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ANAESTHESIA BY THE *n*-ALKANES

A COMPARATIVE STUDY OF NERVE IMPULSE BLOCKAGE AND THE PROPERTIES OF BLACK LIPID BILAYER MEMBRANES

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Summary

1. The suppression of the propagated action potential in the squid giant axon and in the frog sciatic nerve, by saturated solutions of the *n*-alkanes from *n*-pentane to *n*-decane, has been examined. A progressive loss in the activity of the alkane was found as the chain length increased, *n*-nonane being effectively inert. The concentration dependence of the suppression of the action potentially *n*-pentane was also measured.

2. The effects of the alkanes on lipid bilayers were determined using black film techniques. For both phosphatidylcholine-cholesterol and monoolein-cholesterol bilayers in alkane-saturated aqueous phases, the bilayer thickness decreased by several angstrom units on passing from *n*-pentane to *n*-decane, from which it was concluded that the adsorption also decreased. The concentration dependence of the thickness and adsorption changes for *n*-pentane were examined.

3. A close correlation is shown to exist between the nerve results and those for a phosphatidylcholine-cholesterol bilayer, suggesting that the site of action of the alkane is in a lipid bilayer region of the nerve membrane.

4. The presence of cholesterol in the bilayer, at levels apparently comparable to those in axon membranes, is essential for the above correlation to hold.

5. A molecular mechanism by which the alkane may inhibit the nerve impulse is proposed. The essential feature is that a thickening of the lipid part of an axon membrane through adsorption of alkane reduces the stability of the ionic channels formed during electrical excitation.

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Introduction

There is a great deal of evidence to suggest that neutral (non-ionic) anaesthetics act at hydrophobic sites in cell membranes [1—4]. Recent discussion has tended to focus on the nature and location of these sites, in particular, whether they are in the lipid bilayer or the protein parts of the membrane [1,2,5—9]. The purpose of this paper is, first, to present some observations which favour the interior of the bilayer as the most likely site of action and secondly, to point out that if this is correct there is a simple mechanism by which ion conduction through membrane pores, and hence impulse propagation, may be inhibited.

A serious difficulty in trying to understand the behaviour of anaesthetics in physico-chemical terms arises from the fact that many anaesthetic molecules are complex and may contain at least two functional groups. In an attempt to avoid this difficulty the present work has been concerned solely with the *n*-alkanes. These compounds also have the advantages that their behaviour in lipid bilayers has been well studied and that they form a homologous series which, as will be shown, yields an important piece of information.

Both the *n*-alkanes and the *n*-alkanols have been examined for their anaesthetic activity [1,10,11]. In each of these homologous series it was found that, as the chain length of the molecules increased, the activity declined and eventually disappeared. Thus, while *n*-pentane and *n*-butanol are anesthetics, *n*-decane and *n*-tridecanol are not. This loss of potency is not reflected in a loss of lipid solubility and the results are therefore not in line with the Meyer-Overton correlation which, although somewhat rough, holds for many other anaesthetics [1,2]. In order to account for the inactivity of decane it has been suggested either that its vapour pressure and/or water solubility are so low that it never reaches equilibrium with the site of action, or that owing to the presence of aggregates or microdroplets its thermodynamic activity is substantially lower than is indicated by its solubility. However, experiments mentioned by Mullins [1] and evidence to be described in this paper indicate that the first suggestion is unlikely to be correct. Also, new data on decane solubility (Table I) confirm older results and thus lessen the likelihood that artefacts such as emulsion formation are important. Rather, it seems that some other factor, possibly molecular size should be considered [1].

Experiments on black lipid films have shown that the bilayer thickness is strongly dependent on the size of the *n*-alkane solvent used in their formation, the smaller solvent molecules yielding the thicker films [13]. It has also been shown that the alkane solvent tends to reside mainly in the centre of the bilayer [14] and that the larger molecules, such as hexadecane, are selectively excluded from the films [13,14]. In bilayers which contain no cholesterol this size effect is noticeable only for molecules between decane and octadecane, the former not being appreciably excluded, and there is no correlation with the cut-off in anaesthetic potency. When bilayers contain cholesterol at levels comparable to those found in nerve membranes [15] all that has so far been shown is that decane is strongly excluded [13,16]. This isolated observation was nevertheless sufficient to suggest that in cholesterol-containing membranes there might be a very good correlation between alkane uptake and anaesthetic potency.

Accordingly experiments have been carried out, using a range of *n*-alkanes, to determine the thickness and alkane-uptake of both phospholipid and monoglyceride films containing cholesterol. Also, since the existing data are for whole animals, it was thought desirable to confirm the existence of the chain-length cut-off in the anaesthetic activity of the *n*-alkanes by means of simple experiments on the squid giant axon and the frog sciatic nerve. In addition, the effect of pentane at different concentrations has been examined in black films and nerve preparations. In the Discussion, the link betweeen alkane adsorption into the bilayer and anaesthetic potency is examined. It is argued that the thickening and increase in tension which alkanes produce in bilayers may produce a de-stabilization of the ionic channels formed during electrical excitation.

Methods and Materials

Black film measurements

Black lipid films were formed in a vertical cell, using the syringe method of supplying lipid solution to the hole in the PTFE partition. The essential features of the apparatus have been described elsewhere [17]. An added refinement was a tight-fitting PTFE lid to the cell to minimize loss by evaporation of the more volatile alkanes. The film-forming solutions were made by dissolving egg yolk phosphatidylcholine or monoolein plus, in some experiments, cholesterol in the required alkane solvent. In the phosphatidylcholine systems the phospholipid was at 7 mM and the cholesterol at 21 mM. Monoolein was used at 6 mM; when used with cholesterol, this was at 15 mM. The aqueous solution was 0.1 M NaCl.

Care was taken to ensure that the alkane under consideration was at equilibrium throughout the whole system comprising the film, its associated bulk lipid and the aqueous phase. To achieve this, the lipid solution and the aqueous phase, were placed in contact for some hours prior to the experiment.

The film capacity was measured by means of a Wayne Kerr Universal Bridge at a frequency of 1 kHz. Details have been given previously [17]. The a.c. signal applied across the film was ca. 5 mV (peak-to-peak). Film area was monitored by inspection through one tube of a binocular microscope containing a calibrated graticule, a light source being attached to the other tube. The temperature throughout the experiments was 20 ± 1°C.

Squid axon measurements

The experiments on squid were performed in the Centro de Biofisica, I.V.I.C., Caracas. The hindmost giant axon from the stellate ganglion was dissected from the mantles of the squid *Dorytheutis plei* and carefully cleaned of all surrounding fibres and most of the loose connective tissue. The dissected axons were mostly about 400 μm in diameter and 8 cm long. The axons were cannulated at the ganglion end and mounted vertically in a perspex cell similar to that described by Baker, Hodgkin and Shaw [18]. Stimulating electrodes were situated near the lower end of the axon. A glass micropipette, 75 μm in diameter, filled with 0.5 M KCl was inserted longitudinally into the axon to a distance of about 4 cm. The micropipette had a floating 25 μm platinum wire inside and was connected to the external circuit via an Ag/AgCl electrode. The

external reference electrode which dipped into the solution bathing the axon was also Ag/AgCl. Resting and action potentials were monitored with an oscilloscope (Tektronix 7313) from the output of a high impedance intracellular probe amplifier system (Mentor N-950) to the inputs of which the electrodes were connected. Artificial sea water (440 mM NaCl/10 mM KCl/50 mM $MgCl_2$/10 mM $CaCl_2$/0.1 mM EDTA/Tris, 10 mM, pH 7.6) and test solution were allowed to flow into the lower part of the chamber and were removed by suction from the top. The supply was from a raised separating funnel, through PTFE tubing. Flow rates were normally at least 10 ml per min; at this level the results showed no dependence on the flow rate. Test solutions were prepared by dilution of artificial sea water saturated with alkane and this, in turn, was prepared by equilibrating artificial sea water with liquid alkane for ca. 24 h with gentle stirring, a method shown to be satisfactory in earlier work on alkane solubility (Dagger, F. and Haydon, D.A., unpublished). The temperature was 18°C. Action and resting potentials in artificial sea water were normally ca. 110 mV and −60 mV respectively.

Sciatic nerve measurements

Sciatic nerves were obtained from large specimens of *Rana temporaria*, except for the pentane-saturated Ringer experiments in which, since *R. temporaria* were not available, *Rana pipiens* were used. The freshly excised nerves were desheathed in the middle of their length for a distance of 1—2 cm and then mounted across a bathing chamber in a perspex block. The free nerve ends trailed into two flanking chambers, one of which was used for stimulating and the other for recording. The nerve passed from one chamber to the next through narrow slits cut in the top of the dividing septae. A small piece of Ringer-soaked tissue was placed over the nerve where it was exposed to air, and this was in turn covered with Vaseline.

The nerve was stimulated at 6 Hz and monophasic compound action potentials were obtained from a platinum electrode at the end of the nerve, recording with respect to a reference electrode in the bathing chamber. Frog Ringer (115 mM NaCl/2.5 mM KCl/1.8 mM $CaCl_2$/1.0 mM $MgCl_2$/10 mM Tris · Cl buffer, pH 7.4) was saturated with alkane, as described in the previous section, and lower alkane concentrations, when required, were obtained by dilution. These solutions were run into the bathing chamber from a separating funnel, as in the squid experiments, a constant level being maintained by means of a suction pipe. Throughout the perfusion the bathing chamber was stirred magnetically at a constant rate. In some instances free alkane was dispersed as a fine emulsion in the Ringer by sonication. Perfusion rates were typically about 1.5 ml per min. A variety of precautions were taken to preclude the evaporation of the more volatile alkanes.

In addition to monitoring the compound action potential during the perfusion, tests were also carried out to detect changes in the tetrodotoxin sensitivity of the compound action potential. At various times, the bathing chamber was drained with a Pasteur pipette, a tetrodotoxin solution of appropriate concentration was introduced and the nerve exposed to the toxin for 4 min (the time scale of the experiment permitting) under constant stirring, thus allowing the maximum inhibition of the compound action potential to occur [19]. The

TABLE I

SOLUBILITIES OF *n*-ALKANES IN SALT SOLUTIONS AT 20°C

	Solubility (mg/l)	
	0.1 M NaCl frog Ringer	Artificial sea water **
n-Pentane	31	22
n-Hexane	7.9	5.5
n-Heptane	2.4	1.6
n-Octane	0.51	0.34
n-Nonane	0.12	0.078
n-Decane	0.042	0.026

* Assumed effectively the same and equal to results of ref. 19 for 0.148 M NaCl.

** Calculated from results of Dagger, F. and Haydon, D.A. (unpublished), using linear interpolation of free energies, from data of ref. 20.

inhibited compound action potential was measured and the alkane perfusion resumed. The compound action potential recovered to its original level in less than 8 min. Sensitivities were expressed as the percentage reduction of the compound action potential by the tetrodotoxin.

The temperature in all experiments was between 18 and 21°C.

The solubility of n-alkanes in electrolyte solutions

Solubility data for *n*-alkanes in water have been reviewed by Bell [12] and are reasonably consistent for the range *n*-pentane to *n*-decane. The effect of electrolytes on the solubility has been examined by Morrison and Billett [20] and discussed by Tanford [21] and, more recently, data have been obtained in this laboratory for *n*-pentane to *n*-decane. All these results suggest that for concentrations below 1.0 mol/l. there is an appreciable decrease in alkane solubility with increasing salt concentration. The measured or estimated solubilities of the alkane in 0.1 M NaCl (black film experiments), frog Ringer and artificial sea water are given in Table I.

Materials

The egg yolk phosphatidylcholine was chromatographically pure and was kindly supplied by Mr. Nigel Miller, Institute of Animal Physiology, Babraham. Monoolein was from Nu Chek Prep (Minnesota, U.S.A.) and was more than 99% pure. The cholesterol was of 'Biochemical Standard' grade from BDH Chemicals Ltd. All the *n*-alkanes were obtained from Koch-Light Laboratories. They were nominally of 'puriss' grade ($\geqslant$99% pure) and were passed through alumina columns before use.

Other reagents were of AR quality and, for the black film experiments, the water was twice distilled.

Results

The effect of n-alkanes on bilayer thickness and composition

The aims of these experiments were to estimate both the thickness of the bilayer and the amount of alkane adsorbed as a function of lipid composition

and alkane concentration. The thickness of the hydrocarbon region of a black film is readily obtained from electrical capacity and area measurements [17]. The only assumption required is that of a dielectric constant for the mixture of non-polar moieties present, and this has been concluded to be close to 2.1 for both phospholipid and monoglyceride chains, as well as for the ring structure of cholesterol [13,17]. In fact, a value of 2.14 has been assumed throughout this work.

The capacity per unit area and thickness results are shown in Figs. 1—4. In order to give a complete picture of both cholesterol-free as well as cholesterol-containing membranes, some earlier data have been included in the first two figures. The main point of interest in Figs. 1 and 2 is that the strong dependence of capacity and thickness on alkane chain length which was already known to exist in monoglyceride and phosphatidylcholine membranes [13] is shifted, in the presence of cholesterol, to much shorter chain lengths. Figs. 3 and 4 reveal how films formed with hexadecane, which are almost as thin as solventless bilayers [13], become thicker in increasing concentrations of pentane and heptane. Owing to the difficulty of preventing evaporation the thicknesses in the pentane systems should be regarded as lower limits.

The amounts of alkane present in the various black films may be estimated by the methods of either Cook et al. [16,17] or Pagano et al. [22,17]. Neither is satisfactory for measuring the alkane content directly, the former for thermodynamic reasons and the latter owing to the fact that microlenses are often present. In each method the lipid adsorption is estimated and, from a knowledge of the total hydrocarbon volume per unit area of film (numerically equal to the thickness) and assumptions as to the partial molar volumes of the lipids, the alkane content is found by difference. The finer details have been discussed

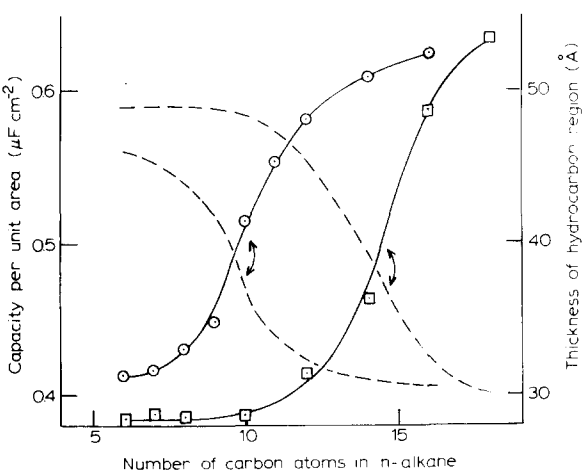

Fig. 1. The variation in electrical capacity per unit area, and hydrocarbon layer thickness of black lipid films formed with different *n*-alkanes. ⊡, capacity of monoolein films in absence of cholesterol (the points for decane-octadecane are taken from ref. 13); ⊙, capacity of monoolein films containing cholesterol/monoolein = 0.55. The dashed curves show the thickness variations, and are related to the full curves as indicated by the arrows. The aqueous phase was 0.1 M NaCl which had been equilibrated with the film-forming solution.

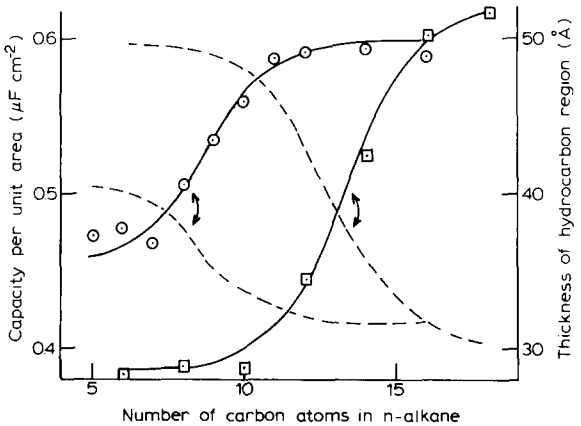

Fig. 2. The variation in electrical capacity per unit area, and hydrocarbon layer thickness of black lipid films formed with different *n*-alkanes. ⊡, capacity of egg phosphatidylcholine films in absence of cholesterol (the points for decane-octadecane are taken from ref. 13); ⊙, capacity of egg phosphatidylcholine films containing cholesterol/phosphatidylcholine = 0.4. The dashed curves show the thickness variations and are related to the full curves as indicated by the arrows. The aqueous phase was as in Fig. 1.

elsewhere [17]. Where both techniques have been applied to the same type of black film good agreement has been found [22,17]. In the present systems it is known that neither the amount of phosphatidylcholine nor monoolein adsorbed into a black film are appreciably dependent on the chain length of the alkane used as a solvent [16,22]. It is also known that the adsorption of *n*-alkanols at alkane/water interfaces is not sensitive to the chain length of the alkane [23]. It will be assumed therefore that, to a good approximation, the amounts of

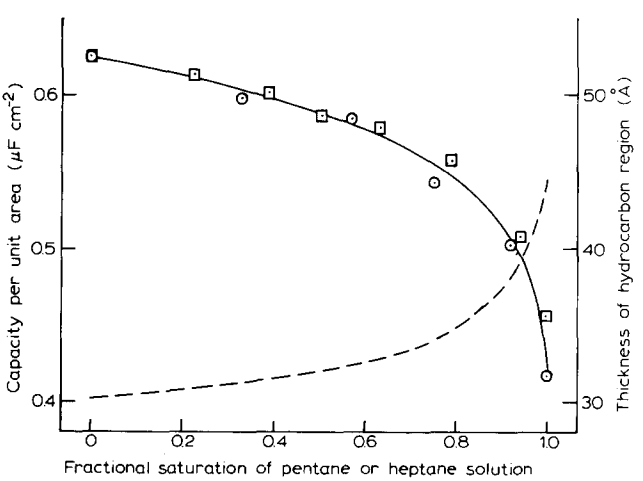

Fig. 3. The influence of *n*-pentane and *n*-heptane on the electrical capacity per unit area and hydrocarbon layer thickness of monoolein-cholesterol-hexadecane films. Capacity in *n*-pentane, (⊡) and *n*-heptane, (⊙). The dashed curve indicates the thickness variation. The film-forming and aqueous phases were equilibrated before the experiments. Bulk partition coefficients for the hydrocarbons were assumed independent of concentration. For *n*-pentane, the capacities are possibly erroneously high and the thicknesses low owing to the difficulty of ensuring equilibrium when using volatile solvents.

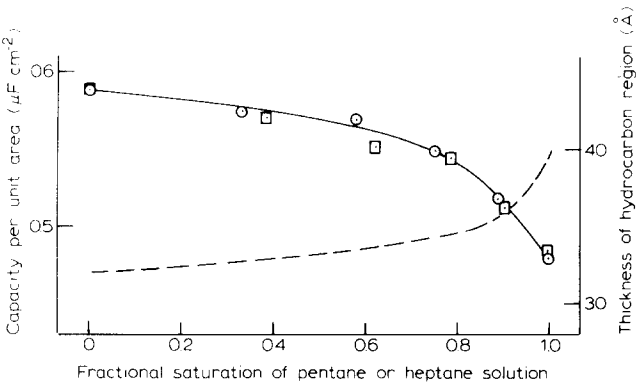

Fig. 4. The influence of *n*-pentane and *n*-heptane on the electrical capacity per unit area and hydrocarbon layer thickness of egg phosphatidylcholine-cholesterol-hexadecane films. Capacity in *n*-pentane, (□) and *n*-heptane, (○). The film forming and aqueous phases were equilibrated before the experiments. As described for Fig. 3, volatility may have affected the pentane data.

phosphatidylcholine + cholesterol and monoolein + cholesterol present in the bilayers were invariant with respect to the alkane used. This being so, changes in the volume of alkane adsorbed are proportional to the changes in membrane thickness and, from a knowledge of the lipid composition for one system, the actual volume of alkane present can be estimated for all of them.

For the cholesterol-free membranes, the numbers of monoolein and egg phosphatidylcholine molecules per cm^2 of membrane were $5.1 \cdot 10^{14}$ and $3.3 \cdot 10^{14}$. respectively [13]. In the monoolein-cholesterol systems there were ca. $3.5 \cdot 10^{14}$ molecules per cm^2 of monoolein and $1.9 \cdot 10^{14}$ molecules per cm^2 of cholesterol, giving a mol ratio cholesterol:monoolein of 0.55 [22], whilst in the phosphatidylcholine-cholesterol systems there were ca. $3.0 \cdot 10^{14}$ molecules per cm^2 of phosphatidylcholine and $1.2 \cdot 10^{14}$ molecules per cm^2 of cholesterol, i.e. cholesterol:phosphatidylcholine = 0.4 [16].

Comparison of the thickness of the phosphatidylcholine-cholesterol-hexadecane black film with the thickness of a leaflet of similar lipid composition in the lamellar phase, as calculated from X-ray diffraction data [13], suggests that the black film contains some 9% (v/v) hexadecane. For lower alkanes, down to undecane, Fig. 2 indicates that this situation changes very little, but that from undecane down to pentane the maximum volume of alkane that can be adsorbed increases to ca. 36%.

The effect of n-alkanes on the propagated action potential of the squid giant axon

Solutions of pentane caused a rapid and complete elimination of the propagated action potential at concentrations greater than 80% saturation (Fig. 5). During the disappearance of the action potential, both the rate of rise and the conduction velocity, as well as the peak height, declined (Fig. 6). The resting potential was not greatly affected. On initial contact with the alkane, the axons tended to depolarize by 2—3 mV but subsequently recovered and, in the final steady alkane concentration the axons were often hyperpolarized by 1—2 mV. In the short term, all the effects were reversible. There was also a strong depen-

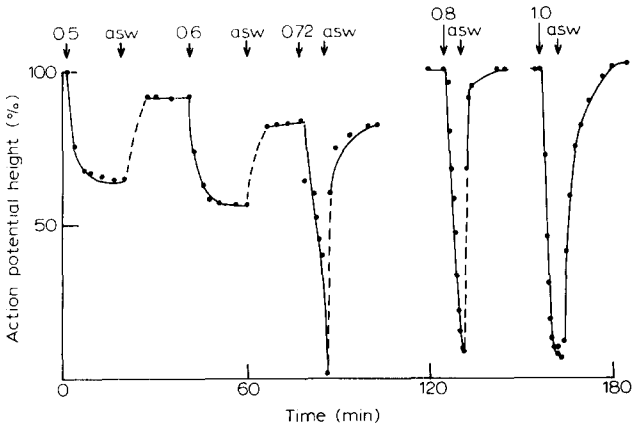

Fig. 5. The suppression of the action potential in the squid giant axon by *n*-pentane at various concentrations. Three different axons were used in this record. The arrows mark the introduction of pentane solution of the degree of saturation indicated, or of artificial sea water (asw).

dence of blocking potency on pentane concentration in the range 50—70% saturation.

Higher alkanes had progressively less influence on the action potential (Fig. 7) until, with octane- or nonane-saturated solutions, no significant effect was detectable. A possible explanation for these latter observations is that, owing to their very low solubility, insufficient alkane reached the axon membrane. However, this appears extremely unlikely, for two reasons. Firstly a volume of octane equal to the total amount of lipid in the axon preparation (including Schwann cell and mitochondria) would have been present in less than 5 ml of perfusing solution, whereas the axon was exposed to 1000 ml. Secondly, the time constant for the octane to saturate the lipid membranes of the axon and

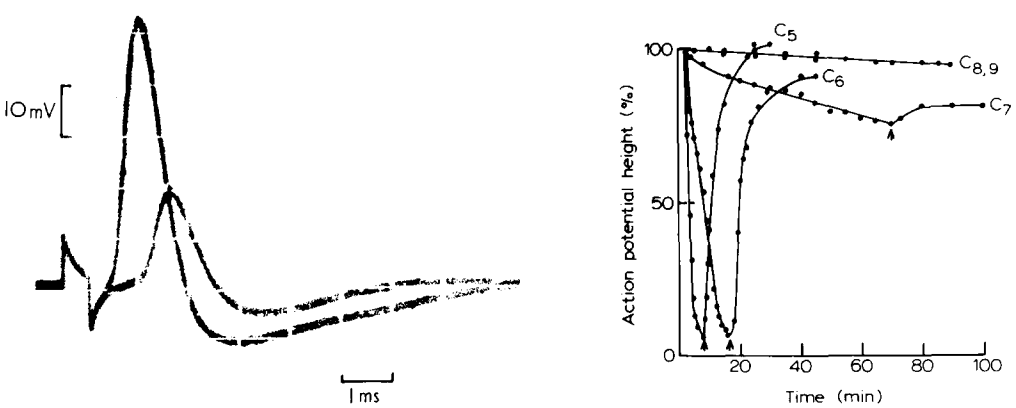

Fig. 6. Two traces of the propagated action potential in the squid giant axon both recorded during exposure to 0.9 saturated *n*-pentane in artificial sea water. The lower trace was recorded approximately 90 s after the upper one. The action potential disappeared within 8 min of contact with the *n*-pentane solution.

Fig. 7. Blockage of the propagated action potential in squid giant axons by saturated solutions in artificial sea water of the *n*-alkanes from *n*-pentane (C_5) to *n*-nonane (C_9). The arrows indicate a switch to alkane-free sea water.

Schwann cell, assuming the diffusional resistance to be equivalent to a layer of water 50 μm in thickness, is approximately 8 min. If the octane were appreciably anaesthetic, therefore, its full effect should have been seen well within the time of an experiment, i.e. >1 h.

The experiments of Fig. 7 confirm the existence of a cut-off in anaesthetic potency with increasing chain length and, in this preparation, the loss of activity occurs between hexane and octane. The anaesthetic potency of *n*-heptane was difficult to assess. It produced a rather slow fall in action potential height which was only partially reversible. The axons therefore appeared to suffer some damage in this system. Similarly irreversible effects were occasionally noted in pentane and hexane systems when axons were exposed to the alkane for longer periods than in Fig. 7.

In the early stages of the application of pentane, hexane and heptane solutions to the axons, a single stimulus quite often produced a train of impulses lasting from several seconds in the case of pentane to several minutes for heptane and dying out with damped oscillations back to the resting potential (Fig. 8). These effects always ceased well before the alkane produced its maximum suppression of the action potential and thus seemed to arise only at the very beginning of the penetration of the alkane into the axon. No comparable effects were observed when the alkane was washed out of the axon. A possible explanation for this hypersensitivity will be discussed later.

The effect of the n-alkanes on the frog sciatic nerve compound action potential

A difficulty in the use of the sciatic nerve to test for anaesthetic activity in the longer chain alkanes arises from the fact that the nerve preparation is likely to contain of the order of a milligram of lipid, and that many litres of alkane-saturated solution may therefore be needed to produce impulse-blocking concentrations in the axons. Moreover the control nerves declined unpredictably in their response to a stimulus after 15 h, thus limiting the duration of a useful experiment and hence also the volume of alkane solution to which the nerve could be conveniently exposed. In order to introduce sufficient alkane at reasonable flow-rates, emulsions, prepared by sonicating small amounts of alkane into the Ringer, were in some instances substituted for simple solutions. In all

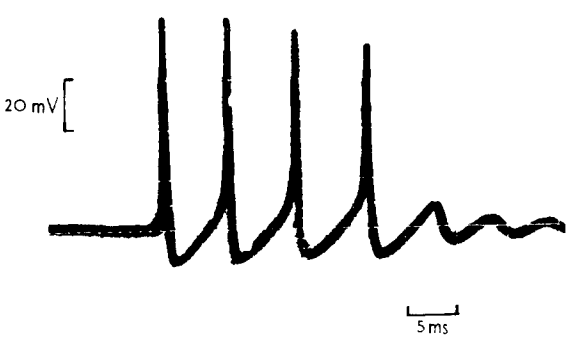

Fig. 8. A train of impulses which outlasted the stimulus in the squid giant axon during exposure to 0.7 saturated *n*-pentane in artificial sea water. The record was taken approximately 2 min after contact with the alkane solution. The resting and action potentials before introduction of the alkane were —60 and 108 mV respectively. At the onset of the repetitive firing the resting potential changed to —58 mV.

systems this procedure produced a more rapid suppression of the compound action potential which was independent of the flow and stirring rates.

The results for the alkanes pentane to decane are shown in Fig. 9. While, as for the squid axon, the longer chain molecules are clearly less effective than the shorter ones, it is again necessary to consider the extent to which diffusion time may account for the observed variations. A simple diffusional model indicates that the times for the alkanes to reach equilibrium with a membrane some distance inside the bundle of axons should be inversely proportional to both their water solubility and to their diffusion coefficients. Owing to the complex structure of the sciatic nerve, estimates of absolute diffusion times are likely to be highly inaccurate but, if it is assumed that in the pentane system, for example, the suppression of the compound action potential is purely diffusion limited, reasonably accurate upper limits for the diffusion times in the other systems should be calculable. The time constant for pentane was approximately 1 min and hence the corresponding values for hexane, heptane, octane, nonane and decane should have been about 5, 17, 80, 300 and 1500 min, respectively.

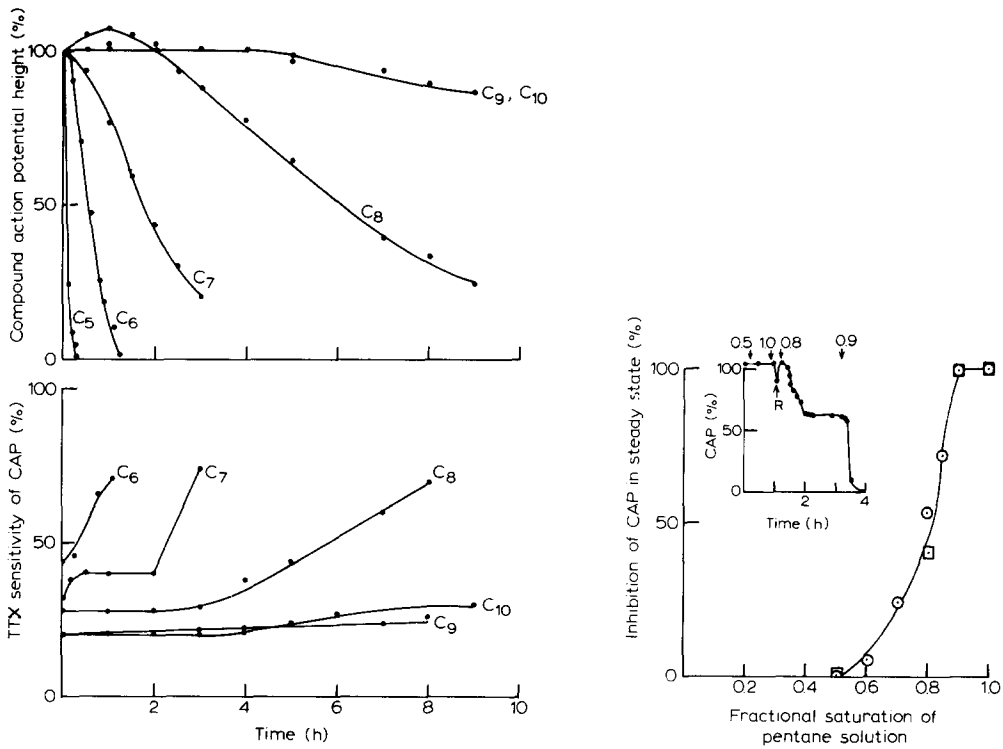

Fig. 9. The blockage of the compound action potential (CAP) in the desheathed frog sciatic nerve by saturated solutions (dilute emulsions) of the *n*-alkanes from pentane to decane. The lower graph shows the reduction of the compound action potential after 4 min exposure to tetrodotoxin (TTX) (2—7 μg/l) at various stages of the suppression of the impulses. Further details of this assay are given in ref. 19.

Fig. 10. The suppression of the compound action potential (CAP) in the desheathed frog sciatic nerve by *n*-pentane solutions. The "equilibrium" curve was obtained by experiments, as in the inset, on two nerve preparations, (⊙ and ⊡). The arrows indicate the introduction of pentane solutions of various degrees of saturation. *R* denotes a switch to pentane-free Ringer.

For all the alkanes except decane, the compound action potentials declined with time constants considerably larger than these estimates and it is concluded that there is a genuine loss of anaesthetic potency with increasing chain length. In fact, it is probable that the decline of the compound action potentials in the presence of nonane and decane does not represent a true anaesthetic effect at all. Thus, firstly, it was noted in squid axon experiments that the presence of free alkane even though it were, like decane, anaesthetically inactive, substantially accelerated the deterioration of the nerve. Secondly, the near coincidence of the nonane and decane curves in Fig. 9 suggests the operation of some non-specific effect unrelated to chain length.

When emulsions rather than true solutions of alkane were used, the blockage of the compound action potential could never be reversed, presumably owing to the difficulty of removing the final traces of liquid hydrocarbon. Even for solutions, e.g. of pentane, the nerve could not be revived once the compound action potential had completely disappeared. At lower levels of inhibition, however, partial or total reversibility could be achieved. Fig. 10 shows the concentration dependence of compound action potential block for dilutions of pentane-saturated Ringer. The relationship is quite steep, the first effects being observed at 60% saturation and total inhibition at 90% saturation. This curve was obtained from several experiments of the type shown in the inset to Fig. 10, i.e. perfusion with successively more concentrated pentane solutions, noting the steady state inhibition achieved in each case.

As the effects of the alkanes on the compound action potential might conceivably have been due to an inhibition of the sodium pumps in the axons, a sciatic nerve was exposed for 4 h to 50 μM ouabain. Despite continuous stimulation no decline in the compound action potential occurred until 15 h later.

Discussion

The effects of the n-alkanes on impulse propagation

The existence of a decline in anaesthetic potency with increasing chain length, in the *n*-alkanes, noted previously in whole animals [1,10], is confirmed by the present results for isolated nerve preparations. For the squid giant axon the decline is clearly between *n*-pentane and *n*-octane and, for the sciatic nerve, though a little less clearly, between *n*-pentane and *n*-nonane.

The action of the alkanes appears to be two-fold. There is a reversible blockage of the impulse which may properly be called anaesthesia and, for more prolonged exposures to the alkane, especially at high concentrations, there is an irreversible blockage, the occurrence of which is somewhat irreproducible from one nerve preparation to the next. An irreversible action has been reported for other anaesthetics [1,2]. In the course of the reversible blockage of the action potential, both the conduction velocity and the maximum rate of rise of the potential decrease and it is concluded that the maximum sodium current is therefore also decreased [24]. The rise in tetrodotoxin sensitivity of the sciatic nerve (Fig. 9), especially when considered in conjunction with the constancy of the resting potential in the squid axons, supports the notion that the alkane inhibits the functioning of the sodium channels. Voltage clamp experiments, which will be described elsewhere, have confirmed this conclusion but, at the

same time, have revealed that the potassium channel is also affected.

The tendency for impulses to arise spontaneously during the early stages of alkane treatment resembles the effect produced by abnormally low Ca^{2+} concentrations in the external solution [25,26]. No attempt has yet been made to discover how the alkanes produce this effect but it may be significant that the repetitive firing was observed only when the alkane was penetrating the axon and not during washing out or in the final equilibrium state. Thus, a higher concentration of the alkane on the outside than on the inside of the membrane seems necessary. It may be speculated therefore that, in line with the mechanism proposed for low Ca^{2+}, the differential interaction of the alkaline on the two sides of the membrane lowers the internal electric field. This could occur through a change in the dipole part of the surface potential on one side [27]. Although there is no evidence as yet that the alkanes affect the dipole potentials to the required extent, it should be remembered that these potentials are so large (ca. 400 mV) [27] that a change of only 5% would probably be sufficient to account for the observations.

Changes in bilayer adsorption as an explanation of the chain length dependence of impulse blockage

Ideally, in order to show the relationship between alkane uptake and impulse blockage, the adsorption of alkane into the axon membrane should have been measured. This would have been very difficult both in experimental technique and in the interpretation of the data, and was therefore not attempted. However, direct measurements on human erythrocytes have been carried out (Dagger, F. and Haydon, D.A., unpublished) and, although they are limited in accuracy and concentration range, they show clearly that for a biological membrane containing an appreciable amount of cholesterol (cholesterol : phospholipid 0.89 : 1 [28]), the adsorption of the alkanes (at identical concentrations relative to saturation) decreases sharply for homologues larger than *n*-pentane and is a few percent (v/v) only for *n*-decane.

In the experiments with black films formed with different chain length alkanes, the membrane thicknesses and compositions correspond to equilibrium adsorption into a lipid bilayer from a near-saturated aqueous phase. That there is a considerable decrease in this adsorption with increasing chain length was originally reported some time ago [13]. The new and possibly very significant fact revealed by the present results is that this adsorption is strongly dependent on the cholesterol content of the bilayer. In comparing these results with the nerve experiments it is obviously desirable to have some knowledge of the cholesterol content of the axons concerned. For the sciatic nerve there is no useful information but, for both the stellar nerve of the squid *Dosidicus gigas* and the garfish olfactory nerve, analyses show that the cholesterol : phospholipid mol ratio is in the region of 0.33 : 1 [15,29] and therefore close to the ratio of 0.4 : 1 deduced for the black films of Fig. 2.

From the evidence discussed above it seems reasonable to conclude that the variation of alkane adsorption with chain length found in both human erythrocytes and lipid bilayers occurs also in the nerve axon. Certainly, there is a striking correlation between the decline in anaesthetic potency with chain length and decreasing adsorption into a phosphatidylcholine-cholesterol black

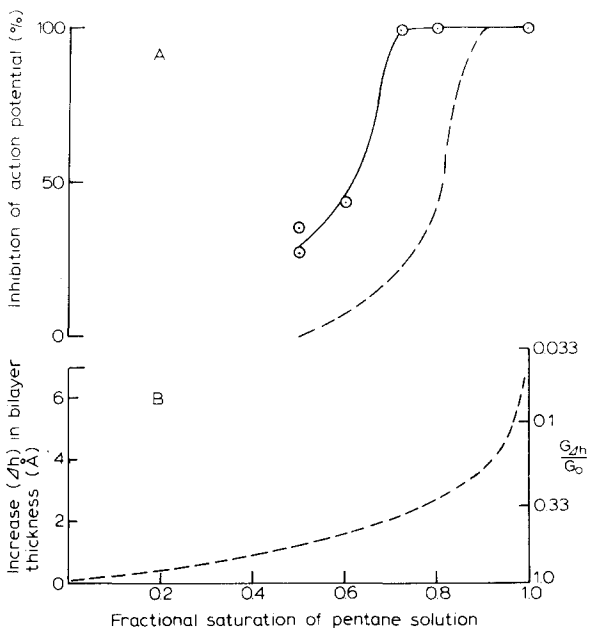

Fig. 11. A comparison of the percentage inhibition of the action potential by *n*-pentane solutions (A) with the thickening of a phosphatidylcholine-cholesterol-hexadecane black film by pentane (B). The full curve (⊙) in A is for the squid giant axon and is derived from the data of Fig. 5 and other similar records. The dashed curve in A is for the sciatic nerve and is as in Fig. 10. In B, the right hand ordinate shows the factor by which the conductance (G) of a gramicidin-containing membrane would be reduced, according to Eqn. 1, by the observed thickness change.

film. There is also a correlation (Fig. 11) between anaesthetic potency and adsorption for pentane at different concentrations. These correlations obviously support the notion that the interior of a lipid bilayer is the site of action of the hydrocarbon anaesthetics. If these anaesthetics act in a protein it is necessary to accept that there is a similarity with respect to alkane binding between the protein site and the bilayer interior. It is also of interest that the decline in anaesthetic potency with alkane chain length occurs between *n*-pentane and *n*-decane not only in the squid axon and sciatic nerve preparations, but also when the righting reflex of the mouse is used as the end-point [10,11]. The excitability of an isolated nerve is determined by the voltage-dependent sodium and potassium channels, but the susceptibility of these to anaesthetics is not considered to be high enough for their inhibition to underlie general anaesthesia [2]. If, therefore, the cut-off in *n*-alkane activity is to be accounted for in terms of protein site interactions then a further similarity must exist between the proteins involved in local and general anaesthesia. At present it seems more reasonable to think of the bilayer as the common factor.

A membrane thickness-tension hypothesis for the blockage of the impulse by hydrocarbons

If the site of action of the alkanes in a nerve membrane is a lipid bilayer region it is necessary to consider how the ionic channels involved in impulse propagation might be influenced. Both the sodium and potassium channels of a nerve membrane have relatively large unit conductances (between 3 and 12 ·

10^{-12} S [30,31] and are widely believed to take the form of pores through the membrane. In this respect these channels exhibit broad similarities with the polypeptide gramicidin A [33]. As emphasized earlier in this paper, one of the bilayer parameters influenced by the alkanes and which correlates with impulse blockage, is the thickness. Obviously if the membrane thickness increased significantly it could become increasingly difficult for an ionic channel to link the two aqueous phases or to continue to function owing to the change in the stresses to which it is subjected. In other words, in thicker membranes the stability of open ionic channels might well decline.

Such a mechanism has already been proposed to account for the conductance properties of gramicidin in black films of different thicknesses [27,34]. In these systems, as the bilayer thickness increases, the equilibrium which exists between conducting and non-conducting gramicidin is shifted strongly to the non-conducting side. This occurs to an extent which can be semi-quantitatively accounted for by assuming that in order that the channel entrances should be exposed simultaneously to both aqueous phases, the lipid deforms or 'dimples' (Fig. 12). Thus, when the hydrocarbon region in the bilayer becomes thicker than the length of the channel (ca. 28 Å [35,36]) the standard free energy of channel formation becomes more positive by an amount equal to the surface free energy change in the formation of the dimple. This surface free energy change may be crudely estimated in macroscopic terms from the surface area increase and membrane tension. Empirically, the constant K of equilibrium between conducting and non-conducting states for gramicidin in a monoolein-decane black film is related to film thickness (h) by an equation of the form

$$K \approx A \exp\left(-\frac{h}{B}\right) \tag{1}$$

where A and B are constants, the latter being approximately 2.2 Å [28]. Eqn. 1 is consistent with the dimpling hypothesis if a value of about 3 dyne/cm is

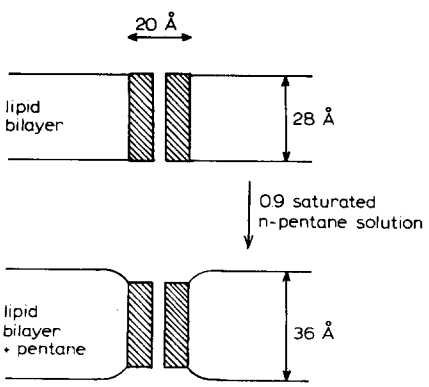

Fig. 12. The proposed thickness-tension model. The shaded region represents a gramicidin channel situated in a monoolein-cholesterol lipid bilayer. It is assumed in the upper diagram that the bilayer thickness is equal to the channel length. The lower diagram shows the change in thickness of the bilayer which occurs in a pentane solution of concentration sufficient to block the action potential (0.9 saturated). The deformation or dimpling of the bilayer involves a surface free energy change which tends to reduce the stability of the open channel.

assumed for the bilayer surface tension, and the channel has dimensions as shown in Fig. 12. The experimental value of the tension is, in fact, very close to 3 dyne/cm [13,37].

Such numerical tests are not readily applied to the nerve membrane, but it is of interest to consider the data of Fig. 11, in which a 60% saturated pentane solution roughly halves the peak height of the squid action potential and increases the thickness of a phosphatidylcholine-cholesterol bilayer by 1.6 Å. If Eqn. 1, which was obtained for gramicidin in a monoolein-decane membrane, is applied to the sodium channel, a two-fold diminution of the maximum sodium current should have occurred, in reasonably good agreement with a 50% depression of the spike amplitude. The relative sensitivities of gramicidin and the sodium channel to thickness changes are, on the dimpling model, determined by their size (especially their outside diameters) and by the effective surface tensions produced by the adsorption of alkane. The tensions produced in the axon membrane are not known but, in the most closely related artificial membrane, a phosphatidylcholine-cholesterol bilayer, equilibration with *n*-decane yields a surface tension of ca. 1 dyne/cm [16]. Lower alkanes which partition more strongly into the bilayer should produce larger tensions. It should also be remembered that since the opening and closing of the sodium channel takes place within a few milliseconds, dynamic rather than equilibrium tension might be more relevant, and would certainly be larger. The molecular weight of the sodium channel has been estimated at 229 000 daltons [38] indicating that it is substantially larger than gramicidin and could thus require a dimple of larger area to expose its entrances. It is concluded that if the lengths of the sodium (or potassium) channels are similar to that of gramicidin then the thickness-tension model could account quantitatively for the blockage of the impulse by small alkanes. The fact that gramicidin channels are formed by a dimerization process is not necessarily relevant since it is of no consequence for the model whether the ion channel is broken in the middle or by the openings being pulled away from the membrane surfaces. Should the length of the nerve channels be substantially greater than the normal thickness of the membrane, the detailed model proposed for gramicidin would seem less applicable. However, thickness and tension changes could still be important because they would stress the channels or change their equilibrium positions and hence affect their stability.

A definitive test of the thickness-tension idea is not easy to devise, but the model does require that biological membranes as well as artificial bilayers should be thickened by the anaesthetic *n*-alkanes. These molecules are, as mentioned earlier, strongly adsorbed by erythrocyte membranes (Dagger, F. and Haydon, D.A., unpublished) and, as the membrane area shows, if anything, a decrease, a thickness increase is indicated. Perhaps more significant is that preliminary experiments by Haydon, Kimura and Urban have shown the capacity of the squid giant axon membrane to be reversibly lowered from 1.0 to 0.85 $\mu F/cm^2$ by a saturated solution of pentane. A 1 kHz signal of amplitude 1 mV was used, the membrane being at its normal resting potential. The most obvious explanation for this effect is that pentane thickens the bilayer regions of the axon membrane but, as the molecular basis of the original 1 $\mu F/cm^2$ is not properly understood, such a conclusion would be premature.

The relevance of the thickness-tension hypothesis to impulse blockage or anaesthesia caused by molecules other than *n*-alkanes is not yet clear. It is tempting to assume that all non-ionic anaesthetics share a common mechanism, but there is no conclusive evidence for this. Nevertheless the *n*-alkanols, as well as the *n*-alkanes, show a cut-off in anaesthetic potency with increasing size (*n*-tridecanol is inert [11]), and the lengthening of the side chains on barbiturates also produces a complete loss of hypnotic potency [39]. This suggests a common site of action for these molecules. It has been found (unpublished data) that ethanol and diethylether at anaesthetic concentrations both reduce the capacity per unit area of monoolein-hexadecane black films. As these molecules can only raise the dielectric constant of the bilayer interior they must be causing a thickness increase. The reversal of anaesthesia by pressure has been reported for a large range of anaesthetics including ethanol and diethylether [40,8]. The reversal has been accounted for by supposing that a vital hydrophobic region, expanded by the anaesthetic, is compressed back to its original dimensions. This is consistent with the thickness-tension hypothesis and with there being to some extent a common mechanism in many systems. In a sense the thickness-tension model is an extension of the critical volume hypothesis [41]. However, its advantage is that a specific molecular mechanism is proprosed by which anaesthetics reduce electrical excitability.

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References

1 Mullins, L.J. (1971) in Handbook of Neurochemistry (Lajtha, A., ed.), Vol. VI, pp. 395—421, Plenum Press, New York
2 Seeman, P. (1972) Pharmacol. Rev. 24, 583—655
3 Schneider, H. (1968) Biochim. Biophys. Acta 163, 451—458
4 Seeman, P., Roth, S. and Schneider, H. (1971) Biochim. Biophys. Acta 225, 171—184
5 Boggs, J.M., Yoong, T. and Hsia, J.C. (1976) Mol. Pharmacol. 12, 127—135
6 Lee, A.G. (1976) Nature 262, 545—548
7 Woodbury, J.W., D'Arrigo, J,S. and Eyring, H. (1975) in Molecular Mechanisms of Anaesthesia (Fink, B.R., ed.), Vol. 1, pp. 253—275, Raven Press, New York
8 Miller, K.W., Paton, W.D.M., Smith, R.A. and Smith, E.B. (1973) Mol. Pharmacol. 9, 131—143
9 Richards, C.D. (1976) Nature 262, 534
10 Führer, H. (1921) Biochem. Z. 115, 235—261
11 Brink, F. and Posternak, J.M. (1948) J. Cell. Comp. Physiol. 32, 211—233
12 Bell, G.H. (1973) Chem. Phys. Lipids 10, 1—10
13 Fettiplace, R., Andrews, D.M. and Haydon, D.A. (1971) J. Membrane Biol. 5, 277—296
14 Andrews, D.M., Manev, E.D. and Haydon, D.A. (1970) Special Disc. Faraday Soc. No. 1, 46—56
15 Chacko, G.K., Villegas, G.M., Barnola, F.V., Villegas, R. and Goldman, D.E. (1976) Biochim. Biophys. Acta 443, 19—32
16 Cook, G.M., Redwood, W.R., Taylor, A.R. and Haydon, D.A. (1968) Kolloid-Z. u.Z. für Polymere 227, 28—37
17 Fettiplace, R., Gordon, L.G.M., Hladky, S.B., Requena, J., Zingsheim, H.P. and Haydon, D.A. (1975) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 4, pp. 1—75, Plenum Press, New York
18 Baker, P.F., Hodgkin, A.L. and Shaw, T.I. (1962) J. Physiol. 164, 330—354
19 Levinson, S.R. (1975) Ph.D. Dissertation, Univerity of Cambridge
20 Morrison, T.J. and Billett, F. (1952) J. Chem. Soc. 3819—3822

21 Tanford, C. (1973) The Hydrophobic Effect, pp. 9—10, John Wiley and Sons, New York
22 Pagano, R.E., Ruysschaert, J.M. and Miller, I.R. (1972) J. Membrane Biol. 10, 11—30
23 Aveyard, R. and Briscoe, B.J. (1972) J. Chem. Soc., Faraday I, 68, 478—491
24 Hodgkin, A.L. (1967) The Conduction of the Nervous Impulse, Liverpool University Press
25 Frankenhaeuser, B. and Hodgkin, A.L. (1957) J. Physiol. 137, 218—244
26 Huxley, A.F. (1959) Ann. N.Y. Acad. Sci. 81, 221—246
27 Haydon, D.A. (1975) Ann. N.Y. Acad. Sci. 264, 2—16
28 Bar, R.S., Deamer, D.W. and Cornwell, D.G. (1966) Science 153, 1010—1012
29 Camejo, G., Villegas, G.M., Barnola, F.V. and Villegas, R. (1969) Biochim. Biophys. Acta 193, 247—259
30 Almers, W. and Levinson, S.R. (1975) J. Physiol. 247, 483—509
31 Conti, F., DeFelice, L.J. and Wanke, E. (1975) J. Physiol. 248, 45—82
32 Armstrong, C.M. (1974) Q. Rev. Biophys. 7, 179—210
33 Hladky, S.B. and Haydon, D.A. (1972) Biochim. Biophys. Acta 274, 294—312
34 Haydon, D.A. and Hladky, S.B. (1972) Q. Rev. Biophys. 5, 187—282
35 Urry, D.W., Goodall, M.C., Glickson, J.D. and Mayers, D.F. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1907—1911
36 Veatch, W.R., Fossel, R.T. and Blout, E.R. (1974) Biochemistry 13, 5249—5256
37 Requena, J. and Haydon, D.A. (1975) Proc. Roy. Soc. Lond. A, 347, 161—177
38 Ellory, J.C. and Levinson, S.R. (1973) Nature 245, 122—123
39 Mautner, H.G. and Clemson, H.C. (1970) in Medicinal Chemistry (Burger, A., ed.), 3rd Edn., Pt. II, pp. 1365—1383, Wiley-Interscience, New York
40 Johnson, F.H. and Flagler, E.A. (1950) Science 112, 91—92
41 Mullins, L.J. (1954) Chem. Rev. 54, 289—323