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Temperature-dependence of the action of nerve blocking agents and its relationship to membrane-buffer partition coefficients: thermodynamic implications for the site of action of local anaesthetics

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1 The local anaesthetic action of a series of nerve blocking agents was examined at temperatures between 4°C and 20°C in isolated sciatic nerves from cold-adapted frogs. Cooling alone had little effect on the amplitude of the action potential but the conduction velocity was decreased and the duration increased. Cooling had little effect on the local anaesthetic action of the short chain alkanols but that of the long chain alkanols, benzyl alcohol, benzocaine and pentobarbitone was markedly enhanced.

2 The partition of both short and long chain alkanols and of pentobarbitone into a liposome suspension of similar composition to axonal membrane lipids was reduced by cooling from 40°C to 4°C.

3 The results are not compatible with the lipid hypotheses of anaesthetic action. The implications for the nature of the site of action are discussed.

Introduction

Since the work of H.H. Meyer and E. Overton at the beginning of the century, it has been accepted that there is a striking correlation between the potency of anaesthetic agents and their oil-water partition coefficient (see for example Miller, Paton, Smith & Smith, 1972). This has led to a widespread assumption that the action of anaesthetics is the result of their interaction with the lipid moiety of nerve membranes (e.g. Meyer, 1937; Mullins, 1954; Seeman 1972; Miller, Paton, Smith & Smith, 1973; Pringle, Brown & Miller, 1981; Janoff, Pringle & Miller, 1981). The various hypotheses of anaesthetic action based on such interactions all require that the effect of an anaesthetic be directly related to the number or volume fraction of anaesthetic molecules dissolved in the membrane lipids (Smith, 1974). They therefore predict that the potency of anaesthetics should decrease with cooling as it is known that the partition coefficient of anaesthetics between lipid membranes and the surrounding medium is directly related to the temperature (e.g. benzyl alcohol: Colley & Metcalfe, 1972; *n*-butanol and *n*-propanol: Katz & Diamond, 1974). This prediction has already been tested for general anaesthesia in goldfish acclimatised to various temperatures (Cherkin & Catchpool, 1964) and for the local anaesthetic action of benzyl alcohol and butanol on the lateral olfactory tract of the guinea-pig (Richards, Martin, Gregory, Keightley, Hesketh, Smith, Warren & Metcalfe, 1978). In both cases the results were, in the main, in direct contradiction to the predictions of the lipid hypotheses.

In this paper we test this prediction further by examining the effect of temperature on the potency of ten substances that have a local anaesthetic action on the sciatic nerve of cold-adapted frogs and comparing the changes in anaesthetic potency with changes in the lipid-buffer partition coefficient for a model membrane system. Preliminary communications have been given to the Physiological Society (Bradley & Richards, 1981; 1983).

Methods

Anaesthetic effects on action potential amplitude

Compound action potentials were recorded from frog isolated sciatic nerves mounted in a perspex chamber with silver stimulating and recording electrodes. The chamber was mounted on a plate cooled

by the Peltier effect which allowed the temperature to be progressively reduced from room temperature (c.20°C) down to 1–2°C. The temperature of the nerve was monitored by a miniature thermistor probe. The nerves were incubated in frog Ringer solution of composition (mM):NaCl 120, KCl 1.8, $CaCl_2$ 1.1, $NaHCO_3$ 16, glucose 5, equilibrated with 95% O_2, 5% CO_2, pH 7.15–7.25 over the temperature range employed in this study. The action potential was first recorded with the nerve immersed in frog Ringer solution at room temperature, using a stimulus that was supramaximal for the large early component of the compound action potential (4–6 V, 100 μs) and the action potentials were recorded with a Neurolog amplifier and averager (Digitimer Ltd.) and plotted on a chart recorder for subsequent analysis. The temperature was progressively reduced to 1–2°C at a rate of cooling of approximately 0.5°C min^{-1} and responses were recorded at intervals of 1°C. The preparation was returned to room temperature to confirm the stability of the response.

The frog Ringer solution was then replaced by a solution of anaesthetic in Ringer, the concentration of anaesthetic being chosen to give a depression of the action potential of approximately 25% of the value recorded at room temperature and the nerve was then left until the response had attained a steady level. The progressive cooling and rewarming to room temperature was then repeated. Care was taken to maintain a supramaximal stimulus at all times as both cooling and the presence of an anaesthetic can lead to an elevation of threshold (Richards *et al.*, 1978). When the minimum temperature had been reached the nerve was maintained at that temperature for 10–20 min to ensure that equilibrium had been reached between the nerve and the surrounding fluid. No time-dependent change in the action potential was seen during this period nor was there any significant hysteresis in the effect of the various anaesthetics on rewarming. From these observations we conclude that the anaesthetic bound to the nerve was in equilibrium with the bathing medium throughout the complete cycle of cooling and warming.

Recordings were subsequently analysed by measurements of amplitude, latency to peak and area (amplitude × width at half amplitude) of the large early component of the compound action potential.

Estimates of partition coefficients

Partition coefficients were determined for butanol (80 mM), pentanol (30 mM), octanol (3 mM), and sodium pentobarbitone (0.2 mM) dissolved in 0.9% NaCl with 20 mM Tris buffer, pH 7.6. The lipid phase was prepared from phosphatidyl choline (egg lecithin), phosphatidyl serine (from bovine brain) and cholesterol films in a molar ratio of 9:1:5 (2 phospholipid: 1 cholesterol) (Richards & White, 1981) which had been dispersed with a probe sonicator for 5 min. The resulting liposomes were used for partition coefficient measurements by membrane dialysis; a 1% suspension being used for measurement of the partition coefficient of octanol and a 5% suspension being used for the other agents. Each agent was subjected to dialysis for 24 h at temperatures of 4°C, 22°C, and 37°C. The partition coefficients for the alkanols were determined by extraction of the aqueous and lipid samples with a fixed volume of hexane followed by gas chromatography of these extracts. The amounts of alkanol in the extracts were related to the original aqueous concentrations by means of calibration curves. The partition coefficient of pentobarbitone was determined by estimating the quantity of ^{14}C-labelled pentobarbitone in each phase.

Some difficulties were experienced with the gas chromatography of pentanol as a component of the lipid was extracted by the hexane and interfered with the pentanol peak. In view of this the partition data for pentanol should be regarded as an indication of the trend with temperature rather than an expression of the absolute values.

Results

The majority of the frogs used in our experiments had been kept in a cold room (4°C) for 1–2 months and cooling the sciatic nerve in frog Ringer solution containing no anaesthetic was found to produce little change in the amplitude of the compound action potential, though it was considerably broadened and its conduction velocity decreased (see Figure 1).

The nerve blocking action of butanol (40 mM) judged by its depression of action potential amplitude was little affected by cooling from 19.5°C to 2°C (Figure 1c). In contrast, the action of benzyl alcohol (10 mM) was strongly potentiated (Figure 1b). Thus the action of these two alcohols on the frog sciatic nerve is similar to that described by Richards *et al.* (1978) for their actions on the lateral olfactory tract of the guinea-pig.

In addition to the above agents, the effects of cooling on the nerve blocking potency of the following compounds was tested: ethanol (500 mM), *n*-propanol (100 mM), *n*-pentanol (15 mM), *n*-hexanol (5 mM), *n*-heptanol (2 mM), *n*-octanol (1.5 mM), *n*-nonanol (1 mM), benzocaine (0.5 mM) and sodium pentobarbitone (4 mM). These concentrations were, in most cases, similar to those previously described as being required to cause nerve block in frog sciatic nerve (Seeman, 1972).

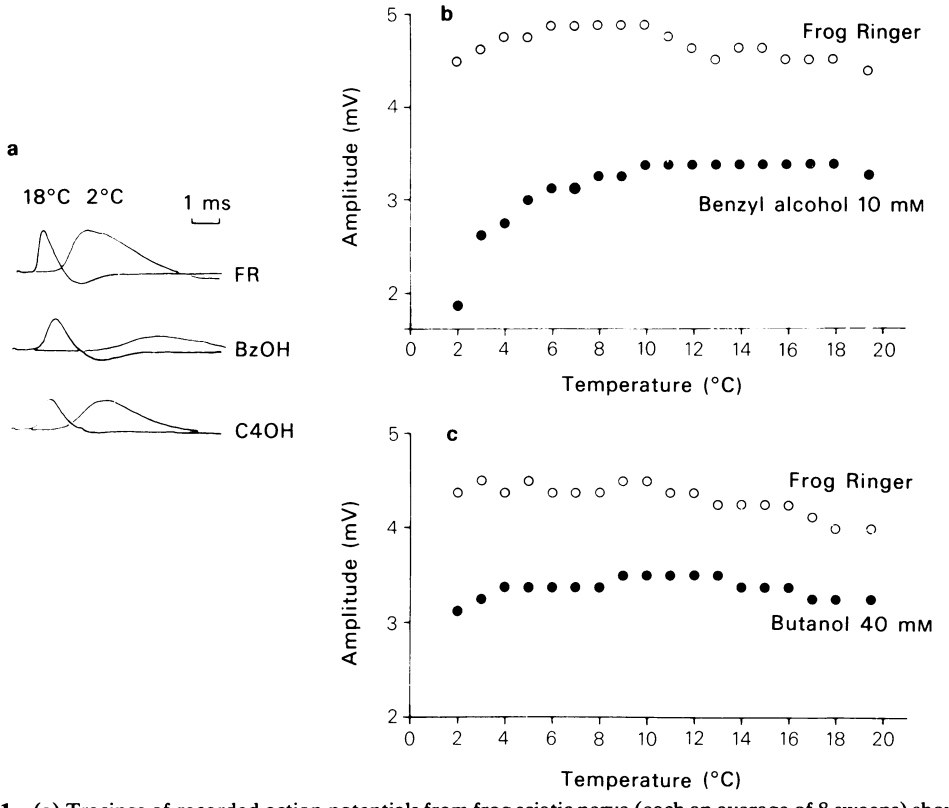

Figure 1 (a) Tracings of recorded action potentials from frog sciatic nerve (each an average of 8 sweeps) showing superimposed potentials at 18°C and 2°C. Upper pair shows the effect of cooling in frog Ringer solution with no added anaesthetic. Middle pair shows the effect of cooling in Ringer containing 10 mM benzyl alcohol (BzOH). Lower pair shows the effect of cooling in Ringer containing 40 mM *n*-butanol (C_4OH). (b) Changes in action potential amplitude on progressive cooling from 19.5°C to 2°C in frog Ringer solution alone (○) and in frog Ringer solution containing 10 mM benzyl alcohol (●) (The results are from one experiment.) (c) Changes in action potential amplitude in frog Ringer solution (○) and in 40 mM butanol (●) on progressive cooling. (The results are from one experiment.)

The effects of cooling on anaesthetic potency for all the agents tested in the present study are summarised in Figure 2 which shows the increase in potency on cooling from 18°C to 2°C, expressed in terms of the changes in amplitude of the compound action potential. No change in potency was observed for ethanol or propanol, only a slight increase in potency for butanol and pentanol and a marked increase in potency was observed for the remaining agents (the alkanols from hexanol to nonanol; benzyl alcohol, benzocaine and pentobarbitone). This increase in potency was evident whether one measured the changes in the amplitude, the latency to the peak or the area of the compound action potential. For example Figure 3 shows the change in latency to the peak of the compound action potential in the absence of any anaesthetic, in 40 mM butanol and in 2 mM heptanol. Little increase in potency is seen with cooling in the presence of butanol but a marked steepening of the curve relating temperature to latency was seen when the nerve was cooled in the presence of heptanol. This is consistent with the effect of temperature on the potency of heptanol judged by its ability to depress the amplitude of the action potential (Figure 2).

The effect of temperature on anaesthetic potency was independent of the dose used. The dose-response relationships for pentanol and octanol at 4°C and 20°C are shown in Figure 4 from which it can be seen that pentanol is approximately equipotent at both temperatures, while the potency of octanol is markedly increased at low temperature for all doses tested. Despite the apparent increase in the slope of the dose-response curve for octanol at 4°C, the data of Figure 4 are not sufficiently precise to permit deductions about the mechanism of anaesthetic ac-

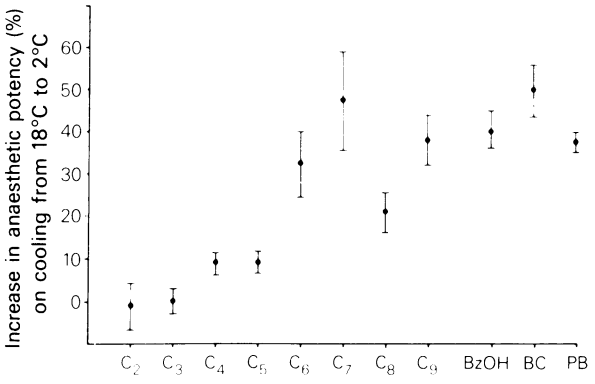

Figure 2 Changes in anaesthetic potency on cooling from 18°C to 2°C for a series of agents. The ordinate scale shows the increase in potency with temperature. (Potency was defined as the amplitude at 18°C less the amplitude at 2°C, each expressed as a percentage of the control values at the two temperatures.) The abscissa scale identifies the nerve blocking agents: alkanols from chain length C_2 (ethanol) to C_9 (nonanol), benzyl alcohol (BzOH), benzocaine (BC) and sodium pentobarbitone (PB). Each point is the mean with the s.e.mean for 3–4 determinations.

tion other than the fact that the potency of octanol at different temperatures does not correlate with the amount bound to membrane lipid (see below).

Figure 5 summarises the changes in anaesthetic potency and in lipid-buffer partition coefficients with cooling for the four agents whose partition coefficients were determined in this study. Figure 5a shows the change in anaesthetic potency as the mean of 3–4 individual experiments on each agent (the amplitude is expressed as a percentage of that in frog Ringer solution at the same temperature). Butanol and pentanol show only a slight increase in potency at the lowest temperatures used (2–4°C), while octanol and pentobarbitone both show a steep and progressive rise in potency as temperature is reduced. In some of the individual experiments with octanol and pentobarbitone, an action potential which was about 70% of the control value at 20°C was virtually extinguished on cooling to 1°C. In all cases the effects were completely reversed on returning the temperature to 20°C.

Figure 5b shows the changes in lipid-buffer partition coefficients for the four agents whose anaesthetic effects were described in Figure 5a. All four agents show a marked decrease in lipid-buffer partition coefficients with cooling over a temperature range in which the anaesthetic potency is either little changed or is markedly increased.

Discussion

For our measurements of the temperature-dependence of the membrane-buffer partition coefficients we have chosen a model membrane system that approximates to the lipid composition of nerve plasma membranes (Chacko, Villegas, Barnola, Villegas & Goldman, 1976). We may therefore expect our

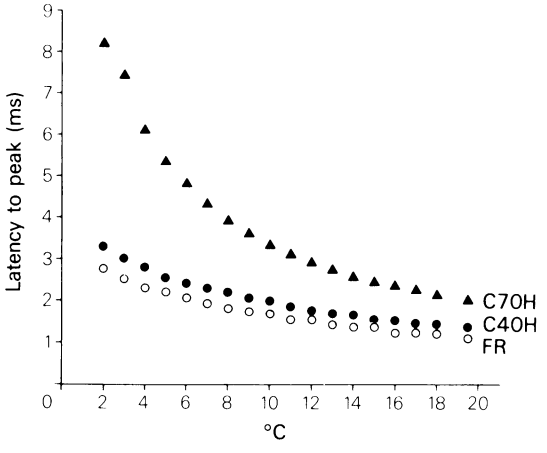

Figure 3 The change in latency to the peak of the compound action potential on cooling from 19.2°C to 2°C: progressive slowing of the action potential in frog Ringer solution (FR) alone (○); similar curve obtained in the presence of 40 mM *n*-butanol (C4OH) (●); much steeper rise in latency with 2 mM heptanol (C7OH) (▲). Both butanol and heptanol reduced the action potential amplitude by 20–25% at 19°C.

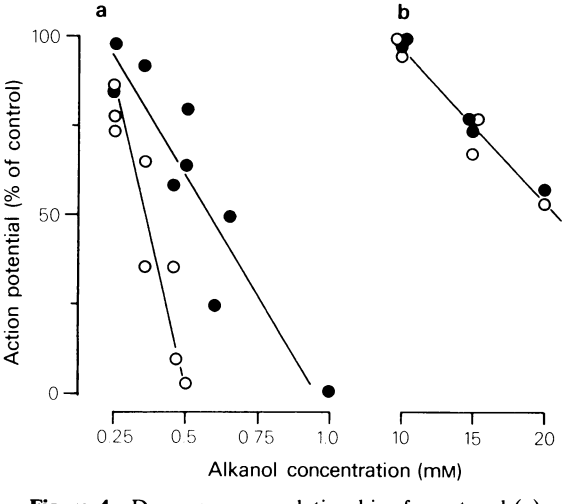

Figure 4 Dose-response relationships for octanol (a) and pentanol (b) at 4°C (○) and 20°C (●). Each point shows the percentage depression of the amplitude of the action potential with respect to the control value at the same temperature for an individual nerve.

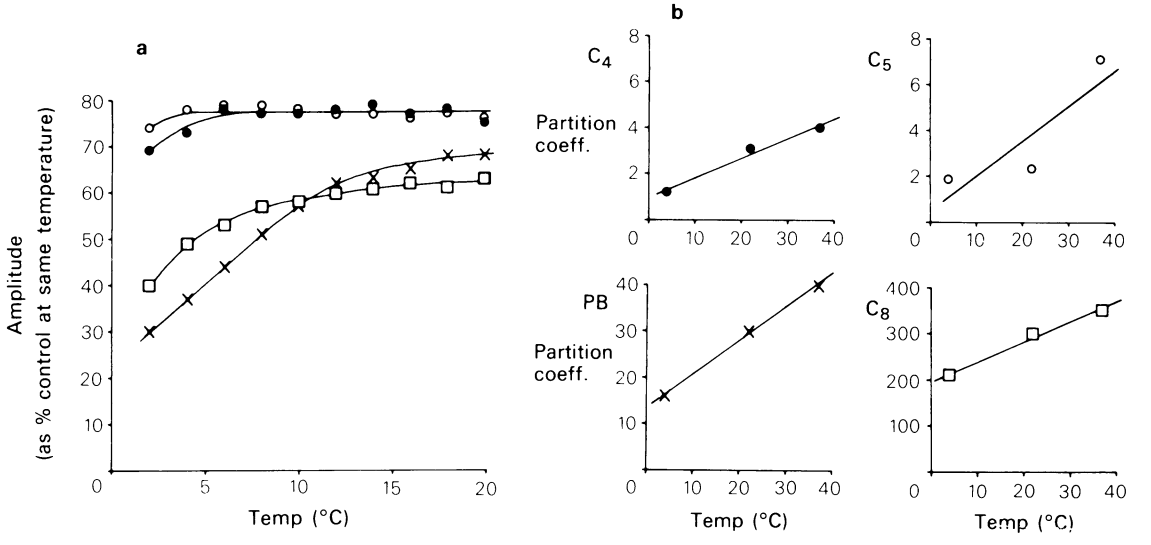

Figure 5 Changes in anaesthetic potency and membrane-buffer partition coefficients with temperature. (a) Action potential amplitude as % control at the same temperature: (●) butanol (40 mM); (○) pentanol (15 mM); (□) octanol (2 mM); (x) pentobarbitone (4 mM). Each point is the mean of 3–4 observations for each agent (b) Changes in membrane-buffer partition coefficient with temperature for butanol (C4), pentanol (C5), octanol (C8) and pentobarbitone (PB). Symbols as in (a). Each point is the mean of 3–4 observations and the regression lines calculated by the method of least squares. The concentrations used are given in the Methods. The s.e.mean for the data plotted in both (a) and (b) is, in all cases, less than 10% of the values shown.

results to have a greater applicability to the problem of anaesthetic action than those studies which are based on olive oil or model membranes consisting of a single species of phospholipid. In agreement with earlier studies (Colley & Metcalfe, 1972; Richards *et al.*, 1978) our results show that the membrane-buffer partition coefficients of *n*-butanol, *n*-pentanol, *n*-octanol and pentobarbitone increase as the temperature is raised from 4°C to 40°C. Although the precise numerical value of the partition coefficient will depend on the exact composition of the membrane lipid (see Miller & Yu, 1977), the direction of the change in partition coefficient with temperature is unlikely to alter with changes in the lipid composition of membranes. For example, Katz & Diamond (1974) found that the partition of *n*-butanol into dimyristoyl lecithin increased with temperature, a result in exact concordance with our results with a more complex lipid mixture.

It is generally agreed that local anaesthetic agents block impulse conduction by decreasing the inward sodium current and that they have little or no effect on potassium current (Taylor, 1959). There has been one study which has examined the temperature-dependence of the local anaesthetic action of benzyl alcohol on membrane currents (Harper, MacDonald & Wann, 1983). These authors noted that both a decrease in temperature and application of benzyl alcohol shifted the steady-state inactivation curve for the sodium conductance in a hyperpolarizing direction. They therefore concluded that the increase in potency of benzyl alcohol was due to a summation of inactivated sodium channels caused by the reduction in temperature and of inactivated sodium channels due to the effect of benzyl alcohol. They also noted that the reduction in the inward sodium current produced by application of benzyl alcohol was independent of temperature between 7°C and 20°C. Unfortunately, no measurements were made with short-chain alkanols (which do not increase in potency at low temperatures) so the reasons for the different effects of temperature on the short-chain and long-chain alkanols are not yet clear.

The detailed implications of the effects of temperature on the potency of anaesthetic agents for specific hypotheses of anaesthetic action have been discussed at length in several recent articles (Richards *et al.*, 1978; Richards, Keightley, Hesketh & Metcalfe, 1980; Franks & Lieb, 1982). It is clear that our results are not compatible with the critical volume hypothesis or with the lipid solubility rule of anaesthesia, since the potency of many anaesthetics either increases or is unchanged under conditions in which the membrane-buffer partition coefficients is decreasing. Both of these hypotheses would require that the potency of the anaesthetics

should follow the same trend as the partition coefficient. Other hypotheses of anaesthesia based on non-specific anaesthetic-lipid interactions, such as the membrane fluidity hypothesis (Metcalfe, Seeman & Burgen, 1968) or the phase transition hypothesis of Trudell (1977), are similarly excluded. As temperature changes the basic physiological properties of the nerve as well as the partition coefficient of nerve blocking agents, it may be objected that the increase in the potency of various anaesthetics with a fall of temperature is the result of a change in the properties of the nerve (see Harper *et al.*, 1983). However, the force of our argument rests not only on the differing directions of the temperature-dependence of potency and partition coefficient changes but also on the fact that a number of agents show no change of potency. It is this difference in behaviour that cannot be readily reconciled with any hypothesis which requires that anaesthesia results from a disturbance of membrane structure that is independent of the chemical nature of the anaesthetic. Our data are, however, consistent with those hypotheses which suggest that different anaesthetics may act on different sites, e.g. the degenerate perturbation hypothesis (Richards *et al.*, 1978) which suggests that different anaesthetics will bind to different sets of sites.

In conclusion, for all four agents we have examined, the enthalpy changes of partition between water and membrane lipid are endothermic. However, for local anaesthetic action the changes in potency with temperature imply either very small enthalpy changes (short chain alkanols) or exothermic enthalpy changes (long chain alkanols, benzyl alcohol, benzocaine and pentobarbitone) (see Appendix 1). As polar interactions decrease with increasing temperature (Miller, 1961) while hydrophobic interactions increase (e.g. Figure 5) our results suggest that the site of anaesthetic action possesses some polar properties, a view also expressed by Franks & Lieb (1978) on the basis of correlations between anaesthetic potency and partition into a number of hydrophobic solvents.

Appendix

The thermodynamic implications of the temperature-dependence of the potency of anaesthetics merit further comment. Consider a simple binding model for anaesthetic action (cf. Franks & Lieb, 1982) in which the equilibrium constant K is described by the relation:

$$K = [sa]/[s - a]\,[a] \qquad 1.$$

where s is the concentration of sites and a the concentration of anaesthetic (note that K is thus proportional to the potency of the anaesthetic). The free energy change ($\triangle G$) that occurs when the anaesthetic binds is given by the well-known thermodynamic relation:

$$\triangle G = -RT\,1n\,K \qquad 2.$$

assuming that the binding is taking place at constant pressure. R is the gas constant and T the absolute temperature. From this relationship it is clear that the greater the potency of an anaesthetic, the greater the free energy change on binding. The heat liberated or absorbed during binding (the enthalpy change $\triangle H$) is related to the equilibrium constant by the relation:

$$(d[1nK]/dT)_p = \triangle H/RT^2 \qquad 3.$$

If we assume that a given degree of anaesthesia or nerve block occurs when a constant fraction of sites has been occupied, equation 3 can be integrated to give the relation:

$$1n\,(a'/a) = \triangle H/R(1/T' - 1/T) \qquad 4.$$

where a and a′ are the concentrations of anaesthetic required to reach a given end point at temperatures T and T′. Thus from dose-response curves at different temperatures the enthalpy changes involved in particular anaesthetic processes can be calculated.

Finally, the free energy change $\triangle G$ is related to the enthalpy and entropy ($\triangle S$) changes by the relation:

$$\triangle G = \triangle H - T\triangle S \qquad 5.$$

From these relations certain consequences follow:
(1) Since the partition coefficients are directly related to the absolute temperature the transfer of alkanols from water to membrane lipid is endothermic with enthalpy values in the range 15–30 KJ mol^{-1}. (Calculated from the data of Figure 5 and equation 4.)
(2) The potency of the long-chain alkanols, benzyl alcohol, benzocaine and pentobarbitone is inversely related to the absolute temperature; so the binding of these agents is exothermic. From the dose-response curves for pentobarbitone (not shown) and octanol (Figure 4) at 4°C and 20°C we calculate that the enthalpy change for the binding of pentobarbitone is about 10KJ mol^{-1} and that for octanol is about 20KJ mol^{-1} (neglecting changes in heat capacity).
(3) From these calculated values of enthalpy changes, it appears that the enthalpy of binding of the longer chain alcohols to their sites of action is of a different sign from that for their binding to membrane lipid.
(4) The potency of the short chain alkanols (C_2–C_5) does not change significantly over the temperature range of our study (2°C–20°C) so from equations 3 and 4 it is clear that the enthalpy changes are very small for the binding of these agents to their target sites. Since they unquestionably do bind to nerves to cause nerve block, any free energy change that occurs must be due in some considerable measure to changes in entropy (equation 5).

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