General anaesthetics and bacterial luminescence

I. The effect of diethyl ether on the in vivo light emission of *Vibrio fischeri*

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The factors which determine the sensitivity of bacterial luminescence to inhibition by the general anaesthetic, diethyl ether, have been investigated. The in vivo luminescent reaction of *Vibrio fischeri* displays a change in sensitivity to this agent during the bacterial growth cycle. This variation is particularly marked during the lag phase of growth and detailed investigations of the effect of potassium cyanide and n-decanal on the potency of ether during this early period are described. The results suggest that fluctuations in the substrate levels available to the light-producing enzyme are responsible for the variation in sensitivity to ether.

**INTRODUCTION**

The light-producing reaction of luminous bacteria has proved of great interest in the investigation of the mode of action of general anaesthetics. It is found that the doses required to reduce the light emission by 50% are similar to those required to produce anaesthesia in mammals (Halsey & Smith 1970; White & Dundas 1970). Thus bacterial luminescence is more sensitive than most of the model systems commonly employed in the study of the mechanisms of general anaesthesia. Furthermore the same relative potencies of anaesthetic substances are observed with the bacterial light reaction as in mammalian experiments indicating that the sites of action of anaesthetics in the two systems may have similar physicochemical natures.

Previous investigators who used luminous bacteria suggested that anaesthetics act by a specific inhibition of the light emitting reaction (Halsey & Smith 1970; Johnson, Van Schowenburg & Van der Burgh 1935). However these early studies did not consider the possible variation of anaesthetic potency during the bacterial growth cycle although Halsey & Smith noted that a variation in response may occur. This paper reports the results of investigations into the effect of the age of batch cultures of *Vibrio fischeri* on the response of its luminescent system to diethyl ether. The variation in response which is observed provides a clue as to how ether inhibits the light emission. *Vibrio fischeri* was chosen as the most

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suitable organism because the light emitting enzyme was readily available. When provided with the correct substrates this enzyme emits light which is spectrally indistinguishable from *in vivo* light emission. This permitted parallel studies to be carried out of the effect of ether on the *in vitro* luminescent reaction. The results of these investigations are presented in the accompanying paper in which a more complete account of the biochemistry of the luminescent reaction is given. For the present purposes it is sufficient to note that the light is produced by the unimolecular decay of an excited intermediate which is formed by the reaction of reduced flavin mononucleotide with the enzyme, luciferase, in the presence of a long-chain aldehyde and oxygen.

**Materials and methods**

(a) Materials

Cultures of *Vibrio fischeri* (National Collection of Marine Bacteria, No. 1281) were obtained from the Torry Research Station, Aberdeen, Scotland. They were grown on an adaptation of Farghaly's medium (Farghaly 1950) containing 0.5% peptone. The peptone, supplied by B.D.H. Chemicals Ltd, contained a 'wide range of high polypeptides and amino acids'. Analytical grade diethyl ether and chloroform were obtained from B.D.H. Ltd. Halothane was obtained from I.C.I. Ltd, Macclesfield, England and methoxyflurane from Abbott Laboratories, Kent, England. The straight chain aldehyde *n*-decanal, was obtained from Koch-Light Laboratories. All other chemicals were analytical grade.

(b) Measurement of light intensity

Luminescent intensity was monitored with a photomultiplier tube (type: E.M.I. 6094B) whose output could be displayed after amplification on a pen-recorder. Experiments with controls used two identical photomultiplier tubes whose output could be displayed alternately on the recorder by using a two-way microswitch.

Difficulty was experienced with luminescent measurements made during the period of maximum light emission because the photomultiplier tube became 'saturated' and would give no further increase in signal output with increasing light intensity. This was overcome by inserting neutral density filters (Kodak Ltd) of known transmittance, between the bacterial sample and the photomultiplier tube.

A standard light source was used to check the apparatus for electrical faults. This was a 'beta-light'; a glass bulb, coated on the inside with a phosphorescent material and filled with tritium.
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(c) The measurement of bacterial growth

The degree of growth of bacterial cultures was estimated by measuring turbidity with a Hilger Light Absorptiometer previously calibrated in terms of total dry cell mass. The calibration plot relating instrument reading to dry cell mass was a straight line over the range of concentrations studied.

(d) Methods of anaesthetic addition

Two methods were employed for the addition of anaesthetics to bacterial suspensions.

(i) The direct addition method

In this method the anaesthetic was added as a saturated aqueous solution from a thermostatically controlled glass syringe to a known volume of bacterial suspension contained in a thermostatted glass vessel. Slow stirring rates were employed which ensured complete mixing of the anaesthetic while minimizing its loss from the suspension. Control experiments indicated that the dilution effect of the added liquid was insignificant and that loss of the volatile anaesthetic was negligible during the time of a single experiment (about 1 min). Removal of the anaesthetic could be achieved by flushing the bacterial suspension with air. This caused the light intensity to return to its original level indicating that no irreversible changes had occurred. This method of anaesthetic addition was very useful for the rapid determination of anaesthetic potency. The accuracy of the method is considered later.

(ii) The flow method

In this method the liquid anaesthetic was introduced at a known rate into the bacterial air supply the flow rate of which was measured by a rotameter. This provided a supply of anaesthetic vapour at a known partial pressure with which the bacterial suspension came to equilibrium. Experiments with this technique frequently lasted up to 30 min and a control sample was employed to correct for natural fluctuations in the light intensity.

Throughout this work potency is expressed as the ED_{50} which is defined as the concentration or partial pressure of anaesthetic required to reduce the light intensity to 50% of its initial value.

(e) Addition of n-decanal and potassium cyanide

n-Decanal is sparingly soluble in water (Hastings et al. 1963) but it may be conveniently introduced in the form of a suspension obtained by shaking 0.1 ml of the aldehyde with 100 ml of distilled water for 30 min (5.4 \times 10^{-3} \text{ M}). A suspension diluted tenfold was used for low n-decanal concentrations. Because of the tendency of n-decanal to undergo autoxidation in aqueous solution
(Hastings et al. 1963) suspensions of aldehyde were made up as required and discarded after use.

Potassium cyanide was used as a 0.06 M aqueous solution which was renewed at regular intervals and kept in a tightly stoppered vessel to minimize loss of HCN.

Results

(a) Growth and luminescence of Vibrio fischeri

The characteristics of growth and luminescence of *Vibrio fischeri* are well documented (Farghaly 1950; Baylor 1949; Calleja & Rogers 1968; Kempner & Hanson 1968; Nealson, Platt & Hastings 1970). However since precise details of growth and luminescence depend on the growth medium and the incubating temperature, it was considered desirable to monitor these characteristics as a function of culture age for the particular conditions employed. 0.5 ml of bacterial culture in its stationary phase was added to 100 ml of growth medium and the growth and luminescence were measured at regular intervals. Figure 1 shows the results for bacterial cultures grown at 28 °C. The normal stages of bacterial growth - the lag phase, the logarithmic phase and the stationary and death phases may be recognized. Figure 2 shows detailed measurements of luminescence during the first 10 h from subculture. The light intensity reaches an initial maximum after 2 h and this is followed by a period of almost exponential decay. The luminescence reaches a minimum after about 6 h, during which time the light intensity has fallen by a factor of ten.† Rapid exponential growth of luminescence then occurs and the intensity increases by a factor of $10^4$ within 3 h.

† The minimum in luminescence depends on the size of the inoculum. If large inoculae are used the minimum may be less distinct or absent.
(b) Effect of n-decanal and potassium cyanide on light emission

It has been shown by Nealson and his co-workers (Nealson et al. 1970) that the addition of n-decanal to luminous bacteria can, under certain circumstances, stimulate light emission. This presumably arises because n-decanal can penetrate the cell membrane and increase the levels of aldehyde factor which is necessary in the luminescent reaction. The response of the light emission to aldehyde therefore provides an approximate indication of the concentration of the natural aldehyde factor available within the cells.

![Graph showing the effect of n-decanal and potassium cyanide on light emission. The graph indicates a marked variation in light intensity during the first 10 h of the growth cycle. Initially, n-decanal causes the light intensity to be decreased by up to 50%, but as the lag phase proceeds, this depression becomes gradually smaller until eventually a stimulation occurs.]

Potassium cyanide is known to block the respiratory cytochromes thus preventing electron transfer to oxygen. This has the effect of making a higher concentration of FMNH$_2$ available to the light emitting reaction and the response to cyanide provides a measure of the level of reduced flavin normally available to the luciferase.

Figure 3 shows the effect of the rapid addition of $2 \times 10^{-4}$ M n-decanal on the light intensity during the first 10 h of the growth cycle. The response displays a marked variation during this period. Initially n-decanal causes the light intensity to be decreased by up to 50%. However as the lag phase proceeds this depression becomes gradually smaller until eventually a stimulation occurs. Maximum
stimulations of up to 500% are observed during the period of minimum light emission. Smaller concentrations of aldehyde cause proportionately lower degrees of stimulation. The response to aldehyde is transitory, maximum intensity being reached within 5 s. This is followed by a slow decay of luminescence with a half-life of about 3 min.

![Figure 3. Effect of KCN and n-decanal on luminescence during the lag phase. O, No additions; ⊗, 0.0011 M KCN; ●, 2 × 10⁻⁴ M n-decanal; ×, 0.0011 M KCN followed by 2 × 10⁻⁴ M n-decanal.](image)

The response to 0.0011 M KCN is biphasic. Within 10 s of addition light intensity is increased by up to 20%. This is followed by a depression to an intensity as low as 10% of the initial value. The final response to KCN is shown in figure 3 which also shows the response of the light emission to the successive addition of KCN and n-decanal. After the full effect of cyanide has occurred the subsequent addition of aldehyde gives stimulation to an intensity which is practically constant throughout the early growth cycle. The intensity reached is roughly equal to that of the maximum unperturbed luminescence during the lag phase. As observed in the absence of cyanide, the response to aldehyde is transitory.

(c) Variation of the potency of ether

Dose against response curves for ether were measured at intervals over the complete growth cycle using the direct addition technique. ED₅₀’s were obtained from these curves and the variation of potency over the growth cycle is shown.
in figure 4. Various broadly defined stages during the growth cycle may be identified as of interest:

(i) 30 min after subculture,
(ii) about 5 h after subculture,
(iii) the period when the intensity is close to its maximum value, i.e. from the end of the exponential phase to the start of luminescent decay (10-20 h),
(iv) the death phase – over 24 h.

The salient features of figure 4 are:

• the appearance of a maximum in ED$_{50}$ during stage (ii),
• during stage (iv) ED$_{50}$ increases to at least double the value observed in stage (iii),
• hyperactivity (the stimulation of light emission by low concentrations of anaesthetic) is observed only in stages (ii) and (iv),
• the average ED$_{50}$ of ether during stage (iii), i.e. $0.022 \pm 0.001$ M may be calculated, using Henry’s Law, to be equivalent to a partial pressure of $0.033 \pm 0.006$ atm (1 atm $\approx 10^5$ Pa).

(d) Effect of ether during the early growth cycle

The ED$_{50}$ of ether was measured at regular intervals during the first 10 h of the growth cycle, by using the flow method. The results shown in figure 2 confirmed that the potency passes through a distinct maximum after about 5 h, the greatest ED$_{50}$ being observed during the period of minimum light emission. In addition, the appearance of hyperactivity is confined to the same period.

An accurate dose–response curve for ether was also determined for bacterial cultures at stage (iii) of growth using the flow method. The curve was then
re-plotted in the form percentage response against log (partial pressure of ether) which was linear for responses between 20 and 80%. The best straight line was computed by the method of least squares and the intercept at 50% gave the ED$_{50}$ for ether as 0.030 atm (standard deviation 0.001). This value is similar to those obtained by other workers (Halsey & Smith 1970; White & Dundas 1970) who used *photobacterium phosphoreum* (0.026 ± 0.002 and 0.0198 respectively). This value obtained by the flow method agrees closely with that observed using the direct addition technique (0.033 ± 0.006) thus confirming the accuracy of the latter technique.

(e) The effect of n-decanal and KCN on the potency of ether

Since the addition of n-decanal or KCN produced significant effects on light intensity during the early growth cycle, it was of particular interest to study the effect of these compounds on the potency of ether during the same period. It was not possible to use the flow method of anaesthetic addition for the following reasons: (i) the work of Nealson and his co-workers (Nealson *et al.* 1970) suggests that the transitory effect of n-decanal arises because this compound is consumed by the bacteria. The concentration of aldehyde would therefore be uncertain during the long period required for the flow method. (ii) Following the addition of KCN, hydrogen cyanide would be readily lost from solution and therefore the concentration of cyanide would also be subject to some uncertainty. For these reasons the direct addition method was used.

The potency of ether in the presence of $2 \times 10^{-5}$ M n-decanal was determined at regular intervals during the early growth cycle. The aldehyde was added first and when its maximum effect had been reached a dose–response curve for ether was rapidly measured. (This took approximately 30 s.) From these curves values

![Figure 5. Effect of $2 \times 10^{-5}$ M n-decanal on the ED$_{50}$ of ether during the early growth cycle. O, ED$_{50}$; •, ED$_{50}$ with n-decanal; H, hyperactivity.](http://rspb.royalsocietypublishing.org/Downloaded from http://rspb.royalsocietypublishing.org/ on December 29, 2017)
of $ED_{50}$ were obtained. Figure 5 shows that the effect of $2 \times 10^{-5} \text{M} \text{ n-decanal}$ is to remove the maximum in $ED_{50}$, the potency of ether remaining essentially unchanged throughout the early growth cycle when n-decanal is present.

Figure 6 shows the effect of varying the n-decanal concentration on the $ED_{50}$ for ether using bacterial cultures during the period of minimum light emission.

![Figure 6. Effect of different n-decanal concentrations on the $ED_{50}$ of ether at the time of minimum light emission. H, hyperactivity.](image)

The addition of aldehyde removes the hyperactivity caused by ether which is characteristic of bacterial cultures at this stage of growth, and the $ED_{50}$ falls from 0.032 M to as low as 0.017 M. At higher aldehyde concentrations the $ED_{50}$ increases reaching 0.055 M at $10^{-4}$ M aldehyde.

Figure 7 illustrates the effect of 0.0011 M KCN on the $ED_{50}$ of ether during the early growth cycle. The considerable scatter of points reflects the large uncertainties inherent in these experiments. These arise because of the difficulty of measuring the effect of ether on luminescence which has already been reduced.
to a low level by the addition of cyanide. In spite of the large experimental errors (± 10%) the results indicate that the addition of 0.0011 m KCN always increases the ED$_{50}$ of ether. Furthermore hyperactivity was always observed in the presence of KCN whether or not it occurred in its absence.

(f) Transient effects of ether on light emission

Use of the direct addition method for the introduction of anaesthetic gives rise to interesting transient effects which cannot be observed when the flow method is employed. Figure 8 illustrates these effects for bacterial cultures in the death phase. The addition of ether initially causes a rapid depression of the luminescence which is followed by a much slower recovery. The recovery phenomenon (which will be referred to as secondary stimulation) is not caused by loss of anaesthetic from the bacterial suspension. It only occurs to a significant extent during the lag phase and the death phase.

Secondary stimulation is sensitive to the addition of potassium cyanide – during the lag phase 0.0011 m KCN enhanced the magnitude of the recovery phenomenon.

(g) Additional observations

Although this study was mainly concerned with the effect of ether in the early growth cycle, the following observations were also made.

(i) The potencies of the general anaesthetics chloroform, halothane and methoxyflurane displayed a variation over the growth cycle similar to that observed for ether.
(ii) The addition of $2 \times 10^{-5} \text{M} n$-decanal removes the hyperactivity in bacterial cultures produced by low concentrations of halothane and methoxyflurane.

(iii) $0.0011 \text{M} \text{KCN}$ raises the ED$_{50}$'s of these compounds during the same period of growth.

(iv) The addition of $2 \times 10^{-5} \text{M} n$-decanal to death phase bacterial cultures not only removes hyperactivity and lowers the ED$_{50}$ of ether but also frequently stimulates light emission.

**Discussion**

It is clear from the results presented that the response of bacterial luminescence to general anaesthetics is a complex function of the age of the bacterial culture. This complexity, which was largely overlooked in earlier studies, manifests itself in three ways:

(a) the variation in anaesthetic potency;

(b) the occurrence of transient effects when anaesthetic is added as an aqueous solution ('secondary stimulation');

(c) the occurrence of hyperactivity which is confined to the lag phase and death phase.

The detailed investigation of the response of lag phase bacterial cultures to ether provides important evidence for the cause of the variation in potency. The high ED$_{50}$'s and hyperactivity of the lag phase are closely associated with a period of very low light emission (figure 2). This drop in luminescence following subculture has been described by other workers. Kempner & Hanson (1968) attributed its occurrence to a dialysable inhibitor present in their nutrient broth growth medium. They concluded that the inhibitor, whose identity was not elucidated, is completely destroyed by the time exponential luminescent growth has begun. Furthermore, if the medium is first ‘conditioned’ by allowing growth to proceed up to the start of the exponential phase followed by centrifugation and removal of the cells and re-inoculation with fresh bacteria, no drop in luminescence is observed. More recent work (Eberhard 1972) has also suggested the presence of an inhibitor in a complete medium containing tryptone and yeast extract. This inhibitor, the identity of which was not elucidated, is said to repress luciferase synthesis.

The results of the present study do not support the idea that the fall in light intensity is caused by the inhibition of enzyme activity. It has been shown that the addition of a long chain aldehyde during the period of low light emission will stimulate the luminescence. Also potassium cyanide, which alone produces a transitory stimulation followed by a strong inhibition, makes the bacteria capable of powerful luminescent emission on the subsequent addition of aldehyde (figure 3). These results suggest that the drop in luminescence is caused not by a loss or inhibition of enzyme activity but by a shortage of reduced flavin and aldehyde factor.
These conclusions are in substantial agreement with those reached by Nealson and his co-workers (Nealson et al. 1970), who studied the MAV strain of *Vibrio fischeri* in both complex and minimal medium. Responses to potassium cyanide and n-decanal were obtained which were very similar to those observed in this work. Also these workers demonstrated by an *in vitro* luminescent assay that extractable luciferase levels remained unchanged throughout the early growth cycle. Since the high ED$_{50}$'s observed during this period coincide with the fall in light intensity, it is natural to suppose that the variation of anaesthetic potency arises as a result of the variation of the levels of reduced flavin and aldehyde factor available to the luminescent reaction. The effects of potassium cyanide and n-decanal on the potency largely substantiate this hypothesis. Thus during the period of low light intensity, 2 × 10$^{-5}$ M n-decanal lowers the ED$_{50}$ of ether and removes its hyperactive effects (figure 5). In addition a range of aldehyde concentration produces a biphasic change in the potency of ether, low concentrations of aldehyde giving lower ED$_{50}$'s, while higher levels give higher ED$_{50}$'s (figure 6). Throughout the early growth cycle 0.0011 M potassium cyanide causes substantial increases in ED$_{50}$ and produces hyperactivity whether or not it occurs in the absence of cyanide (figure 7).

The most plausible explanation consistent with these observations is that the variation of anaesthetic potency during the early growth cycle is caused by variations in the levels of substrates available to the light emitting enzyme. This is consistent with the conclusions of earlier workers (Halsey & Smith 1970; Johnson et al. 1935) who suggested that anaesthetics reduce the light intensity by a specific inhibition of the luminescent reaction. In addition, the phenomena of hyperactivity and secondary stimulation appear to be related to substrate levels and it is likely that they are caused by similar mechanisms. Indeed it is not implausible that it is the variation of the magnitude of these phenomena that is responsible for the observed changes in anaesthetic potency.

In this work using the intact bacterial system, it has not been possible to determine the point in the complex luminescent reaction at which ether is acting. This is largely due to an inability to vary the levels of reduced flavin and aldehyde factor in any precise manner. In the accompanying paper investigations using the *in vitro* luminescent reaction are described. These have enabled the mechanism by which ether perturb the luminescent reaction to be further elucidated.

**References**


