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NERVE IMPULSE BLOCKAGE IN SQUID AXONS BY *n*-ALKANES: THE EFFECT OF AXON DIAMETER

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SUMMARY

1. The anaesthetic effects of aqueous solutions of the *n*-alkanes pentane to nonane on the propagated action potential of squid axons have been investigated for a range of axon diameters.

2. By the use of small axons (approx. 200 μm diameter) to minimize effects due to long diffusion times and alkane depletion it was found that *n*-pentane and *n*-hexane caused a rapid reversible inhibition of the impulse, while higher homologues had progressively less effect, *n*-nonane being apparently inert.

3. The rate of action potential decline due to the *n*-alkanes was found to be strongly dependent on axon diameter. For *n*-hexane, *n*-heptane and *n*-octane the rate of decline was inversely proportional to the square of the axon diameter.

4. The mechanisms which may underly the increased sensitivity of small axons to impulse blockade by *n*-alkanes are discussed. A quantitative comparison is made between the effects of *n*-hexane, *n*-heptane and *n*-octane on the action potential. It is argued that this supports the idea of a real decline in anaesthetic potency on ascending the homologous series, rather than an effect due to long diffusion times and solution depletion.

INTRODUCTION

There has recently been some interest in the anaesthetic properties of the *n*-alkanes. One reason for this is the apparent diminution in their anaesthetic potency with increasing chain length. Thus, the small *n*-alkanes, such as *n*-pentane, are potent anaesthetics but higher homologues such as *n*-nonane and *n*-decane are apparently inert (Fuhner, 1921; Mullins, 1971; Seeman, 1972; Haydon, Hendry, Levinson & Requena, 1977*a*). This behaviour is an exception to the Meyer Overton correlation and has been explained on the basis of decreased adsorption of the longer *n*-alkanes into the lipid bilayer portion of cell membranes (Haydon *et al.* 1977*a*). The observations that the anaesthetic alkanes increase both the thickness and tension of artificial lipid bilayers has led to the idea that such factors may be important in producing the blockage of nervous impulses (Haydon *et al.* 1977*a,b*). In this respect the longer *n*-alkanes should be inactive because their adsorption into the bilayer is insufficient to cause significant alterations in bilayer thickness or tension. One

important difficulty with this line of argument lies in demonstrating that the inactivity of *n*-nonane and *n*-decane is an equilibrium effect and not due to long diffusion times or depletion of alkane from solution. Thus the solubilities in sea water of the longer alkanes are quite low (*n*-pentane, 306 μM; *n*-decane, 0·18 μM; Haydon *et al.* 1977*a*) and there is much material in an axon preparation, both in the membranes and in the axoplasm, that could bind hydrocarbons. Rough calculations show that, for *n*-decane, the equilibration time could well exceed that for the natural deterioration of an axon under experimental conditions.

The present study is an attempt to investigate further the decline in potency of the higher alkanes and to distinguish as far as possible between effects due to alkane depletion and different diffusion times on the one hand, and effects due to the lower potency of the alkane at the axonal membrane on the other. We have employed the axons radiating from the stellate ganglion of the squid having diameters in the range 160–800 μm. Small cleanly dissected squid axons constitute a good preparation for minimizing effects due to depletion and diffusion. Nevertheless even using these small fibres it has not been found possible to measure equilibrium potencies for the alkanes. The results do, however, allow a quantitative comparison of the anaesthetic effects of different alkanes.

A second phenomenon of relevance in the present experiments is the greater susceptibility to block by local anaesthetics of small diameter nerve fibres. This observation has been extensively investigated using myelinated nerves and has important clinical applications, for example in allowing the preferential block of pain fibres rather than motor nerves (Nathan & Sears, 1963; de Jong, 1970; Franz & Perry, 1974; Staiman & Seeman, 1977). The finding that small myelinated nerves are blocked before large ones by local anaesthetics has been confirmed on a number of occasions (see for example de Jong, 1970). The effect of axon size on the susceptibility of unmyelinated axons has not been systematically investigated. The experiments reported here help to quantify the effect of unmyelinated axon size.

A number of theories have been advanced to account for the increased susceptibility of small nerves to block by local anaesthetics. Some are based on non-equilibrium diffusion effects (e.g. Franz & Perry, 1974) while others regard the differential block as an equilibrium phenomenon (Nathan & Sears, 1963; Staiman & Seeman, 1977). Attempts to distinguish between such theories have not proved completely successful. In part this is because the preparations employed have involved bundles of nerve fibres with extremely complex diffusion geometry. Another problem has been the use of imprecise measurements of nerve activity, such as the extracellular recording of the compound action potential. In the present study a number of these difficulties have been overcome by intracellular measurement of the action potential in finely cleaned single axons.

METHODS

The experiments were performed with axons dissected from the mantles of freshly killed *Loligo forbesi*. The axon diameters were in the range 160–800 μm. After careful fine dissection and cleaning, these diameters were measured by means of a calibrated graticule; the value taken being the average of six readings taken at regular intervals along 1 cm of axon.

The axons were mounted in the four-chamber perspex cell shown in Fig. 1. Stimulation of the

nerve was accomplished by extracellular platinum electrodes in chambers C and D. The stimulating pulse lasted 400 μs and the threshold voltage required was usually about 0·2 V. For an experiment the stimulus strength was set at three times threshold. No attempt was made to seal the compartments from each other with grease or any agent which might absorb alkane from solution. The resting potential and propagated action potential were monitored in chamber B using intracellular micro-electrodes. These were micropipettes pulled by means of a Kopf 700C vertical pipette puller and then filled with 3 M-KCl. The tip resistance of each micropipette was measured and only those with resistance in the range 5–20 MΩ were employed in the experiments. The circuit

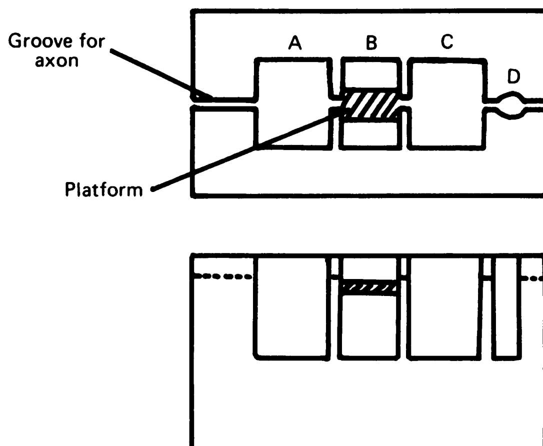

Fig. 1. Schematic diagram of the Perspex cell used in these experiments. Stimulating electrodes were placed in chambers C and D while anaesthetic solution was applied to chamber B. The recording micro-electrode was inserted into the axon in chamber B where it was supported by a Perspex platform. The volume of chamber B was 3·5 ml and within it lay 1·1 cm of axon.

was completed by sealing a silver–silver chloride wire into the micropipette and using a silver–silver chloride electrode in bath B as reference potential. These silver electrodes were connected to a high input impedance amplifier for the measurement of potential difference. The membrane potential was displayed on a storage oscilloscope and readings taken from this trace.

The bathing solution of artificial sea water (ASW) had the following composition (mM): NaCl, 430; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50; Tris base, 10. The pH of the solution was adjusted to 7·4 by addition of HCl. Saturated solutions of alkanes were made up by equilibration of ASW with an excess of the appropriate alkane. For the larger alkanes at least 2 d with continuous stirring were allowed for this to occur. Solutions of lower alkane concentration were obtained by dilution of a saturated solution with ASW immediately prior to an experiment.

The solution was supplied to chamber B of the cell through Teflon tubing. Flow rates of about 10 ml min^{-1} were employed. The solution passed through a cooling system prior to entry into the cell by which the temperature in chamber B was maintained at 6 ± 1 °C. At constant temperature there was no dependence of nerve blocking effect on solution flow rate as long as the flow rate was above 4 ml min^{-1}. Solution was removed from chamber B by suction into a Teflon tube which maintained a constant volume of fluid in the chamber. The position of the Teflon tube supplying alkane solution to chamber B was systematically altered within chamber B to look for effects due to local concentration differences. Its position had no influence on the rate of decline of the action potential.

The propagated action potential entered the recording chamber from chamber C and passed on towards chamber A. These two neighbouring chambers contained alkane-free ASW and one important question concerned the extent to which the impulse in these solutions was recorded by

the electrode in chamber B. To examine this question the position of the micro-electrode along the segment of axon in chamber B was systematically varied. As long as the electrode was inserted more than 3 mm from the partition with chamber C there was no effect of electrode position on the rate of action potential decline. Experiments were therefore performed with the micro-electrode inserted more than 5 mm from the partition.

RESULTS

Resting potentials measured at the beginning of each experiment were typically in the range −55 to −65 mV. Axons with resting potentials more positive than −55 mV were rejected. The resting potential was monitored throughout each

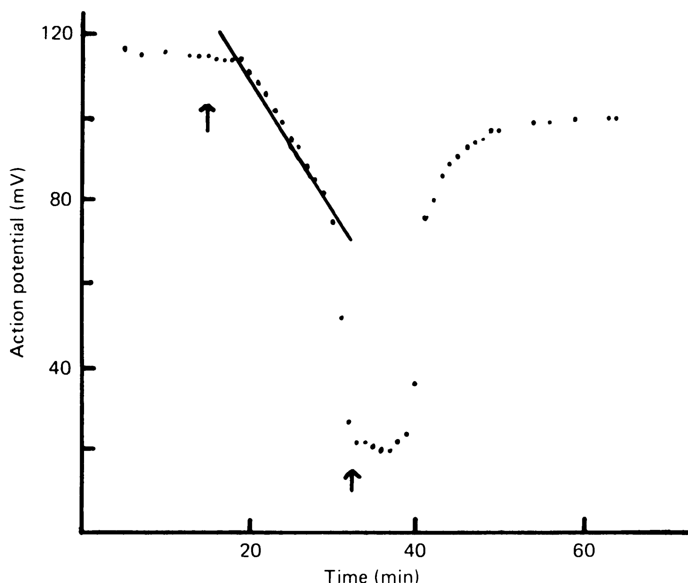

Fig. 2. The effect of saturated *n*-hexane on the amplitude of the action potential. Arrows represent the times of solution changes to *n*-hexane and back to ASW. Axon 19, diameter 710 μm. The line was drawn by eye and used to estimate the initial rate of action potential decline.

experiment and remained constant to within ±2 mV during application of the test alkanes. Prolonged application of one of the longer alkanes, such as *n*-octane, produced an irreversible loss of the action potential without significantly affecting the resting potential. Thus it was not found necessary with any axon to abandon the experiment due to loss of the resting potential during the application of test solutions.

The action potential height was usually found to be between 80 and 110 mV. This height was monitored and recorded once per minute during an experiment. As found by previous workers *n*-pentane and *n*-hexane in saturated solution produced a rapid and reversible inhibition of the action potential (Haydon *et al.* 1977*a*). Longer homologues had progressively less effect and *n*-nonane was apparently inert. *n*-Octane and *n*-heptane were intermediate in effect, producing a slow inhibition of the action

potential which was only partially reversible and to an extent which varied from one axon to another. A typical experiment involving the application of saturated *n*-hexane is shown in Fig. 2. It can be seen that the initial rate of decline of the action potential was nearly linear. There followed a complete inhibition which was reversible on switching the solution to ASW.

With all alkanes tested, prolonged exposure (> 1 h) produced a gradual irreversible decline of the action potential. Even with *n*-hexane solutions on small axons it was

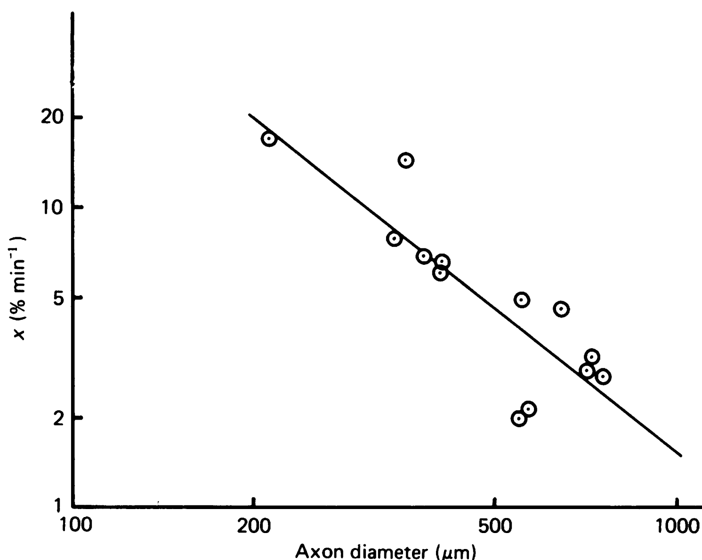

Fig. 3. The relationship between axon diameter (d) and the initial rate of action potential decline x (% min^{-1}) in the presence of saturated *n*-hexane. The line is drawn by linear regression and has a slope of $-1{\cdot}6$ ($r = 0{\cdot}85$).

not possible to achieve a stable reversible degree of block. Accordingly it does not appear possible to measure the equilibrium anaesthetic potency of these molecules. However, the rate of initial decline of the action potential did appear to be a reproducible quantity and some useful conclusions can be drawn by examining this quantity as a function of alkane concentration and of axon diameter.

For all active alkanes it was found that small axons were considerably more sensitive to impulse blockade than large axons. Fig. 3 is a logarithmic plot of the initial rate of action potential decline (x) against axon diameter (d) for saturated solutions of *n*-hexane. The line is plotted by linear regression and has a slope of $-1{\cdot}6$. Figs. 4 and 5 show similar data for saturated solutions of *n*-heptane and *n*-octane respectively. The line for *n*-heptane has a slope of $-1{\cdot}9$, while that for *n*-octane has a slope of $-2{\cdot}1$. Thus, for three *n*-alkanes, the rate of action potential decline is approximately inversely proportional to the square of the axon diameter.

The effects of alkane concentration on the rate of action potential decline were investigated for *n*-hexane and *n*-heptane. Fig. 6 shows the rate of action potential decline *versus* alkane saturation for these two molecules. Each axon employed in this

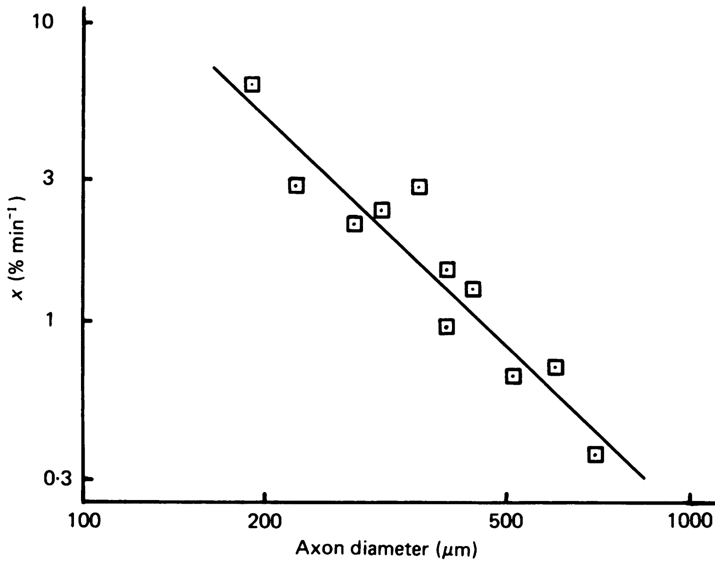

Fig. 4. The relationship between axon diameter (d) and the initial rate of action potential decline x (% min^{-1}) in the presence of saturated n-heptane. The linear regression line has a slope of $-1{\cdot}9$ ($r = 0{\cdot}94$).

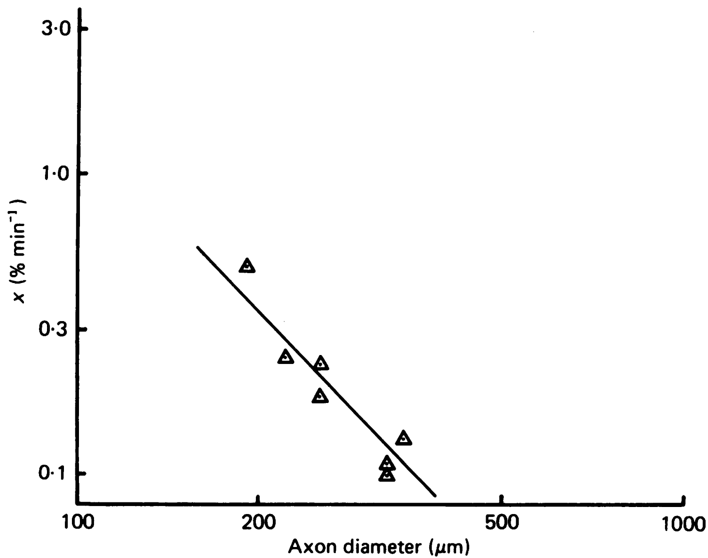

Fig. 5. The relationship between axon diameter (d) and the initial rate of action potential decline x (% min^{-1}) in the presence of saturated n-octane. The linear regression line has a slope of $-2{\cdot}1$ ($r = 0{\cdot}93$).

experiment was exposed first to a saturated alkane solution and then to one or more dilute solutions. The results are plotted as the rate of action potential decline for a dilute solution divided by that for a saturated solution on the same axon. In this way, the problem of the variation in susceptibility for different axons has been circumvented. From Fig. 6 it can be seen that there is a linear relationship between the rate of action potential decline and alkane concentration.

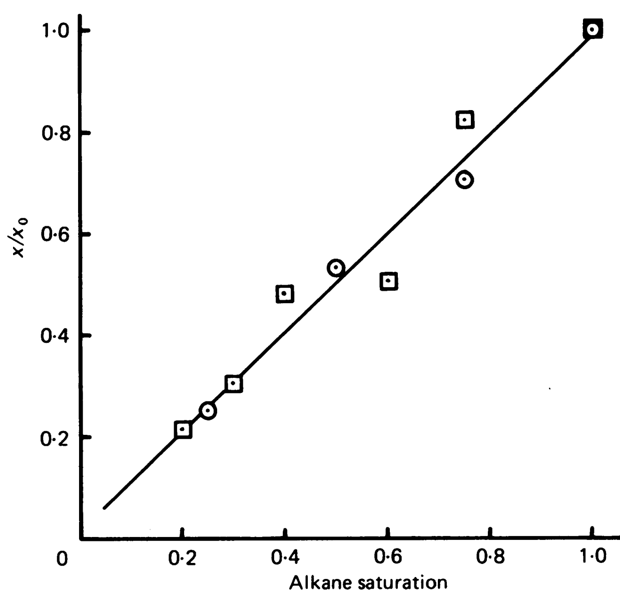

Fig. 6. The initial rate of action potential decline as a function of the fractional saturation of aqueous solutions of alkanes *n*-hexane (⊙) and *n*-heptane (⊡). The ordinate is plotted as the rate for a test solution (x) divided by the rate for a saturated solution of that alkane on the same axon (x_0). These data are from five separate axons. The linear regression line has a slope of 0·97 ($r = 0{\cdot}98$).

DISCUSSION

Two questions will be discussed with respect to the present results. The first concerns the explanation for the increased susceptibility of small axons to local anaesthetics. The second enquires as to whether there is a genuine decline in the local anaesthetic activity of the *n*-alkanes on ascending the homologous series. It seems intuitively most likely that the rate of action potential decline observed here is limited by radial diffusion of alkane into the axon. Differences between large and small axons probably relate to differences in radial diffusion rates. Nevertheless a number of other possible explanations for these data need to be considered briefly.

Franz & Perry (1974) suggested that longitudinal diffusion effects may underly the differential susceptibility to procaine of myelinated nerves of varying size. In their model the 'critical length' of a fibre which needed to be exposed to local anaesthetic was longer for fibres of larger diameter (and larger internodal distance). In the

experiments reported here the intracellular action potential has been measured within a segment of axon directly exposed to the anaesthetic solution. The micro-electrode position was established to be sufficiently far from the unexposed axon so that measurements were not significantly affected by currents due to excitation of the anaesthetic-free membrane. Therefore the present dependence of action potential decline on axon diameter cannot be ascribed in any obvious manner to a critical length effect.

Another possibility is that the height of the action potential in small axons is more susceptible to changes in the size and voltage- and time-dependence of the inward sodium current. The small *n*-alkanes are known to reduce $\bar{g}_{Na}$ and produce a hyperpolarizing shift along the voltage axis of the Hodgkin–Huxley h_{∞} parameter (Haydon, Requena & Urban, 1980; Haydon & Kimura, 1981). It is possible that small axons may exhibit a greater decline in action potential height than large axons due to the same alterations in these parameters. This is made more likely by indirect evidence that the control value of $\bar{g}_{Na}$ may be lower in small axons (Stein & Pearson, 1971). Such an effect of axon diameter is difficult to evaluate quantitatively but a contribution of this nature to the increased susceptibility of small fibres observed here cannot be excluded.

A crude model based on radial diffusion can provide an explanation for the present data. One essential feature of any satisfactory model appears to be the presence of 'sinks' for alkane adsorption both inside and outside the axon itself. Thus, the effect of saturated *n*-octane (3·0 μM) on the squid nerve, has a time course of hours and is much longer than that of tetrodotoxin at similar concentrations (Keynes, Bezanilla, Rojas & Taylor, 1975). This observation is consistent with the idea that numerous hydrophobic sinks for *n*-alkane adsorption are present. Outside the axon these sinks comprise Schwann cell membranes and organelles (Adelman, Moses & Rice, 1977) while the inside of the axon contains mitochondrial membranes and axoplasmic proteins which may also adsorb *n*-alkane. The affinity of axoplasmic proteins for *n*-alkanes is unknown but significant binding of *n*-alkanes to haemoglobin has been observed (Wishnia, 1969). If a simple model is assumed in which the axon and its associated sheath behaves as a homogenous solid cylinder then the radial diffusion time to each cylindrical plane within the structure varies as the square of the total cylinder diameter (Carslaw & Jaeger, 1947; Rud, 1961). Thus the assumption of internal sinks is consistent with the square-law relationship observed in these results. Indeed without intra-axonal sinks it is difficult to construct a diffusion model with the required strong dependence on axon diameter.

In order to examine the implications of the present results for the phenomenon of a decline in alkane potency with increasing chain length it is necessary to make some quantitative comparison of the anaesthetic effects of different alkanes. The data suggest that the rate of action potential decline (x) is proportional to the concentration of the alkane (c) and inversely proportional to the square of the axon diameter (d). This may be expressed by the empirical equation:

$$x = \frac{k_{n}c}{d^2}$$

where k_n is a constant which will take a particular value for each alkane under test. (k_6 for hexane, k_7 for heptane, etc.). The value of k_n is a measure of the anaesthetic potency of the alkane after the effects due to concentration and axon diameter have been removed. An idea of the relative values of k_n can be obtained by plotting log (x) against log (c/d^2), for n-hexane, n-heptane and n-octane. This is done in Fig. 7 in which the lines are drawn by linear regression but are constrained to be of slope = 1. From this the ratios of $k_6:k_7:k_8$ are found to be 1·0:0·72:0·24.

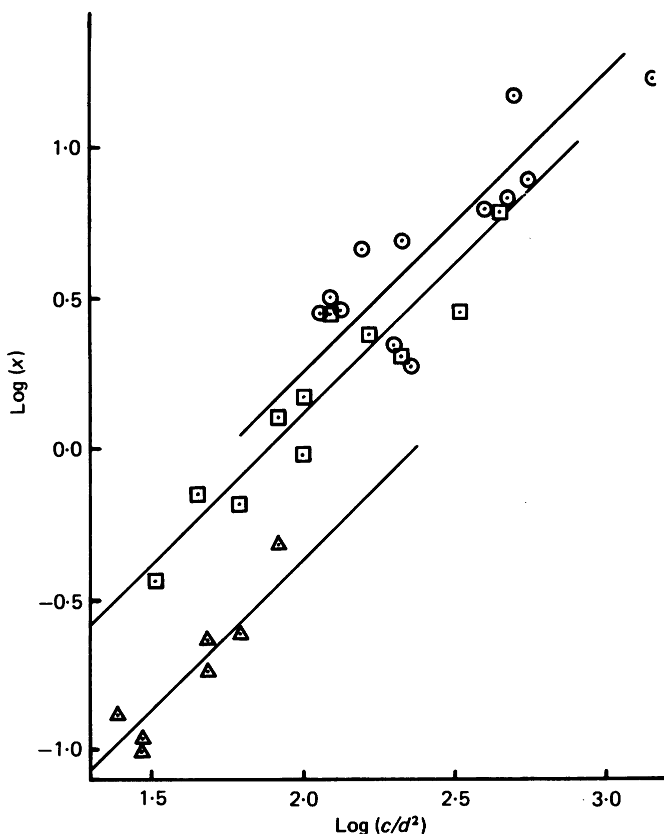

Fig. 7. A quantitative comparison of the anaesthetic effects of n-hexane (⊙), n-heptane (⊡) and n-octane (△). The ordinate is log (x) while the abscissa is log (c/d^2). c is the alkane concentration in μM, d is axon diameter in mm. The lines are drawn with slope = 1. See text.

The value of k_n is not simply a function of the anaesthetic potency of the alkane at its site of action. k_n should also be a function of two other parameters for each alkane: the diffusion coefficient and the size of the diffusion sink for depletion of alkane solution. The diffusion constants in water at 4 °C for the n-alkanes methane to pentane vary as the inverse of the square roots of their molecular weights (Bonoli & Witherspoon, 1969). Extrapolation of this trend can be used to estimate the ratios

of diffusion constants for *n*-hexane, *n*-heptane and *n*-octane and gives values of 1·0:0·93:0·87. These differences are insufficient to explain the observed variation in k_n values. The diffusion sinks in the axon preparation may be considered in two groups: aqueous compartments and hydrophobic regions. The solubility of *n*-octane in the aqueous compartments will be lower than that of *n*-heptane or *n*-hexane. Furthermore studies on artificial lipid bilayer membranes of appropriate composition (Haydon *et al.* 1977 *a*) suggest that *n*-octane partitions to a lesser extent than *n*-heptane into the centre of the bilayer. Accordingly the sink available for *n*-octane should be smaller than that for *n*-heptane or *n*-hexane. It follows that differences between k_n values due to sink effects alone would put $k_6 < k_7 < k_8$ and so these effects are in the reverse direction to those observed.

In conclusion this study has allowed a quantitative comparison of the local anaesthetic potencies of *n*-hexane, *n*-heptane and *n*-octane which supports the concept of a genuine decline in potency on ascending the series. The arguments for this conclusion are necessarily qualitative and not fully compelling. This lack of precision stems from two sources. First it has not proved possible to measure equilibrium anaesthetic potencies for the larger anaesthetic alkanes – even using small axons. Secondly the pattern of diffusion of an alkane into a single axon is likely to be extremely complex. The presence of numerous sinks for alkane adsorption both inside and outside the axonal membrane itself with an array of unknown partition coefficients precludes a complete description of such a process.

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