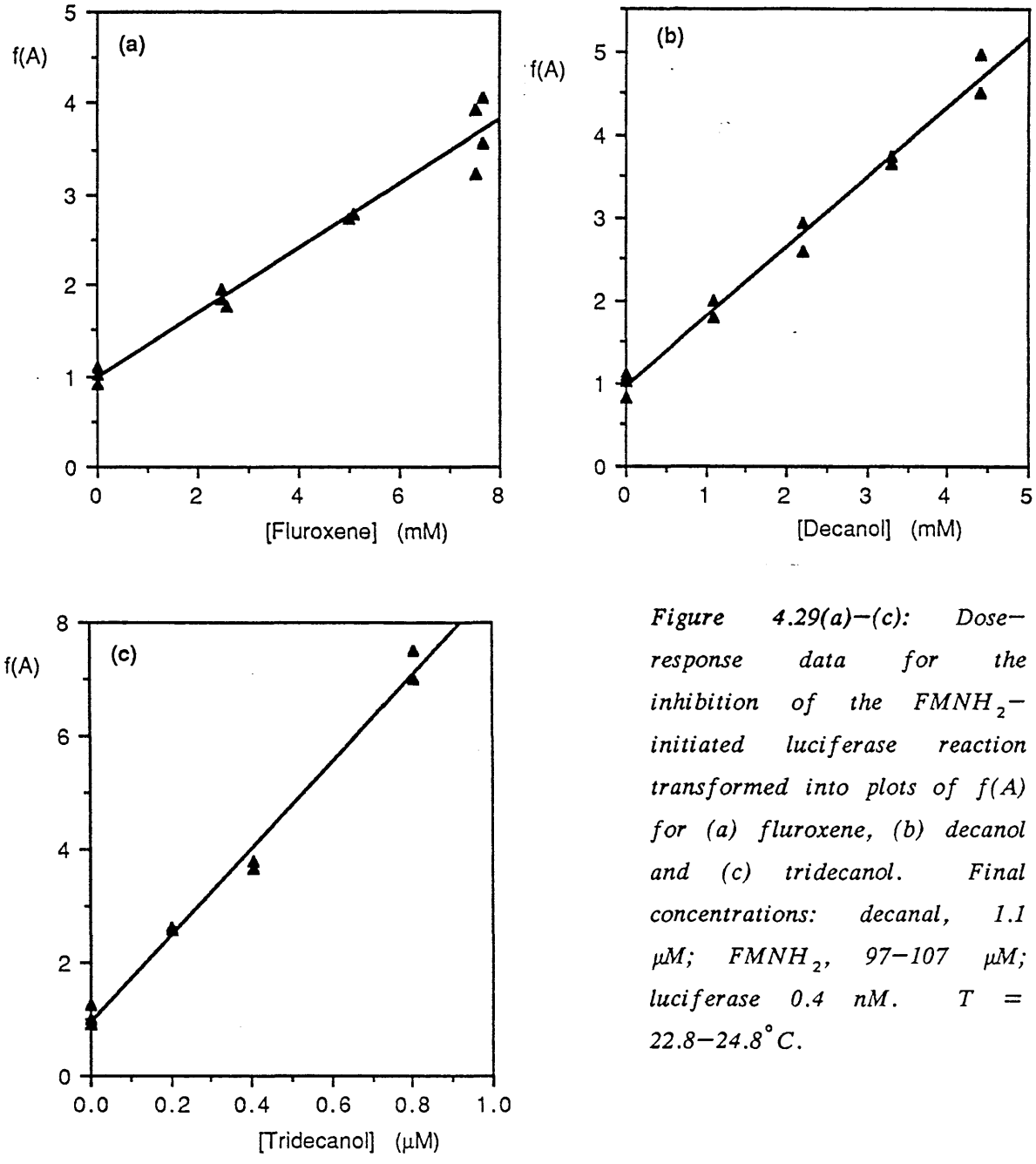


Figure 4.29(a)–(c): Dose–response data for the inhibition of the $FMNH_2$–initiated luciferase reaction transformed into plots of f(A) for (a) fluroxene, (b) decanol and (c) tridecanol. Final concentrations: decanal, 1.1 μM; $FMNH_2$, 97–107 μM; luciferase 0.4 nM. T = 22.8–24.8°C.

By rearranging this equation it is possible to define:

$$f(A) \equiv \left[1 + \frac{[S]}{K_m}\right]\frac{v_0}{v_i} - \frac{[S]}{K_m} = 1 + \frac{[A]}{K_i} \qquad (4.19)$$

This analysis permits the anaesthetic dissociation constant, K_i, to be calculated from dose–response data obtained at a fixed decanal concentration. The value of f(A) may be calculated for each anaesthetic concentration using equation (4.19) and when plotted as a function of that concentration yields a straight line of slope $1/K_i$.

Examples of such plots are given in Figures 4.29(a)–(c) for fluroxene and decanol, anaesthetics which retard the luminescence decay like most other agents, and tridecanol, which produces biphasic luminescent decay kinetics. Clearly the data fall on straight lines of intercept 1. The K_i values determined from the gradients of the lines (which are calculated by the method of least squares) had a typical standard error of 5%.

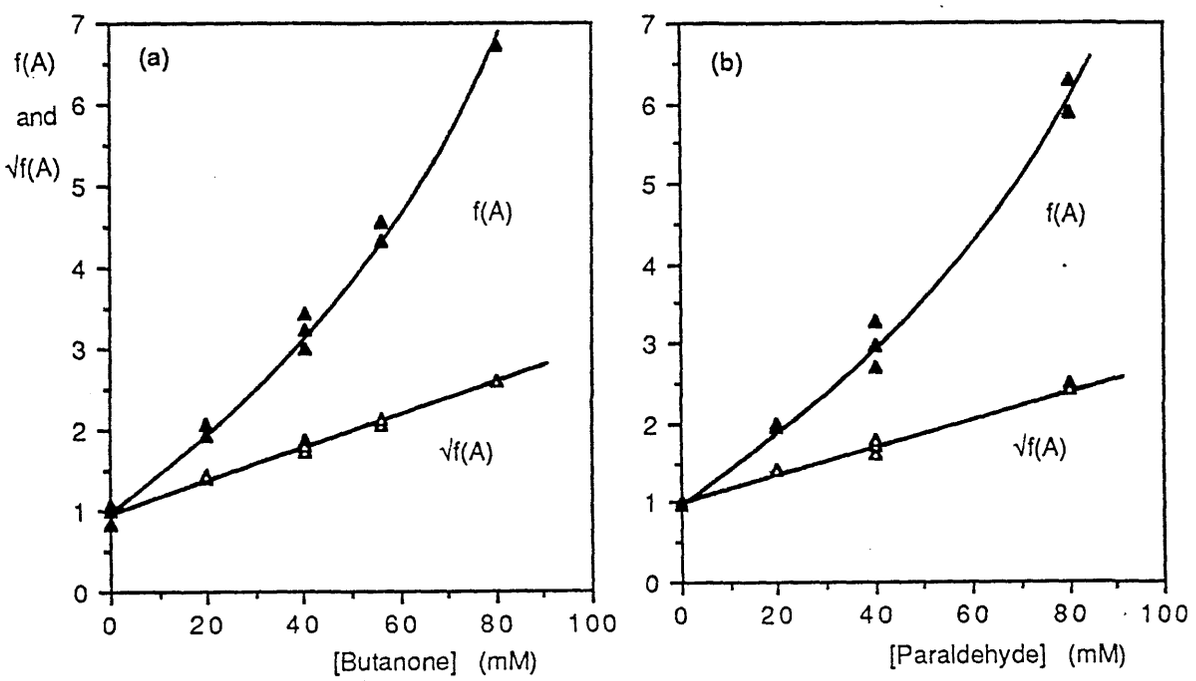
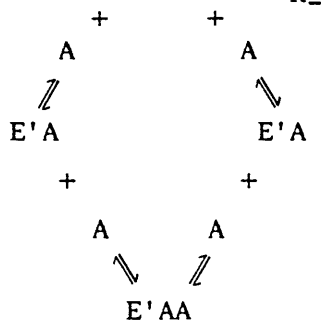


Figure 4.30: Plots of f(A) and √f(A) for (a) butanone and (b) paraldehyde. Assays were performed by the $FMNH_2$–initiation method. Final concentrations: decanal, 1.1 μM; $FMNH_2$, 96–108 μM; luciferase, 0.4 nM. T = (a) 22.6°C; (b) 25.1°C.

In some cases, as in Figures 4.30(a) and (b) for butanone and paraldehyde, the plot of f(A) is parabolic so that √f(A) is a linear function of [A], within experimental error. This behaviour may be explained using a model which was developed by Franks and Lieb (1984) to account for similar observations of the inhibition of firefly luciferase by small anaesthetics. The model assumes that two anaesthetic molecules can bind to the enzyme but that the binding of only one molecule is sufficient to inhibit catalysis. The modified reaction scheme is:

$$E' + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E'S \xrightarrow{k_2} E_r + \text{photon} + P \qquad (4.20)$$



where the dissociation constants of each anaesthetic binding step are equal. For this scheme:

$$f(A) \equiv \left[1 + \frac{[S]}{K_m}\right]\frac{v_0}{v_i} - \frac{[S]}{K_m} = \left[1 + \frac{[A]}{K_i}\right]^2 \qquad (4.21)$$

so that:

$$\surd f(A) = 1 + \frac{[A]}{K_i} \qquad (4.22)$$

Thus, when two molecules bind, K_i is calculated as the inverse of the gradient of $\surd f(A)$ plotted as a function of [A]. Although the above model provides the simplest explanation of the data, it is probably naive to assume that the dissociation constants for the interaction of the first and second anaesthetic molecules are identical. It should be noted that, if f(A) and $\surd f(A)$ values up to an f(A) of around 6 (equivalent to about 70% inhibition) are calculated assuming that the two dissociation constants are *not* equal, it becomes evident that they would have to differ by almost a factor of 10 before the difference between them showed up as significant non-linearity in the $\surd f(A)$ plot.

The anaesthetic potency of particular agents is normally quantified in terms of the dose required to anaesthetise half of a group of animals under test. It is therefore desirable to compute a corresponding parameter for the effect of anaesthetics on the rate of luciferase-catalysed reactions. Conventionally the concentration of anaesthetic which reduces the maximum initial rate by 50%, the ED_{50}, is used. However, since the mechanism of inhibition is competitive, the ED_{50} is, of course, dependent on the concentration of the aldehyde substrate. It is convenient to calculate, as a standard ED_{50}, the concentration of inhibitor required to depress the maximum initial rate by 50% in the presence of a concentration of decanal which is equal to its K_m. For $[S] = K_m$ and $[A] = ED_{50}$, equation (4.19) becomes:

$$f(ED_{50}) = 2 \cdot \frac{v_0}{\frac{1}{2}v_0} - 1 = 1 + \frac{ED_{50}}{K_i} \qquad (4.23)$$

which gives:

$$ED_{50} = 2K_i \qquad (4.24)$$

If two anaesthetic molecules bind then:

$$ED_{50} = (\sqrt{3} - 1)K_i \qquad (4.25)$$

It should be noted that it is possible to calculate this standard ED_{50} even with inhibition data which were obtained in the presence of a decanal concentration not equal to its K_m.

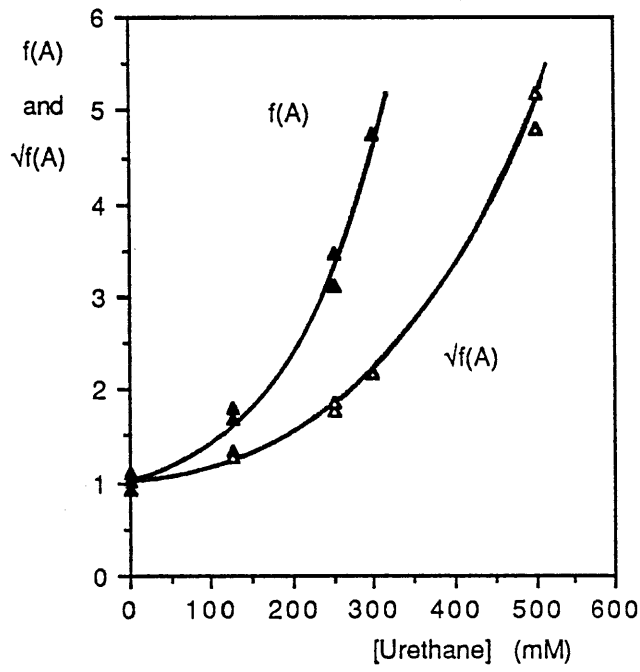


Figure 4.31: Plots of f(A) and √f(A) for urethane. Assays were performed by the $FMNH_2$–initiation method. Final concentrations: decanal, 0.85 μM; $FMNH_2$, 97 μM; luciferase, 0.4 nM. T = 24.0°C.

Those agents which cause a net increase in the peak intensity of the light output (and butanol which does not affect it) at low concentrations cannot be analysed rigorously by this method. Consequently, their ED_{50} concentrations were determined directly from dose–response data collected with the final decanal concentration set equal to 0.85 μM (= K_m). It is nonetheless interesting to observe

that over the range of concentrations of acetone and propanol which do inhibit, the f(A) plot rises more steeply than a parabola, indicating that more than two molecules of these agents may be binding. The f(A) curve for urethane (Figure 4.31) which does not cause any excitation, is similarly steep. In contrast, the inhibition by both chloroform and butanol follows a parabolic f(A) curve; it seems that just two molecules of these agents can bind to luciferase.

The dissociation constants and ED_{50} concentrations of a large number of different anaesthetics were determined according to the analysis described in this section and are presented in Tables 4.2(a)−(d). The information in theses tables constitutes a comprehensive profile of the response of the bacterial luciferase enzyme to general anaesthetics. In the next chapter this profile is compared with anaesthesia data for whole animals and the implications of this comparison for the nature of the physiological site or sites of anaesthetic action are discussed.

Tables 4.2(a)−(d) (following pages): Dissociation constants (K_i) and ED_{50} concentrations determined for the inhibition of bacterial luciferase by general anaesthetics: (a) n−alcohols, (b) n−alkanes, (c) halogenated anaesthetics and (d) miscellaneous anaesthetics. The calculation of these parameters from experimental data is given in the text. The ED_{50} is the concentration required to depress the maximum rate of the luciferase reaction in the presence of a decanal concentration equal to the K_m of this substrate (0.85 μM) and at saturating levels of $FMNH_2$ (≃100 μM). Luciferase assays were performed with the $FMNH_2$−initiation method. $T = 22.5-25.5^{\circ}C$. n is the number of anaesthetic molecules which can bind and inhibit luciferase. (*) indicates that stimulation was observed at low concentrations. (•) indicates that biphasic luminescence decay was observed. Unmarked agents inhibited the maximum rate (peak intensity) apparently in proportion to the reduction in the luminescence decay constant that they caused. Where values of K_i are not given, the f(A) curve was steeper than parabolic; in these cases the ED_{50} was determined by interpolation of the dose−response curve.

Table 4.2(a): K_i and ED_{50} Values for N-alcohols.

Agent	K_i	ED_{50}	n
Ethanol*	---	1.55 ± 0.12 M	>2
Propanol*	---	0.544 ± 0.020 M	>2
Butanol*	---	128 ± 35 mM	2
Pentanol	2.99 ± 0.19 mM	5.97 ± 0.38 mM	1
Hexanol	0.377 ± 0.013 mM	0.753 ± 0.026 mM	1
Heptanol	33.4 ± 4.4 μM	66.7 ± 8.7 μM	1
Octanol	4.28 ± 0.33 μM	8.55 ± 0.66 μM	1
Nonanol	4.04 ± 0.26 μM	8.07 ± 0.51 μM	1
Decanol	1.17 ± 0.09 μM	2.34 ± 0.17 μM	1
Undecanol*	0.385 ± 0.019 μM	0.769 ± 0.038 μM	1
Dodecanol*	0.253 ± 0.023 μM	0.506 ± 0.047 μM	1
Tridecanol*	0.160 ± 0.018 μM	0.320 ± 0.036 μM	1
Tetradecanol*	0.179 ± 0.004 μM	0.358 ± 0.007 μM	1
Pentadecanol*	0.251 ± 0.010 μM	0.502 ± 0.020 μM	1
Hexadecanol*	0.28 ± 0.07 μM	0.56 ± 0.13 μM	1

Table 4.2(b): K_i and ED_{50} Values for N-alkanes

Agent	K_i	ED_{50}	n
Propane	6.99 ± 0.23 mM	5.05 ± 0.17 mM	2
Pentane	0.292 ± 0.016 mM	0.584 ± 0.032 mM	1
Hexane	29.5 ± 3.3 μM	59.0 ± 6.6 μM	1
Heptane	5.35 ± 0.25 μM	10.7 ± 0.05 μM	1
Octane	0.985 ± 0.015 μM	1.97 ± 0.03 μM	1
Nonane	0.385 ± 0.013 μM	0.770 ± 0.025 μM	1
Decane*	0.131 ± 0.025 μM	0.262 ± 0.049 μM	1
Undecane*	0.0504 ± 0.0070 μM	0.108 ± 0.017 μM	1
Dodecane*	0.038 ± 0.017 μM	0.076 ± 0.033 μM	1

Table 4.2(c): K_i and ED_{50} Values for Halogenated Hydrocarbons

Agent	K_i (mM)	ED_{50} (mM)	n
Chloroform*	---	20.6 ± 3.0	1
Halothane	5.70 ± 0.90	11.4 ± 1.8	1
Methoxyflurane	2.07 ± 0.22	4.14 ± 0.44	1
Fluroxene	2.79 ± 0.04	5.59 ± 0.08	1
Enflurane	5.25 ± 0.45	10.5 ± 0.9	1
Isoflurane	6.6 ± 0.9	13.2 ± 1.8	1

Table 4.2(d): K_i and ED_{50} Values for Miscellaneous Agents

Agent	K_i (mM)	ED_{50} (mM)	n
Diethyl Ether	32.5 ± 1.9	23.8 ± 1.4	2
Urethane	---	261 ± 24	>2
Acetone*	---	503 ± 45	>2
Butanone	50.5 ± 1.1	37.0 ± 0.8	2
Paraldehyde	53.7 ± 1.9	39.3 ± 1.4	2
Benzyl Alcohol	6.85 ± 0.45	13.7 ± 0.9	1
Adamantanol	1.2 ± 0.4	2.3 ± 0.8	1
Barbital	15.7 ± 1.7	31.4 ± 3.4	1
Pentobarbital	9.5 ± 0.2	18.9 ± 0.3	1

CHAPTER 5

THE ANAESTHETIC SENSITIVITY OF BACTERIAL LUCIFERASE: COMPARISON WITH WHOLE ANIMALS

5.1 Introduction: Does Bacterial Luciferase Resemble the Anaesthetic Target in Animals?

The ED_{50} concentrations and dissociation constants presented for 39 anaesthetic agents in Tables 4.2(a)−(d) contain a considerable amount of information about the properties of the anaesthetic binding site on bacterial luciferase. When the potencies ($\equiv 1/ED_{50}$) of these agents as luciferase inhibitors and as general anaesthetics are compared, as in Figure 5.1, the similarities and differences between the luciferase enzyme and the physiological site of general anaesthetic action become evident. In this chapter, the properties of the anaesthetic binding site on luciferase, revealed by the inhibition data, are analysed in detail. The possible reasons why these properties may account for the form of the correlation in Figure 5.1 between luciferase and the physiological anaesthetic target are discussed.

[Before proceeding, I should like to point out that it remains a matter of considerable debate whether there are one or more different types of anaesthetic binding site in the central nervous systems of living creatures. Certainly it seems unlikely that molecules as structurally diverse as xenon, halothane, ketamine and alfaxalone all act at the same site. Nonetheless, there are grounds for believing that a large group of relatively simple anaesthetic compounds may indeed have a common site of action. The evidence for this view consists mainly of the correlations between the anaesthetic potency of simple compounds and their partition coefficients for the transfer between water and amphiphilic solvents (*e.g.* olive oil (Meyer, 1901; Overton, 1901) and *n*−octanol (Franks and Lieb, 1978)). Additionally, recent evidence shows that the affinity of a single protein pocket, on firefly luciferase, for a relatively large group of agents correlates well with anaesthetic potency (Franks and Lieb, 1984). However, the notion of a single site of action, even for relatively simple molecules is by no means proven and is not

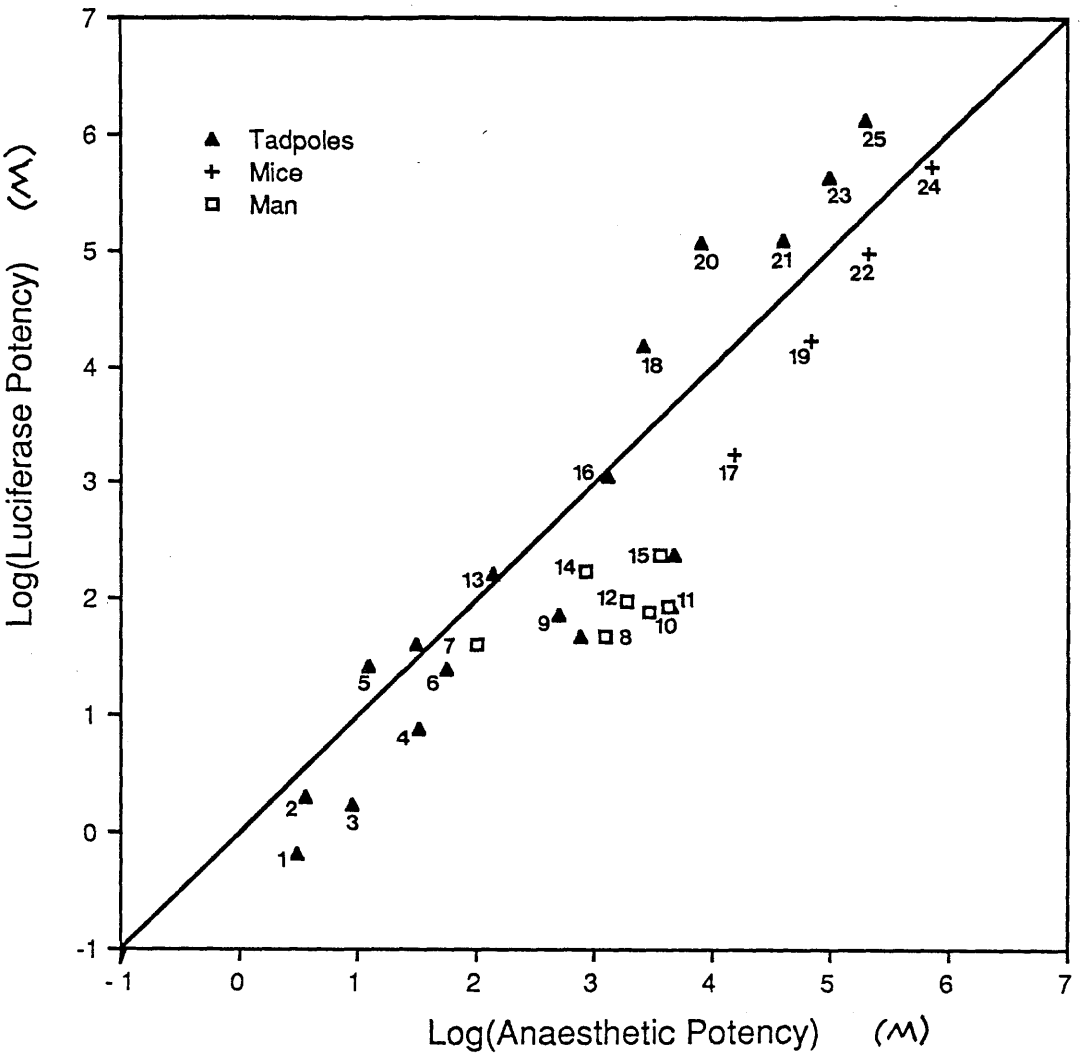


Figure 5.1: Comparison of the potencies of a diverse range of agents as inhibitors of bacterial luciferase from Vibrio harveyi and as general anaesthetics. The line is a line of identity. Potency is defined as the reciprocal of the aqueous ED_{50} *concentration (taken from Table 4.2). Anaesthetics are referred to as follows: 1, ethanol; 2, acetone; 3, propanol; 4, butanol; 5, butanone; 6, paraldehyde; 7, diethyl ether; 8, chloroform; 9, benzyl alcohol; 10, isoflurane; 11, halothane; 12, enflurane; 13, pentanol; 14, fluroxene; 15, methoxyflurane; 16, hexanol; 17, pentane; 18, heptanol; 19, hexane; 20, octanol; 21, nonanol; 22, heptane; 23, decanol; 24, octane; 25, undecanol. Sources of whole animal data: [Man] Steward et al. (1973) – compounds 7, 8, 10, 11, 12, 14, 15. [Mice] Führer, 1921 – compounds 17, 19, 22, 24. [Tadpoles] Vernon (1913) – compounds 2, 5, 6, 7, 8. Meyer and Hemmi (1935) – compounds 1, 3, 4, 13, 16, 18, 20, 21, 23, 25. Kita et al. (1981) – compounds 9, 11, 15. Doses quoted as anaesthetising partial pressures,* P_{50} *(atms), were converted to aqueous* ED_{50} *concentrations using the method described in the legend to Figure 1.3. Anaesthetising gaseous concentrations of alkanes were converted to aqueous concentrations using the method cited in Franks and Lieb (1984).*

universally accepted (Halsey *et. al.*, 1978; Richards *et. al*, 1978). In this chapter, although I will, for convenience, usually refer to *the* site of anaesthetic action in animals, that should not be taken to preclude the possibility that there are several sites.]

It is clear from Figure 5.1 that, although there is a broad correlation between luciferase and anaesthetic potencies over about six orders of magnitude, certain groups of agents deviate very significantly from this correlation. Thus the more potent anaesthetics, which are generally the most apolar, tend, on the whole, to be the more potent luciferase inhibitors, suggesting that the hydrophobic natures of the two binding sites are roughly similar. However, long-chain alcohols are more potent and alkanes and halogenated agents are less potent as luciferase inhibitors than as general anaesthetics. These groups lie respectively above and below the line of identity drawn as a guide on Figure 5.1. Therefore in detail, bacterial luciferase does not appear to be a particularly good model of this target. This result is unexpected since the ability of anaesthetics to depress the light output from whole luminous bacteria (albeit of species other than *Vibrio harveyi*) correlates much better with anaesthetic potencies (Figure 1.3). In the next chapter, evidence will be presented to suggest that species differences and the presence of anaesthetic targets other than luciferase are responsible for the dissimilar anaesthetic sensitivities of purified *Vibrio harveyi* luciferase and luminous bacteria. For the time being I want to concentrate on the details of the inhibition data obtained in this study for bacterial luciferase. I proceed now to a discussion of the groups of anaesthetics which are distinguishable by their location relative to the line of identity in Figure 5.1.

5.2 Small Anaesthetics

Most of the relatively small anaesthetics tested in this study were observed to inhibit bacterial luciferase at concentrations close to those which induce general anaesthesia in animals. They are acetone, butanone, diethyl ether, paraldehyde, pentanol and hexanol. Ethanol, propanol and butanol are all substantially less effective on luciferase than on animals; to a large extent, this is due to the fact that they either excite (ethanol and propanol) or have no effect on (butanol) luciferase activity at low concentrations, which partially masks their affinity for the inhibitory binding site on luciferase. If the ED_{50} concentrations of these alcohols are "corrected" for this masking effect (so that $1/ED_{50}$ is a truer measure of the

affinity for the aldehyde pocket), their inhibitory potencies correlate much better with their anaesthetic potencies. Thus, to small and relatively polar anaesthetics the binding environment of bacterial luciferase appears similar to that found at the anaesthetic target. These agents probably fit quite easily into the substrate binding site on luciferase, having cross–sectional dimensions which are comparable to those of the endogenous substrate, a long–chain aliphatic aldehyde. Paraldehyde is somewhat exceptional in that it is a cyclic molecule. However, despite its misleading name, paraldehyde is in fact an ether compound and, as Figure 5.2 shows, has structural features which are closely homologous to diethyl ether. Given this similarity, it does not seem surprising that a site which can accomodate diethyl ether would also bind paraldehyde.

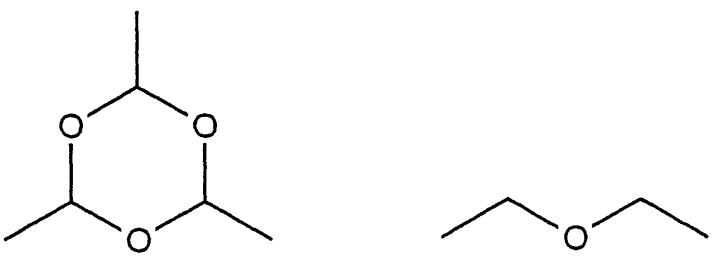


Figure 5.2: The structures of paraldehyde and diethyl ether.

This result gives an indication that those proteins in the body which normally bind ligands containing sizeable portions of methylene chain (*e.g.* fatty acid processing enzymes) may be affected by clinical levels of small anaesthetics. Other proteins of this kind, such as the receptors sites of the secondary messengers diacylglycerol (Berridge, 1985) and (metabolites of) arachidonic acid (Piomelli *et al.*, 1987), are to be found in the central nervous system. Perhaps small anaesthetics produce unconsciousness by blocking the binding sites of these messenger compounds.

5.3 *N*—alcohols

The aqueous concentrations of *n*—alcohols longer than hexanol which half inhibit luciferase are considerably less than the concentrations which anaesthetise half of a population of tadpoles. Octanol provides perhaps the most striking example of this difference: its ED_{50} concentration on luciferase is only 8.55 μM; fifteen times as much (130 μM) is required to induce anaesthesia!

Why do long—chain alcohols appear to bind tighter to luciferase than to the site of anaesthetic action in animals? *N*—alcohols are very simple molecules, consisting of an apolar methylene chain with a polar hydroxyl group at one end. Since they are almost identical in structure to the endogenous long—chain aliphatic aldehyde substrate of the enzyme, alcohols seem particularly well suited to take advantage of the binding pocket of luciferase. The greater affinity of bacterial luciferase for such molecules may reflect the tighter binding of either the apolar chain or the polar head group, or both, to the enzyme than to the physiological anaesthetic target. Meighen and Mackenzie (1973) found that the secondary hydroxyl groups on the ribityl side—chain of the flavin substrate appear to play a role in aldehyde binding. They showed that deletion of these hydroxyl groups caused a seven—fold increase in the Michaelis constant for the interaction of *n*—octanal with the luciferase—peroxyflavin intermediate. This increase reflects the reduced affinity of the intermediate for octanal, although is also seems to be partly due to faster catalytic rate constants in the absence of these hydroxyl groups. Since the flavin substrate is known to bind very close to the aldehyde binding pocket (see section 2.4), it seems likely that there is a direct interaction, possibly a hydrogen bond, between the flavin hydroxyl groups and the polar head group (CHO) on the aldehyde. If that is so, a similar interaction with the polar head group on alcohols may well contribute to the observed high affinity of the enzyme for alcohols. In order to probe further the role of the methylene chain, the hydrophobic portion of alcohol molecules, in the interactions between alcohols and the luciferase pocket, it is useful to consider the plot of the logarithms of the alcohol ED_{50} concentrations against chain length (Figure 5.3). For comparison the ED_{50} concentrations for general anaesthesia are also included on this graph.

The ED_{50} curves for general anaesthesia and luciferase inhibition are qualitatively similar. As one ascends the homologous series of *n*—alcohols, there is a general decline in the ED_{50}, a consequence of the hydrophobic natures of the sites of action. This decline in the ED_{50} stops at around C_{11} for anaesthesia and C_{13} for luciferase inhibition. Thereafter the ED_{50} concentrations are more or less

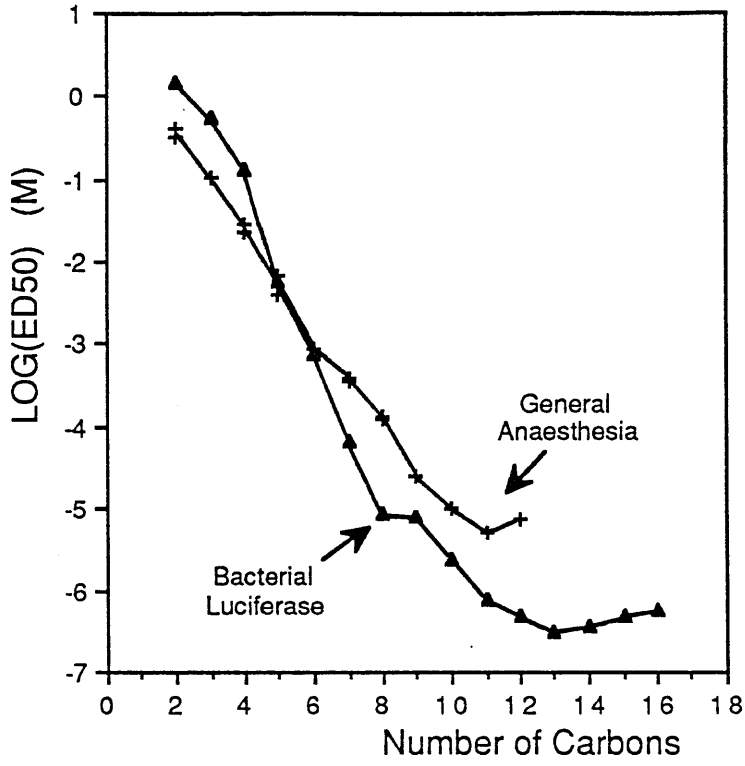


Figure 5.3: The ED_{50} concentrations of n–alcohols for inhibition of bacterial luciferase and for general anaesthesia as functions of chain length. Luciferase ED_{50} concentrations are taken from Table 4.2(a). Anaesthesia data for tadpoles are from Vernon (1913) and Meyer and Hemmi (1935).

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constant but then, quite abruptly, all anaesthetic or inhibitory potency disappears. Thus undecanol and dodecanol, which are approximately equipotent, are the most potent anaesthetics but tetradecanol has no anaesthetic activity at all (Meyer and Hemmi, 1935; Pringle *et al.*, 1981). Similarly, tridecanol and tetradecanol are the most potent luciferase inhibitors but hexadecanol is almost ineffective. (The ED_{50} values of pentadecanol and hexadecanol, shown on Figure 5.3, were determined by extrapolation from the inhibition observed at concentrations close to saturation for these agents). The abrupt loss of anaesthetic or inhibitory potency of the longest members of the series in known as the cut–off effect (section 1.1).

It is also evident from Figure 5.3 that, despite an overall, qualitative similarity, there are distinct quantitative differences in the ED_{50} curves for anaesthesia and luciferase inhibition. The drop in the ED_{50} as one ascends the series for luciferase inhibition is substantially greater than for anaesthesia. In addition, octanol and nonanol are equipotent as luciferase inhibitors, giving rise to a "kink" in the ED_{50}

curve – a feature which is much more pronounced than the small fluctuations in the differences between the anaesthetic ED_{50}'s of adjacent alcohols. The implications of these findings will be considered later in this section. Firstly, however, I want to discuss the molecular interpretation of the cut–off effect.

5.3(a) The Cut–Off

The cut–off may be understood if the thermodynamic activities for inhibition and anaesthesia are plotted against chain length (Figure 5.4). The thermodynamic activity may be calculated as the ratio of the ED_{50} to the saturated concentration for compounds which are not very soluble in water (Brink & Posternak, 1948). Figure 5.4 shows that the activities for anaesthesia and inhibition are very low for medium–sized alcohols; but for longer alcohols they suddenly shoot up and the point is soon reached beyond which the concentration required to produce a 50% effect is greater than the maximum achievable aqueous concentration (*i.e.* thermodynamic activity > 1). Thus, in the case of bacterial luciferase, although the concentrations for C_{13} to C_{16} required to cause 50% inhibition are more or less equal (at around 0.4 μM), the continuous fall in solubility with increasing chain length renders alcohols longer than pentadecanol impotent. Even at saturation (0.1

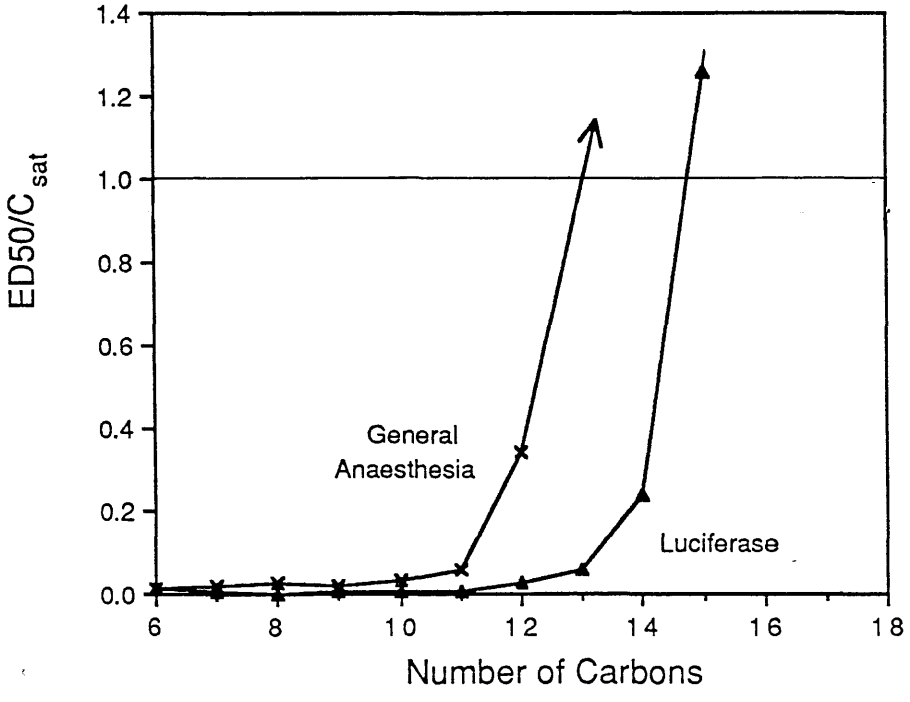

Figure 5.4: Thermodynamic activities (ED_{50}/C_{sat}) of n–alcohols for luciferase inhibition and general anaesthesia. The ED_{50} concentrations used are the same as in Figure 5.3. Alcohol solubility data (at 25°C) were taken from Bell (1973).

μM), hexadecanol causes only 16% inhibition. The highest measureable thermodynamic activity for tadpole anaesthesia is 0.34, for dodecanol. However the thermodynamic activity for tridecanol must exceed 1 since a saturated solution of this alcohol is not sufficient to causes anaesthesia in 50% of a population of tadpoles (Pringle *et al.*, 1981)

The cut-off occurs because the ED_{50} reaches a limiting minimum value. Why does the ED_{50} level off in this way? For the inhibition of luciferase by alcohols longer than butanol, the ED_{50} concentration is simply related to the dissociation constant, K_i: $ED_{50} = 2K_i$. Thus, the luciferase cut-off results from the fact that the increase in binding affinity with increasing chain length begins to tail off beyond C_{11} so that, eventually, alcohol moleclues longer than C_{13} do not bind any tighter to the pocket on the enzyme. It is not difficult to imagine how this might occur for alcohols binding to a protein pocket of finite volume. The site on luciferase appears to have room only for a limited number of methylene groups (somewhere between eleven and thirteen); when longer molecules bind, their extra methylene groups, not being accomodated in the pocket, protrude into water and make little or no contribution to the strength of binding.

A protein binding site thus provides a simple and plausible explanation for the cut-off effect. Bacterial luciferase is in fact the second protein for which the inhibitory potencies of the *n*-alcohols cut-off beyond a certain chain length. This has also been observed, apparently for the same reasons, with firefly luciferase (Franks and Lieb, 1984). The result reported here therefore strengthens the hypothesis that the disappearance of general anaesthesic potency for the longest members of the *n*-alcohol homologous series is a consequence of the fact that anaesthetics act by binding to protein sites in the central nervous system. The observation that the alcohol cut-off for anaesthesia occurs at a shorter chain length than for luciferase inhibition may simply reflect the smaller volume of the anaesthetic target, which appears to accomodate, at most, eleven methylene groups. Lipid theories of the mechanism of general anaesthesia have yet to credibly account for the cut-off effect. Experimental evidence suggests that there is no cut-off in the solubility of long-chain aliphatic compounds in artificial lipid bilayers. The partition coefficients for the transfer of *n*-alcohols (Franks and Lieb, 1986) and *n*-alkyltrimethylammoniun ions (Requena and Haydon, 1985) from water into cholesterol-containing bilayers increase steadily for as far as data is available (up to C_{15} and C_{18} respectively). The more complex lipid environment of real biological membranes may produce an alcohol solubility cut-off – although this seems unlikely. Alternatively, it may be that the critical perturbation of the bilayer caused

by alcohols may itself disappear as the chain length increases. It has in fact been shown that the ability of alcohols to disorder a lipid bilayer declines to zero for C_{20}; but this decline is too slow to account for the abrupt cut−off observed in anaesthesia (Pringle *et al.*, 1981). Thus, currently, the protein model of the anaesthetic target provides the simplest explanation of the cut−off.

It should be noted that the ED_{50} curve for luciferase inhibition begins to level off at the alcohol chain length at which biphasic luminescence decay is first observed. The change from monophasic to biphasic decay of the light output from alcohol−inhibited luciferase reactions occurs at C_{11}. As a result, the meaning of the difference between the ED_{50} values of these two subgroups of alcohols is not totally clear. In addition, the advent of biphasic decay suggests that the levelling off of the ED_{50} curve, which leads to the cut−off effect, may possibly be an artefact associated with the single−shot assay method used to measure inhibition. At first sight, it is conceivable that a cut−off might not have been observed if a method which allowed continuous turnover of luciferase had been employed to determine the inhibitory effect of alcohols. However, since the alcohols from C_{12} to C_{16} appear to act in a very similar manner (all inducing biphasic luminescence decay) it seems very likely that *their* ED_{50} concentrations may be compared meaningfully with one another. Thus in all probability, the levelling off of the alcohol ED_{50} curve and the subsequent cut−off in potency are genuine effects. This conclusion may be supported using a completely different type of experiment to measure the dissociations constants for alcohols binding to the luciferase−peroxyflavin intermediate. These experiments, which rely on the stabilising rather than the inhibitory effects of *n*−alcohols, and the light that they shed on both the cut−off and the reasons why long−chain alcohols produce biphasic luminescence decay kinetics will be discussed fully in the next chapter.

5.3(b) Apparent Methylene Group Binding Energies

The general pattern of increasing potency terminated by an abrupt cut−off as one ascends the homologous series of *n*−alcohols, which is observed for both general anaesthesia and luciferase inhibition, suggests a promising degree of similarily between the sites of action of these two processes. Nonetheless, closer inspection of the data reveals distinct differences between luciferase and the physiological anaesthetic target. In particular, the slope of the ED_{50} curve between C_4 and C_8 is steeper for luciferase inhibition than for anaesthesia. It also contains a prominent horizontal "kink" between C_8 and C_9 which is much greater than any of the fluctuations in

the anaesthetic ED_{50} curve. The slopes of these curves are a measure of the increase in the free energy of binding as one ascends the alcohol series. In the case of luciferase inhibition, since the dissociation constant of the inhibitor is known, it is possible to calculate the standard free energy of binding per mole of alcohol. This is given by the equation:

$$\Delta G^{\circ} = -RT\ln(1/K_i) \qquad (5.1)$$

where $1/K_i$ (= [E'A]/([E'][A]) – equation 4.15) is the association constant. R is the universal gas constant (= 8.314 J K^{-1} mol^{-1}) and T the temperature in degrees Kelvin. The difference in binding energy between adjacent members of the series is:

$$\Delta\Delta G^{\circ} = \Delta G^{\circ}_{n+1} - \Delta G^{\circ}_{n} = -RT\ln[K_{i(n)}/K_{i(n+1)}] \qquad (5.2)$$

$\Delta\Delta G^{\circ}$ is therefore the binding energy gained by the addition of a single methylene (CH_2) group. Since $ED_{50} = 2K_i$ for alcohols longer than butanol, it would equally have been possible to calculate $\Delta\Delta G^{\circ}$ values for these agents using ED_{50} data. Dissociation constants are, of course, not available for the interaction of alcohols with the physiological anaesthetic target, but if the apparent methylene group binding energies are calculated using ED_{50} values instead in equation 5.2, it is possible, at least, to make a fair comparison with bacterial luciferase. These data are summarised for C_5 to C_9 in Table 5.1. Methylene binding energies were not calculated for the addition of CH_2 groups to decanol and undecanol since these additions brought about a change in the inhibited reaction kinetics. The relative magnitudes of the K_i values for decanol, undecanol and dodecanol may not therefore be attributed solely to changes in binding energy. Beyond dodecanol the levelling off of K_i values begins – a feature discussed in the previous section.

Except for $C_8 \rightarrow C_9$, the apparent binding energy of each additional methylene group is larger (that is, more negative) for luciferase. Thus these groups bind more strongly to the luciferase enzyme than to the anaesthetic target; this tighter binding obviously contributes to the greater overall affinity of luciferase for long–chain alcohols (although the interactions of the polar hydroxyl group may also be a factor in this high affinity).

If the anaesthetic target is the lipid portion of neuronal membranes, the

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Table 5.1: Apparent methylene group binding energies for n-alcohols. $\Delta\Delta G^\circ$ *was calculated using equation 5.2, as explained in the text.* K_i *values for alcohols binding to luciferase were taken from Table 4.2(a). For general anaesthesia,* $\Delta\Delta G^\circ$ *was determined using alcohol* ED_{50} *concentrations for tadpoles: (*) Vernon (1913); (†) Meyer and Hemmi (1935).*

n	Bacterial Luciferase $\Delta\Delta G^\circ$ (kJ mol^{-1})	General Anaesthesia $\Delta\Delta G^\circ$ (kJ mol^{-1})
5	-5.12	-3.73*
6	-5.99	-2.29*
7	-5.08	-2.45*, -2.61†
8	-0.14	-4.02†
9	-3.06	-2.23†

comparative methylene binding energies of luciferase and the target contain no new information about it. Nonetheless, it is worth noting that the free energy per methylene group for the transfer of alcohols between buffer (25 mM glycylglycine, pH 7.8) and cholesterol-containing lipid bilayers is -3.63 kJ mol^{-1} at 25°C (Franks and Lieb, 1986). This is very similar to the value of $\Delta\Delta G^\circ$ calculated for the partitioning of alcohols between water and hexadecane, which is -3.51 kJ mol^{-1} at 20°C (Aveyard and Mitchell, 1969). The thermodynamics of the partitioning of methylene groups between water and hydrocarbon solvents have been studied in detail for alkane solutes (Davis *et. al*, 1974; Tanford, 1980; Abraham, 1982). The conclusions drawn from these studies may be extended to account for alcohol partitioning data since the free energy per CH_2 group calculated for the transfer of alkanes between water and hexane is -3.74 kJ mol^{-1} at 25°C (using data from Abraham (1982)) – very similar to the values quoted above for alcohol methylene groups. Tanford (1980) has argued that the free energy of transfer of alkanes from water to hydrocarbon solvents is derived entirely from the changes that occur in the water structure during this transfer. The introduction of an alkane solute into water involves a reorganisation of the water structure in the vicinity of the solute molecule. This reorganisation results in a large drop in entropy (Tanford 1980; Abraham, 1982), the magnitude of which increases slightly with chain length. It also entails an enthalpic change associatied with alterations in the hydrogen bonding network between water molecules. The sign and magnitude of this enthalpy change are strongly dependent on chain length. Alkanes shorter than hexane appear

to cause a strengthening of the hydrogen bonds within water – longer alkanes have the opposite effect (see Table IV of Abraham, 1982). The precise reasons for the changes in ΔH and ΔS for water/hydrocarbon partitioning of methylene groups are not yet fully understood. However, since these changes appear to be due to the effect of the methylene chain on water, it seems reasonable to suppose that they also contribute to the apparent methylene binding energy for the interaction of alcohols with hydrophobic protein binding sites. One might therefore expect a contribution from these processes of around -3.5 kJ mol^{-1} at 25°C to the overall apparent methylene binding energy. The observation that $\Delta\Delta G^{\circ}$ is even more negative than this for some of the methylene groups binding to luciferase indicates that additional enthalpic and/or entropic terms contribute favorably to the binding energy in this protein pocket.

There are several possible such terms to be considered. In all probability, docking of a ligand at a protein pocket displaces water molecules from the pocket into bulk water. This exchange may well contribute to a favorable increase in entropy if the entropy gained as the water molecules are released from the site exceeds that which is given up by the ligand which loses degrees of translational and rotational freedom. The enthalpic change resulting from the displacement of water will depend on the the relative strengths of the bonds that water molecules make at the pocket and in bulk water. I have noted that water molecules can arrange themselves around the apolar surface of alkanes shorter than hexane into a 'bonding network in which the hydrogen bonds are stronger than in bulk water. However, it seems unlikely that water molecules within the confined space of a protein pocket would be free to adopt a configuration around the apolar surfaces of that pocket which enabled stronger hydrogen bonds than are formed in bulk water. Consequently, the displacement of water molecules from apolar surfaces may well be enthalpically favorable. On the other hand, the displacement of water molecules from interactions with polar regions of the site is likely to be less enthalpically favorable and may even be unfavorable. Additionally, the ligand may make better Van der Waals interactions at the luciferase site than in water – contributing to a favorable drop in enthalpy. Or it may stabilise a conformation of the protein which has a lower free energy.

The relative contributions of each of these possible enthalpic and entropic terms to the methylene binding energies calculated for the interaction of alcohols with luciferase cannot be determined at present. The precise molecular details of this interaction require much further investigation. However, given that the endogenous substrate for bacterial luciferase is a long–chain aldehyde, a distinct possibility is

that the methylene binding energies in Table 5.1 are relatively large because the luciferase pocket is narrow and apolar. A saturated aliphatic alcohol would fit snugly at such a site and would therefore be very effective at clearing water molecules from it. This displacement, as was argued above, could produce favorable enthalpic and entropic changes. Thus far, the evidence for this view of the binding pocket on luciferase is hardly conclusive and, indeed, it is not difficult to think of alternative explanations for the data. However, further evidence, particularly from studies of the binding of haolgenated anaesthetics and cycloalcohols (see sections 5.5 and 6.1), will be presented in support of the notion of a narrow, apolar binding site on bacterial luciferase.

If the site of action of general anaesthesia, as seems possible, is a protein pocket of some kind, there are several feasible interpretations of the observation that the apparent methylene binding energies are lower for the anaesthetic target than for the luciferase enzyme. It may be that the target is simply wider than the luciferase pocket so that alcohols binding there do a poor job of displacing water molecules, thus yielding a lower methylene binding energy. Alternatively, the general anaesthetic target may simply be less polar (in the regions where CH_2 groups bind) than the luciferase pocket. The difference in apparent methylene binding energies may also be explained if one assumes that luciferase contains a pre-formed binding but that the anaesthetic target is "induced" by apolar ligands which can stabilise a conformation of the protein containing an apolar cavity, to which they bind. When they dissociate the cavity collapses. In such a case there would be no free energy contribution to ligand binding from the displacement of water and such a site might therefore appear to bind methylene groups less well than luciferase.

The addition of a methylene group to octanol does not make any net contribution to binding strength ($\Delta\Delta G^\circ \simeq 0$ for C8 $\rightarrow$ C9). Apparently the standard free energy gained by removing this additional methylene group from water is offset by the energy cost of introducing the extra length of methylene chain into the pocket. This might result from the presence of a local polar region, or patch, within the luciferase pocket. It may be that alcohols up to octanol make little or no contact with this patch but that the extra length of nonanol makes contact inevitable – an interaction which costs energy (possibly by disturbing hydrogen bonds between water molecules and the patch). In comparison, the region beyond the polar patch appears to be relatively apolar since the addition of a methylene group to nonanol makes a contribution of -3.06 kJ mol^{-1} to the binding energy. Alternatively, nonanol and longer molecules may induce an energetically costly conformational shift in the protein as they bind. Further comment on this feature

will be given immediately below.

5.3(c) $\alpha,\omega-N-$alkyldiols

The binding site on luciferase was probed further using the homologous series of $\alpha,\omega-n-$alkyldiols. These compounds consist of an aliphatic methylene chain with a polar hydroxyl group at either end. They are competitive inhibitors of bacterial luciferase (*e.g.* see Figure 5.5 for 1,7−heptanediol). As was observed with

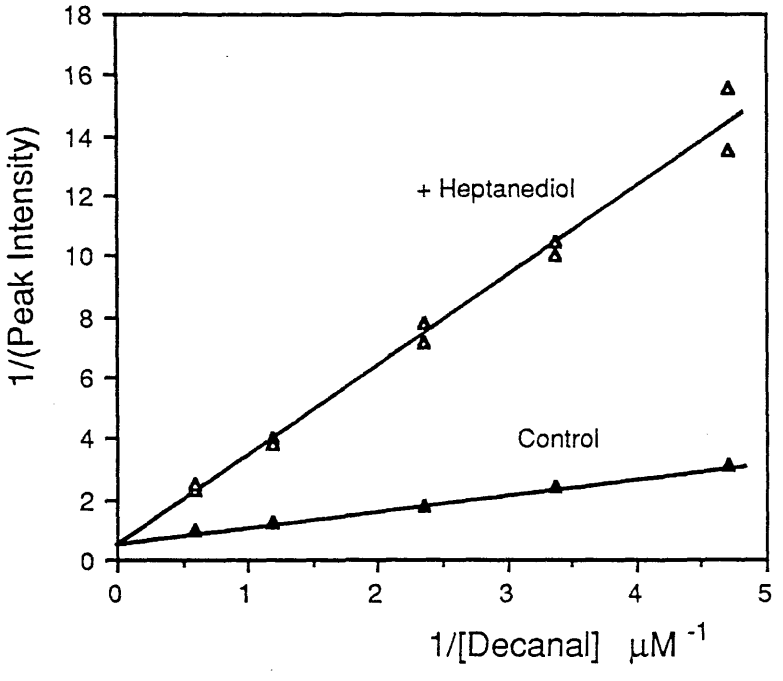

Figure 5.5: Lineweaver−Burk plot showing purely competitive inhibition of the $FMNH_2$−initiated luciferase reaction by 1,7−heptanediol. Final concentrations: decanal, 0.21−1.7 μM; $FMNH_2$, 102 μM; luciferase, 0.4 nM. $T = 24.9^{\circ}C$.

$n-$alcohols, medium length molecules in this series retard the decay of luminescence apparently in proportion to the reduction in peak intensity. This relationship is shown in Figure 5.6 for 1,7−heptanediol and 1,10−decanediol. Long−chain diols, however, behave slightly differently from the corresponding alcohols. 1,12−dodecanediol, 1,14−tetradecanediol and 1,16−hexadecanediol all induce biphasic luminescence decay − like long−chain $n-$alcohols − but the decay constant of the initial phase of this decay, instead of increasing with dose, is approximately constant (see Figure 5.7 for C_{12} and C_{14} diols). Thus these diols appear to act like

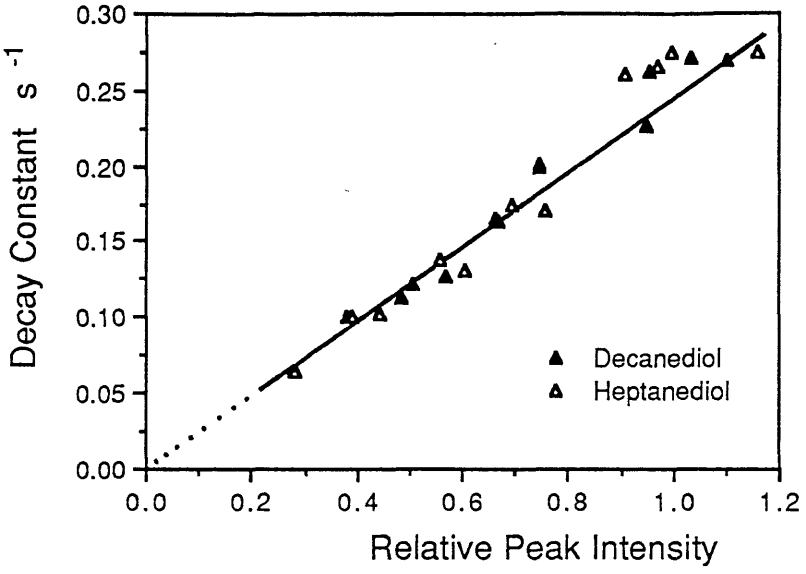

Figure 5.6: The depression of the maximum rate (peak intensity) of the $FMNH_2$–initiated luciferase reaction by α,ω–n–alkyldiols shorter than C_{11} is apparently proportional to the concomitant reduction in the luminescence decay constant. In this regard, these agents act like most other anaesthetics – see Figure 4.17. Data are for 1,7–heptanediol and 1,10–decanediol. Final concentrations: decanal, 0.85 μM; $FMNH_2$, 97–103 μM; luciferase, 0.4 nM. T = 24.8°C.

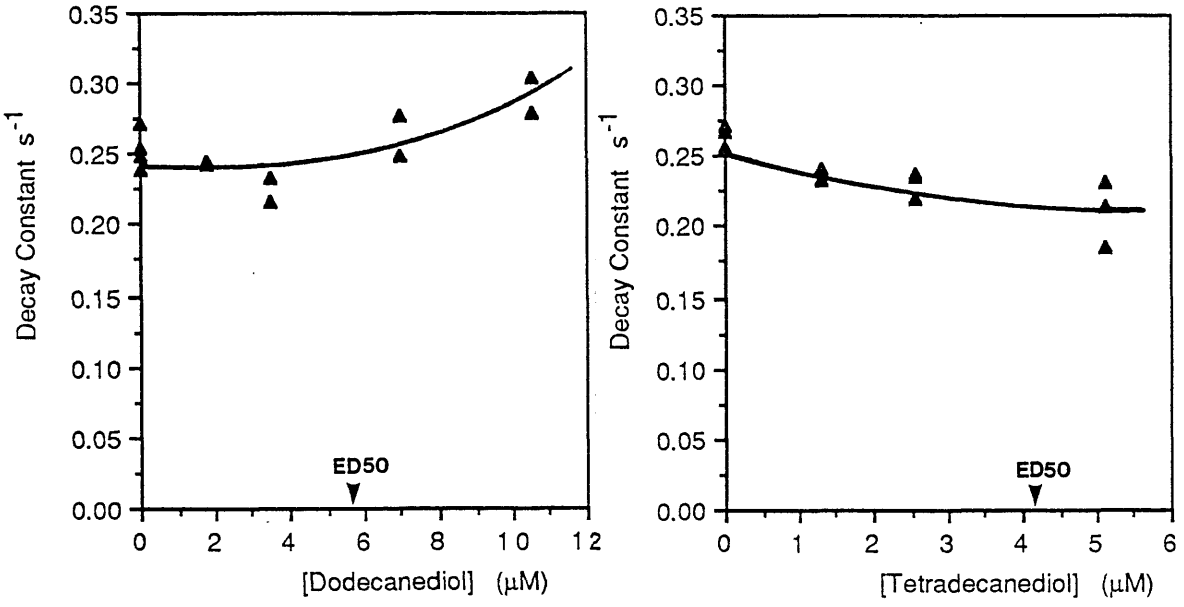

Figure 5.7: The decay constant of the initial phase of the biphasic luminescence decay observed with (a) 1,12–dodecanediol and (b) 1,14–tetradecanediol is not greatly affected by the diol concentration. The ED_{50} concentrations for depression of the peak intensity are indicated by arrowheads. Final concentrations: decanal, 0.85 μM; $FMNH_2$, 97–101 μM; luciferase, 0.4 nM. T = 25.0°C.

undecanol. No explanation for this difference between the effects of long–chain alcohols and diols on luminescence decay has been elucidated.

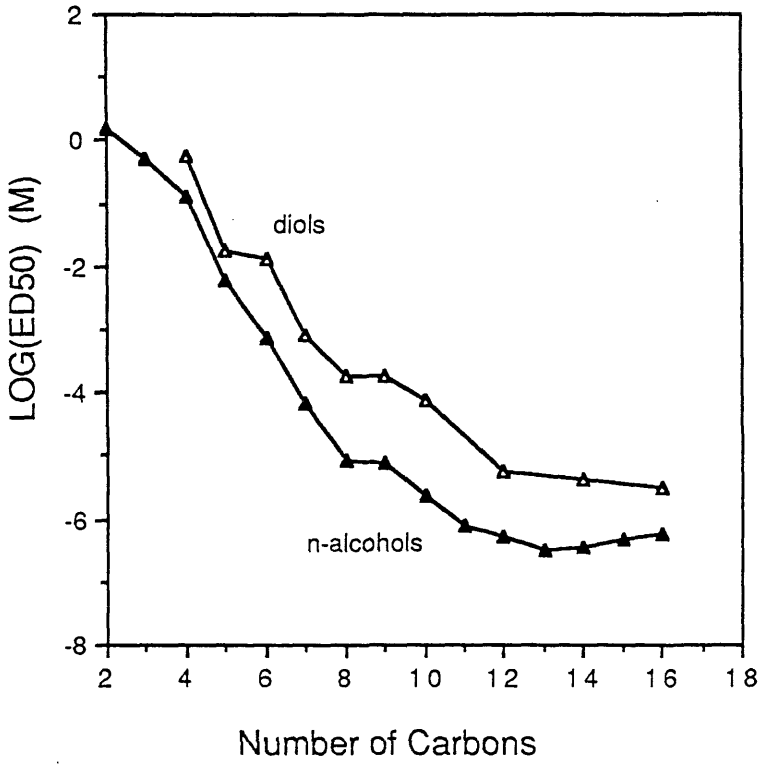

Figure 5.8: ED_{50} concentrations for inhibition of the $FMNH_2$–initiated bacterial luciferase reaction by α,ω–n–alkyldiols and n–alcohols as functions of chain length. The data are taken from Tables 5.2 and 4.2(a).

The ED_{50} concentrations of the diols are summarised in Table 5.2 and their logarithms are plotted against chain length in Figure 5.8. It is interesting to note that the ED_{50} curve follows a very similar trend to that for *n*–alcohols, which is also shown in the figure, suggesting that the members of the two series are binding in the same way at the same site on luciferase. The displacement of the diol curve above the alcohol curve reflects the cost of binding an additional hydroxyl group in the relatively apolar luciferase pocket. It is notable that there is a kink in the diol ED_{50} curve between C_8 and C_9. In section 5.3(b) it was suggested that the kink in the alcohol ED_{50} curve, which is also observed between C_8 and C_9, might be due to the presence of a polar patch in the pocket or to conformational strains induced by the binding of ligands longer than C_8. It is interesting to note that the kink in alcohol and diol ED_{50} curves appears at the same methylene chain length

rather than for molecules with the same overall length. This observation may be used to distinguish between the two models which have been suggested to account for the kink. It might be expected that, if a conformational strain is responsible for the reduction of methylene binding energy, the kink would appear for molecules of the same length. The fact that it appears for molecules of the *same methylene chain length* suggests rather that a polar patch is responsible for the relatively small apparent methylene binding energies observed for $C_8 \rightarrow C_9$. This reasoning needs to be tested by further experimental work – see p146, section 5.4(c) for additional comments.

Table 5.2: Dissociation constants (K_i) and ED_{50} concentrations determined for the inhibition of bacterial luciferase by α,ω–n–alkyldiols. All procedures and conditions were as described in the legend to Table 4.2.

Agent	K_i (± s.e.)	ED_{50} (± s.e.)	n
1,4-butanediol	782 ± 37 mM	572 ± 27 mM	2
1,5-pentanediol	88.2 ± 0.5 mM	176 ± 1.0 mM	1
1,6-hexanediol	68.0 ± 0.3 mM	136 ± 0.5 mM	1
1,7-heptanediol	403 ± 18 μM	806 ± 9 μM	1
1,8-octanediol	93.5 ± 0.5 μM	187 ± 9 μM	1
1,9-nonanediol	94.9 ± 0.4 μM	190 ± 7 μM	1
1,10-decanediol	39.3 ± 0.17 μM	78.6 ± 3.3 μM	1
1,12-dodecanediol*	2.81 ± 0.10 μM	5.62 ± 0.20 μM	1
1,14-tetradecanediol*	2.02 ± 0.18 μM	4.10 ± 0.35 μM	1
1,16-hexadecanediol*	1.55 ± 0.17 μM	3.09 ± 0.34 μM	1

(*) indicates that biphasic luminescence decay kinetics were observed.

The ED_{50} concentrations of 1,5–pentanediol and 1,6–hexanediol are also almost equal, producing a second kink between C_5 and C_6. However this does not seem to represent a second possible polar patch in the luciferase pocket. Collander (1954) has shown that there is only a small difference between the oil/water partition coefficients for these two agents. It therefore seems possible that the kink in the luciferase ED_{50} curve caused by these compounds may reflect their solubility behaviour rather than some structural feature of the luciferase binding pocket. Finally, the levelling off of the ED_{50} curve which was observed for alcohols is repeated with the diols – leading also to a cut–off effect for this homologous series.

5.4 *N*−alkanes

Like the *n*−alcohols, the homologous series of *n*−alkanes are competitive inhibitors of bacterial luciferase and, in all probability, also associate with the enzyme at the aldehyde binding pocket. They inhibit the enzyme at concentrations which are about an order of magnitude lower than required for inhibition by the corresponding alcohols. However, in contrast to alcohols, alkanes are actually less potent as inhibitors of bacterial luciferase than as general anaesthetics. The data points for pentane to octane on Figure 5.1 all lie below the line of identity.

5.4(a) The Amphiphilic Nature of the Luciferase Binding Pocket

Why does bacterial luciferase have a lower affinity for alkanes than the physiological site of anaesthetic action? In Figure 5.9 the logarithms of the alkane and alcohol ED_{50} concentrations [taken from Tables 4.2(a) and (b)] for bacterial luciferase inhibition have been plotted against the number of carbon atoms in each molecule. On average, alkanes bind about 10 times tighter than the corresponding alcohols. In contrast, the partition coefficients for the transfer of alkanes from water into hexadecane, a pure hydrocarbon, are 10,000 times greater than for alcohols (Stein, 1985). The luciferase pocket is evidently much less hydrophobic than hexadecane. It is not possible to make a simple comparison of the affinities of the physiological anaesthetic target for alcohols and alkanes since anaesthesia data for these series of compounds have not been determined on the same species of animal. However, as a guide, it may be noted that the alkane ED_{50} concentrations determined on mice are 60−90 times lower than the ED_{50} concentrations of the corresponding alcohols acting on tadpoles. On the basis of this evidence, the luciferase binding pocket appears somewhat less hydrophobic than the physiological anaesthetic target.

The observation that alkanes appear to bind less well to luciferase than to the general anaesthetic target seems therefore to result from the greater level of contact between them and polar features in the enzyme pocket. Evidence for such polar features on luciferase was given in section 5.3. It was suggested that secondary hydroxyl groups on the ribityl side−chain of $FMNH_2$ might hydrogen−bond to the polar head group of *n*−alcohols binding to luciferase. In addition, the equipotency

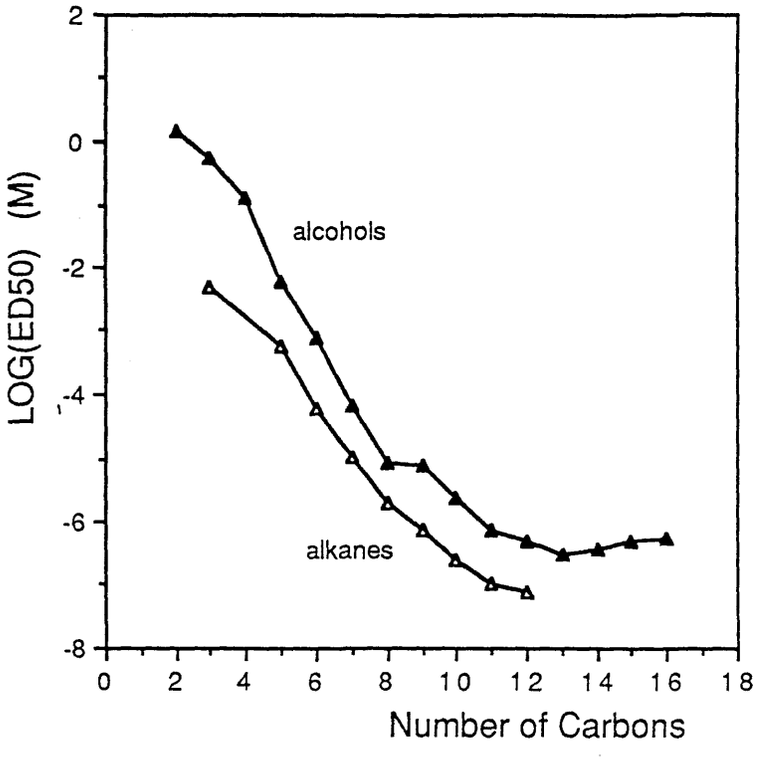

Figure 5.9: ED_{50} concentrations for inhibition of the $FMNH_2$-initiated luciferase reaction by n-alkanes and n-alcohols as functions of chain-length. The data are taken from Tables 4.2(a) and (b).

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of octanol and nonanol (and 1,8-octanediol and 1,9-nonanediol) pointed to the presence of a second possible polar region within the luciferase pocket.

Further information on the amphiphilic natures of the anaesthetic target and the luciferase pocket may be deduced from a slightly altered thermodynamic perspective. It is instructive to calculate the partial pressures, P_{50}, of these agents which produce a 50% effect. This may be done using the equation derived in Appendix 1:

$$P_{50} = \frac{RT.ED_{50}}{\lambda} \qquad (5.3)$$

P_{50} is the partial pressure (in atmospheres) which is in equilibrium with the aqueous ED_{50} concentration (moles per litre). R is the univeral gas constant (0.08206 lit atm deg^{-1} mol^{-1}), T the temperature (degrees Kelvin) and λ the water/gas partition coefficient expressed as a ratio of molar concentrations. The derivation of this equation assumes that the alcohol and alkane vapours behave ideally and that a thermodynamic equilibrium has been established. P_{50} values were calculated for alcohol anaesthesia of tadpoles and for the inhibition of luciferase by both alkanes

and alcohols. The anaesthesia of mice by alkanes was originally determined using alkane vapours (Führer, 1921). This information is summarised in Tables 5.3(a) and (b).

Table 5.3(a): Partial pressures (P_{50}) of n–alcohols and n–alkanes required to inhibit bacterial luciferase by 50%. The data were calculated from aqueous ED_{50} concentrations and water/gas partition coefficients using equation 5.3. Luciferase data were taken from Table 4.2. The water/gas partition coefficients used were for dilute solutions at 25°C and were calculated from the tabulated data in the following sources: [alcohols] Butler et al. (1935); [alkanes] Hine and Mookerjee (1975).

	Alcohol	Alkane	Alkane P_{50}
n	P_{50}(atm)	P_{50}(atm)	Alcohol P_{50}
5	7.77×10^{-5}	7.33×10^{-1}	9,434
6	1.16×10^{-5}	1.07×10^{-1}	9,224
7	1.27×10^{-6}	2.20×10^{-2}	17,323
8	2.07×10^{-7}	6.35×10^{-3}	30,676

The striking result from this calculation is that, from the gas phase, alkanes bind much less well to luciferase and the anaesthetic target than alcohols. This is a reversal of the result obtained in the aqueous phase, so to speak. Thus alcohols inhibit luciferase at partial pressures which are nearly 10,000 times lower than the inhibiting partial pressures of the corresponding alkanes. How may this result be interpreted? In the gas phase it is unlikely that there are any interactions between either alcohol or alkane molecules; although hydrogen bonds can form between the hydroxyl groups of alcohol molecules, such interactions will be extremely rare at the very low partial pressures considered here. Binding of alkanes and alcohols from the gas phase to the luciferase site is associated with a number of enthalpic and entropic changes. These probably include the displacement of water molecules from the site into bulk water, the immobilisation of the ligand and the formation of Van der Waals contacts. However, the magnitude of the enthalpic and entropic contributions of these processes to the free energy of binding are likely to be similar for alkanes and alcohols of the same chain length. Therefore it appears that the vastly superior affinity of the luciferase pocket for alcohols is a consequence of the

ability of their hydroxyl groups to make strong bonds there. This implies that the site contains polar features.

Table 5.3(b): Partial pressures (P_{50}) of alcohols and alkanes required to induce general anaesthesia in 50% of a population of animals. Alcohol P_{50} values were calculated with equation 5.3 using tadpole ED_{50}'s obtained at 18°C by Vernon (1913). These P_{50}'s are probably slightly overestimated (by 30–40%) because water/gas partition coefficients at 25°C were used in the calculation. Alkane P_{50} values were calculated from the gaseous ED_{50}'s (quoted as mol lit^{-1}) determined for mice (T = 37°C) by Führer (1921).

	Alcohol	Alkane	Alkane P_{50}
n	P_{50}(atm)	P_{50}(atm)	Alcohol P_{50}
5	5.34×10^{-5}	1.12×10^{-1}	2,090
6	1.36×10^{-5}	3.95×10^{-2}	2,904
7	6.49×10^{-6}	1.58×10^{-2}	2,427
8	3.01×10^{-6}	7.89×10^{-3}	2,613

This line of argument may be supported by considering the transfer of alkanes and alcohols from both hexadecane and *n*–octanol to the gas phase. Hexadecane/gas and *n*–octanol/gas partition coefficients were calculated as the product of solvent/water and water/gas partition coefficients and are summarised in Tables 5.4 (a) and (b).

Tables 5.4(a)–(c) (over): Water/gas ($\lambda_{w/g}$), hexadecane/gas ($K_{hd/g}$) and octanol/gas ($K_{oct/g}$) partition coefficients for (a) n–alcohols and (b) n–alkanes. Partition coefficients are expressed as ratios of molar concentrations. Values of $\lambda_{w/g}$ for n–alcohols are taken from Butler et al. (1935); those for n–alkanes are from Hine and Mookerjee (1975). $K_{hg/g}$ and $K_{oct/g}$ were calculated as the product of water/gas and solvent/water partition coefficients [taken from Stein (1985)]. (c) Ratios of $K_{hd/g}$ and $K_{oct/g}$ for corresponding alcohols and alkanes.

Table 5.4(a): Partition Coefficients - Alcohols

n	$\lambda_{w/g}$	$K_{hd/g}$	$K_{oct/g}$
2	4720	27	2,260
3	3540	117	7,740
4	2860	238	21,700
5	1880	766	47,200
6	1590	2040	170.000
7	1290	7590	---

Table 5.4(b): Partition Coefficients - Alkanes

n	$\lambda_{w/g}$	$K_{hd/g}$	$K_{oct/g}$
2	0.0490	3.31	2.89
3	0.0347	10.7	7.95
4	0.0263	37.2	----
5	0.0195	135	----
6	0.0135	490	240
7	0.0120	1550	953

Table 5.4(c): Partition Coefficient Ratios

	Alcohol $K_{hd/g}$	Alcohol $K_{oct/g}$
n	Alkane $K_{hd/g}$	Alkane $K_{oct/g}$
2	8.2	780
3	11.0	970
4	6.4	---
5	5.7	---
6	4.2	708
7	4.9	---

Alcohols partition only about six times better into hexadecane than the corresponding alkanes. The difference in the partitioning of alcohols and alkanes of a given chain length may simply be due to dipole−induced dipole interactions between the OH groups on the alcohol and hexadecane. In contrast, the alcohol hydroxyl group confers a big (about 800−fold) advantage over alkanes in the partitioning between the gas phase and the amphiphilic solvent, octanol. It is the formation of hydrogen bonds between the polar hydroxyl groups of alcohol solutes and the octanol solvent which confers this advantage. These comparisons indicate that the notion of polar features at the luciferase binding pocket provides a very plausible explanation for the differences in P_{50} values for alkanes and alcohols interacting with the enzyme.

Table 5.3(b) − on page 140 − shows that the anaesthetising partial pressures for alcohols are around three orders of magnitude less than the P_{50} for the corresponding alkanes. Whether the site of action is a lipid or a protein it seems reasonable to assume that all effects except polar interactions will be more or less equal for alcohols and alkanes. Therefore, as with luciferase, the simplest account of the differences in P_{50} values appears to be the amphiphilic nature of the anaesthetic binding site. The smaller difference between alcohol and alkane P_{50}'s for anaesthesia than for luciferase inhibition reaffirms the conclusion, drawn above, that the anaesthetic target is more apolar overall than the luciferase binding site.

It is important to realise that the descriptions of the luciferase pocket and the physiological anaesthetic binding site which are relevant to the above arguments must include any water molecules at the water/site interface with which bound ligands interact. This point may be emphasised by calculating the binding energy of the alcohol hydroxyl group at the site which can be done using the equation:

$$\Delta\Delta G^{\circ}_{OH} = -RT.\ln\left[\frac{P_{50}(\text{alkane})}{P_{50}(\text{alcohol})}\right] \qquad (5.3)$$

This equation is analagous to equation 5.2 which was applied to calculate apparent methylene binding energies. To be precise, $\Delta\Delta G^{\circ}_{OH}$ is the difference between the binding energy contributed by an alcohol hydroxyl group and the binding energy of the hydrogen on the end of the alkane of the same chain length. Using the data in Tables 5.3(a) and (b), $\Delta\Delta G^{\circ}_{OH}$ works out at 22.6−25.5 kJ mol^{-1} for luciferase but at only 19 kJ mol^{-1} for the physiological anaesthetic target (bearing in mind that alcohol and alkane anaesthesia data were not determined on the same species of animal). A likely explanation for these large binding energies is that alcohol

moleclues binding to luciferase and to the anaesthetic target in animals are oriented with their hydroxyl group at the water/site interface. The fact that $\Delta\Delta G^{\circ}_{OH}$ is greater for luciferase indicates that the hydroxyl group of alcohols bound to the enzyme pocket may be less constrained in their interactions at the water/site interface or possibly that there is a superior level of contact between this hydroxyl group and other polar features of the luciferase pocket. It may be that the polar features of the aldehyde binding site on luciferase which were suggested earlier (*i.e.* secondary hydroxyls on the flavin substrate or the polar patch indicated by the equipotency of C_8 and C_9 alcohols and diols) play a role in this regard.

5.4(b) The Cut−Off

A cut−off in anaesthetic potency is observed as one ascends the homologous series of *n*−alkanes and, as with the alcohol cut−off, this phenomenon is mimicked by bacterial luciferase. The alkane anaesthetic cut−off for mice occurs around C_{10} (Mullins, 1954), whereas the ability of alkanes to inhibit luciferase disappears rapidly beyond C_{11} − tridecane and tetradecane are totally inactive as inhibitors. This behaviour is illustrated in Figure 5.10 where the thermodynamic activities of

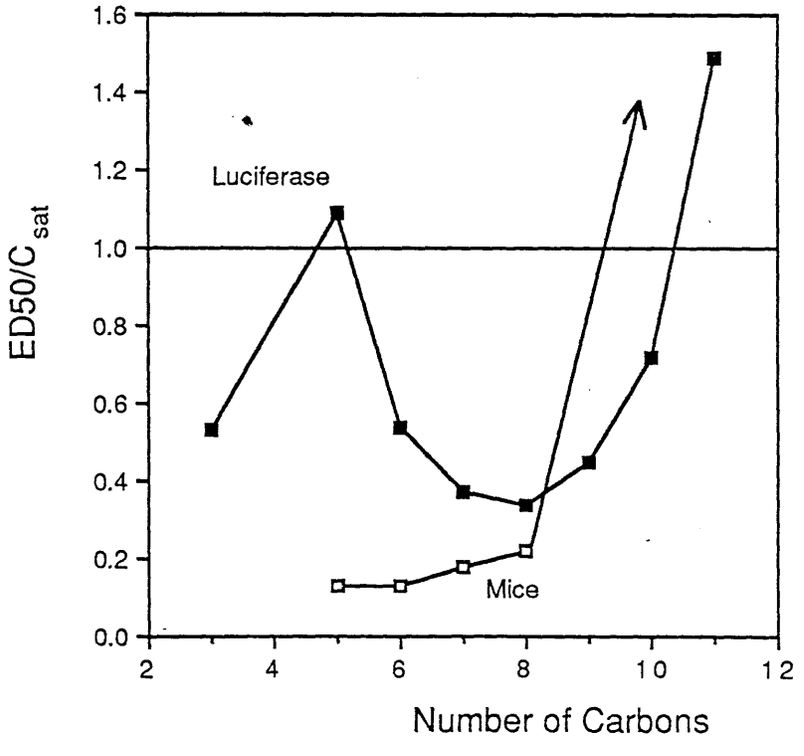

Figure 5.10: Thermodynamic activities (ED_{50}/C_{sat}) of n−alkanes for inhibition of luciferase and general anaesthesia. Luciferase data are from table 4.2(b). Anaesthesia data [mice]: Führer (1921). Alkane solubilities are from Bell (1973).

anaesthesia and inhibition by alkanes [again calculated as ED_{50}/C_{sat}, because the solubilities of these agents are low (Brink and Posternak, 1948)] are plotted against chain length. These activities are much higher than the activities of the corresponding alcohols, an observation which helps to explain why the cut-off occurs at a shorter chain length for alkanes than for alcohols. Although the alcohol ED_{50} curve begins to level off at the same chain length as for alkanes (around C_{11} – see Figure 5.9), the alcohol cut-off is not immediate because the ED_{50} at this point is still a small fraction of the solubility. In contrast, since *n*-alkanes inhibit luciferase at a much higher fraction of their aqueous solubilities than alcohols (*i.e.* at higher thermodynamic activities), the alkane cut-off occurs almost as soon as the ED_{50} curve begins to level off. In common with alcohols, the levelling off in the ED_{50} curve coincides with the appearance of biphasic decay of the light output of inhibited reactions. However, it will be shown in the next section that the alkane cut-off for luciferase is nonetheless a real effect.

Although alkanes induce anaesthesia at lower thermodynamic activities than they inhibit bacterial luciferase, the anaesthetic potency cut-off occurs at an even shorter chain length. On the basis of the reasoning given above to account for the different cut-off points of alkanes and alcohols acting on luciferase, one might expect *this* cut-off to appear at a longer chain length than for luciferase inhibition. However, it is possible to explain this discrepancy if one assumes, simply, that the anaesthetic target has a smaller volume that the luciferase binding pocket. This is consistent with the estimate of the relative sizes derived from the alcohol cut-off data (although, again, it should be borne in mind that the anaesthesia data for alkanes and alcohols were determined on different animals – mice and tadpoles respectively). To date there is little experimental evidence to suggest an abrupt cut-off in the lipid perturbation caused by alkanes which are thought by some workers to be responsible for general anaesthesia. Haydon and his colleagues (1977) observed that the ability of *n*-alkanes to absorb into (and thicken) lipid-cholesterol bilayers declines to an insignificant level beteewn C_7 and C_{11} – indicative of a cut-off. However, as was discussed in Chapter 1, the evidence in support of membrane expansion as the mechanism of general anaesthesia remains controversial. The ability of bacterial luciferase to imitate the cut-off in alkane anaesthesia thus adds further to the argument that anaesthetics act by direct interaction with protein targets in the central nervous system.

5.4(c) Apparent Methylene Group Binding Energies

Apparent methylene group binding energies were calculated for $C_5 \rightarrow C_8$ alkanes using luciferase dissociation constants and anaesthetic ED_{50} concentrations for mice in equation 5.2. The results of these calculations are given in Table 5.5. Methylene binding energies were not determined for the addition of CH_2 groups to nonane and decane since these transitions involved changes in the inhibition kinetics which obscure the meaning of the differences in the K_i values for nonane, decane and undecane.

Table 5.5: Apparent methylene group binding energies for n–alkanes. $\Delta\Delta G^\circ$ was calculated using equation 5.2, as described in section 5.3(b). K_i values for alkanes binding to luciferase were taken from Table 4.2(b). $\Delta\Delta G^\circ$ for general anaesthesia was calculated using ED_{50} concentrations for mice: Führer (1921), $T = 37^\circ C$.

n	Bacterial Luciferase $\Delta\Delta G^\circ$ (kJ mol^{-1})	General Anaesthesia $\Delta\Delta G^\circ$ (kJ mol^{-1})
5	-5.66	-3.88
6	-4.22	-2.98
7	-4.18	-3.21
8	-2.32	---

The first point to note is that the apparent CH_2 binding energies for luciferase in Table 5.5 are quite similar to the $\Delta\Delta G^\circ$ values calculated from alcohol dissociation constants (see Table 5.1). This supports the assumption that alkanes and alcohols bind to the same site on the luciferase enzyme.

It is also of interest to note that although, overall, alkanes appear to bind less well to luciferase than to the anaesthetic target in mice (suggesting that the enzyme pocket is more polar), certain methylene groups appear to bind tighter to luciferase. As was stated in section 5.3, the number and variety of the enthalpic and entropic changes which accompany protein–ligand interactions make a molecular interpretation of the relatively large $\Delta\Delta G^\circ$ values observed for alkanes binding to luciferase rather

difficult. Nevertheless, it was argued that this result might simply reflect the narrowness and apolarity of the regions within the luciferase pocket to which these CH_2 groups bind – a view which will be supported by evidence presented in sections 5.5 and 6.1. It follows that the luciferase binding site may well consist of distinct polar and apolar regions. Such a model, though somewhat speculative, can account for both the large apparent methylene binding energies and the relatively low overall affinity of the site of alkane molecules. The presence of discrete polar features in the luciferase pocket has already been suggested by the role of hydroxyl groups on the flavin side chain in aldehyde binding (Meighen and Mackenzie, 1973) and by the equipotency of C_8 and C_9 alcohols and diols. Other polar zones may also exist; for example, the water/site interface discussed in section 5.4(b). Additionally, the water molecules which fill those parts of the site not occupied by the ligand would constitute a polar region which, depending on the geometry of the pocket, may well be localised.

If the equipotency of C_8 and C_9 alcohols and diols may be taken to infer the existence of a polar patch in the luciferase pocket (as discussed in section 5.3(c)), one is obliged to ask why octane and nonane are not also equipotent. Inspection of the apparent CH_2 binding energies in Table 5.5 reveals that the addition of a CH_2 group to octane contributes only half as much binding energy as the preceding CH_2 additions. Thus there is a small kink in the alkane ED_{50} curve at this point and, although it is a pale shadow of the kink in the alcohol and diol ED_{50} curves, it may be argued, tentatively, that this feature is consistent with a polar patch. One can imagine that alcohols and diols might be anchored at a particular location in the pocket by their hydroxyl groups in such a way that their methylene chains do not reach the polar patch unless they contain more than eight CH_2 groups. Alkanes, not being anchored by any hydroxyl group, may "rattle" around within the pocket (along with water molecules bound there), keeping to a mainly apolar zone but making energetically costly contacts with the polar patch from time to time (possibly by disturbing the hydrogen bonds of water molecules interacting with it). However nonane may be too long to avoid contact with the polar patch, even temporarily, and causes more disturbance than shorter alkanes. The methylene group added to octane might thus appear to bind relatively weakly.

Nevertheless it must be emphasised that the precise details of alkane, alcohol and diol binding must await further investigation. I have tried to show that a plausible explanation of the inhibition data for these agents is that the luciferase pocket may be narrower and more polar than the anaesthetic target, although it also seems to contain regions which are actually more apolar than the anaesthetic target.

More evidence for this view will be presented in the next section and the next chapter.

5.5 Halogenated Anaesthetics

The halogenated anaesthetics halothane, chloroform, methoxyflurane, enflurane, isoflurane and fluroxene appear on Figure 5.1 as a cluster of points lying well below the line of identity. These agents are all considerably less potent as inhibitors of bacterial luciferase than as general anaesthetics. In fact, they comprise the group of inhibitors which correlates least well, in terms of potency, with anaesthesia data. A detailed comparison of the ED_{50} concentrations determined for luciferase inhibition and calculated for anaesthesia in man is given in Table 5.4. The ratio of luciferase to human ED_{50} concentrations ranges from 5 for fluroxene to 48 for halothane.

Table 5.6: Comparison of ED_{50} concentrations of halogenated anaesthetics for inhibtion of bacterial luciferase and for general anaesthesia. Luciferase ED_{50} concentrations are taken from Table 4.2(c). Anaesthetic ED_{50} concentrations for man [Steward et al., 1973] were calculated from partial pressure data as described in the legend to Figure 1.3.

Agent	Bacterial Luciferase ED_{50} (mM)	General Anaesthesia ED_{50} (mM)	Ratio
Halothane	11.4	0.24	48
Isoflurane	13.5	0.32	41
Enflurane	10.5	0.52	20
Methoxyflurane	4.14	0.28	15
Chloroform	11.8†	0.79	15
Fluroxene	5.59	1.14	5

† *For the purposes of this comparison, the ED_{50} of chloroform has been crudely "corrected" by subtracting the concentration of this anaesthetic which produces maximum stimulation (i.e. 20.6−8.8 = 11.8 mM).*

In view of their relative ineffectiveness, it should be emphasised that halogenated anaesthetics inhibit luciferase in exactly the same way as other agents which are very potent inhibitors of the enzyme. It was pointed out in section 4.5 that methoxyflurane is a pure competitive inhibitor – just like *n*–decanol, which is one of the most effective luciferase inhibitors. Moreover, all the halogenated anaesthetics belong to that class of inhibitors which reduce the rate constant for the decay of luminescence apparently in proportion to the depression of the maximum rate (peak intensity) that they cause [*e.g.* see Figure 4.17 for methoxyflurane and halothane]. Chloroform is somewhat exceptional in that it causes a small degree of excitation of the maximum ~~initial~~ rate at low concentrations; but otherwise it acts in exactly the same way as the other halogenated agents. Even if the chloroform ED_{50} is "corrected" for this excitation, as in Table 5.6, it is still much less effective as a luciferase inhibitor than as a general anaesthetic.

Since halogenated anaesthetics are among the most volatile agents which were used in this study, it was important to test whether their surprisingly high ED_{50} concentrations were not due simply to evaporation during the course of the experiment. In section 3.2(b), it was noted that halothane was observed not to evaporate from the glass syringe used to deliver aliquots of a halothane solution over a thirty minute period. However a further test of the experimental handling procedures was also performed in order to check that significant evaporation was not occurring in the time between delivery of the anaesthetic solution to the reaction vial and initiation of the reaction. With the kind assistance of Guy Moss, the sensitivity of both firefly and bacterial luciferases to the *same* halothane solution was investigated. In the experiment two assays of the inhibition caused by this halothane solution were performed on each enzyme. The first assay on firefly luciferase was followed by one on bacterial luciferase and then this sequence was repeated. Bacterial luciferase assays were performed by injecting 2.5 ml of a 304 μM solution of catalytically reduced $FMNH_2$ into a vial containing 10 μl of luciferase solution, 1.0 ml of *n*–decanal solution and 4.0 ml of buffer or a saturated buffer solution of halothane. Final concentrations were: $FMNH_2$, 101 μM; luciferase, 0.3 nM, *n*–decanal, 0.852 μM (= K_m); halothane, 9.3 mM. All solutions were prepared in 50 mM phosphate buffer, pH 7.0. Firefly luciferase assays were identical in form. 2.5 ml of 6.0 mM ATP was injected into a vial containing 15 μl of firefly luciferase solution, 1.0 ml of a solution of the substrate luciferin, 3.75 ml of buffer and either 0.25 ml of phosphate buffer or 0.25 ml of the same halothane solution as before. Final concentrations were: ATP, 2.0 mM; luciferase, 3 nM; luciferin, 15 μM (= K_m); halothane, 0.58 mM. All solutions for the firefly assays were made up in 25 mM *n*–glycylglycine, pH 7.8 except the halothane solution which was

prepared in the 50 mM phosphate buffer.

The ED_{50} concentrations calculated from the results of this simple test were 0.4 and 14 mM for firefly and bacterial luciferase respectively. These values are consistent with previous determinations of the halothane ED_{50} for the two enzymes [0.39 ± 0.01 mM for firefly (Franks and Lieb, 1984) and 11.4 ± 1.4 mM for bacterial luciferase (this thesis)] and confirm that no appreciable evaporation was occurring as a result of the experimental protocol used for volatile agents. The high ED_{50} values determined on bacterial luciferase for the halogenated anaesthetics therefore reflect the genuine insensitivity of this enzyme to such compounds.

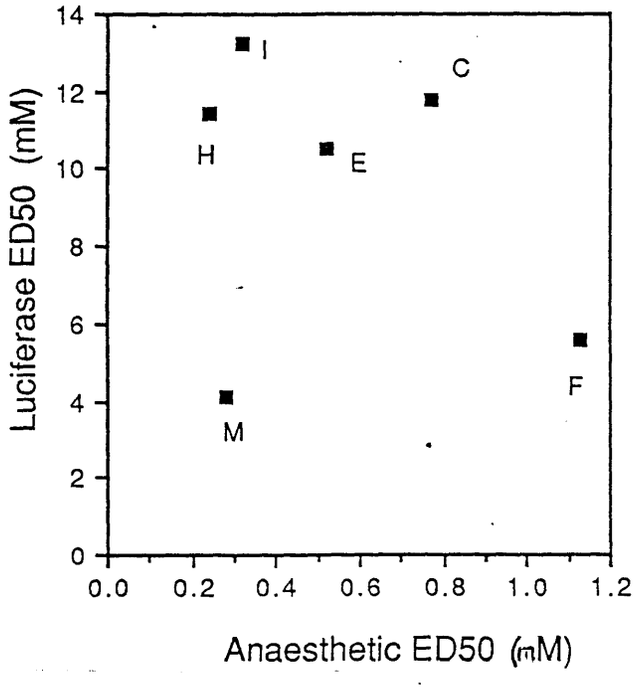

Figure 5.11: There is no correlation between the ED_{50} concentrations of bulky halogenated anaesthetics for inhibition of bacterial luciferase and for general anaesthesia. The data are taken from Table 5.6. Anaesthetics: M, methoxyflurane; H, halothane; E, enflurane; I, isoflurane; F, fluroxene; C, chloroform.

Why does bacterial luciferase discriminate so strongly against halogenated anaesthetics? In previous sections, consideration of the details of the inhibition of luciferase by alcohols and alkanes suggested that the binding site on the enzyme contains distinct polar and apolar regions. It is conceivable that the lack of correlation between general anaesthetic and luciferase ED_{50} concentrations of

halogenated agents, shown in detail in Figure 5.11, might arise because they bind to regions of differing polarity within the pocket. However, such a model would necessarily be very sophisticated and it is not possible to develop it in any detail without much further investigation. In any case, a much simpler account of the lack of correlation in Figure 5.11 may be available. It was also noted in previous sections that the relatively large apparent methylene binding energies of alcohols and alkanes may reflect the narrow dimensions of the luciferase pocket. A common feature of the halogenated anaesthetics is their bulkiness. It is not difficult to imagine that molecules containing one or more bulky fluorine, chlorine or bromine groups would be sterically hindered from docking at a site which is a good fit for methylene chains. This point can be illustrated using CPK space filling models of the aldehyde substrate, *n*−decanal, and the anaesthetics halothane, fluroxene, methoxyflurane and diethyl ether, shown in the photograph in Figure 5.12. Clearly a pocket which has evolved to accomodate decanal would quite conceivably be too narrow for such anaesthetics to bind easily. It is noticeable that fluroxene, which consists of a narrow chain surmounted by a bulky CF_3 group, binds relatively well (compared with its affinity for the anaesthetic target in the central nervous system) whereas halothane, which has perhaps the most "globular" proportions of these agents, binds very poorly indeed.

Also included in Figure 5.12 are diethyl ether and methoxyflurane. Ether binds apparently as well to luciferase as to the general anaesthetic target, but methoxyflurane binds fifteen times less well. It seems likely that this difference may be explained by the presence of large chlorine and fluorine groups of methoxyflurane. Other bulky anaesthetics were also observed to be poor inhibitors of luciferase (see Table 4.2(d)). Benzyl alcohol, for example, has an ED_{50} of 13.7 mM, nearly seven times greater than the anaesthetic dose for tadpoles (2 mM; Kita *et al.*, 1981). A much larger compound, pentobarbital, was even less effective on luciferase; its ED_{50} concentration, determined by extrapolation to be 31 mM (at pH 7.0) is almost 200 times the concentration required to anaesthetise tadpoles (0.16 mM at pH 7.4; Firestone *et al.*, 1986).

This result implies that the halogenated anaesthetics meet with relatively little steric hindrance at the physiological anaesthetic target. Obviously the fluid hydrocarbon region of the lipid bilayer within nerve cell membranes, which has been postulated as a site of anaesthetic action (Miller, 1985), would easily accomodate bulky, apolar agents. However, recent results also show that some protein sites, unlike bacterial luciferase, may be equally accomodating. An anaesthetic binding site which does not discriminate against halothane, methoxyflurane or chloroform has

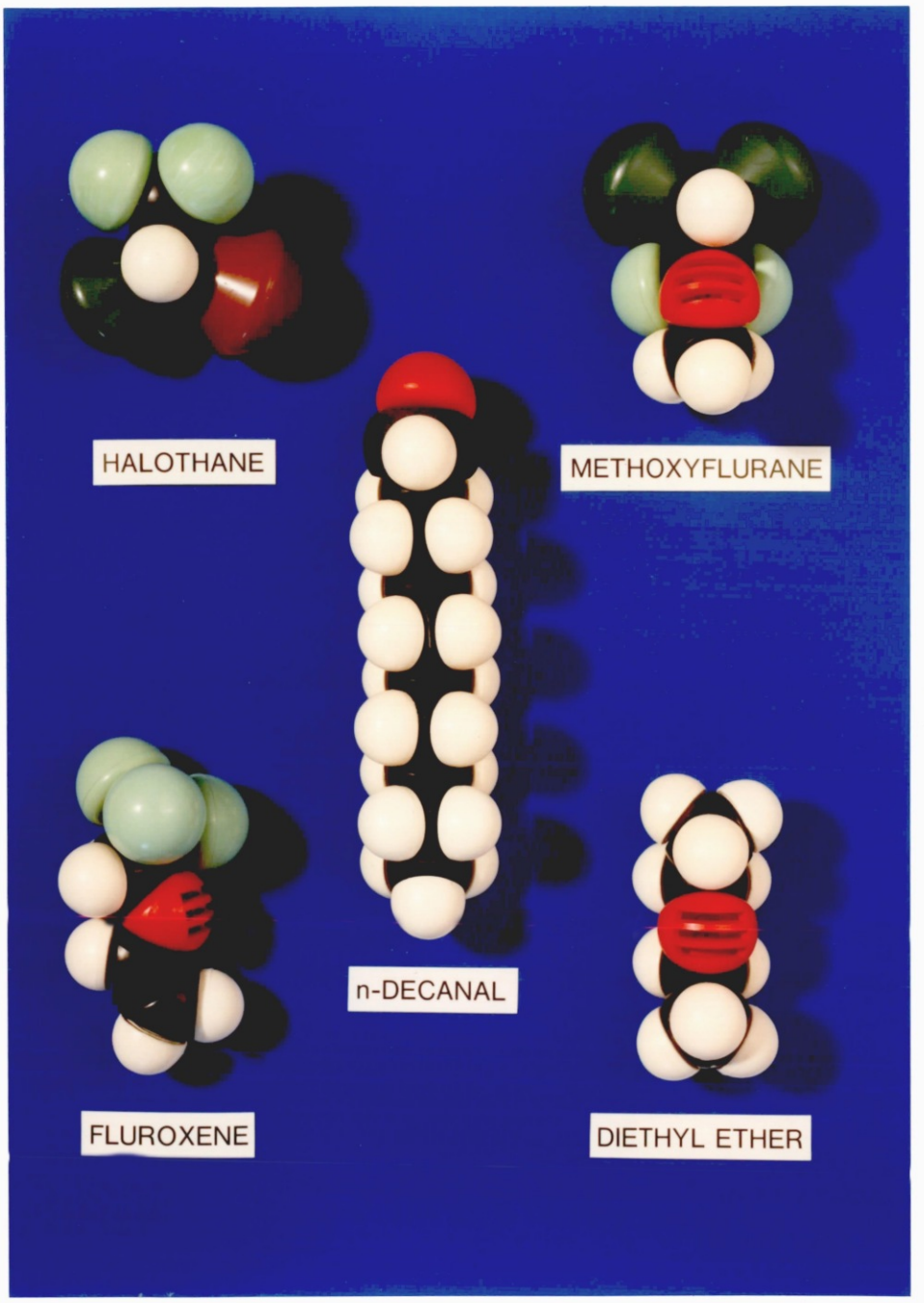

Figure 5.12: Photograph of CPK models of the aldehyde substrate used in this work, n–decanal, and the anaesthetics halothane, methoxyflurane, fluroxene and diethyl ether. Atoms are colour–coded as follows: black – carbon; white – hydrogen; red – oxygen; light–green – fluorine; dark–green – chlorine; brown – bromine.

been found on firefly luciferase (Franks and Lieb, 1984); indeed these anaesthetics inhibit firefly luciferase at concentrations close to those required to induce anaesthesia. The comparative anaesthetic and inhibitory potencies of halogenated anaesthetics thus cannot distinguish between possible lipid and protein sites of action within the central nervous system. The results discussed in this chapter appear to indicate that the physiological anaesthetic target, if it is a protein, differs in details of geometry and polarity distribution from the binding pocket on bacterial luciferase.

CHAPTER 6

FURTHER INVESTIGATIONS OF THE ANAESTHETIC BINDING SITE ON BACTERIAL LUCIFERASE

Several aspects of the results of inhibiton experiments with general anaesthetics, discussed in the previous chapter, were investigated further. The geometry of the luciferase pocket was probed with a simple set of experiments involving the homologous series of cycloalcohols. In addition, evidence confirming the validity of the cut−off effect for luciferase was provided by a study of the ability of *n*−alcohols to stabilise the lifetime of the luciferase−peroxyflavin complex. This study also led to a straightforward explanation for the appearance of biphasic luminescence decay in the presence of alcohols longer than decanol and alkanes longer than nonane. Finally, the possible reasons for the discrepancies between the ED_{50} concentrations determined in this project for *Vibrio harveyi* luciferase and those reported in the literature for *Vibrio fischeri* luciferase and for whole bacteria were examined. All of these investigations are described in this chapter.

6.1 Probing the Geometry of the Luciferase Pocket with Cycolalcohols

In section 5.5 it was suggested that the relatively low affinity of bacterial luciferase for halogenated anaesthetics could be ascribed to steric hindrance at the aldehyde binding site. This model of the site is consistent with other, circumstantial evidence. The endogenous substrate of luciferase is a long−chain aliphatic aldehyde and one might reasonably expect the binding site on the enzyme to be specific for this type of compound. Since such aldehydes are composed only of a single polar head−group on a long, hydrophobic methylene chain, this specificity might easily be achieved if the binding site is similarly long, narrow and hydrophobic. Moreover, the relatively large apparent methylene binding energies calculated for alcohols and alkanes may conceivably be attributed to the interaction of these groups with narrow, apolar regions of the enzyme pocket.

However, it may be that this view of the pocket is oversimplified. Various strands of evidence, described in the previous chapter, point to the presence of distinct polar regions within the aldehyde binding site. As was mentioned in section 5.5, the luciferase ED_{50} values for halogenated anaesthetics might reflect interactions with regions of differing polarity in this site. Isoflurane, enflurane, methoxyflurane and fluroxene all contain a polar oxygen group, a feature which is not possessed by either halothane or chloroform. It is conceivable that the interaction between halogenated anaesthetics and the luciferase binding pocket is dominated, not by steric factors, but by the variety of polar and apolar interactions that these agents may make in the enzyme pocket. Although this latter explanation seems unlikely in view of the fact that anaesthetics which are relatively small and polar, *e.g.* diethyl ether, inhibit luciferase at concentrations which are close to those required for anaesthesia, it was nonetheless desirable to attempt to distinguish between it and the steric hypothesis.

This was done using the homologous series of cycloalcohols. These saturated alicyclic compounds contain a single secondary alcohol group. Chemically, they are similar to primary *n*−alcohols but structurally they are quite different. I have argued that the reason why *n*−alcohols bind so well to the pocket is that they can slot easily into it. Such a site (whether it be a shallow trench or a deep hole in the protein) would only partially accomodate the discoid proportions of cycloalcohols and would therefore be expected to bind them less well.

The inhibition of luciferase by cycloalcohols (C_6, C_7, C_8, C_{10} and C_{12}) was studied in detail. Like almost every anaesthetic agent used in this work, they were shown to be competitive inhibitors of the enzyme. In Figure 6.1 this is illustrated for cyclooctanol on a double reciprocal Lineweaver−Burk plot. Moreover, cycloalcohols all act to reduce the luminescence decay constant apparently in proportion to the depression of the peak intensity that they cause. This behaviour is shown for cyclohexanol and cyclododecanol in Figure 6.2 and is identical to the behaviour shown for methoxyflurane, halothane, *n*−heptanol and paraldehyde in Figure 4.17. Thus there appears to be nothing exceptional about the interaction of these agents with luciferase. [Note that the action of cyclododecanol, which causes a retarded simple exponential decay of luminescence, is qualitatively different from that observed with *n*−dodecanol; this alcohol induces biphasic luminescence decay, one phase of which is accelerated and the other greatly retarded by increasing the concentration − see Figures 4.22, 4.23(c) and 4.24]. Cycloalcohol K_i and ED_{50} values are given in Table 6.1.

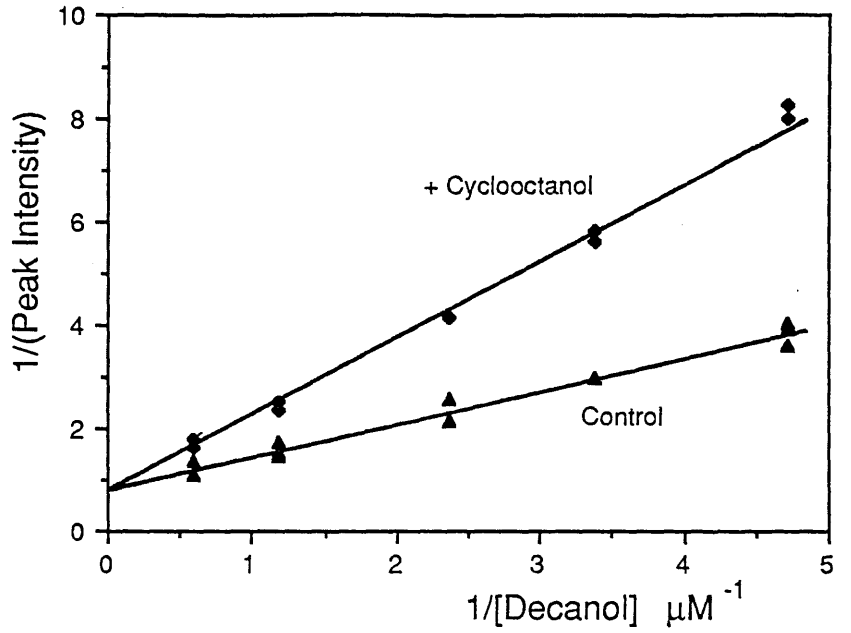

Figure 6.1: Lineweaver–Burk plot showing purely competitive inhibition of the $FMNH_2$–initiated luciferase reaction by cyclooctanol. Final concentrations (in 7.51 ml): decanal, 0.21–1.7 μM; $FMNH_2$, 102 μM; luciferase, 0.4 nM. T = 24.9°C.

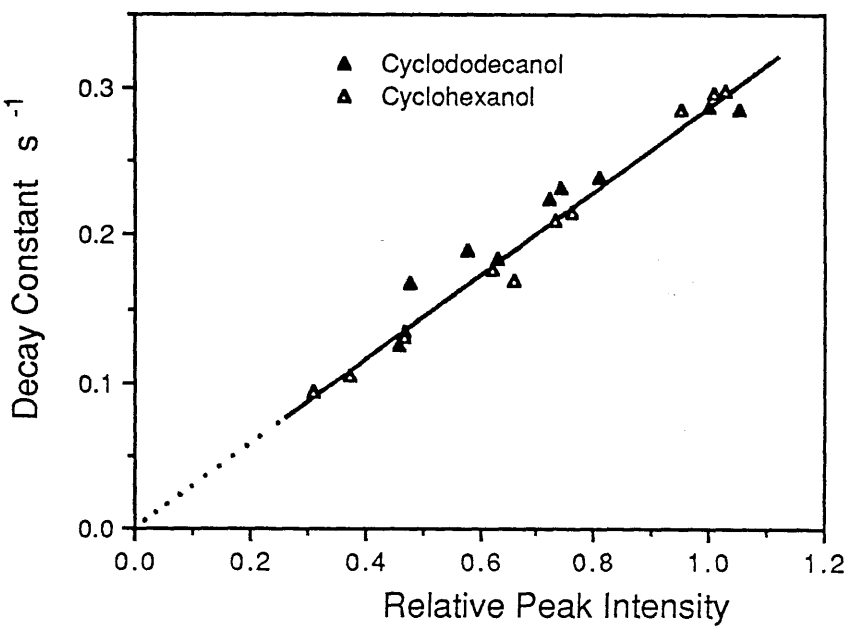

Figure 6.2: The depression of the maximum rate of the $FMNH_2$–initiated luciferase reaction by cycloalcohols is apparently proportional to the concomitant reduction in the luminescence decay constant. Data shown are for cyclohexanol and cyclododecanol, the shortest and longest cycloalcohols used. Final concentrations: decanal, 1.1 μM; $FMNH_2$, 100 μM; luciferase, 0.4 nM. T = 25.2°C.

Table 6.1: Dissociation constants (K_i) and ED_{50} concentrations determined for the inhibition of bacterial luciferase by cycloalcohols. All procedures and conditions were as described in the legend to Figure 4.2.

Agent	K_i (± s.e.)	ED_{50} (± s.e.)	n
Cyclohexanol	9.24 ± 0.42 mM	18.5 ± 0.8 mM	1
Cycloheptanol	4.96 ± 0.66 mM	9.92 ± 1.3 mM	1
Cyclooctanol	2.46 ± 0.35 mM	4.92 ± 0.69 mM	1
Cyclodecanol	91.0 ± 7.6 μM	182 ± 15 μM	1
Cyclododecanol	69.3 ± 1.7 μM	139 ± 3 μM	1

As luciferase inhibitors, cycloalcohols are between 25 and 1000 times less potent than the corresponding *n*−alcohols. However, since ring−closure of saturated hydrocarbons is known to increase aqueous solubility (Davis *et al.*, 1974; McAuliffe, 1966), the lower potency of cycloalcohols is, at least in part, due to their greater solubility. It is possible to compensate, in some measure, for this effect by

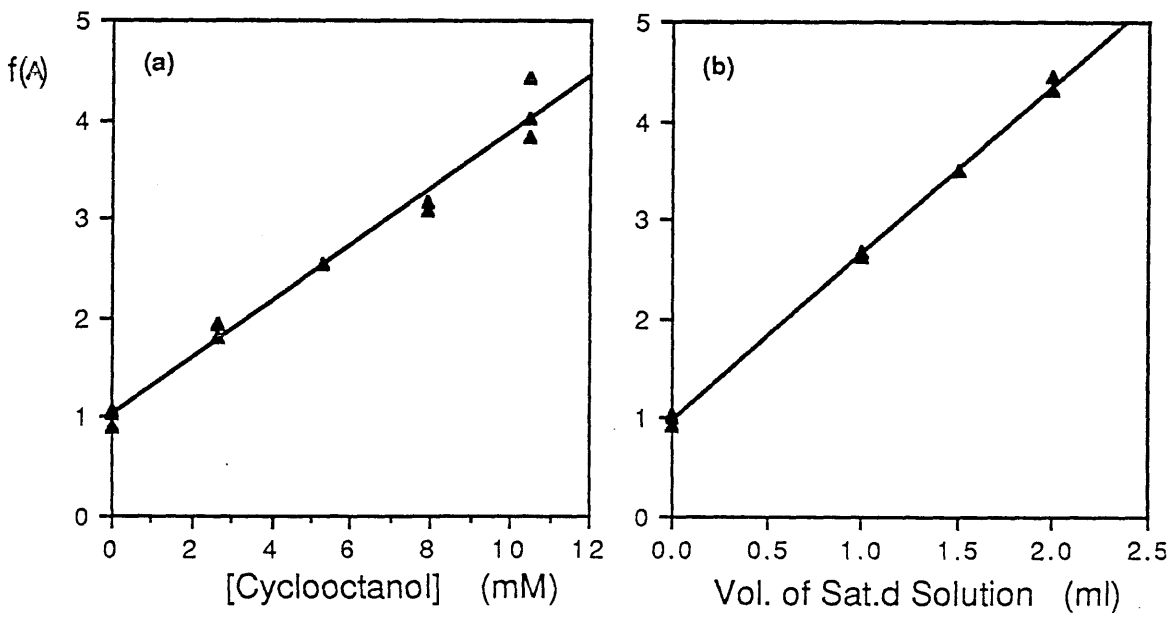

Figure 6.3: f(A) dose−response data for the inhibition of luciferase by (a) a standard cyclooctanol solution of known concentration and (b) a saturated solution of cyclooctanol. The volumes on the abscissa in (b) are the amounts of saturated solution diluted in a total volume of 7.51 ml. Final concentrations: decanal, 0.85 μM; $FMNH_2$, 101 μM; luciferase, 0.4 nM. T = 24.8°C.

comparing the values of the ratios ED_{50}/C_{sat} for the corresponding members of the two series. Since accurate aqueous solubility data are not available in the literature for cycloalcohols (to my knowledge), these were determined by the novel experimental method described in section 3.4(c). Dose–response data were taken for a saturated solution and a standard solution (of known concentration) of each agent. These data are shown for cyclooctanol as f(A) plots in Figure 6.3. Each of these plots yields a K_i value and an ED_{50} concentration. The solubility, C_{sat}, may be calculated as:

$$C_{sat} = \frac{7.51\ (ml)}{V_{50}\ (ml)} \times ED_{50}\ (M) \qquad (6.1)$$

where V_{50} is the volume of the saturated solution (in 7.51 ml total) which inhibits luciferase by 50% (at [decanal] = 0.85 μM = K_m). The method was tested with *n*–hexanol and *n*–octanol, for which reliable solubility data already exist. Values of 56.3 ± 6.2 and 4.73 ± 0.44 mM respectively were obtained for the solubilities of these alcohols and agree well with literature values of 61 and 4.5 mM (Butler, 1933; Bell, 1973). The aqueous solubilities of the cycloalcohols are summarised in Table 6.2 and their logarithms are plotted against chain length in Figure 6.4 along with solubility data for *n*–alcohols and cycloalkanes.

Table 6.2: Aqueous solubilities (C_{sat}) of cycloalcohols and values of ED_{50}/C_{sat} for the inhibition of luciferase by cycloalcohols and n–alcohols. Measurement of C_{sat} is described in the text. Cycloalcohol and n–alcohol ED_{50} concentrations are taken from Tables 6.1 and 4.2(a). The aqueous solubilities of n–alcohols at 25°C are taken from Bell (1973).

Number of Carbons	Cycloalcohol C_{sat}	Cycloalcohol ED_{50}/C_{sat}	*N*-alcohol ED_{50}/C_{sat}
6	169 ± 11 mM	0.11	0.011
7	108 ± 9 mM	0.092	0.0039
8	44.6 ± 2.7 mM	0.11	0.0019
9	---	---	0.0067
10	1.18 ± 0.09 mM	0.15	0.0074
11	---	---	0.0092
12	0.377 ± 0.034 mM	0.37	0.023

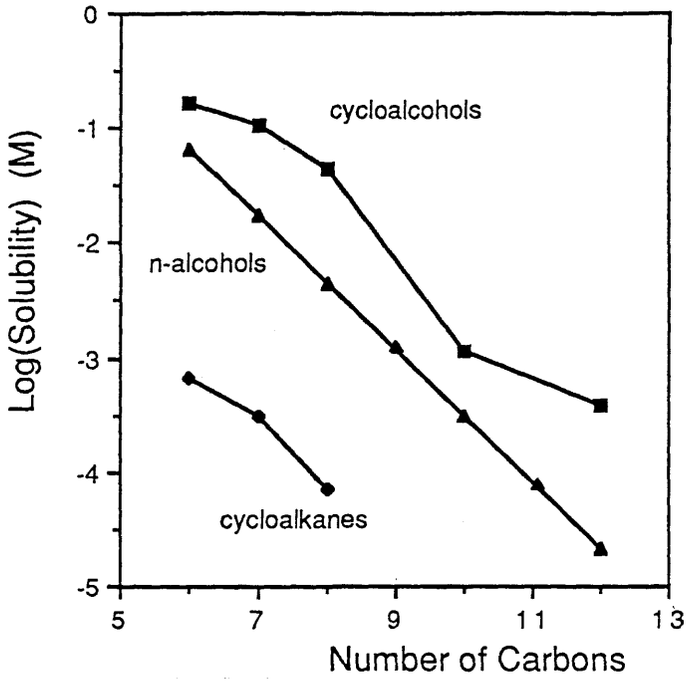

Figure 6.4: The dependence on chain length of the aqueous solubilities of cycloalcohols determined in this work, compared with the solubilities of n−alcohols (Bell, 1973) and cycloalkanes (McAuliffe, 1966). T = 25°C for all three series.

The form of the cycloalcohol data in Figure 6.4 differs from similar plots of the solubilities of straight chain saturated hydrocarbon series which are usually linear with a negative gradient (Bell, 1973). In the case of *n*−alkanes and *n*−alcohols, the gradients of such plots are −0.64 and −0.58 respectively (Bell, 1973), which correspond to reductions in solubility by factors of 4.4 and 3.8 for each additional methylene (CH_2) group. In contrast, a CH_2 group added to a cycloalcohol (except between C_8 and C_{10}) causes approximately only a 2−fold drop in solubility. Between C_8 and C_{10}, solubility actually drops by almost a factor of 38 (averaging at 6.1 per CH_2 group). The solubilities of cyclohexane, cycloheptane and cyclooctane (660 μM, 310 μM and 71 μM respectively (McAuliffe, 1966)), also plotted on Figure 6.4, show a pattern of decline which is broadly similar to that observed for cycloalcohols up to C_8. Davis *et al.* (1974), reviewing the solubilities of cycloalkanes up to cyclooctane, concluded that the small incremental drop in C_{sat} could be attributed to the lower solvent−accessible area of cyclic hydrocarbons. The explanation for the large drop in solubility between C_8 and C_{10} is not obvious. It may be that the increased floppiness of molecules longer than cyclooctanol is in some way responsible. Further comment on this feature, which was reproducible, must await a more detailed investigation.

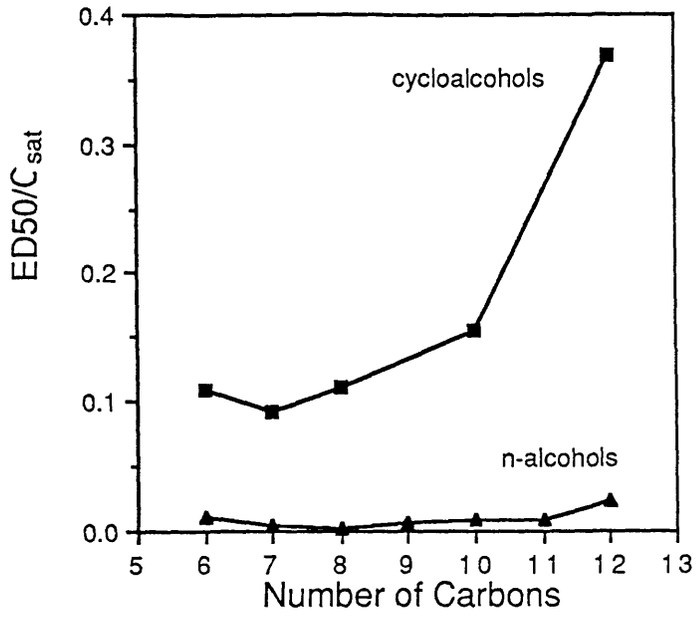

Figure 6.5: Comparison of the values of ED_{50}/C_{sat} for cycloalcohols and n−alcohols determined for the inhibition of the $FMNH_2$−initiated luciferase reaction. Even when compared on this basis, the n−alcohols are much more potent. The data were taken from Table 6.2.

The ratios ED_{50}/C_{sat} for cycloalcohols and *n*−alcohols are given in Table 6.2 and plotted in Figure 6.5. Obviously, even when compared on this basis, the cycloalcohols still appear much less effective than *n*−alcohols as inhibitors of the enzyme. This result therefore supports the notion that the anaesthetic binding site on luciferase is long and narrow. It is not difficult to imagine that cycloalcohols might bind poorly to such a site. For example, if it is trench−like, cycloalcohols would not be able to bury all of their hydrophobic surface area in the pocket; if the pocket is more like a borehole then, again, only a partial interaction may be allowed between protein and cycloalcohol. While the precise conformation of the luciferase pocket remains to be elucidated, it seems clear that its geometry strongly favours long, narrow ligands.

In the previous section, inspection of the comparative affinities of luciferase and the physiological anaesthetic target for a range of general anaesthetics indicated that the target places less rigorous steric constraints on the binding of anaesthetics than does bacterial luciferase. That being so, one would predict that cycloalcohols should be more potent as anaesthetics than as inhibitors of luciferase. The anaesthetising

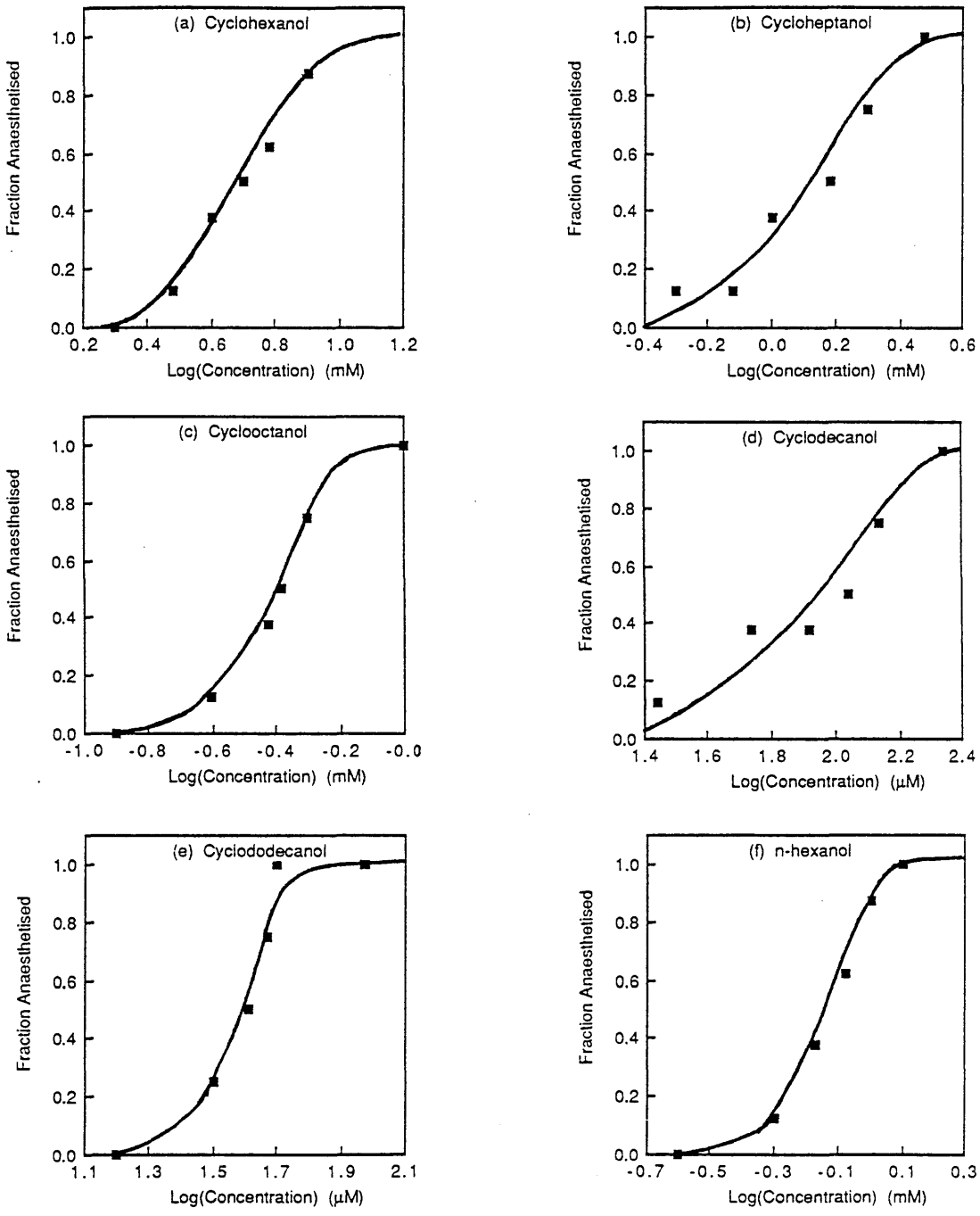

Figure 6.6: Dose–response data showing the anaesthetising effect of (a) cyclohexanol, (b) cycloheptanol, (c) cyclooctanol, (d) cyclodecanol, (e) cyclododecanol and (f) n–hexanol on Xenopus laevis tadploes. Eight tadpoles were used at each concentration. T = 23 ± 1°C.

concentrations (ED_{50}) of cycloalcohols were determined on tadpoles using the method described in section 3.4(d). Dose–response data for the five cycloalcohols used in this study are given in Figures 6.6(a)–(e). As a test of the method employed to assay the response of tadpoles to anaesthetising agents, the ED_{50} of *n*–hexanol was also determined [Figure 6.6(f)]. The data were analysed according to the method of Waud (1972). The ED_{50} concentrations and slopes of the dose–response curves are presented in Table 6.3.

Table 6.3: ED_{50} concentrations and slope values for general anaesthesia of tadpoles by cycloalcohols and n–hexanol.

Agent	ED_{50} (± s.e.) (mM)	Slope (± s.e.)
Cyclohexanol	4.98 ± 0.47	3.9 ± 1.2
Cycloheptanol	1.29 ± 0.17	2.8 ± 0.8
Cyclooctanol	0.390 ± 0.033	4.9 ± 1.7
Cyclodecanol	0.084 ± 0.014	2.1 ± 0.7
Cyclododecanol	0.0384 ± 0.0024	7.4 ± 0.4
n-hexanol	0.73 ± 0.05	6.1 ± 1.8

An ED_{50} concentration for *n*–hexanol of 0.73 ± 0.05 mM was obtained which compares with literature values of 0.9 mM (Vernon, 1913) and 0.7 mM (Pringle *et al.*, 1980). There is some variation in the slopes but, as no correlation with chain–length is apparent, this is probably due to random experimental errors. The tadpole ED_{50} concentrations of cycloalcohols are plotted along with those determined for luciferase inhibtion in Figure 6.7. As predicted, they are much more potent as anaesthetics (2–12 times) than as luciferase inhibitors. In addition, Table 6.4 presents the values of ED_{50}/C_{sat} for anaesthesia of tadpoles by cycloalcohols and *n*–alcohols. Evidently, when the different solubilities of the corresponding members of these two series are taken into account, there is little difference in their relative effectiveness, *i.e.* there is little steric discrimination at the target underlying anaesthesia. These data provide a striking contrast to the comparative ED_{50}/C_{sat} values given in Table 6.2 for the inhibition of bacterial luciferase by cycloalcohols and *n*–alcohols. Thus, if the physiological site of anaesthetic action is indeed a

protein, it seems clear that it is broader than the luciferase pocket.

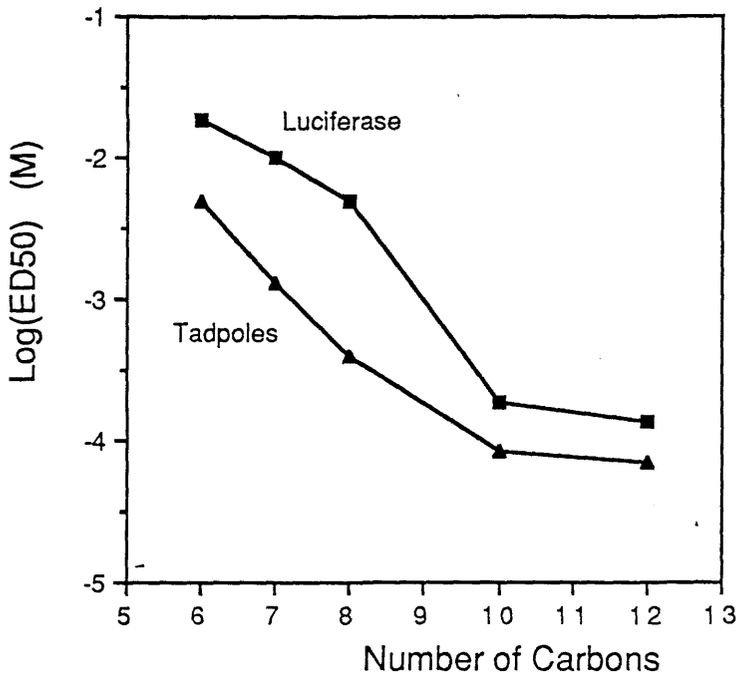

Figure 6.7: Comparison of cycloalcohol and n–alcohol ED_{50} concentrations for inhibition of bacterial luciferase and general anaesthesia. Cycloalcohols are invariably more potent as anaesthetics than as inhibitors of the enzyme.

Table 6.4: Values of ED_{50}/C_{sat} for general anaesthesia induced by cycloalcohols and n–alcohols. Cycloalcohol solubilities are taken from Table 6.2; n–alcohol solubilities are from Bell (1973). Cycloalcohol ED_{50} concentrations are taken from Table 6.3; n–alcohol ED_{50} concentrations are from: Vernon (1913) – hexanol; Meyer and Hemmi (1935) – heptanol to dodecanol.

Number of Carbons	Cycloalcohol ED_{50}/C_{sat}	*N*-alcohol ED_{50}/C_{sat}
6	0.029	0.014
7	0.012	0.022
8	0.009	0.029
9	---	0.023
10	0.071	0.032
11	---	0.054
12	0.10	0.34

6.2 The Stabilisation of the Luciferase–Peroxyflavin Intermediate by *N*–alcohols

In the presence of *n*–alcohols longer than decanol the light output from the luciferase–catalysed reaction decays in a biphasic manner; shorter alcohols, in contrast, simply retard the exponential luminescence decay. The difference between the actions of these two sub–groups of alcohols means that it is difficult to make a meaningful comparison between their ED_{50} concentrations. In particular, the advent of biphasic luminescence decay at C_{11} (undecanol) also raises the possibility that the cut–off in the inhibitory activity of long–chain alcohols, which results from the levelling off of the ED_{50} curve that begins at around the same point, may be an artefact associated with the decay kinetics. Since the observation that luciferase appears to mimic the anaesthetic cut–off for alcohols (and alkanes) is potentially one of the most important findings of this work, it is clearly worthwhile to attempt to remove any doubt as to the validity of the luciferase cut–off. Experiments were therefore performed to observe the ability of *n*–alcohols to stabilise the luciferase–peroxyflavin complex. This allows alcohol dissociation constants to be determined without recourse to measurements of the inhibition of luminescence. The results of these experiments, which are described and interpreted in this section, confirm the cut–off effect for luciferase and provide a simple explanation of the biphasic nature of the luminescence decay in the presence of long–chain alcohols.

6.2(a) Stabilisation Experiments

The luciferase–peroxyflavin complex, which has a lifetime of around 20 seconds at 20°C (Hastings *et al.*, 1985), is stabilised by a variety of hydrocarbon compounds (Baumstark *et al.*, 1979; Tu, 1979). Tu (1979) showed that long–chain alcohols, carboxylic acids and the methyl esters of these acids all reduce the rate of decay of the intermediate. In some cases, the stabilisation achieved is quite dramatic: for example, a saturated solution of *n*–dodecanol was observed by Tu to increase the intermediate lifetime by two orders of magnitude.

The experimental method used to observe the decay of intermediate II, described in section 3.4(b), derives from the protocol adopted by Tu (1979). The intermediate was prepared by rapid injection of 400 μl of catalytically reduced $FMNH_2$ into a cuvette containing a 10 μl droplet of luciferase. Inhibitor and buffer solutions were then added in different proportions in order to achieve a range of inhibitor concentrations without affecting the final concentration of enzyme or flavin [0.55 nM and 100 ± 3 μM (s.d.) respectively]. At intervals a small aliquot

(typically 0.4 ml) of the intermediate was diluted in phosphate buffer (to give a total volume of 5.0 ml) and the luminescent activity of the sample assayed by injection of 2.5 ml of n−decanal. The final concentration of decanal was usually 10 or 20 μM. The resulting peak intensity is a measure of the intermediate concentration at the time the aliquot was taken.

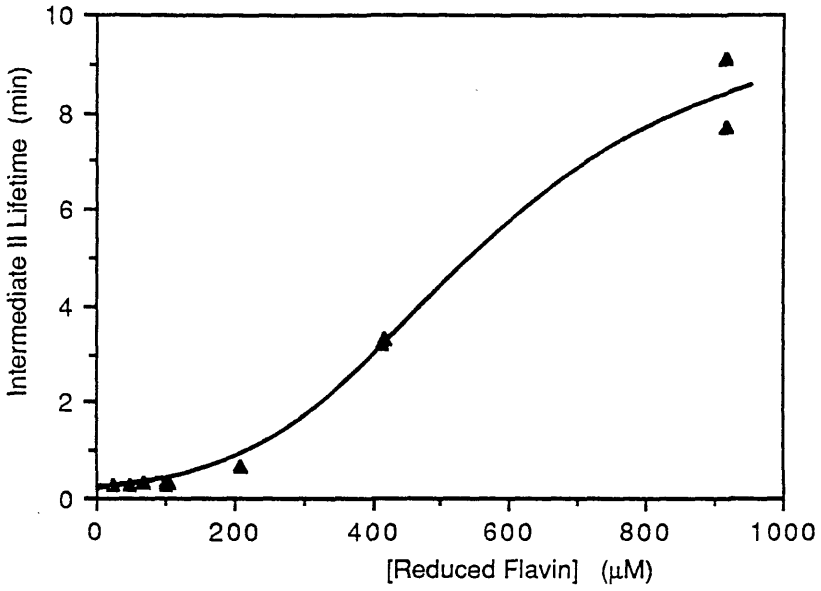

Figure 6.8: The lifetime of Intermediate II increases dramatically as the flavin concentration used to prepare the intermediate is increased. The lifetime was calculated as the reciprocal of the rate constant for the decline of luminescent activity (assayed by the aldehyde initiation method) in a series of aliquots taken from a sample of intermediate. Flavin concentrations on the abscissa are the calculated final concentration, Luciferase concentration in intermediate samples: 35 nM. T = 24.5−25.0°C.

6.2(b) Preliminary Experiments: The Effect of $FMNH_2$ on the Lifetime of Intermediate II

In early experiments to determine appropriate conditions for the observation of the stabilisation caused by alcohols, it became apparent that $FMNH_2$ itself has a profound stabilising effect on the intermediate lifetime − a finding that has not been reported elsewhere. This effect can be seen in Figure 6.8 where the lifetime of intermediate II is plotted as a function of the calculated final flavin concentration. (See below for the method of calculation of this lifetime). Since $FMNH_2$ is rapidly

autoxidised [$t_{\frac{1}{2}} \simeq 0.1$ sec (Gibson and Hastings, 1962)], it seemed possible that the products of the autoxidation of reduced flavin, namely FMN and hydrogen peroxide might be responsible for the observed stabilisation. However, the rate constant for the decay of intemediate II (= 1/lifetime) was unaffected by the addition of FMN or H_2O_2 – as shown in Figure 6.9. This result is not easy to explain. One might suggest that a second $FMNH_2$ molecule binds to the luciferase–peroxyflavin intermediate and stabilises it. However, since at high flavin concentrations intermediate II has a lifetime close to 8 minutes (at 25°C) – far in excess of the lifetime of $FMNH_2$ in solution – it would be necessary to postulate that the binding of this second reduced flavin molecule is almost irreversible.* Meighen and Hastings (1971) found that only a single flavin molecule was involved in the luminescent reaction. However, in NMR studies Vervoort *et al.* (1986b) observed a second $FMNH_2$ molecule to be loosely associated with the enzyme. The binding of this

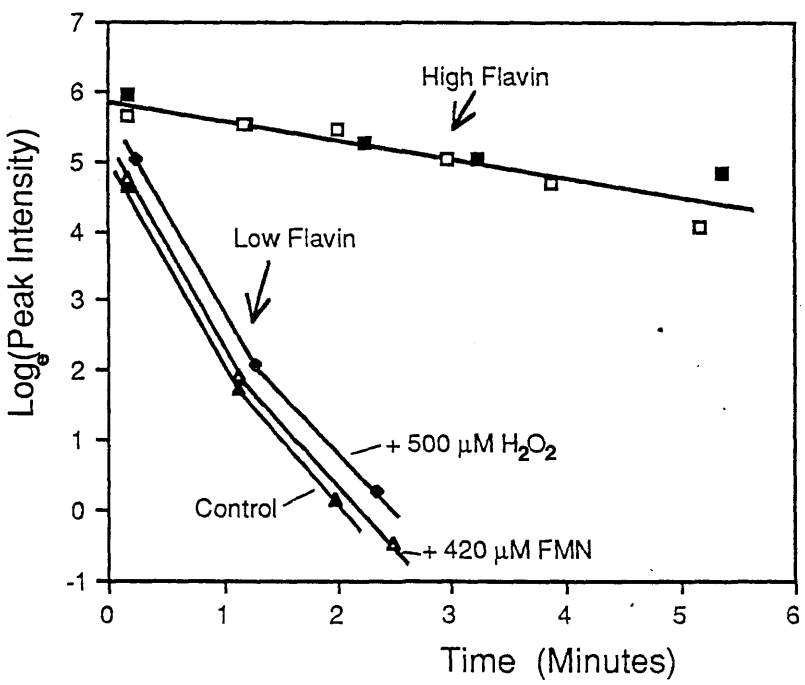

Figure 6.9: The lack of any stabilising effect of FMN or H_2O_2 on intermediate II. The stabilisation observed when a high $FMNH_2$ concentration (412 µM) is used to prepare the intermediate is not reproduced when similarly high concentrations of FMN (420 µM) or H_2O_2 (500 µM) are added to a sample of intermediate prepared at a low $FMNH_2$ concentration (109 µM). [Bracketted concentrations give the final concentrations in 400µl of intermediate]. The data show the decline as a function of time in the natural log of the peak intensity evoked when 2.5 ml of 30 µM decanal is added to a 40 µl aliquot of intermediate diluted in 4.96 ml of assay buffer. Luciferase concentration (in 400 µl): 27 nM. T = 24.2°C.

*Added Note: For [$FMNH_2$] > 240 µM, the O_2 conc., excess $FMNH_2$ persists in solution; one need not insist on irreversibility of flavin binding to explain stabilisation.

second molecule was said to be non−specific − similar to the flavin interaction with hydrophobic regions on bovine serum albumin. It therefore seems most unlikely that such a weak interaction could be responsible for the stabilisation of intermediate II. The precise explanation of this phenomenon requires further investigation. In any case, the intermediate lifetime is almost independent of the flavin concentrations below 100 μM. Reduction of the flavin concentration from 100 to 25 μM results in a small 8% drop in the lifetime of the intermediate. (Figure 6.10). Furthermore, in the presence of 40 μM decanol the lifetime of the intermediate is unaffected by variation of flavin concentrations below 100 μM. The stabilisation due to alcohols was therefore measured in the presence of a final concentration of 100 μM, since this is also the flavin concentration at which inhibition experiments were performed.

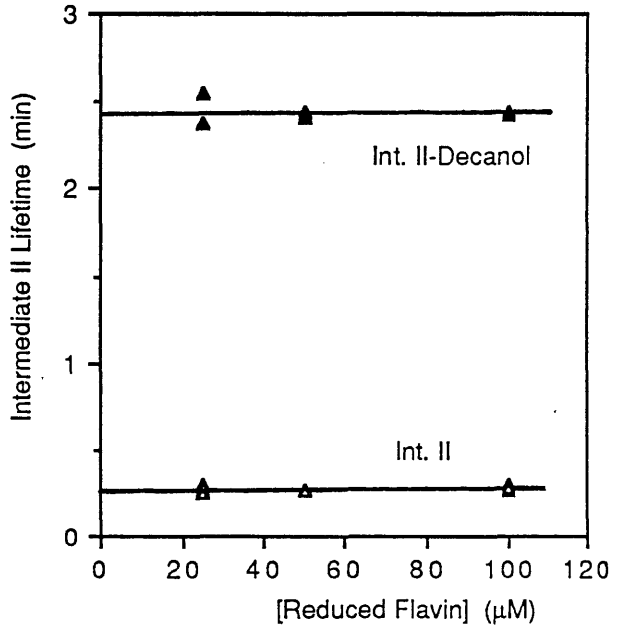

Figure 6.10: Up to 100 μM, the lifetime of intermediate II increases only slightly with flavin concentration (Open triangles). In the presence of 40 μM decanol (solid triangles), the lifetime of intermediate II is independent of the flavin concentration. Procedures and conditions were essentially as described for Figure 6.9. $T = 25.0^{\circ}C$.

6.2(c) Analysis of Results of Stabilisation Experiments

The stabilising effect of pentadecanol is shown in Figure 6.11, where the time−dependence of the logarithm of the luminescent activity of aliquots from samples of intermediate II is shown for a number of concentrations of the alcohol. The decay is exponential at least down to 10% of the original signal. The decay rate of control samples was so fast that the concentration of intermediate II dropped to 2% of its original level after one minute. The control decay constant was therefore determined from only two data points taken within the first minute (faster assaying not being possible) but the results of such determinations proved to be very reproducible. The variation of the gradients in Figure 6.11 reflects the dependence

on the pentadecanol concentration of the rate constants for the decay of the

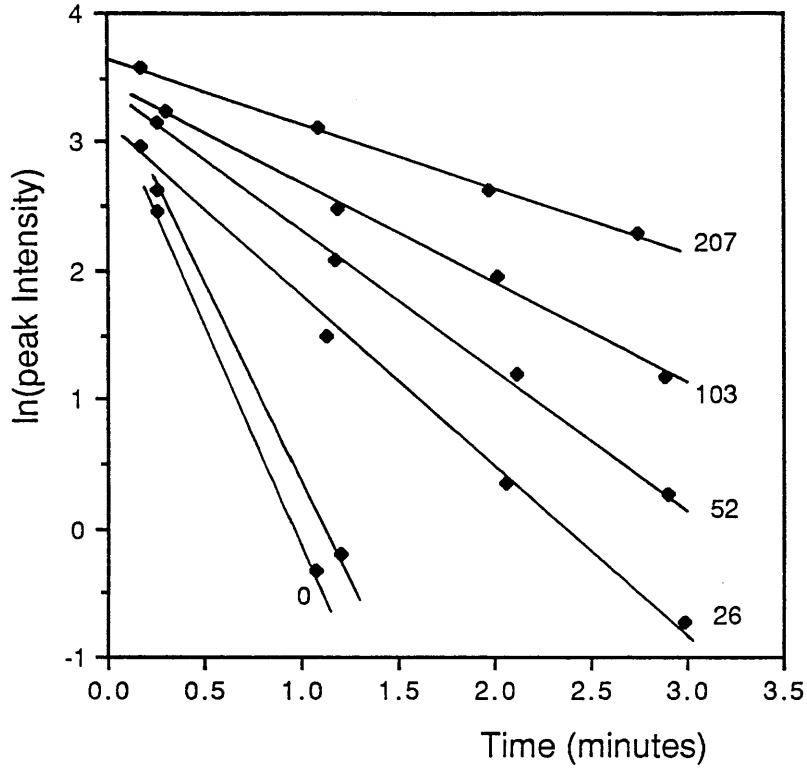

Figure 6.11: Stabilisation of intermediate II by pentadecanol. The data show the time–dependent decline in the natural logarithm of the luminescent activity of aliquots from samples of intermediate prepared in the presence of a range of pentadecanol concentrations. Final concentrations (in 2.01 ml of intermediate): pentadecanol, 0, 26, 52, 103 and 207 nM; $FMNH_2$, 97 μM; luciferase, 0.55 nM). Luminescent activity was assayed by injecting 2.5 ml of 60 μM decanal into 0.4 ml aliquots diluted in 4.6 ml assay buffer. T = 25.1°C.

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luminescent activity of intermediate II. These decay constants (λ) were determined by the method of least squares. The relationship between the inverse of λ and the alcohol concentration is shown for octanol and pentadecanol on Figures 6.12(a) and (b). The hyperbolic form of these curves may be interpreted using the model reaction scheme developed by Tu (1979):

$$\begin{array}{ccccc} E' & + & A & \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} & E'A \\ \downarrow k_0 & & & & \downarrow k_1 \\ E + P & & & & E + P + A \end{array} \qquad (6.1)$$

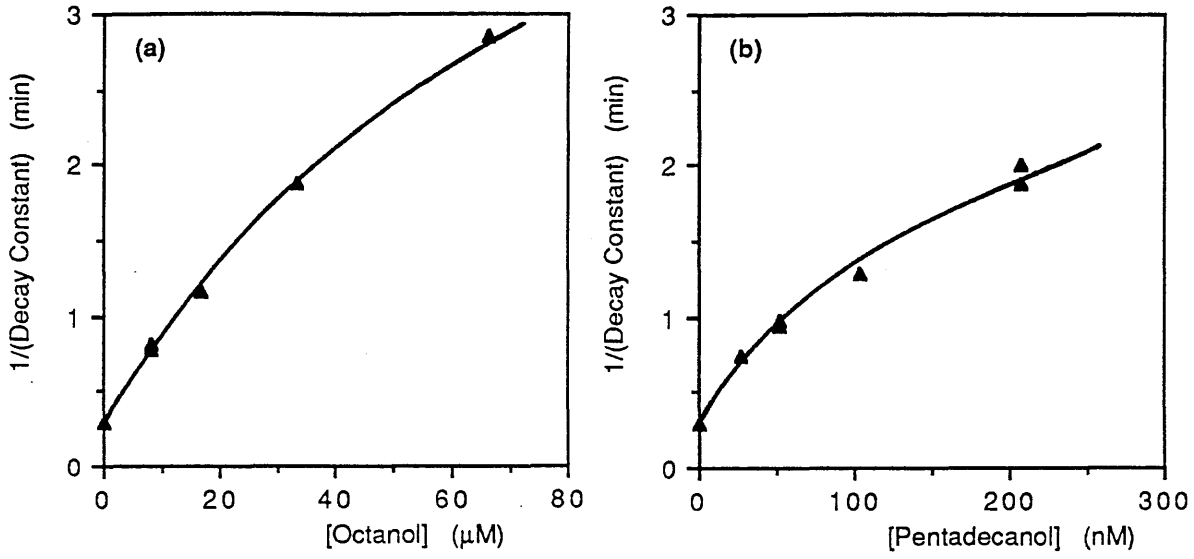

Figure 6.12: The stabilisation of intermediate II by (a) octanol and (b) pentadecanol as functions of alcohol concentration. Procedures and conditions were as described in the legend to Figure 6.11.

E' is the luciferase–peroxyflavin intermediate and E'A is the complex of this intermediate with the stabilising alcohol, A. P represents the products of decay, FMN and H_2O_2. k_0 and k_1 are respectivley the rate constants for the spontaneous decay of E' and E'A, where $k_0 \gg k_1$. k_{on} and k_{off} are the rates of association and dissociation for the interaction between intermediate II and the alcohol. It is assumed that k_{on} and k_{off} are both faster than the decay constants k_0 and k_1. This allows an equilibrium constant K_D to be defined as:

$$K_D \equiv \frac{k_{on}}{k_{off}} = \frac{[E'][A]_f}{[E'A]} \qquad (6.2)$$

where $[A]_f$ is the free alcohol concentration. If the alcohol concentration is greatly in excess of the total luciferase concentration (as in all my experiments), then $[A]_f \simeq [A]$. At a given time, t, the total concentration of intermediate II remaining is:

$$[E']_{tot} = [E'] + [E'A]$$

$$= [E']\left[1 + \frac{[A]}{K_D}\right] \qquad (6.3)$$

The rate of change of $[E']_{tot}$ is:

$$\frac{d}{dt}[E']_{tot} = -k_0[E'] - k_1[E'A] \qquad (6.4)$$

(assuming a fast equilibrium). Using equations 6.2 and 6.3 to substitute for [E'] and [E'A], equation 6.4 becomes:

$$\frac{d}{dt}[E']_{tot} = \frac{-(k_0 + k_1[A]/K_D)}{(1 + [A]/K_D)} \cdot [E']_{tot} \qquad (6.5)$$

Multiplying the right hand side of this equation above and below by K_D and integrating yields:

$$\log_e\left[\frac{[E']_{tot}}{[E']^{\circ}_{tot}}\right] = \frac{-(k_0K_D + k_1[A])}{K_D + [A]} \cdot t = -\lambda t \qquad (6.6)$$

where $[E']^{\circ}_{tot}$ is the intermediate concentration at time t = 0. The fractional concentration $[E']_{tot}/[E']^{\circ}_{tot}$ is directly proportional to the peak intensity of assays of the luminescent activity of diluted aliquots of the intermediate. Although the interval between sampling and assaying for luminescent activity and the alcohol concentration both affect the observed peak intensity, as long as these are the same for each assay of aliquots from a particular preparation of intermediate, they will not distort the proportional relationship. The decay constants calculated from the gradients of plots of $\log_e$(Peak Intensity) *vs.* [A] (*e.g.* Figure 6.11) are therefore given by the equation:

$$\lambda = \frac{k_0K_D + k_1[A]}{K_D + [A]} \qquad (6.7)$$

The lifetime of intermediate II (τ) is simply the reciprocal of this decay constant so that:

$$\tau = \frac{1}{\lambda} = \frac{K_D + [A]}{k_0K_D + k_1[A]} \qquad (6.8)$$

which accounts for the hyperbolic form of the plots of τ against alcohol concentration shown in Figures 6.12(a) and (b). It would have been possible to fit this equation to the data in order to determine estimates of k_1 and K_D. However, a more convenient method is to transform this equation into a linear relationship so

that uncomplicated methods of linear regression may be applied. Such a transformation may be made if an accurate value of k_0 is available and this presents no problem since k_0 can be precisely determined by multiple direct measurements of the rate of decay of intermediate in the controls. (k_0 was determined to be -3.72 ± 0.07 at 25°C). The difference between the stabilised lifetime and that of the controls may be defined as:

$$\Delta\tau \equiv \frac{1}{\lambda} - \frac{1}{k_0} = \frac{K_D + [A]}{k_0K_D + k_1[A]} - \frac{1}{k_0} \qquad (6.9)$$

$$= \frac{[A](k_0 - k_1)}{(k_0K_D + k_1[A]).k_0} \qquad (6.10)$$

Inverting 6.10 gives:

$$\frac{1}{\Delta\tau} = \frac{k_0^2K_D}{(k_0 - k_1)} \cdot \frac{1}{[A]} + \frac{k_0k_1}{(k_0 - k_1)} \qquad (6.11)$$

Thus a plot of $1/\Delta\tau$ against $1/[A]$ gives a straight line of the form $y = mx + c$ wherein:

$$m = \frac{k_0^2K_D}{(k_0 - k_1)} \quad \text{and} \quad c = \frac{k_0k_1}{(k_0 - k_1)} \qquad (6.12)$$

The data can be fitted to equation 6.11 using the method of weighted least squares. The weights of each data point were taken as the inverse of the square of the standard error (s.e.) in $1/\Delta\tau$ (Topping, 1962); standard errors were calculated from the standard errors in $1/\lambda$ (themselves derived from the least squares fit to the decay of luminescent activity in graphs such as Figure 6.11) by the equation:

$$s.e.(1/\Delta\tau) = \frac{d(1/\Delta\tau)}{d(1/\lambda)} . s.e.(1/\lambda) \qquad (6.13)$$

(Wilkinson, 1960). This gives:

$$s.e.(1/\Delta\tau) = \frac{[s.e.(1/\lambda)]^2}{\left[\frac{1}{\lambda} - \frac{1}{k_0}\right]^4} \qquad (6.14)$$

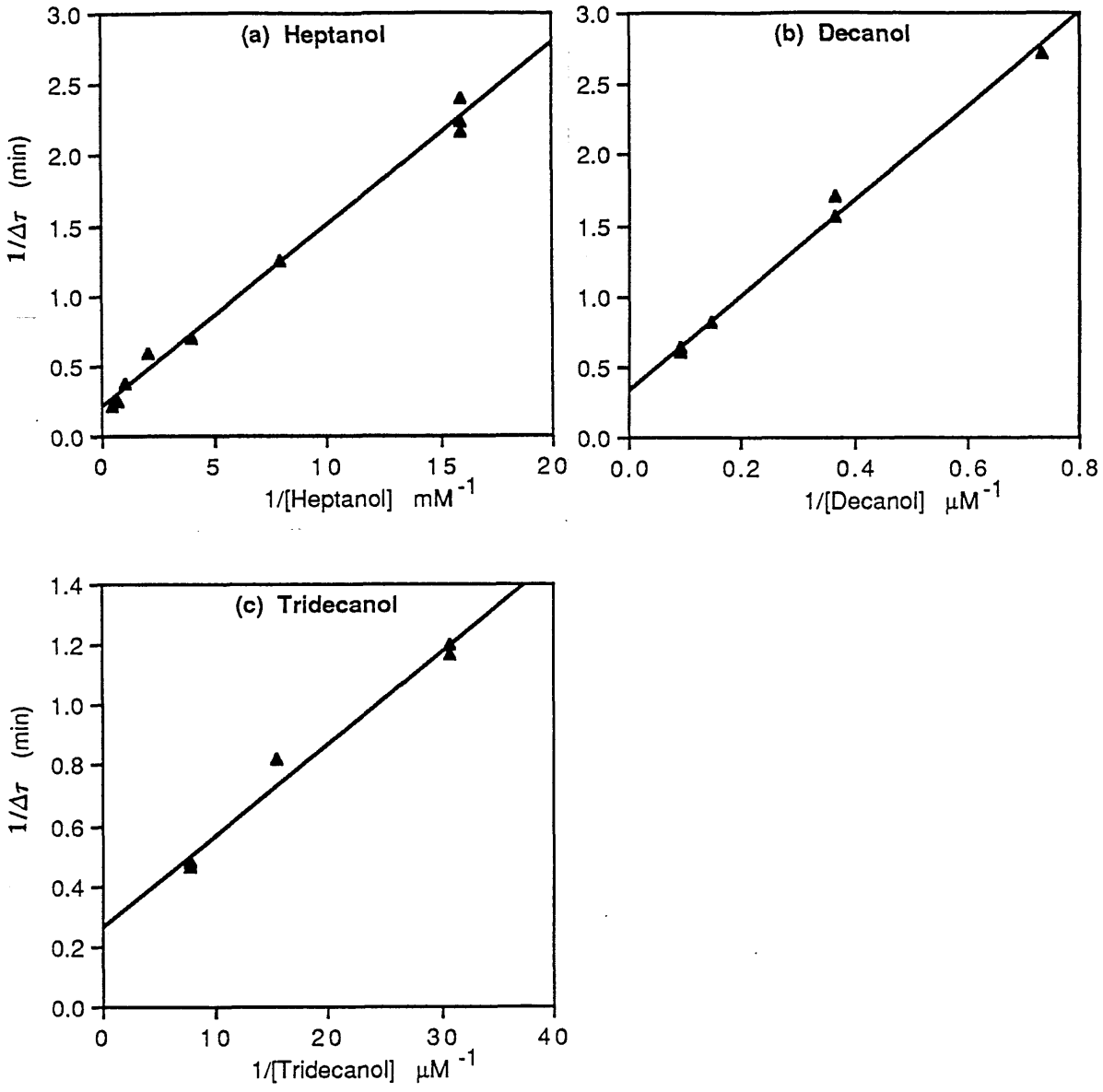

Figure 6.13: Example plots of $1/\Delta\tau$ against the reciprocal of the alcohol concentration for (a) heptanol, (b) decanol and (c) tridecanol. Within experimental error, the data all lie on straight lines – indicating that the model developed in the text is an adequate description of the process of stabilisation. Experimental conditions were essentially as described in the legend to Figure 6.11.

Since $\frac{s.e.(1/\lambda)}{[1/\lambda - 1/k_0]}$ was approximately constant, the weights were therefore taken as equal to $(1/\lambda - 1/k_0)^2$. This analysis was applied to the stabilisation data obtained for each agent. Figures 6.13(a)–(c) for heptanol, decanol and tridecanol indicate that plots of $1/\Delta\tau$ against 1/[A] are indeed satisfactorily linear. The best fit gradient, m, and intercept, c, (and their standard errors) were calculated with a BASIC computer program and these parameters may be used to calculate k_1 and K_D by rearranging

equation 6.12. Thus:

$$k_1 = \frac{k_0 c}{k_0 + c} \qquad \text{and} \qquad K_D = \frac{(k_0 - k_1)m}{k_0^2} \qquad (6.15)$$

The values of k_1 and K_D are summarised for alcohols from C_6 to C_{16} in Table 6.5. The stabilisation of intermediate II by undecane and halothane was also investigated. Data for these agents are included in Table 6.5. Halothane caused only a small degree of stabilisation even at concentrations close to its solubility limit (17.5 mM). The k_1 and K_D values for halothane are therefore only estimates.

The rate constants for the decay of the luciferase–peroxyflavin–alcohol complex, k_1, show no systematic variation with chain length (Figure 6.14). The fluctuations which are apparent may simply be due to random experimental error – although the values of k_1 determined for octanol and undecanol appear to be exceptionally low. The dissociation constants, K_D, for alcohols binding to intermediate II are plotted against chain length in Figure 6.15. The dissociation constants obtained by Tu (1979) using a similar experimental method are also included (as an inset) in Figure 6.15 as are the dissociation constants, K_i, determined in this present study from inhibition experiments. Several interesting features are apparent. Firstly, note that Tu's results differ in value but not in form from the K_D values measured in this work. This may be due to differences in our experimental methods. A final concentration of 0.1% bovine serum albumin was present in Tu's intermediate solution which may account for the fact that his dissociation constants for decanol, dodecanol and tetradecanol are all higher than observed here. The fact that Tu's result for octanol is *lower* than reported here is more difficult to explain.

The similarities and differences in the K_i and K_D curves in Figure 6.15 are of particular interest. From hexanol to decanol K_i and K_D are more or less equal – even to the extent that the kink between C_8 and C_9 is reproduced in the K_D curve. Beyond C_{10} the curves diverge rapidly; between C_{10} and C_{12} the values of K_D drops by nearly two orders of magnitude. This decline halts abruptly at C_{12} and there is no significant difference between the dissociation constants (K_D) of dodecanol, tridecanol, tetradecanol and pentadecanol. In the following sub–sections the implications of these features are discussed in full.

Table 6.5: Dissociation (K_D) and decay (k_1) constants determined for complexes of intermediate II with n–alcohols, undecane and halothane. T = 25°C.

Agent	K_D (± s.e.)	k_1 (± s.e.) (min^{-1})
Hexanol	222 ± 7 μM	0.449 ± 0.028
Heptanol	32.2 ± 2.6 μM	0.225 ± 0.123
Octanol	4.76 ± 0.03 μM	0.077 ± 0.007
Nonanol	4.03 ± 0.08 μM	0.536 ± 0.008
Decanol	0.746 ± 0.039 μM	0.408 ± 0.069
Undecanol	0.130 ± 0.007 μM	0.134 ± 0.085
Dodecanol	8.74 ± 2.0 nM	0.369 ± 0.085
Tridecanol	7.11 ± 0.37 nM	0.288 ± 0.059
Tetradecanol	7.03 ± 1.02 nM	0.401 ± 0.172
Pentadecanol	7.91 ± 0.93 nM	0.573 ± 0.082
Hexadecanol	64 ± 9 nM	0.457 ± 0.068
Undecane	1.80 ± 0.34 nM	0.541 ± 0.132
Halothane	21 mM	0.36

Control Decay Constant: k_0 = 3.72 ± 0.07 min^{-1} at 25°C

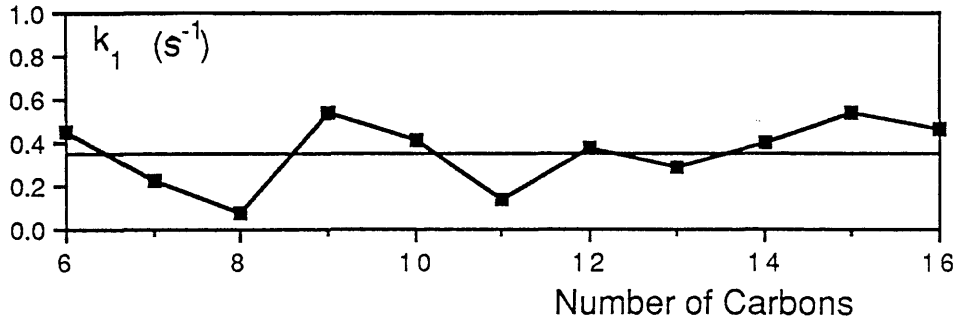

Figure 6.14: The variation in k_1 (the decay constant of the intermediate II–alcohol complex) for alcohols from hexanol to hexadecanol. The solid horizontal line represents the average value of k_1 = 0.35 min^{-1}.

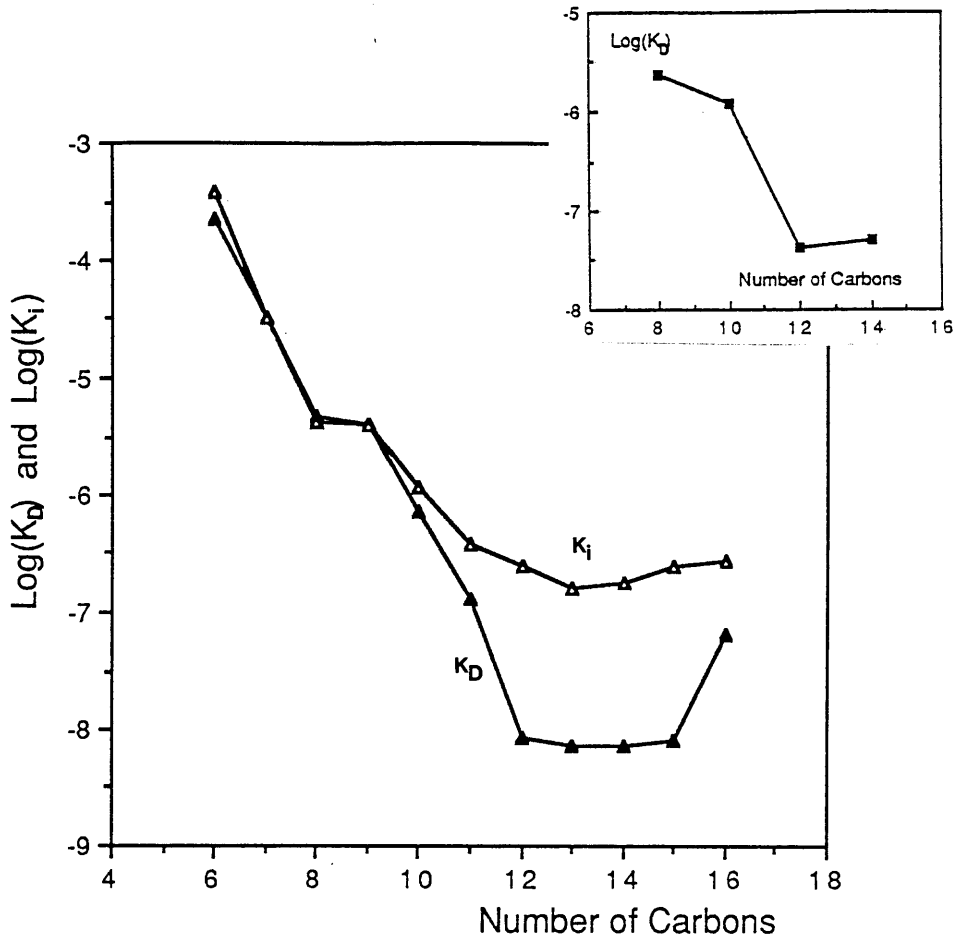

Figure 6.15: Comparison of K_D and K_i. Solid triangles: dissociation constants (K_D) for the interaction of alcohols with intermediate II determined by stabilisation experiments. Open triangles: dissociation constants (K_i) derived from experiments to measure the inhibition by n−alcohols of the luciferase reaction in vitro. The data were taken from Tables 6.5 and 4.2(a). Inset: dissociation constants (K_D) for the interaction of alcohols with intermediate II determined by Tu (1979).

6.2(d) Confirmation of the Cut−Off Effect for Bacterial Luciferase

The K_D curve in Figure 6.15 levels off for alcohols longer than dodecanol. Since there is no observed difference in the mechanism of stabilisation by alcohols of different lengths, it may be concluded that this trend genuinely reflects the inability of alcohols longer than dodecanol to bind any tighter to the luciferase−peroxyflavin complex. It therefore seems likely that the parallel levelling off in the K_i curve, which results ultimately in the luciferase cut−off, occurs for the

same reasons. In section 5.3 I reasoned that since long-chain alcohols (> C_{11}) act in the same way on the luminescent reaction, all inducing biphasic decay of the light output, their K_i values may be compared with one another. Consequently, I concluded, the levelling off, and hence the cut-off, are real effects; this reasoning is borne out by the measurements of long-chain alcohol dissociation constants for binding to intermediate II. Presumably, therefore, the alkane cut-off which also coincides with the appearance of biphasic luminescence decay, is also a real effect.

Even so, the precise effect of the advent of biphasic luminescence decay on the measurement of the inhibition of the luciferase reaction by alcohols and alkanes of increasing chain length remains to be elucidated. A further question as to why the K_D values of long-chain alcohols and alkanes are around 30 times less than the corresponding K_i must also be addressed. These problems will be considered in the next subsection where it will be shown that they do not affect the finding that the luciferase cut-off is genuine.

Previously (section 5.3) the observation that alcohols longer than a critical length do not bind any tighter to luciferase, even though they are increasingly hydrophobic, was interpreted in terms of an interaction with a binding pocket of finite volume. Thus, judging from Figure 6.15, dodecanol appears to fill the pocket on the luciferase-peroxyflavin intermediate; the additional methylene groups of longer alcohols may therefore protrude into water from where they can make no contribution to the binding energy. If this is the case, dodecanol may define the length and volume of the luciferase pocket as approximately 16 Å and 225 ml/mol respectively. Finally, it only remains to be emphasised that the luciferase cut-off provides a simple explanation for the observation of a cut-off in anaesthetic potency as one ascends the homologous series of *n*-alcohols and *n*-alkanes and thus supports the concept of a protein site of anaesthetic action in the central nervous system.

6.2(e) Binding of Long-Chain Alcohols to Intermediate II – An Explanation of the Biphasic Luminescence Decay Induced by Long-Chain Inhibitors

The K_i and K_D values of *n*-alcohols, which are more or less equal from C_6 to C_{10}, diverge quite suddenly thereafter. The K_i for undecanol is 3 times greater than its K_D. For dodecanol there is a factor of 29 between these constants and a similar factor differentiates the K_i and K_D values of tridecanol, tetradecanol and pentadecanol. Significantly, the alcohol for which the divergence begins, undecanol,

is also the first alcohol to induce biphasic decay in the light output of the luciferase reaction; it may be argued that this is not coincidental.

I propose that the difference in K_i and K_D results from the much greater affinity of the luciferase–peroxyflavin intermediate for long–chain alcohols (and alkanes) than the luciferase enzyme on its own. Consider an $FMNH_2$–initiated assay of the luciferase reaction in the presence of a long–chain alcohol. Prior to initiation, the reaction vial contains luciferase in equilibrium with the substrate, *n*–decanal, and the long–chain alcohol (LHS of Figure 6.16). The luciferase enzyme is present on its own and in complexes with either *n*–decanal or the long–

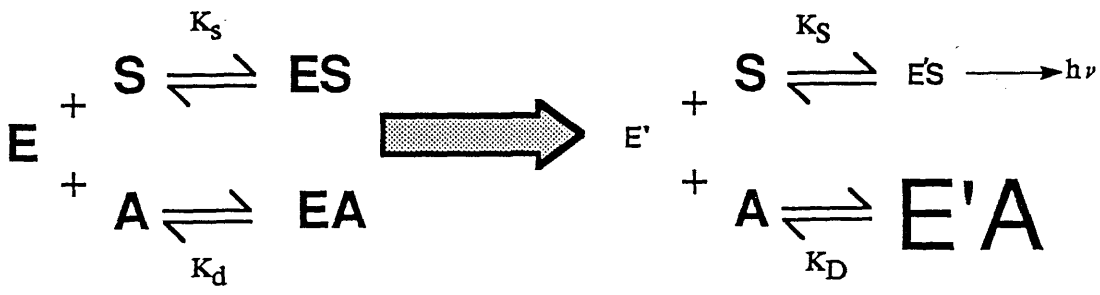

Figure 6.16: *Schematic represenation of the shift in binding equilibrium of long–chain alcohols upon conversion of luciferase (E) to the luciferase peroxyflavin, intermediate II (E'). S and A are n–decanal and a long–chain alcohol respectively. They form complexes with luciferase (ES and EA) with dissociation constants K_S and K_d. Upon initiation of the reaction, E converts to E'; the new dissociation constants of the interaction of S and A with E' are K_S and K_D. Since $K_D/K_d \ll K_S/K_S$, the equilibrium shifts in favour of the E'A complex. The relative sizes of ES, EA, E'S and E'A in the diagram give an indication of this shift. The precise values of K_S, K_S and K_d are unknown. However, K_S is likely to be close to the value of the Michaelis constant for decanal. Later in this section, it will be pointed out that K_d is probably close to, but somewhat greater than K_i, the dissociation constant derived from inhibition experiments.*

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chain alcohol. [The possibility that the aldehyde substrate and competitive inhibitors can bind to the enzyme in the absence of $FMNH_2$ has recently been demonstrated (Holzman and Baldwin, 1981, 1983)]. The relative concentration of the luciferase–alcohol complex depends on the affinity of the enzyme for the alcohol (as well as on its affinity for the aldehyde and the concentrations of decanal and

alcohol present). Upon injection of $FMNH_2$, those luciferase molecules which are complexed with decanal immediately proceed along the reaction pathway, producing photons and hence a rapid rise in luminescence. At the same time, however, luciferase is converted to the luciferase–peroxyflavin form. This conversion is extremely rapid since the rate constants for the reaction of $FMNH_2$ with luciferase and the subsequent reaction of O_2 with the luciferase–$FMNH_2$ complex are both about 2×10^8 $M^{-1}s^{-1}$ (*V. fischeri*; Hastings and Gibson, 1963) and since neither the concentration of $FMNH_2$ nor that of O_2 were rate–limiting under experimental conditions. As a result of the conversion, the affinity for the long–chain alcohol is greatly enhanced; this enhancement exceeds any increase in affinity for the substrate, *n*–decanal. The binding equilibrium is thus shifted heavily in favour of the intermediate II–alcohol complex, a process which causes depletion of the relative proportions of the free intermediate and the intermediate complexed with decanal (RHS of Figure 6.16). Soon thereafter, the rapidly increasing luminescence reaches a maximum and then declines. Since this decline is strongly influenced by the extra binding, following conversion, of the long–chain alcohol at the high affinity site on intermediate II, one would predict that the rate constant associated with this initial phase of luminescence decay should be faster than in the absence of long–chain alcohols and that it should increase with alcohol concentration. Figure 4.23(c), which illustrates the dependence of this rate constant on the concentration of dodecanol present, shows that this is indeed the case. Following this initial phase, a new equilibrium between intermediate II, decanal and the long–chain alcohol is established. In inhibition experiments, the alcohol concentrations used ranged typically from 1 to 6 times the value of K_i – which corresponds to about 30 to 180 times the K_D. Since the decanal concentration was normally around 0.85 μM (= K_m), the new equilibrium is dominated almost entirely by the intermediate II–alcohol complex. The rate of the reaction and hence the rate of luminescence decay under these conditions are thus limited by the rate at which intermediate II is released from its complex with the alcohol. This is what I have referred to as the final phase of the luminescence decay (section 4.4).

Two simple predictions derive from this explanation of the biphasic nature of the luminescence decay in the presence of long–chain alcohols. Firstly, if the biphasic decay of luminescence does indeed result from the very different affinities of luciferase and intermediate II for long–chain alcohols, one would expect to see only exponential decay of the luminescence evoked by the injection of *n*–decanal into a vial containing intermediate II in equilibrium with a long–chain alcohol. In this case, since the conversion of luciferase to the high affinity form (intermediate II) is complete before initiation of the luminescent reaction, one would not expect to

see the initial fast rise and fall of luminescence which is associated with the shift in binding affinities that accompanies this conversion (in reactions initiated by $FMNH_2$). Secondly, one would also predict that, under conditions of equal luciferase, reactant and alcohol concentrations, the decay of luminescence in the decanal–initiated reaction would proceed at the same rate as the final phase of luminescence decay in an $FMNH_2$–initiated reaction.

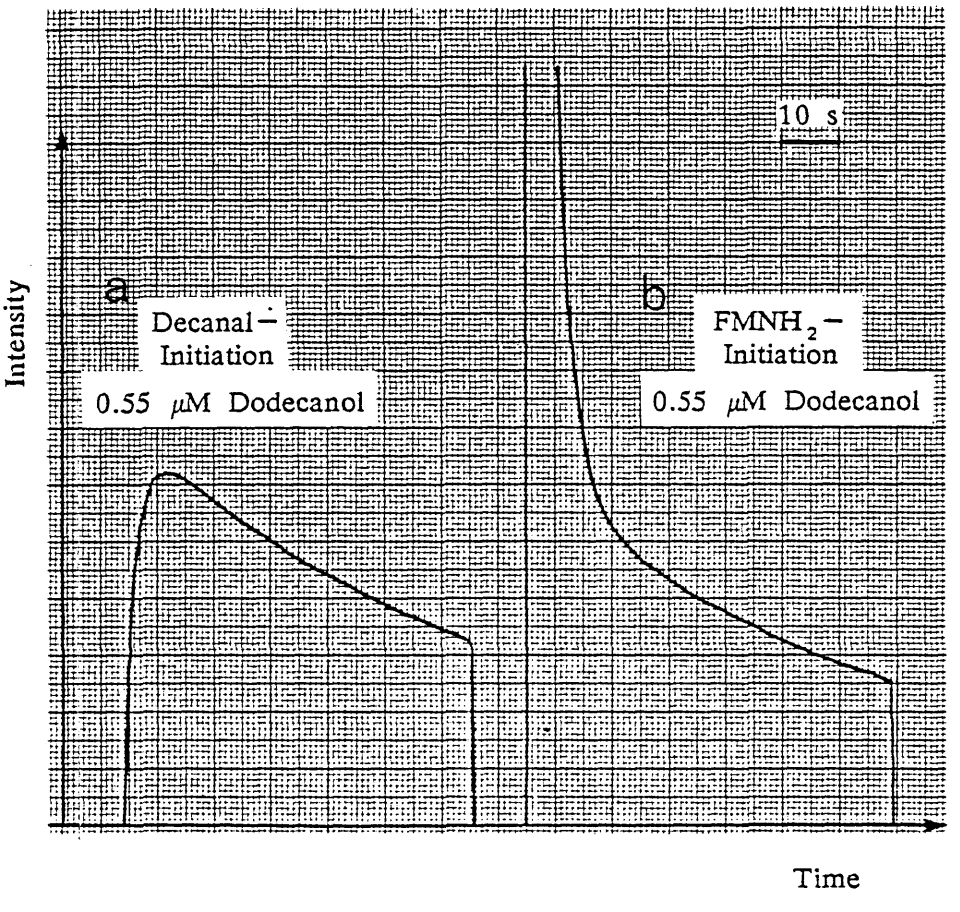

Figure 6.17: Comparison of the time–dependence of the luminescent output of the in vitro luciferase reaction initiated by (a) decanal and (b) $FMNH_2$ in the presence of a final dodecanol concentration of 0.55 μM. The profiles are traced from chart recorder output. Decanal–initiation: a sample of intermediate II was prepared and allowed to equilibrate with dodecanol and the luciferase reaction started by injecting decanal. $FMNH_2$–initiation: luciferase was equilibrated with decanal and dodecanol and the reaction initiated by injection of $FMNH_2$. Final concentrations were identical in the two cases: decanal, 0.85 μM; $FMNH_2$, 110 μM; luciferase, 0.2 nM. $T = 24.5^{\circ}C$. Note that the intensities of the two signals were not recorded on the same scale. The chart recorder gain was adjusted for the $FMNH_2$–initiated assay to give an output level in the final phase of decay which was comparable to the output recorded in the decanal–initiated assay. As a result, the peak of the $FMNH_2$–initiated assay was off–scale.

These predictions were tested experimentally for dodecanol. In $FMNH_2$–initiated assays, 2.5 ml of 329 μM $FMNH_2$ was injected into a vial containing 2.5 ml of 2.56 μM decanal, 2.5 ml of a buffer solution of dodecanol and 10 μl of a stock solution of luciferase. By the decanal–initiation method, 2.5 ml of 2.56 μM decanal was injected into a solution of intermediate II which had been prepared by the injection of 2.5 ml of 329 μM $FMNH_2$ into a vial containing 2.5 ml of a dodecanol solution and 10 μl of luciferase. Final concentrations were: luciferase, 0.2 nM; $FMNH_2$, 110 μM; decanal, 0.85 μM (= K_m). Final dodecanol concentrations ranged from 0 to 0.55 μM. (See sections 3.4(a) and (b) for further experimental details). Figure 6.17 shows the luminescence decay of the two different assay methods in the presence of 0.55 μM dodecanol. The profile of the flavin initiated reaction is characteristically biphasic; the large initial spike (off–scale) is followed by a very slow decay phase. As predicted, the decay of luminescence in the reaction initiated by decanal is exponential. Furthermore, the decay rate of this reaction appears to be very similar to the decay rate of the final phase in the flavin–

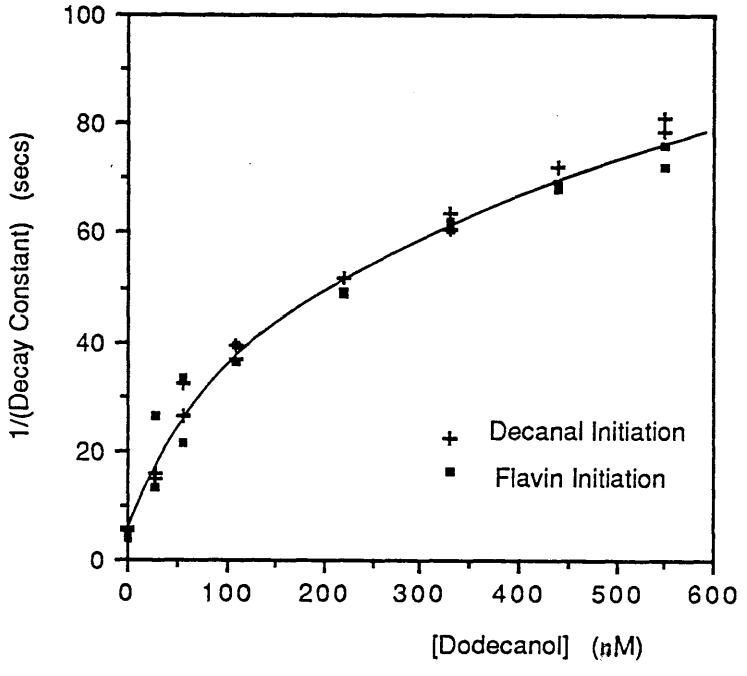

Figure 6.18: The decay constant of the final phase of decay of the $FMNH_2$–initiated reaction varies in exactly the same way with dodecanol concentration as the decay constant measured for decanal–initiated reactions. Experimental conditions, other than the order of addition of substrates, were identical for both assay methods – see legend to Figure 6.17 for details. This result was predicted by the hypothesis proposed to account for the appearance of biphasic luminescence decay in luciferase reactions initiated by $FMNH_2$ in the presence of long–chain alcohols.

initiated reaction. The rate constants associated with these decays were determined at a number of dodecanol concentrations by a simple regression analysis of logarithmic plots of the luminescence as a function of time. The dependence of the reciprocals of these decay constants on the dodecanol concentration is shown in Figure 6.18. It is quite clear from this graph that the decay of luminescence which follows the injection of decanal into a solution of intermediate II in equilibrium with dodecanol occurs at the same rate as the late phase of luminescence decay in the $FMNH_2$–initiated reaction. The second prediction is thus also borne out. Hence, the experimental evidence strongly supports the hypothesis that luciferase has a lower affinity for *n*–alcohols than the luciferase–peroxyflavin intermediate. The observation that the K_D of undecane is also much less than its K_i value (by a factor of 30) indicates that the same hypothesis accounts for the biphasic luminescence decay induced by long–chain alkanes. Additionally, this latter result infers that the hydroxyl group on alcohol molecules plays no special role in the ability to stabilise intermediate II.

Reinterpretation of the Meaning of K_i for Long–Chain Alcohols and Alkanes.

Although the model developed for the analysis of inhibition experiments in section 4.6 assumes that K_i is the dissociation constant for the binding of the inhibitor to intermediate II, it is evident from the results of stabilisation experiments that this has to be modified for alcohols longer than decanol. In fact, according to the explanation of the biphasic decay induced by long–chain alcohols, outlined above, it appears that the K_i may more closely reflect the dissociation constant for the binding of these agents to the luciferase enzyme on its own. In the $FMNH_2$–initiated assays which were used to measure inhibition of long–chain alcohols, the peak intensity is controlled both by the equilibrium between luciferase and its complexes with decanal and the alcohol prior to initiation and by the rate at which the equilibrium shifts in favour of the intermediate II–alcohol complex following the transformation of luciferase into intermediate II (which has a much higher affinity for the alcohol). This latter process will render the peak intensity less than it would otherwise have been. Thus the value of K_i is actually lower than the true luciferase–alcohol dissociation constant. In all probability, since the difference between K_i and K_D for C_{12} to C_{15} alcohols is approximately constant ($\simeq$ 30, see Figure 6.15), the K_i values parallel the true dissociation constants. It is not possible, however, to determine the magnitude of the difference between them. In any case, this result indicates that the advent of biphasic kinetics does not introduce any artefactual difficulties into the observation of a cut–off in the inhibitory potencies of long–chain alkanes and alcohols. Similar considerations and reasoning apply also to alkanes and the alkane cut–off.

Why does the luciferase–peroxyflavin intermediate bind long–chain hydrocarbons so much tighter than the luciferase enzyme on its own?

There is evidence to suggest that the binding of $FMNH_2$ stabilises a conformation of luciferase in which the aldehyde pocket has a high affinity for hydrophobic inhibitor molecules. Holzman and Baldwin (1981) showed that the affinity of *V. harveyi* luciferase for 2,2–diphenylpropylamine, which competes with the aldehyde substrate, was enhanced in the presence of reduced flavin. The notion that this is the result of a conformational change arises because the same effect was observed simply by raising the phosphate concentration (Holzman and Baldwin, 1982). In addition, it has been shown that phosphate protects *V. harveyi* luciferase against proteolytic attack by trypsin and chymotyrpsin and that the anion enhances the thermal stability of luciferase (Holzman and Baldwin, 1980). Both of these findings have been interpreted as due to conformational stabilisation. It is known that high phosphate antagonises the binding of $FMNH_2$ (Meighen and Mackenzie, 1973) – presumably by binding to the site of attachment of the phosphate moiety at the end of the ribityl side–chain on flavin. Thus the binding of flavin might be expected to induce the same conformational stabilisation as phosphate. It may be that the stabilised conformation also has a much higher affinity for alcohols and alkanes.

An alternative or additional reason for the tighter binding of long–chain alcohols and alkanes to intermediate II may be that the flavin substrate, when bound, forms part of the aldehyde binding site. Such an alteration to this site might conceivably aid the binding of hydrocarbon compounds. The possible role of the secondary hydroxyl groups in the ribityl side–chain of the flavin substrate in the interaction between alcohols and intermediate II has already been noted (section 5.3). However, these groups are unlikely to be involved in the differential affinities of luciferase and intermediate II for *n*–alcohols since the corresponding difference in the affinities for undecane, which contains no polar hydroxyl group, is the same (factor of 30). There is considerable experimental evidence which is consistent with the notion of adjacent flavin and aldehyde binding sites on *Vibrio harveyi* luciferase – a feature which is implicit in the fact that the enzyme catalyses a reaction between these compounds. Fried and Tu (1984) showed that the affinity labeling probe, 2–bromo–[1–^{14}C] 1–decanal could not bind to the enzyme in the presence of the aldehyde substrate; but when it did (in the absence of aldehyde), the modified enzyme had no affinity for $FMNH_2$. This result was repeated with the photo–activated labeling probe, 1–diazo–2–oxoundecane (Tu and Henkin, 1983). In other studies inactivation of luciferase by both *n*–ethylmaleimide (Nicoli *et al.*, 1974) and ethoxyformic anhydride (Cousineau and Meighen, 1976) was impeded by

the presence of either the aldehyde or flavin substrates.

6.2(f) The Stabilisation of Intermediate II by Short−Chain Alcohols

The divergence between K_i and K_D increases beyond decanol and is responsible for the biphasic nature of the kinetics of luminescence decay in the presence of long−chain alcohols. For alcohols from hexanol to decanol, however, the corresponding values of K_i and K_D are very similar. This is also true for halothane and probably extends to all agents which do not produce biphasic kinetics. There are two possible explanations for this observation. It may be that there is little or no difference between the dissociation constants of short−chain ($< C_{10}$) alcohols for luciferase and for intermediate II. Perhaps only long−chain alcohols are able to take advantage of the apparent changes in the aldehyde binding site which occur with the formation of intermediate II. An alternative explanation may be that short−chain alcohols do indeed bind tighter to intermediate II than to luciferase but that this increase in affinity is not as great as the increase in affinity for the substrate, *n*−decanal. If this is the case, the observed absence of biphasic luminescence decay with these agents follows from the fact that conversion from luciferase to intermediate II (upon injection of $FMNH_2$) would favour the complex with the substrate, rather than the intermediate II−alcohol complex [opposite to the case of long−chain alcohols − see section 6.2(e)]. The action of short−chain alcohols would simply be to cause a general depression of the reaction, reducing the peak intensity and the decay rate. This explanation naturally explains why K_i and K_D diverge beyond C_{10} when *n*−decanal is used as a substrate. One possible way to test it would be to observe whether the use of an aldehyde substrate which is longer or shorter than decanal produces a corresponding shift in the alcohol chain length at which biphasic luminescence decay first appears. However, this has yet to be attempted.

Whatever the precise reason for the similarity between their K_i and K_D values, it is of interest that short−chain alcohols (and halothane) cause significant stabilisation of intermediate II at concentrations in the vicinity of their inhibitory ED_{50} concentrations. For example, an ED_{50} concentration of heptanol (66.7 μM) was calculated, using equation 6.7, to reduce the decay rate of intermediate II by 3−fold. The stabilisation caused by such inhibitors can account for a result which was noted in section 4.4. Figure 6.19 shows the effect on the peak intensity and the luminescence decay constant of the $FMNH_2$−initiated luciferase reaction of (i) a reduction in the decanal concentration from 1.10 μM and (ii) an increase in the

concentration of heptanol, a competitive inhibitor, in the presence of 1.10 μM decanal. According to a simple−minded view of competitive inhibition one would predict that the data points in Figure 6.19 should all lie on the same line, since the

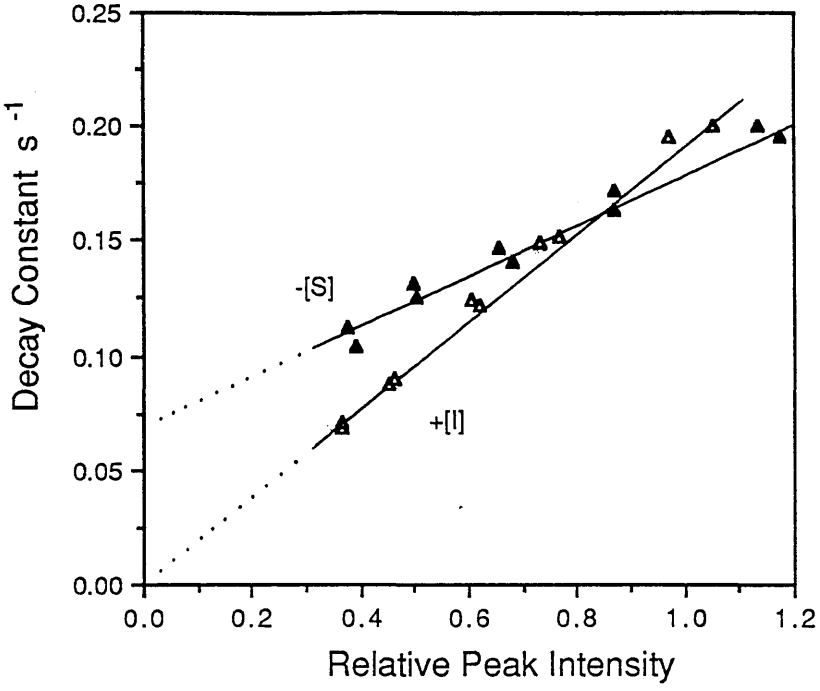

Figure 6.19: Reducing the substrate concentration (−[S]) does not have the same effect on the relationship between the maximum rate (peak intensity) and the luminescence decay constant as increasing the concentration of heptanol, a competitive inhibitor (+[I]). The relative peak intensities have been normalised so that a decanal concentration of 1.1 μM at 25°C gives unit peak intensity. Closed triangles: data points correspond to decanal concentrations from 1.67 to 0.25 μM − (the peak intensity at 1.1 μM was determined, for normalisation purposes, by interpolation). T = 23.3°C. Open triangles: data points correspond to heptanol concentrations from 0 to 0.14 mM (in the presence of 1.1 μM decanal). T = 25.0°C. Final concentrations of $FMNH_2$ and luciferase were 95 and 0.4 nM respectively.

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addition of a competitive inhibitor normally only causes an effective reduction of the substrate concentration [by a factor of $(1 + [I]/K_i)^{-1}$, where [I] is the inhibitor concentration and K_i its dissociation constant]. Clearly, this is not the case with bacterial luciferase. As the substrate concentration is reduced to zero the luminescence decay constant approaches a non−zero value; yet as the inhibitor concentration is increased the decay constant appears to go to zero. This difference

may be attributed to the stabilising effect of inhibitors.

Since each luciferase molecule turns over only once, the luminescence decay rate is influenced by the luminescent reaction rate and the rates of non-productive decay of intermediate II and other dark side-reactions. As the substrate concentration is lowered, the proportion of intermediate II molecules not complexed with substrate rises. Therefore the contribution of the rate of decay of intermediate II to the luminescence decay rate rises accordingly. From Figure 6.19 it is apparent that as [S] → 0 (*i.e.* as $v_{pk} \rightarrow 0$) the luminescence decay constant approaches a limiting value of around 0.068 s^{-1} (or 0.086 s^{-1} in the case of the "faster" enzyme – see section 3.1). These values are close to the observed rate constant for the decay of intermediate II in comparable conditions (k_0 = 3.72 min^{-1} = 0.062 s^{-1}; note that all stabilisation experiments were performed using the "faster" enzyme).

In contrast, as the inhibitor concentration is increased, the proportion of intermediate II molecules complexed with an inhibitor molecule rises. This proportion approaches unity as the inhibitor concentration becomes very high and the luminescence decay constant should approach the decay constant of the intermediate II-inhibitor complex. From stabilisation experiments it is known that the rate constant for such complexes (k_1) averages at 0.35 min^{-1} = 0.006 s^{-1}. Figure 6.19 shows that the estimated luminescence decay constant at high inhibitor concentrations (*i.e.* as $v_{pk} \rightarrow 0$) probably approaches this value. Thus it is not precisely correct to state that the reduction in the peak intensity caused by most inhibitors is proportional to the retardation of luminescence decay that they cause, although I have suggested in previous sections (see Chapter 4) that this is *apparently* so.

Finally, since lowering the substrate concentration raises the proportion of free intermediate II complexes, and hence the rate of spontaneous (non-productive) decay of these complexes, the total light output of the luciferase reaction falls. The decline in the quantum yield of $FMNH_2$-initiated reactions as a function of the decanal concentration is shown in Figure 6.20(a). Although raising the concentration of an inhibitor has the same effect on the peak intensity as lowering the substrate concentration, it actually helps to preserve intermediate II from decay. Consequently, as Figure 6.20(b) shows, the quantum yield of the luciferase reaction is actually conserved as the concentration of hexanol increases (and the peak intensity falls).

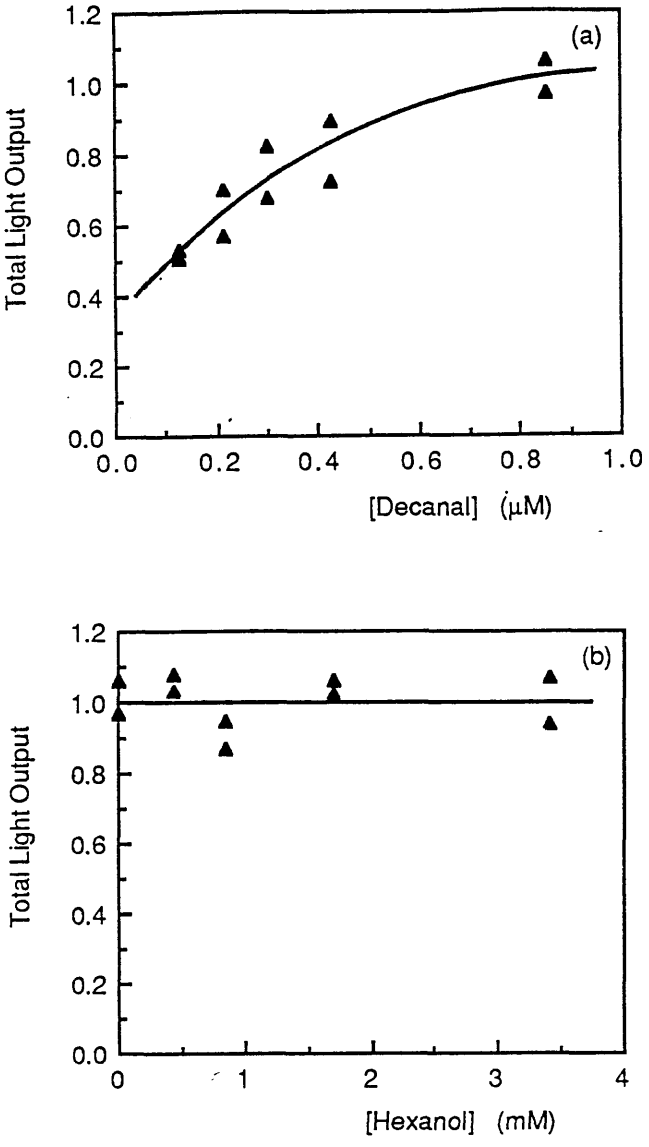

Figure 6.20: Variation of the total light output from $FMNH_2$–initiated luciferase reactions as functions of (a) substrate concentrations [0.13–0.85 μM decanal] and (b) the concentration of a competitive inhibitor, hexanol, in the presence of 0.85 μM decanal. The magnitude of the total light output has been normalised so that an uninhibited reaction with 0.85 μM decanal gives unit total light output. Final concentrations: $FMNH_2$, 92 μM; luciferase, 0.4 nM. $T = 25.9^{\circ}C$.

6.3 Investigation of the Anaesthetic Sensitivity of *Vibrio fischeri* Luciferase and the NADH:FMN Oxidoreductase from *Vibrio harveyi*

The anaesthetic sensitivity reported in this thesis for luciferase purified from *Vibrio harveyi* bacteria differs quite significantly from the results obtained with *Vibrio fischeri* bacteria and its partially purified luciferase extracts. This difference is apparent in Table 6.6 where the ED_{50} concentrations determined for *V. harveyi* luciferase are compared with a large body of previously published ED_{50} data for *V. fischeri* bacteria and luciferase and for *Photobacterium phosphoreum* bacteria. The concentrations of part of the homologous series of *n*–alcohols required to depress the luminescence from *Bacillus fischeri* bacteria are also included in the table.

Table 6.6: ED_{50} concentrations for the depression of the light output from V. harveyi, V. fischeri, Ph. phosphoreum and Bacillus fischeri luciferases and bacteria. ED_{50} values are given in mM.

	Luciferases		Bacteria			
	V.h.[1]	*V.f.*[2]	*Ph.ph.*[3]	*Ph.ph.*[4]	*V.f.*[5]	*B.f.*[6]
Agent	24°C	21°C	21°C	25°C	28°C	
Chloroform	20.6	2.14	2.15	2.03	2.09	
Halothane	11.4	1.73	0.52	0.41	0.90	
Methoxyflurane	4.14	1.54	--	0.48	0.79	
Diethyl Ether	23.8		31.0	13.7	22.0	
Urethane	261					330
Ethanol	1550					460
Propanol	544					140
Butanol	128					31
Pentanol	5.97					8.0
Hexanol	0.753					2.0
Heptanol	0.0667					0.30
Octanol	0.00855					0.07

Sources of data: [1]V. harveyi luciferase, (this thesis). [2]V. fischeri luciferase, (Adey et al., 1976). [3]Ph. phosphoreum bacteria, (Halsey and Smith, 1970). [4]Ph. phosphoreum bacteria, (White and Dundas, 1970). [5]V. fischeri bacteria, (Middleton, 1973). [6]Bacillus fischeri bacteria, (Taylor, 1934) – ED_{50}'s were estimated from Taylor's graphical data. Where anaesthetic doses were quoted as partial pressures, they were converted to aqueous concentrations using the method described in the legend to Figure 1.3.

The comparison between the different luminescent systems represented in Table 6.6 is certainly interesting. Notice that the inhibitory effect on *V. harveyi* luciferase of chloroform, halothane and methoxyflurane, all bulky halogenated agents, is much less than on *V. fischeri* or *P. phosphoreum* bacteria or *V. fischeri* luciferase. In complete contrast, *V. harveyi* luciferase is much more sensitive to alcohols longer than butanol (which cause no excitation of this enzyme) than *Bacillus fischeri* bacteria. Additionally, diethyl ether and urethane, two relatively small anaesthetics

(which, unlike small alcohols, cause no excitation of *V. harveyi* luciferase) are more or less equipotent on all the systems included in the table.

A detailed interpretation of these similarities and differences is not easy because of the variation in experimental conditions. Nonetheless, it seems possible that real structural differences between the luciferase enzymes from *V. harveyi* and *V. fischeri* species are involved. In addition, the discrepancy between the anaesthetic sensitivity of *V. fischeri* luciferase and whole bacteria suggests that there may be anaesthetic targets other than luciferase within luminescent bacteria. As a step towards a systematic investigation of these possiblities, the anaesthetic sensitivities of *V. harveyi* and *V. fischeri* luciferases were determined under similar experimental conditions. The effect of halothane on the NADH:FMN oxidoreductase enzyme, which is part of the luminescent apparatus of whole bacteria, was also investigated.

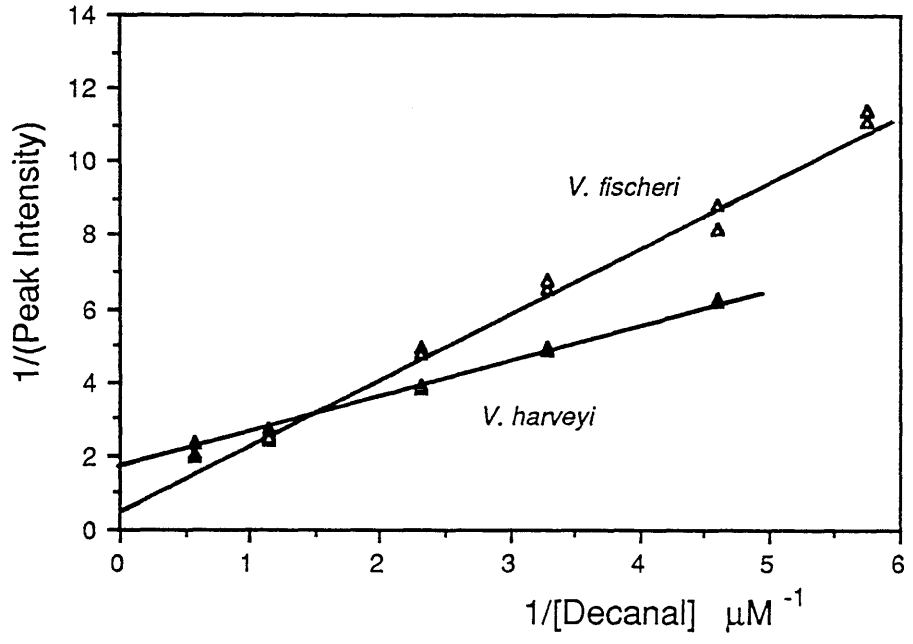

Figure 6.21: Lineweaver–Burk plots of the maximum rate (peak intensity) and decanal concentration for $FMNH_2$–initiated reactions catalysed by V. harveyi and V. fischeri luciferase. Final concentrations: $FMNH_2$, 99 μM; luciferases, ≃ 2 nM. T = 25.1°C.

6.3(a) Comparison of *V. harveyi* and *V. fischeri* luciferases

Partially purified luciferases from *V. harveyi* and *V. fischeri* were obtained from Sigma. Assays of luciferase activity were performed by the $FMNH_2$–initiation

method which is described in section 3.4(a). Firstly, the Michaelis constant (K_m) of the aldehyde substrate, *n*−decanal, was determined for each enzyme (at saturating levels of reduced flavin, 99 μM). The results of these experiments are shown in Figure 6.21 as double−reciprocal Lineweaver−Burk plots. The K_m values derived from these plots were 0.67 ± 0.04 and 5.6 ± 1.8 μM for *V. harveyi* and *V. fischeri* luciferases respectively. The result for *V. harveyi* differs only slightly from the K_m determined for the highly purified luciferase which was used in all the experiments described in this thesis (0.85 ± 0.08 μM). Next, the inhibitory ED_{50} concentrations of a halogenated anaesthetic, halothane, and a long−chain alcohol, *n*−decanol were determined under conditions of [decanal] = K_m for each luciferase. The dose−response data for both enzymes and both anaesthetics, transformed into f(A) curves, are presented in Figures 6.22(a)−(d) and the resultant ED_{50} concentrations tabulated below (Table 6.7).

Table 6.7: Comparison of the ED_{50} concentrations for inhibition of V. harveyi and V. fischeri luciferases by halothane and decanol under similar experimental conditions (see legend to Figure 6.22) T = 25.5−26.0°C.

Agent	*V. harveyi* ED_{50}	*V. fischeri* ED_{50}
Halothane	14.1 ± 1.1 mM	6.2 ± 0.3 mM
n-decanol	2.4 ± 0.4 μM	18.5 ± 0.6 μM

The results for *V. harveyi* agree well with the ED_{50} concentrations obtained for purified luciferase [see Tables 4.2(a) and (c)]. Note that the ED_{50} reported here for halothane acting on *V. fischeri* luciferase is nearly four times greater than the ED_{50} obtained by Adey *et al.* (1976) − see column 2 of Table 6.6. This discrepancy may, at least in part, be attributed to differences in experimental protocol. Adey and her coworkers initiated luciferase reactions by injecting 0.5 ml $FMNH_2$ into a vial containing 10 μl of luciferase solution, 0.1 ml of a 0.002% (v/v) dodecanal substrate solution and 1.5 ml of a 0.2% solution of bovine serum albumin (BSA) equilibrated with a known partial pressure of anaesthetic. Significantly, the final concentrations of dodecanal and BSA were 4.6 and 21 μM respectively. Dodecanal can be expected to bind tightly to BSA − [at 2°C and a concentration of 24 μM, four molecules of the long−chain alcohol, dodecanol, bind to each BSA

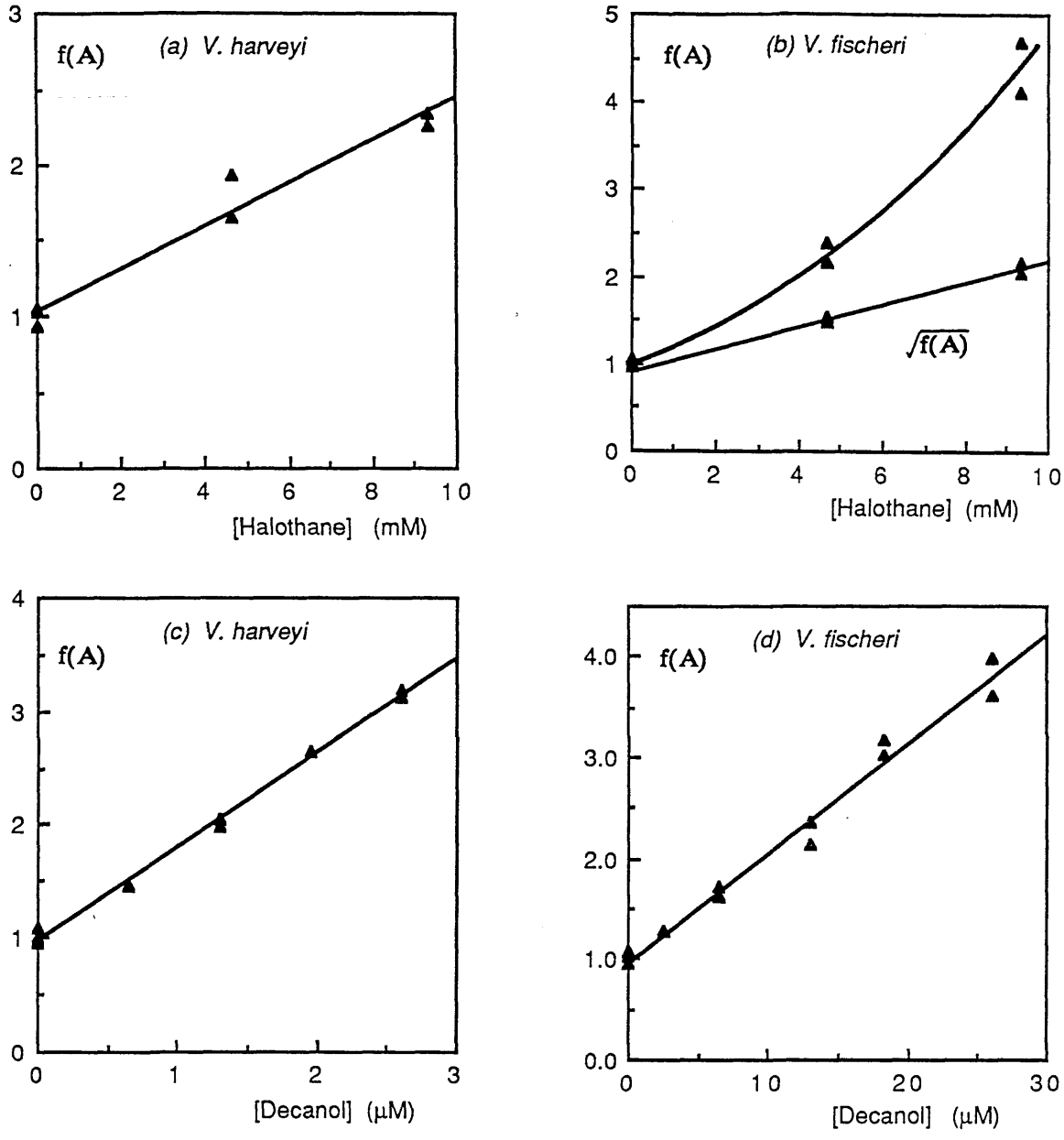

Figure 6.22: Plots of f(A) for the inhibition of V. harveyi and V. fischeri luciferases by halothane and n−decanol. Note that f(A) is parabolic for the inhibition of V. fischeri luciferase by halothane, indicating that two halothane molecules can bind to the enzyme. For calculation of f(A) see section 4.6. Reactions were initiated by injection of $FMNH_2$. Final concentrations: decanal, 0.85 μM (V.h.), 5.6 μM (V.f.) − the K_m in each case; $FMNH_2$, 98 μM; luciferases, ≃ 2 nM. T =25.5−26.0°C.

molecule (Ray *et al.*, 1966)]. This might well have depleted the concentration of aldehyde to below its K_m and simultaneously have displaced anaesthetic from BSA – adding to the free concentration. Using equation 4.8 it may be shown that such experimental conditions would be expected to yield at least a factor of two increase in apparent sensitivity over assays performed with the aldehyde substrate at its K_m and in the absence of BSA.

The results in Table 6.7 make it clear that *V. fischeri* luciferase is more sensitive to halothane (two molecules of which bind to this enzyme) and less sensitive to decanol than *V. harveyi* luciferase. Thus for both of these anaesthetics, *V. fischeri* luciferase bears a closer resemblence to the physiological site of general anaesthetic action. In all probability, some structural difference in the aldehyde binding sites is responsible for the disparate anaesthetic affinities of the two enzymes. Significantly, since only one halothane molecule binds to *V. harveyi* luciferase, its dissociation constant $K_i = ED_{50}/2 = 7.1$ mM (equation 4.24); in contrast, there are two halothane binding sites on *V. fischeri* luciferase so that the dissociation constant for each site is: $K_i = ED_{50}/(\sqrt{3}-1) = 8.5$ mM (equation 4.25). Thus the *affinities* of the two enzymes for halothane are not very different and the greater *sensitivity* of *V. fischeri* luciferase to inhibition by this anaesthetic derives simply from its possession of a second halothane binding site. It seems likely that this second site is adjacent to the first within the aldehyde binding pocket on *V. fischeri* luciferase. The evidence in support of a larger aldehyde binding pocket on this luciferase is that the aldehyde required for optimal activity with *V. fischeri* luciferase, tetradecanal, is four methylene groups longer than decanal, the optimal substrate for *V. harveyi* luciferase (Hastings *et al.*, 1969).

The fact that *n*–decanol binds less well to *V. fischeri* luciferase than to *V. harveyi* luciferase might also result from the greater length of the *V. fischeri* aldehyde pocket. Differences in other structural features may also be involved. Although there is thought to be a large degree of sequence and structural homology between *V. harveyi* and *V. fischeri* luciferases (Baldwin *et al.*, 1979), clear differences have been reported. For instance, *V. harveyi* luciferase is less sensitive to proteolytic inactivation by chymotrypsin and to thermal denaturation than the enzyme from *V. fischeri* (Holzman and Baldwin, 1980), results which may be interpreted in terms of a structural difference. Moreover, Hastings and his coworkers (1969) showed that there are significant differences in the amounts of the polar residue threonine and the apolar residues alanine and isoleucine in the two luciferases. The amino acid composition at the active sites may reflect this difference.

6.3(b) Anaesthetic Sensitivity of the NADH:FMN Oxidoreductase from *V. harveyi*

The results discussed above and those of Adey *et al.* (1976) show that *V. fischeri* luciferase (except possibly in the case of chloroform) is less sensitive to anaesthetics than the whole bacteria. This suggests that there may be other anaesthetic binding sites within the luminescent apparatus. One possibility is the NADH:FMN oxidoreductase (also known as the FMN reductase and the NADH dehydrogenase) which catalyses the reduction of FMN to $FMNH_2$ via the reaction:

$$NADH + H^+ + FMN \xrightarrow{\text{Oxidoreductase}} NAD^+ + FMNH_2$$

(Duane and Hastings, 1975). This enzyme is present in the luciferase preparation supplied by Sigma and its activity may be assayed simply by allowing the $FMNH_2$ that it produces to participate in the light-emitting luciferase reaction. Experiments were performed to observe the effect of halothane on the functioning of the oxidoreductase of *V. harveyi*. 2.5 ml of 295 μM FMN was injected into a vial containing 1 ml of 32.1 μM decanal, 40 μl of 37.9 mM NADH, 4.0 ml of a buffer solution of halothane and 10 μl of 11 mg/ml of the Sigma product containing luciferase and the oxidoreductase. Final concentrations were: FMN, 98 μM; decanal, 4.25 μM, NADH, 0.20 mM. All solutions were made up in 50 mM phosphate buffer, pH 7.0. A high concentration of decanal was used in order to minimise anaesthetic inhibition mediated by binding to the aldehyde site on luciferase. In order to minimise degradation, the NADH stock solution was prepared just prior to the start of an experiment, contained 0.1 mM EDTA and was kept on ice. The halothane solution was prepared as a saturated solution in buffer (section 3.2(b)) Typical reaction profiles in the absence and in the presence of halothane are shown in Figure 6.23. The rise to peak takes about 15 seconds. The decay of luminescence is very slow because the presence of NADH ensures a continued supply of $FMNH_2$, permitting turnover of luciferase – (in 0.2 mM NADH at 25°C the half life of luminescence was estimated to be 30 minutes). At the concentrations of FMN and NADH which were used, 97.9 μM and 0.2 mM respectively, the binding sites of these substrates on the oxidoreductase should be saturated since their dissociation constants have been measured to be about 1.8 and 26 μM (Gerlo and Charlier, 1975; Michaliszyn *et al.*, 1977). However, it should be noted that the luminescence evoked by the injection of FMN was observed to increase linearly with NADH concentration up to at least 2.4 mM – indicating that the NADH binding site was not saturated under my experimental conditions. The rate of the reaction was taken as the maximum intensity.

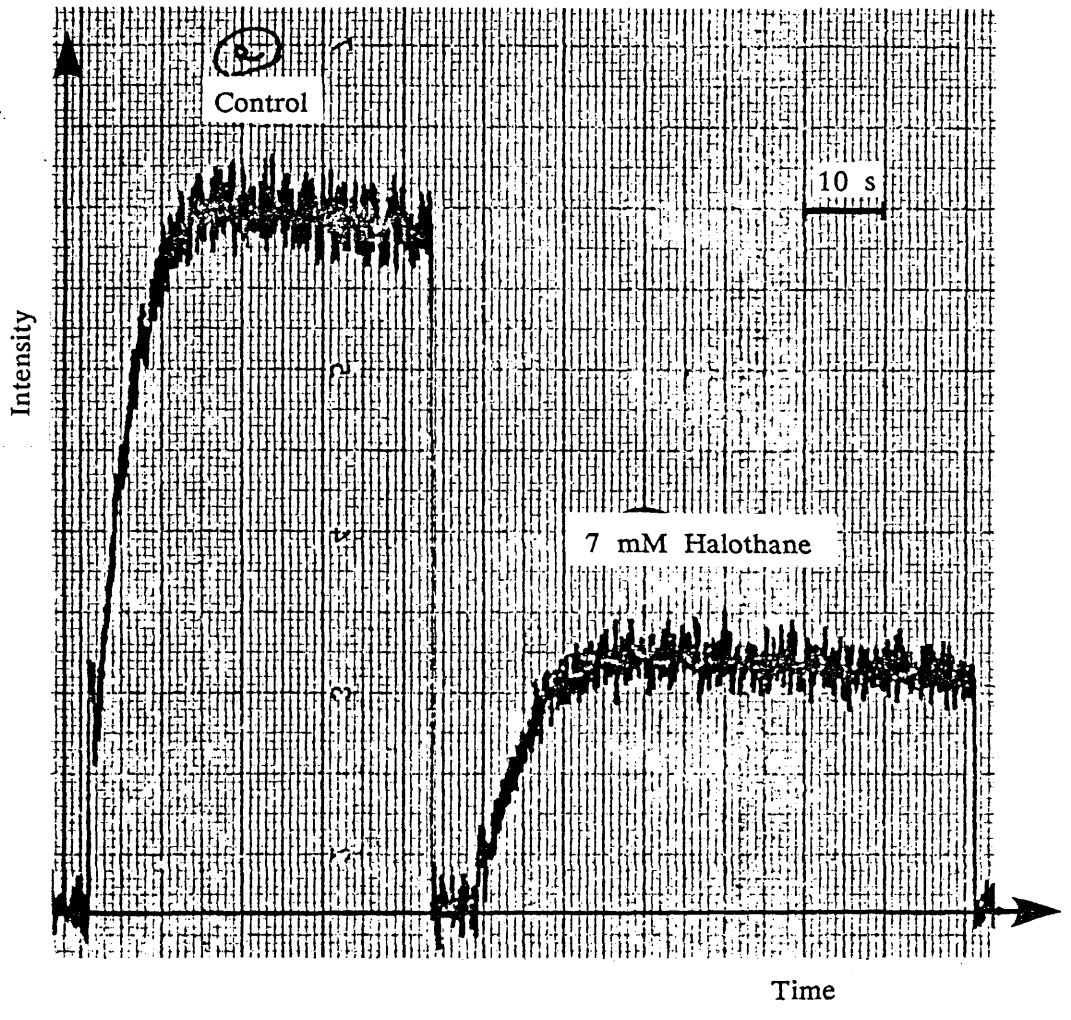

Figure 6.23: The depression of the luminescent output from the coupled luciferase–oxidoreductase reaction by halothane. The figure is a photocopy of a chart recording showing the time–dependence of the intensity of luminescence from such reactions with and without 7 mM halothane. Experimental conditions are given in the text. T = 25.0 °C.

The depression of the maximum intensity caused by halothane is shown in Figure 6.24. Under the experimental conditions described above, the ED_{50} concentration for this anaesthetic acting on the coupled enzyme system is estimated to be 4.6 mM. This result strongly suggests that the NADH:FMN oxidoreductase is sensitive to halothane since it does not seem possible to attribute this degree of inhibition to the luciferase enzyme alone. Using *V. fischeri*, Middleton and Smith (1976) showed that, under experimental conditions similar to those employed here,

only a small percentage (2−4%, *i.e.* 2−4 μM)) of the FMN was reduced at a given time. The halothane ED_{50} concentration for luciferase inhibition, determined by the $FMNH_2$−initiation method at a final flavin concentration of 2.5 μM, was 10.4 ± 1.1 mM, indistinguishable from the result obtained as much higher flavin levels (ED_{50} = 11.4 ± 1.8 mM). Thus the major component of the observed inhibition of the coupled system is most likely due to the effect of halothane on the oxidoreductase enzyme.

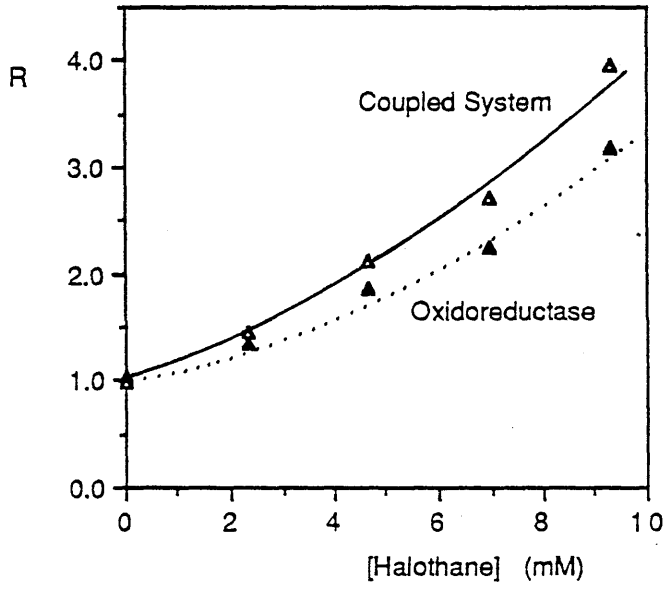

Figure 6.24: Dose−response behaviour of the coupled luciferase−oxidoreductase reaction to halothane. R is the ratio of control (0 mM halothane) to inhibited maximum intensities (I_0 / I_i) at each halothane concentration. The dose−response behaviour calculated according to the method given in the text for the oxidoreductase enzyme alone is shown dotted. Experimental conditions are given in the text. T = 25.0°C.

The halothane sensitivity of the oxidoreductase can be estimated by correcting for the direct inhibition of luciferase which occurs under experimental conditions. In section 4.6, where the analysis of luciferase inhibition was developed, the relationship between the ratio of the control to the inhibited reaction rate (v_0/v_i) to the anaesthetic concentration, [A], was implicit in the equation:

$$\left[1 + \frac{[S]}{K_m}\right]\frac{v_0}{v_i} - \frac{[S]}{K_m} = 1 + \frac{[A]}{K_i} \qquad (6.20)$$

where [S] is the decanal concentration, K_m is its Michaelis constant and K_i is the anaesthetic dissociation constant. This equation may be rearranged to give:

$$\frac{v_0}{v_i} = 1 + \frac{\frac{[A]}{K_i}}{1 + \frac{[S]}{K_m}} \qquad (6.21)$$

Taking [S] = 4.25 μM, K_m = 0.85 μM and K_i = 5.7 mM (the result derived from luciferase inhibition experiments):

$$\frac{v_0}{v_i} = 1 + \frac{[A]}{34.2} \qquad (6.22)$$

Thus the inhibition of luciferase may be calculated at each halothane concentration and this effect can be removed simply by multiplying the ratio of the maximum intensities of the control and inhibited assays ($R = I_0/I_i$) of the coupled reaction by $(v_0/v_i)^{-1}$, calculated using equation 6.22. The resultant inhibition curve for oxidoreductase is shown dotted in Figure 6.24 and yields an estimate of the halothane ED_{50} as 5.5 mM. Thus the oxidoreductase of *V. harveyi* is actually more sensitive to inhibition by halothane than the luciferase enzyme. The mechanism of inhibition of the oxidoreductase appears not to be competitive since raising the NADH concentration from 0.2 to 1.0 mM did not affect the sensitivity of the coupled reaction.

In contrast to the result discussed above, Middleton and Smith (1976) reported that the oxidoreductase from *V. fischeri* bacteria was not affected by an anaesthetising concentration (30 mM) of diethyl ether. However, this result may be erroneous; Middleton and Smith used a fluorometric technique to observe the decline of NADH in a vial containing NADH, FMN and the oxidoreductase but do not appear to have corrected for the substantial degree of non-enzymic degradation of NADH which occurs at room temperature.

It therefore seems evident that the anaesthetic sensitivity of whole bacteria derives from the action of anaesthetics at more than one site. This may well explain why *V. fischeri* luciferase is less affected by anaesthetics than whole bacteria of the same species (see Table 6.5). Similarly, general anaesthesia may also be induced by a set of relatively weak interactions at a number of coupled protein sites, interactions which combine to produce a major effect. Thus, in the quest for possible protein sites of anaesthetic action in the central nervous system, the observation of a relatively small degree of inhibition of a particular protein at anaesthetising concentrations should not necessarily be used as evidence to discard

that target as irrelevant to anaesthesia; it seems important to investigate the network of proteins to which it may belong.

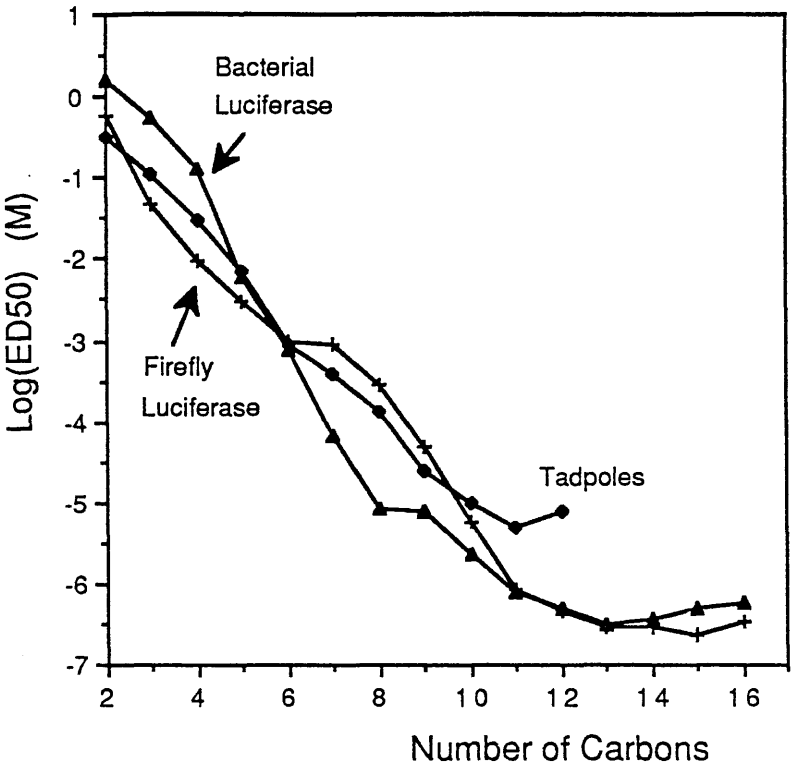

Figure 6.25: Are protein pockets similar to those found on firefly and bacterial luciferases both involved in general anaesthesia? This graph compares the alcohol ED_{50} concentrations of bacterial luciferase (this thesis), firefly luciferase (Franks and Lieb, 1985) and for tadpoles (Vernon, 1913; Meyer and Hemmi, 1935).

The idea of multiple sites of action, all contributing to the overall anaesthetic effect, is not new and has been suggested elsewhere (although not specifically for proteins) as a possible explanation of the different pressure reversal effects observed with some anaesthetic agents (Halsey *et al.*, 1978). The idea receives some support from an intriguing comparison of the ED_{50} concentrations for the inhibition of bacterial and firefly luciferases and for tadpole anaesthesia, plotted in Figure 6.25. The ED_{50} curves for the two luciferases weave around the tadpole ED_{50} curve. This suggests that protein sites similar to bacterial and firefly luciferase may both be involved in anaesthesia.

SYNTHESIS

Although general anaesthetics have been used clinically for more than a hundred and forty years, their molecular mechanism remains a mystery and continues to be the subject of intensive scientific investigation. Recent evidence suggests that sensitive proteins in the central nervous system, rather than the lipid bilayer of neuronal membranes, may be the primary sites of anaesthetic action. In an effort to test this theory, by probing the nature of direct anaesthetic–protein interactions, the anaesthetic sensitivity of a single protein, bacterial luciferase, has been determined *in vitro*. This enzyme catalyses the reaction at the core of the light–emitting process in bioluminescent bacteria. Evidence accumulated over the past seventy years shows that the luminescence from these bacteria and the activity of the luciferase enzyme in particular are sensitive to a number of different anaesthetics. Until now, individual studies of anaesthetic effects on luciferase *in vitro* have used only partially purified enzyme preparations and a handful of anaesthetic agents. The work described in this thesis represents the first attempt to characterise the sensitivity to a broad range of general anaesthetics of highly purified luciferase from a single bacterial species (*Vibrio harveyi*). The resulting pharmacological profile has been compared and contrasted with data on general anaesthesia for whole animals; notable similarities and differences between the anaesthetic binding pocket on the luciferase enzyme and the physiological target of these drugs have been deduced.

Bacterial luciferase was found to be sensitive to inhibition by almost all of the fifty–four anaesthetics tested for an effect. These agents included volatile halogenated anaesthetics, *n*–alcohols, *n*–alkanes, cycloalcohols, ketones and aromatics. They all appear to inhibit luciferase by occupying the hydrophobic binding site of the long–chain aldehyde substrate. Significantly, the inhibitory potencies of *n*–alcohols and *n*–alkanes increase with increasing size, but only up to a certain chain–length (C_{12} for alcohols and C_{10} for alkanes). Thereafter, the ability to inhibit luciferase disappears very rapidly – apparently because there is an upper limit on the affinitites of members of homologous series for the enzyme

pocket. This pattern of increasing potency ending in an abrupt "cut−off" (and the fact that cut−off occurs at a shorter chain length for alkanes than for alcohols) provides a striking parallel to the trend in the anaesthetic potencies of these agents (Meyer and Hemmi, 1935; Führer, 1921; Mullins, 1971). Experiments to measure the ability of alcohols to stabilise the luciferase−peroxyflavin intermediate (II) against spontaneous decay, confirmed that the luciferase cut−off occurs because there is a maximum limit on the strength of binding of alcohols and alkanes to the enzyme. This phenomenon was attributed to the finite volume of the luciferase pocket which allows only hydrophobic ligands up to a critical size to be removed completely from bulk water. Ligands larger than this critical size cannot get their extra hydrophobic surface into the pocket and therefore do not bind any tighter to the enzyme; since the solubility of such ligands continues to diminish with increasing size, the point is soon reached at which only a minority of the luciferase pockets is occupied, even in the presence of a saturated solution of the ligand. Bacterial luciferase is the second protein to display a cut−off effect − Franks and Lieb (1985) found a similar cut−off with firefly luciferase (an unrelated enzyme). The result reported in this thesis therefore provides further strong evidence that the anaesthetic cut−off arises because the site of action in the central nervous system is a protein.

Due to the number and variety of enthalpic and entropic effects to be considered when a ligand binds to a protein, it is not a simple matter to interpret the relative inhibitory potencies of general anaesthetics in terms of the structural features of the binding pocket on bacterial luciferase. However, a relatively simple model can account for most of the data. It is postulated that the anaesthetic binding site (*i.e.* the aldehyde binding pocket) on luciferase is relatively long ($\simeq$ 16 Å) and narrow (with dimensions of width comparable to the width of a methylene chain) and that it contains distinct polar and apolar regions.

The length of the pocket was estimated to be that of *n*−dodecanol, since alcohols longer than this do not bind any tighter to luciferase. The evidence for the narrow dimensions of the site consists primarily of the observations that long−chain alcohols bind well and bulky, halogenated anaesthetics surprisingly weakly to luciferase, in comparison to the affinities of the physiological anaesthetic target and the hydrophobic pocket on firefly luciferase (Franks and Lieb, 1984) for these agents. As a simple test that these differences are indeed due to steric factors, the affinities of cycloalcohols and *n*−alcohols for bacterial luciferase were compared. These compounds are chemically similar but very different in structure. It was found that, even when the greater aqueous solubility of cycloalcohols is taken into account, they bind much weaker to the enzyme − presumably because their discoid

dimensions prevent good contact with the long, narrow lucferase pocket. In addition, the idea of a narrow luciferase pocket provides an economical explanation for the fact that the apparent free energy of binding of methylene groups – calculated from the dissociation constants for alcohols and alkanes binding to luciferase – significantly exceeds the free energy associated with partitioning of such groups into a pure hydrocarbon solvent. The lower apparent methylene binding energies determined for the interaction of alcohols and alkanes at the physiological target suggest, if this site is a protein, that it is somewhat broader than luciferase. This conclusion was supported by the observation that the bulky cycloalcohols are much more potent as anaesthetics than as luciferase inhibitors.

The most hydrophobic anaesthetics tend to be the most potent luciferase inhibitors, indicative of the overall hydrophobic nature of the binding pocket on the enzyme; however, significant polar regions were located either within or close to this pocket. In particluar, calculation and comparison of the partial pressures of alcohols and alkanes required to inhibit luciferase by 50% led to the remarkable result that, from the gas phase, alcohols bind very much tighter (by a factor of about 10^4) to luciferase than do the corresponding alkanes. This implies the presence of strongly polar regions at the luciferase binding site; the main such region is probably the water/site interface. A similar calculation with anaesthesia data produced a similarly remarkable result: that, from the gas phase, alcohols are about 10^3 times more potent as anaesthetics than alkanes of the same chain length. The greater apparent polarity (estimated from the difference between alcohol and alkane binding) of the luciferase site when compared to the physiological anaesthetic target seems to reflect either or both (i) less constraint on the interactions between the hydroxyl group of *n*–alcohols bound to the enzyme pocket and water molecules at the water/site interface or (ii) the presence of a greater number of other polar regions within the luciferase pocket. The possibility of such additional polar regions in this pocket is suggested by the equipotency of C_8 and C_9 *n*–alcohols and α,ω–*n*–alkyldiols and by evidence which indicates that the secondary hydroxyl groups on the flavin substrate form part of the aldehyde (and hence probably the anaesthetic) binding site when the flavin is bound (Meighen and Mackenzie, 1973). Apparently the polar features of the luciferase pocket are responsible for the fact that, overall, alkanes bind less well to it than the the general anaesthetic target – even though alkane (and alcohol) methylene groups (from C_5 to C_9) bind much tighter to the enzyme, a reflection that regions in the enzyme pocket are probably more hydrophobic (as well as narrower) than the physiological target. Thus it seems clear that, if the general anaesthetic target is indeed a binding site on a protein, not only is it broader than the luciferase pocket, but its distribution of polar and apolar regions is

quite different.

Although luciferase is inhibited by a broad range of anaesthetics, they do not all affect the enzyme reaction in precisely the same way. In fact, anaesthetics fall into three major classes, distinguished by their effects on the decay of luminescence in the *in vitro* reaction. Most agents retard the rate constant for luminescence decay in a manner which is apparently (but not exactly) proportional to the depression of the peak intensity that they cause. Experimental evidence shows that this behaviour is due to the ability of anaesthetics to stabilise intermediate II against a spontaneous decay process which does not produce a photon. A number of small and relatively polar anaesthetics were observed to stimulate the peak intensity and the luminescence decay rate at concentrations well below those required for inhibition. This stimulation, which appears to be mediated by interactions at secondary binding sites on the enzyme, is related to an increase in V_{max}. A third class of inhibitor, the long-chain alcohols and alkanes, induce a distinctive biphasic decay of luminescence. In general, the initial phase of this decay is accelerated and the final phase greatly retarded by increasing the inhibitor concentration. An explanation for this behaviour emerged from experiments to measure the stabilisation of intermediate II by long-chain alcohols. It is postulated that formation of intermediate II results in a conformational change in the enzyme which greatly enhances the affinity for long-chain compounds. This hypothesis is supported indirectly by evidence from other laboratories (Holzman and Baldwin, 1980, 1981). Two clear, quantitative predictions deriving from the hypothesis, which relate to expected similarities and differences in the kinetics of luciferase reactions initiated by injections of decanal and $FMNH_2$ in the presence of a long-chain alcohol, were confirmed by experiment.

A surprising finding was that the *in vitro* anaesthetic sensitivity of *Vibrio harveyi* luciferase differs quite substantially from the sensitivities reported for *V. fischeri* luciferase *in vitro* and for whole luminous bacteria *in vivo*. The differences between *V. harveyi* and *V. fischeri* luciferases were confirmed by a direct comparison of the enzymes under similar experimental conditions. The Michaelis constants (K_m) for the aldehyde substrate, decanal, and the affinities for the anaesthetics halothane and decanol were quite distinct for the two enzymes. These results were attributed to structural differences between them, which are also indicated elsewhere in the literature (Hastings *et al.*, 1969; Holzman and Baldwin, 1980). A more detailed comparison of *V. harveyi* and *V. fischeri* may provide further clues as to the properties which confer anaesthetic sensitivity on proteins. Finally and interestingly, the NADH:FMN oxidoreductase from *Vibrio harveyi* was

actually observed to be *more* sensitive to inhibition by halothane than the luciferase enzyme. This indicates that the greater anaesthetic sensitivity of whole bacteria is due to the presence of anaesthetic targets other than luciferase in the luminescent apparatus of the bacteria.

APPENDIX 1

Calculation of Aqueous Concentrations in Equilibrium with Partial Pressures of Anaesthetics.

The ideal gas law states that, for n moles of an anaesthetic gas at partial presure P (atmospheres), temperature T (Kelvin) in a volume V (litres):

$$PV = nRT \qquad (A.1)$$

where R is the universal gas constant (0.08206 lit atm K^{-1} mol^{-1}). The concentration of anaesthetic molecules in the gas phase, C_g, in moles per litre (M) is simply:

$$C_g = \frac{n}{V} = \frac{P}{RT} \quad (M) \qquad (A.2)$$

If the anaesthetic vapour is in equilibrium with the aqueous phase, the aqueous concentration, C_w, is determined by the water/gas partition coefficient at temperature T, λ_T:

$$\lambda_T = \frac{C_w \ (M)}{C_g \ (M)} \qquad (A.3)$$

Thus:

$$C_w = \lambda_T C_g = \frac{\lambda_T . P}{RT} \qquad (A.4)$$

Hence, at a partial pressure P_{50} (atm) which causes a 50% effect, the aqueous concentration, ED_{50} (M), is given by:

$$ED_{50} \ (M) = \frac{\lambda_T . P_{50}(atm)}{RT} \qquad (A.5)$$

It is sometimes useful to use this equation the other way round:

$$P_{50} \ (atm) = \frac{RT . ED_{50}(M)}{\lambda_T} \qquad (A.6)$$

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