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TEMPERATURE DEPENDENCE OF ERYTHROCYTE MEMBRANE EXPANSION BY ALCOHOL ANESTHETICS. POSSIBLE SUPPORT FOR THE PARTITION THEORY OF ANESTHESIA

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SUMMARY

1. Using the sealed-ghost-expansion method, it was found that the membrane area of erythrocyte ghosts increased by 1–2% in the presence of low concentrations of alcohol anesthetics.

2. An increase in temperature enhanced the amount of this drug-induced expansion of the ghost membrane.

3. The ghost membrane/water partition coefficient also increased and could account for the temperature potentiation of the drug-induced ghost expansion in the case of benzyl alcohol. Since homologous alcohols expand erythrocyte membranes and stabilize nerve cell membranes at virtually identical concentrations, the present findings lend support to the classical partition theory of anesthesia.

4. The ghost membrane area also expanded by 0.1% per degree centigrade (in the absence of any drugs) when the temperature was increased.

5. For intact erythrocytes, in contrast with the findings on ghost membranes, it was observed that an elevated temperature diminished the anesthetic's ability to protect erythrocytes against hypotonic hemolysis. Hypotonic hemolysis is a transient phenomenon, involving many events, while ghost volume expansion is a steady-state or equilibrium condition with fewer processes. The drug effects on the ghosts, therefore, were considered to reflect more directly the membrane affinity (or partition coefficient) of the drug.

6. If the pure drug was chosen to represent the standard state, it was found that the anesthetic potency varied inversely with temperature, a conclusion contrary to the classical partition theory. If, however, the standard state was taken to be the infinitely dilute solution, as recommended by SCHNEIDER, the anesthetic action on the ghost membrane was potentiated at higher temperature, in agreement with the partition findings.

INTRODUCTION

It is known that there is a correlation between the oil/water partition coefficients and the potencies of anesthetics[1–15]. By means of temperature variations MEYER[16]

showed that the narcotic potency varied directly with the oil/water partition coefficient for any particular compound. Other studies[15,17–20] on the temperature dependence of anesthesia have been given different interpretations, some supporting the classical partition theory[17–21] and others arguing on thermodynamic grounds[22,23] for a water clathrate or iceberg theory of anesthetic action[24,25].

In a thermodynamic analysis SCHNEIDER[26] has shown that it is difficult to predict the temperature dependence of anesthetic potency using data from model systems (*e.g.* olive oil). As pointed out by others[17,19], an inherent difficulty in interpreting the results of temperature experiments is that it is impossible to know whether the temperature is altering the efficacy of drug action[27,28] despite the perfectly normal behaviour of the animal. The protection and stabilization of erythrocyte membranes by anesthetics is a simple system for testing various theories of anesthesia[29–34] and for examining the temperature dependence of the membrane action of anesthetics.

METHODS

Measurement of drug-induced membrane area expansion

The effect of alcohols on the erythrocyte membrane area was measured by the sealed-ghost-expansion method previously outlined[35]. The effect of temperature was studied as follows. Erythrocyte ghosts were prepared at room temperature (21°) by pipetting 0.5 ml of a stock suspension of human erythrocytes (about $1.8 \cdot 10^6$ cells/ml in 154 mM NaCl (pH 7), 10 mM sodium phosphate buffer) into 10 ml of 51.3 mM NaCl (pH 7), 10 mM sodium phosphate buffer. Approx. 1.5 h later (at which time the ghosts had become sealed) the vials containing ghosts were placed in a water bath regulated at 37 or 10°. After one more hour the cells were monitored only once (0.5-ml sample through an 100-μ aperture) for the control mean cell volume in a Coulter counter Model F using a Coulter mean cell volume computer attachment. An aliquot (always less than 0.1 ml) of alcohol was added and the vial was mixed thoroughly without frothing or bubbling before returning it to the water bath. After another 2 h in the water bath, the cells were monitored for the mean cell volume three or four times consecutively. To maintain the Coulter counter probe and the suspension in the vial at the desired temperature a special lucite jacket was made with side holes for the aperture-monitoring light path and with inlet and outlet pipes for circulating water through the jacket. This lucite jacket remained clamped on the Coulter counter sample platform throughout the experiment and the vials were placed in it without any difficulty. Each drug concentration was tested in triplicate or quadruplicate.

Hypotonic hemolysis experiments

Using the method of hypotonic hemolysis[29–32], the effect of temperature on erythrocyte protection by alcohol anesthetics was studied as follows. A freshly drawn blood sample (from a fasting volunteer) was heparinized (50 units/ml) and the plasma and buffy coat removed after centrifugation. A stock erythrocyte suspension of about $4 \cdot 10^8$ cells/ml was prepared and 0.1-ml aliquots were added to 1.5 ml of hypotonic solution (0.320–0.440% NaCl) buffered at pH 7 by 10 mM sodium phosphate buffer and containing varying concentrations of drug. 5 min later the samples were centrifuged and the absorbance of the hemoglobin in the supernatant was measured at 543 nm. The value of 100% absolute hemolysis was obtained by adding the cells to

buffer not containing any NaCl. For experiments at 37° the stock cell suspension and the 1.5-ml drug solutions were all preincubated in a thermostatically regulated water bath; the pipettes were kept at 37° in a dry oven, and the centrifuge cups, containing water, were preheated in the water bath before each centrifugation. The experiments at 0° were done in a similar fashion except that the bath contained ice, and the pipettes were chilled in the refrigerator to 0°. All values were obtained in sextuplicate at least.

As indicated by MORTENSEN[36] in connection with his thermal fragility test, the final pH of the solution was different at different temperatures; the pH was 7.14 at 0°, 7.0 at 20° and 6.90 at 37°.

Partition coefficients

The chloroform/water partition coefficients for pentanol were determined using [^{14}C]pentanol. 9 ml of distilled water or 10 mM phosphate buffer (pH 7), containing 0.1 μC of [^{14}C]pentanol, were added to a glass-stoppered T-shaped glass tube. The tubes were stoppered after adding 1.5 ml of chloroform and were rocked by a motor-driven device for at least 6 h. The two phases were then removed separately by means of a syringe connected to long polyethylene tubing inserted into a T-tube. Aliquots of 0.2 ml were mixed with a scintillation fluid mixture (Bray's mixture) and the samples counted in a Nuclear Chicago liquid scintillation counter. The partition coefficient is the ratio of the radioactive concentrations in the two phases. Appropriate corrections were made for quenching. The T-shaped partition tubes were immersed during the rocking period in a water bath regulated at 0, 21 or 37°.

The ghost membrane/buffer partition coefficients were determined as described by METCALFE *et al.*[34]. The buffer refers to 10 mM sodium phosphate (pH 7).

MATERIALS

Benzyl alcohol was obtained from British Drug Houses, Poole. 1-Hexanol and 1-heptanol came from Eastman Organic Chemicals, Rochester. 1-Pentanol, 1-nonanol and thymol were supplied by Fisher Scientific Co., N.Y. [^{14}C]Pentanol was from New England Nuclear Corp., Boston.

RESULTS

Effect of temperature on ghost membrane area

The effect of two different temperatures on the mean cell volume at different hypotonic NaCl concentrations is shown in Fig. 1. To obtain these mean cell volume values intact cells were added to preincubated hypotonic NaCl solutions and allowed to swell, hemolyze and seal at the indicated temperature (the ghost-formation method in ref. 35). At 0.9% NaCl the mean cell volume values between 6 and 37° were almost always the same. By means of a microhematocrit method it had also been found previously[37] that the mean cell volume was unaffected by temperature, using incubation times of less than 1 h.

The size of the erythrocyte ghost, however, in the range between 0.3 and 0.4% NaCl was larger at higher temperatures. For example, as shown in Fig. 1 the ghost volume at 20° was 152.5 μ^3, while the mean cell volume at 37° was about 155 μ^3. Since these ghosts appeared as perfect spheres when examined by phase-contrast

microscopy, it can be calculated[37] that this increase in volume is associated with a membrane area expansion of about 1.08%.

Using the sealed-ghost-expansion method, the temperature dependence of the ghost membrane area is shown in Fig. 2. As explained in METHODS, these mean cell

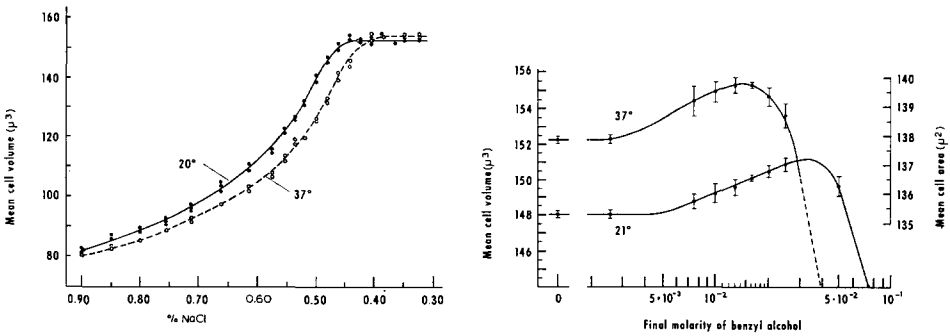


Fig. 1. The mean cell volume of erythrocytes in hypotonic solutions at two different temperatures. Below 0.45% NaCl the cells are all ghosts with a mean area of about 138 μ^2. The ghost area is slightly expanded at higher temperature.

Fig. 2. The effect of temperature on the drug-induced expansion of the ghost membrane. Sealed-ghost-expansion method. Benzyl alcohol was more effective in expanding the membrane at 37° than at 21°. Equal amounts of membrane area expansion (1.00%) occurred at $1.6 \cdot 10^{-2}$ M at 21° and at around $8 \cdot 10^{-3}$ M at 37°. (The increase in temperature alone caused the membrane to expand by 2 μ^2.)

volume values were obtained using cells which were hemolyzed and allowed to seal at room temperature for 1.5 h before changing the temperature. It can be seen from Fig. 2 that the ghost volume and the membrane area of these sealed ghosts increased at higher temperature; the increase is from 148.0 to 152.3 μ^3 which represents a 1.56% increase in membrane area.

By means of both the ghost-formation method (Fig. 1) and the sealed-ghost-expansion method (Fig. 2), therefore, it appears that there was a heat-induced membrane area expansion of the order of 0.1% per degree centigrade.

Effect of temperature on the membrane-expanding potency of anesthetics

The temperature dependence of the anesthetic-induced membrane expansion of ghost membranes was studied using the sealed-ghost-expansion method. It was found that an elevation in temperature enhanced the degree of anesthetic-induced membrane expansion; an example of this is shown in Fig. 2 for benzyl alcohol. In Fig. 2 it is seen that $1.6 \cdot 10^{-2}$ M benzyl alcohol at 21° increased the ghost mean cell volume from 148.0 $\pm$ 0.2 μ^3 (mean $\pm$ S.D.) to 150.2 $\pm$ 0.2 μ^3. The membrane surface area of the spherical ghost of 148.0 μ^3 was 135.31 μ^2 and that of 150.2 μ^3 was 136.66 μ^2; this represented an increase in membrane area of 1.35 μ^2 or 1.00%. At 37° this same concentration of benzyl alcohol increased the ghost mean cell volume from 152.2 $\pm$ 0.2 to 155.3 $\pm$ 0.2 μ^3. The membrane areas of these ghosts were 137.86 and 139.72 μ^2, respectively; this was an increase of 1.86 μ^2 or 1.35%.

For the example shown in Fig. 2, therefore, raising the temperature potentiated the membrane-expanding potency of benzyl alcohol by 1.35%/1.00% or 1.35-fold. Equal amounts of membrane area expansion (1.00%) occurred at $1.6 \cdot 10^{-2}$ M at 21°

and at around $8 \cdot 10^{-3}$ M at 37°. On this basis the increase in temperature has potentiated the drug's expanding potency 2-fold.

Effect of temperature on the lytic potency of anesthetics

Not only was the membrane-expanding potency of the anesthetic enhanced by temperature but the direct hemolytic potency at high drug concentrations was also increased. Fig. 2 shows this for benzyl alcohol concentrations above $3 \cdot 10^{-2}$ M at 21° and above $2 \cdot 10^{-2}$ M at 37°.

Effect of temperature on the anesthetic protection of intact erythrocytes against osmotic hemolysis

There is a voluminous literature describing the protective or stabilizing action of a variety of chemical compounds against hypotonic hemolysis[29–35,38–46]. The procedure employed in all these studies was to add intact erythrocytes to hypotonic solutions containing various concentration of different drugs. As mentioned in the introduction, this phenomenon of membrane protection has many features related to an anesthetic event, and therefore it was of interest to investigate the temperature dependence of this membrane stabilization using this simple and commonly employed procedure.

Using the method of hypotonic hemolysis, the results of a typical experiment on the effect of temperature on erythrocyte stabilization by alcohol anesthetics is shown in Fig. 3 for thymol. Firstly, Fig. 3 shows that in the absence of any drug 50 % osmotic hemolysis occurred at 0.39 % NaCl at 21° and at about 0.345 % NaCl at 37°. The concentration of NaCl eliciting 50 % osmotic hemolysis at 0° was 0.448 %. In other words, the erythrocytes were more fragile at lower temperatures; this is a well-known phenomenon[36,47].

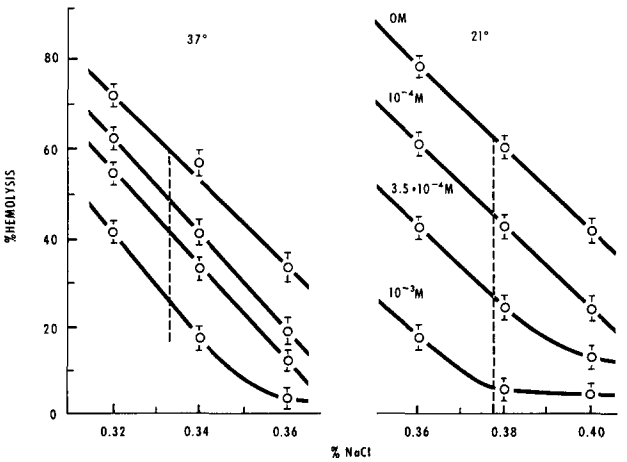


Fig. 3. The effect of temperature on the anesthetic protection of intact erythrocytes against osmotic hemolysis. The protective (or anti-hemolytic) potency of thymol was reduced at higher temperatures. (Note that an increase in temperature alone reduced the osmotic fragility of the cells.)

Secondly, Fig. 3 shows that low concentrations of thymol, ranging from 10^{-4} to 10^{-3} M protected the erythrocytes against hypotonic hemolysis. As shown pre-

viously[29,47], low concentrations of alcohols and anesthetics shift the osmotic fragility curve, while high concentrations have a direct and immediate lytic effect.

Thirdly, it can be seen from the results in Fig. 3 that the anti-hemolytic potency was apparently reduced at higher temperature. Since the osmotic fragility curve is linear between 60 and 30 % absolute hemolysis[37] it is convenient to select 60 % absolute hemolysis as the baseline amount of hemolysis in the absence of any drug. Drawing a vertical line through the fragility curves in Fig. 3 such that the line passes through the 60 % control hemolysis point, it is possible to read off the amount of hemolysis that occurs at different drug concentrations. These results are shown in Fig. 4.

The concentration of anesthetic reducing the amount of osmotic hemolysis from 60 down to 30 % absolute hemolysis may be referred to as the $E.D._{50}$ or the "effective dose causing 50 % reduction in hemolysis". The $E.D._{50}$ value for thymol, for ex-

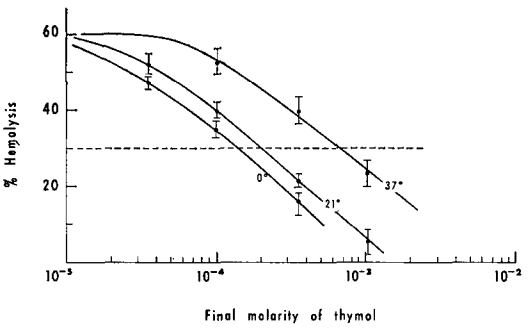


Fig. 4. The effect of temperature on erythrocyte protection by thymol. The $E.D._{50}$ (or effective dose reducing osmotic hemolysis from 60 down to 30 %) for intact cells increases at higher temperatures.

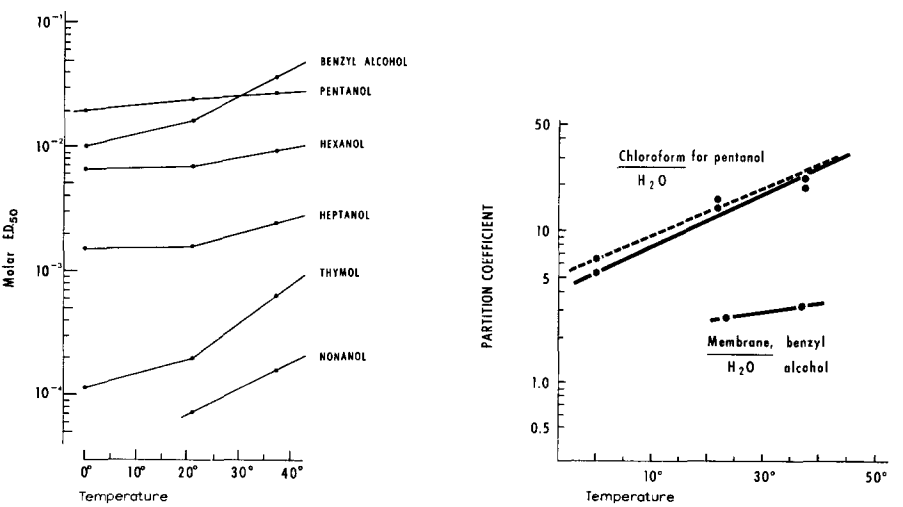


Fig. 5. The effect of temperature on the $E.D._{50}$ values (for intact cells) for four aliphatic and two aromatic alcohol anesthetics. Elevated temperature reduced the protective potency of the anesthetics.

Fig. 6. Effect of temperature on the chloroform/water partition coefficient and the membrane/buffer partition coefficient of the anesthetic alcohols.

ample, at 21° can be obtained from the data in Fig. 4 and is $2 \cdot 10^{-4}$ M. The $E.D._{50}$ values for 6 different alcohol anesthetics, obtained by the method shown in Figs. 3 and 4, are given in Fig. 5 for 0, 21 and 37°.

Effect of temperature on partition coefficients for [^{14}C]pentanol

The effect of temperature on the chloroform/water partition coefficient for pentanol (at $2.45 \cdot 10^{-2}$ M), using [^{14}C]pentanol, is shown in Fig. 6. At 0° the coefficient was about 5, and this rose to around 20 at 37°. Since there is approximately a 1% solubility of chloroform in water (which varies with temperature), these data were corrected for this solubility using the solubility–temperature data given by SEIDELL[48]; the corrections did not change the values greatly. Also shown in Fig. 6 are the membrane/buffer partition coefficients for benzyl alcohol. The present values are higher than those reported previously[34]. It was found that the membrane/buffer partition coefficient for benzyl alcohol at $1.6 \cdot 10^{-2}$ M increased from 2.32 to 2.88 (25% increase) with a rise in temperature.

DISCUSSION

Effect of temperature on the membrane-expanding potency of anesthetics

The significant finding in this study is that, using the sealed-ghost-expansion method, the amount of anesthetic-induced expansion of the membrane area is increased at higher temperatures (Fig. 2). The increase is compatible with the increase in membrane/buffer partition coefficient for benzyl alcohol (Fig. 6 and ref. 34). Such a result, therefore, provides further possible support for the partition theory of anesthesia.

The infinitely dilute solution as the standard state

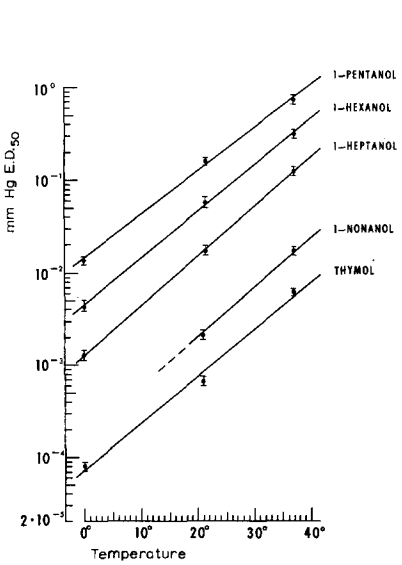
It has been pointed out by SCHNEIDER[26] that the appropriate standard state is that of the infinitely dilute solution. All model systems in the literature (*e.g.* ref. 55) are based on this standard state. On this basis the anesthetic concentration required for ghost membrane expansion diminishes with an increase in temperature (Figs. 2 and 8A); the enthalpy of this process is about −2.5 kcal/mole. For the anesthetic protection of intact erythrocytes against osmotic hemolysis, however, the enthalpy is opposite in sign and is of the order of +1 to +4 kcal/mole (Figs. 5 and 8A).

At present it is unknown why opposite temperature results have been obtained with intact cells and ghosts (see Fig. 8A). The ghost-expansion system more closely represents a true equilibrium than does the intact cell system. This is because the ghosts were incubated with the drug for at least 1.5–2 h before the volumes were measured. The intact cells, on the other hand, were exposed to both the drug and the hypotonic solution at the same time. It is conceivable that the elevated temperatures may have altered the transmembrane penetration rates of drug and water molecules. The rate of cell swelling, therefore, and the rate of membrane disruption would be different at different temperatures. Hence, for intact cells the efficacy[51] of drug action might very well be different at different temperatures.

The pure anesthetic solute as the standard state

Cogent arguments presented by SCHNEIDER[26] explain that the choice of the pure anesthetic as the standard state is a poor one, since the intermolecular forces

and their variations with temperature are different for different compounds. If, however, the pure chemical compound is chosen as the standard state, in accordance with CHERKIN AND CATCHPOOL[15], the anesthetic vapor pressure required to affect the erythrocytes (ghosts or intact cells) rises with temperature (see Figs. 7 and 8B). The



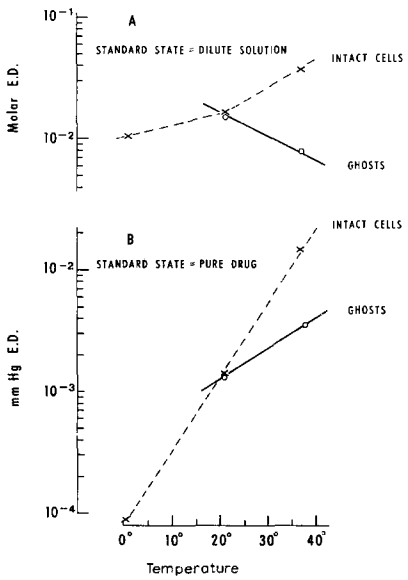


Fig. 7. Relationship of $E.D._{50}$ and temperature. Using the pure anesthetic compound as the standard state, the vapor pressure $E.D._{50}$ (for protection of intact cells) rises steeply with temperature. The vertical bars indicate the standard error of the mean.

Fig. 8. The sign and magnitude of the enthalpy as determined by the choice of the standard state and the choice of the biological anesthetic system. The results here are all for benzyl alcohol. (A) Shows that if the infinitely dilute solution is chosen as the standard state, the enthalpy is -2.5 kcal/mole for ghost expansion but about $+2$ kcal/mole for protection of intact cells. (B) Shows that, choosing the standard state to be the pure chemical compound, the enthalpy values are high, around $+19$ kcal/mole for both ghosts and intact cells. The text explains why the -2.5 kcal/mole value is indicative of the membrane–drug interaction.

enthalpy values obtained from these data are all around + 19 kcal/mole. This compares with $+9$ to $+13$ kcal/mole for goldfish anesthesia[15]. (The anesthetic partial pressures, p, were worked out using FERGUSON'S[22] formula, $p = p_0 \cdot c/c_s$, where p is the partial pressure of the pure anesthetic at a particular temperature, c_s is the concentration of a saturated solution at that temperature, and c is the experimental anesthetic concentration. The values of p_0 and c_s are known[48,52–54].) The main reason for the steep rise in the anti-hemolytic partial pressures in Figs. 7 and 8B is that the vapor pressure of the pure compound (p_0) rises steeply with temperature. Considering these calculations as well as SCHNEIDER'S[26] remarks, it seems that the enthalpy values of around $+19$ kcal/mole are a measure of the enthalpy associated with the transfer of drug solute from vapor to solution rather than of the enthalpy associated with drug transfer from solution to membrane. The results presented in the previous section, dealing with the ghosts and the infinitely dilute solution as the standard state, appear, therefore, to be more meaningful.

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