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MEMBRANE EXPANSION OF INTACT ERYTHROCYTES BY ANESTHETICS

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SUMMARY

1. Low concentrations of the alcohol and phenothiazine anesthetics protected human erythrocytes from hypotonic hemolysis.

2. The reduction in osmotic fragility amounted to a shift of 0.03 g/100 ml NaCl in the presence of a concentration of anesthetic which reduced osmotic hemolysis by 50 %.

3. The anesthetics protected erythrocytes against hypotonic hemolysis over a 1000-fold range in the rate of hemolysis. The drugs do not act by changing the rate of hemolysis.

4. Albumin up to 5.6 % did not reduce the amount of the osmotic hemolysis. Albumin did shift the dose–response curve for chlorpromazine to higher concentrations but only slightly reduced the maximum amount of protection.

5. Low concentrations of the anesthetics did not affect the volumes of the cells in isotonic and moderately hypotonic solutions (down to 0.35 % NaCl).

6 The critical hemolytic relative volume, V_c, was measured by the GUEST AND WING technique. The V_c obtained by a Coulter counter and computer method agreed with the V_c obtained using Van Allen hematocrit tubes. The value for V_c in the absence of any anesthetics was 1.825 and the membrane area of the prehemolytic sphere was about 136 μ^2.

7. The anesthetics increased the V_c. At an anesthetic concentration which reduced osmotic hemolysis by 50 %, the V_c increased to 1.925; at 95 % reduction in hypotonic hemolysis the V_c went up to around 2.02. The corresponding membrane areas were around 141 and 149 μ^2, respectively. At 50 % protection, therefore, the membrane area of the intact erythrocyte expanded by approx. 3 %.

INTRODUCTION

Many types of cells and subcellular membrane-bounded organelles are protected or stabilized by a very wide variety of surface-active compounds, including tranquilizers, antihistamines, fatty acids, alcohols, anesthetics and steroids[1–15]. The general observation is that low concentrations of these compounds (usually from 10^{-8} to 10^{-3} M) protect the cell or the cell organelle from osmotic, mechanical or acid lysis, but that higher concentrations (usually above 10^{-4} or 10^{-3} M) are directly and immediately lytic to the membrane.

Although the mechanism of the drug-induced membrane "stabilization" at low

drug concentration is not known, it is possible that it may be similar to the mechanism of nerve membrane stabilization occurring in anesthesia. This is suggested by the facts that (1) all compounds that have a protective or anti-hemolytic effect on erythrocytes can also act as anesthetics, and (2) there is a 1:1 correlation over a 10000000-fold concentration range between the concentrations of alcohols producing anesthesia (of the frog sciatic nerve or the tadpole reflex) and those producing erythrocyte stabilization[10].

On the basis of the penetration of local anesthetics into lipid monolayers spread at the air–water interface, both SKOU[16] and SHANES[17] suggested that nerve membrane anesthesia was associated with an expansion of the membrane by the anesthetic. The possibility of membrane expansion by anesthetics has also been mentioned by PONDER[18] who suggested that the anti-hemolytic effects of some anesthetics reported earlier by TRAUBE[1] and by JACOBS AND PARPART[2] could be explained by a small increase in the spherical critical hemolytic volume to which the cells swell.

Although it is known that many chemical compounds, including anesthetics, induce shape alterations of erythrocytes and other cells[18–23], the actual changes in membrane surface area have never been accurately measured. Drug-induced increases in V_c, the critical hemolytic volume, have been reported[5,8,12,25], but these have been obtained at only one drug concentration. It was necessary, therefore, to examine a wide range of drug concentrations. In the light of recent findings[26–29], experiments were also designed to test the possibility that the anesthetic-induced protection of red cells against fast hemolysis might be eliminated or reduced in gradual hemolysis.

METHODS

Procedure for determining the shift in osmotic fragility induced by anesthetics

After heparinization (75 units/ml blood) and removing the buffy coat from a freshly drawn blood sample, a stock erythrocyte suspension of approx. $400 \cdot 10^6$ cells per ml was prepared in 154 mM NaCl (pH 7), 10 mM sodium phosphate buffer[10–13]. An aliquot of 0.1 ml of this suspension was pipetted into 1.5 ml of 0.36, 0.38 and 0.40 g/100 ml NaCl (pH 7) containing varying concentrations of anesthetic. After 5 min at room temperature (21–22°) the cells were centrifuged at 1500 × g for 1.5 min, the supernatant removed and the percent hemolysis determined by measuring the absorbance of hemoglobin in the supernatant at 543 nm.

Procedure for determining the osmotic swelling properties and V_c, using microhematocrits and the Guest and Wing method

Fresh blood was heparinized and washed once with 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7). Aliquots of packed erythrocytes were mixed with hypotonic NaCl solutions to give a final hematocrit of 30 % in 154 mM NaCl, 10 mM sodium phosphate buffer (pH 7). The microhematocrit tubes (75 mm × 0.8 mm internal diameter) were centrifuged at 7000 × *g* for 8 min at room temperature, at which time the packed volume was constant and KOEPPE's criterion of translucency[18] was attained. Under these conditions approx. 2 % of the packed cell column is extracellular space[30]. Further details to this procedure are described by SEEMAN *et al.*[27]; the technique is basically a microhematocrit modification of the GUEST AND WING method[31].

For cell suspensions of about 0.3 % (v/v) it was necessary to use Van Allen tubes to obtain the hematocrit values. This method has already been described[31,27].

Determination of the osmotic swelling properties of the erythrocyte using the Coulter counter

Aliquots of 0.5 ml, containing $9 \cdot 10^5$ erythrocytes in 154 mM NaCl (pH 7), were added to 10 ml of hypotonic solutions at pH 7. After 15 min to 1 h the cells were counted and sized with a Coulter counter Model F and mean cell volume computer. The diameter of the aperture was 100μ, the aperture setting was 16, and the attenuation 0.707. Further details can be found in refs. 27 and 29.

Syringe-pump method for carrying out hypotonic hemolysis at different rates

This method has already been described by SEEMAN *et al.*[27].

MATERIALS

1-Hexanol, 1-heptanol and 1-nonanol were obtained from Eastman Organic Chemicals, Rochester. Benzyl alcohol, 1-pentanol and thymol were purchased from Fisher Scientific Co., N.Y. Chlorpromazine hydrochloride (Thorazine) was donated by both Smith Kline and French, Philadelphia and Rhone–Poulenc, Montreal. Crystallized human serum albumin (Cohn's Fraction V) was from Calbiochem, Corp., Los Angeles.

RESULTS

The shift in erythrocyte osmotic fragility brought about by anesthetics

Low concentrations of aliphatic and aromatic alcohol anesthetics protect or stabilize human erythrocytes against osmotic hemolysis. An example of this is shown in Fig. 1 for 1-nonanol.

Under these experimental conditions the osmotic fragility curve was linear between 60 and 30 % hemolysis. It was convenient, therefore, to choose the value of 60 % absolute hemolysis as the control amount of hemolysis in the absence of any anesthetic (see Fig. 2).

The results in Fig. 2 show that for a variety of alcohol anesthetics at a variety of different concentrations the same shift in the osmotic fragility curve was found for the same protective or anti-hemolytic effect produced by the drug. For example, the osmotic fragility curve always shifted by 0.03 g/100 ml NaCl when there was a 50 % reduction in absolute hemolysis (from 60 % hemolysis down to 30 % hemolysis), regardless of which drug was acting.

Drug-induced protection of erythrocytes against osmotic hemolysis occurs in both fast and gradual hemolysis

The data in Fig. 3 show that the protective effect of $1.2 \cdot 10^{-1}$ M benzyl alcohol holds over a wide range in hemolyzing rates. The top line in Fig. 3 indicates the amount of hemolysis in the control experiments where there was no drug. The concentration in the syringe of the NaCl solution (being infused into the hemolyzing suspension) was 0.225% NaCl and the final concentration in the vial at the end of infusion was 0.375 % NaCl. Control hemolysis at 0.375 % NaCl, therefore, was 94 % when the hypotonic solution was injected quickly into the vial, at 100 ml/min; in fact, 5 ml was injected in about 3 sec. This amount of hemolysis was reduced sharply to 43 % when the

infusion rate went down to 0.34 ml/min. Evidence has already been presented[27] that this reduction in hemolysis can be explained by a prelytic leakage of intracellular K^+.

While the control amount of hemolysis dropped at slower hemolyzing rates, the protective effect of benzyl alcolol remained the same.

KATCHALSKY *et al.*[26] found that 1.25 % albumin reduced the osmotic fragility of washed erythrocytes, and that albumin may have retarded the hemolysis rate. The results in Fig. 4 show that under the conditions of the present experiments albumin

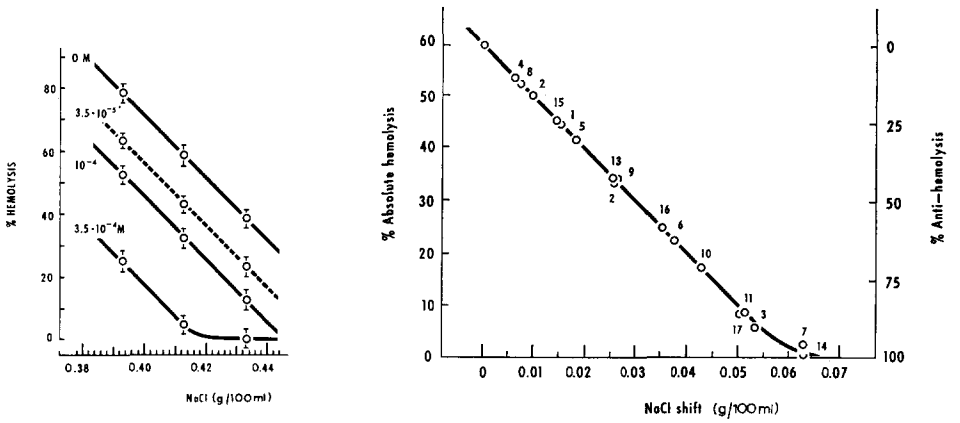

Fig. 1. Effect of nonanol on osmotic fragility, showing that low concentrations of nonanol reduce the osmotic fragility of erythrocytes.

Fig. 2. Shift in osmotic fragility by alcohols. It may be seen that for a variety of alcohol anesthetics at different concentrations, the same shift in the osmotic fragility curve is found for the same protective or anti-hemolytic effect produced by the drug. 1, 2 and 3 represent nonanol, $3.5 \cdot 10^{-5}$, 10^{-4} and $3.5 \cdot 10^{-4}$ M; 4, 5, 6 and 7 are thymol, $3.5 \cdot 10^{-5}$, 10^{-4}, $3.5 \cdot 10^{-4}$ and 10^{-3} M; 8, 9, 10 and 11 are pentanol, 10^{-2}, $2 \cdot 10^{-2}$, $3.5 \cdot 10^{-2}$ and $5 \cdot 10^{-2}$ M; 12, 13 and 14 are hexanol, $2 \cdot 10^{-3}$, $5 \cdot 10^{-3}$ and $2 \cdot 10^{-2}$ M; 15, 16 and 17 are heptanol, $8 \cdot 10^{-4}$, $2 \cdot 10^{-3}$ and $3.5 \cdot 10^{-3}$ M, respectively.

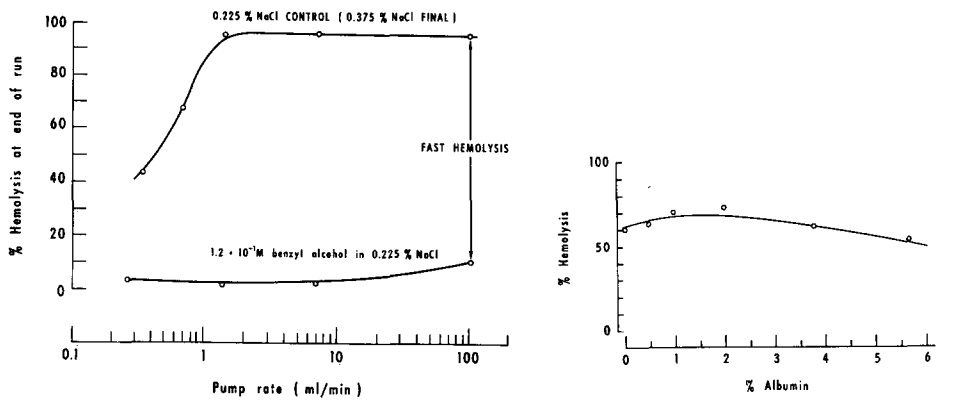

Fig. 3. The drug-induced protection of erythrocytes against osmotic hemolysis *versus* the rate of hemolysis. The control curve (top) shows that as the rate of infusion of hypotonic solution (0.225 % NaCl) is reduced, the amount of hemolysis at the end of the run is also reduced. The protective effect of benzyl alcohol, however, is not affected (bottom line).

Fig. 4. The effect of human serum albumin on the amount of hypotonic hemolysis. These erythrocytes had been washed only once. It appears, therefore, that albumin does not greatly reduce the cell's hemolyzing rate.

had very little, if any, effect on the amount of hypotonic hemolysis. There was a slight drop in hemolysis at 5.6 % albumin. It is possible that the difference between the present results and those of KATCHALSKY *et al.*[26] is that the cells of the latter were washed 4 times.

The results in Fig. 5 show that at all albumin concentrations tested, up to 5.6 % albumin, the protection of erythrocytes by chlorpromazine, a strong local anesthetic, was not abolished. At 5.6 % albumin there was a slight reduction of the maximal protective effect of chlorpromazine. Albumin did shift the chlorpromazine dose–response curve to the right. This is not surprising, insce it is known that albumin adsorbs chlorpromazine[32].

The effect of anesthetics on the critical hemolytic volume

It was found that the alcohol and phenothiazine anesthetics increased the critical hemolytic volume, V_c. An example for nonyl alcohol is shown in Fig. 6. The concentration of 1-nonanol here was $3.4 \cdot 10^{-4}$ M. For 1-nonanol this is rather a high con-

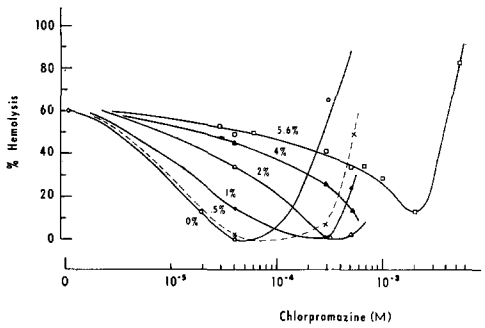

Fig. 5. The effect of human serum albumin in shifting the dose–response curve of chlorpromazine protection of erythrocytes. The maximum amount of cell protection is only slightly affected, however. If albumin reduces the cells hemolyzing rate, therefore, this does not affect the maximal protective action of the anesthetic.

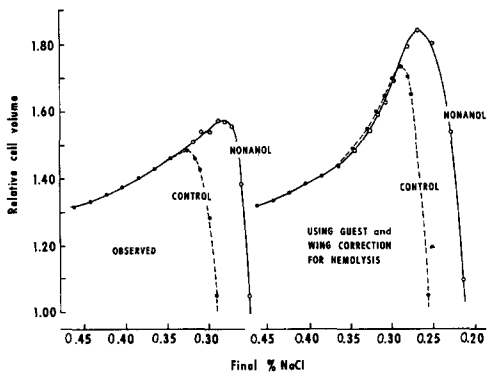

Fig. 6. An example of the increase in critical hemolytic volume brought about by the alcohol anesthetics. The data on the left shows the observed values for the relative cell volumes in solutions of different tonicity, using microhematocrit tubes. The control microhematocrit value for cells in 0.9 % NaCl was 30 % of the packed cell volume. The data on the right shows the relative cell volumes after being corrected for % hemolysis, using the GUEST AND WING method[31].

centration[10]. It is known [8,11], however, that the concentration of anesthetic required to stabilize erythrocytes against hypotonic hemolysis goes up with more concentrated cell suspensions; the concentration of cells in the experiment in Fig. 6 was 30%.

The results in Fig. 6 show three points of interest. (1) The osmotic fragility has been shifted, as already demonstrated in Figs. 1 and 2. (2) The anesthetic had no effect on the osmotic swelling limb of the cell volume curve. It has also been shown by VAN STEVENINCK *et al.*[5] that 10^{-4} M chlorpromazine, while causing erythrocyte protection against hypotonic hemolysis, did not affect the erythrocyte volume. At chlorpromazine concentrations higher than 10^{-4} M, however, KWANT AND VAN STEVENINCK[8] found about a 10% increase in the cell volume in isotonic solution. (3) The third important feature of the data in Fig. 6 is that, using the GUEST AND WING[31] method for correcting the hematocrit for the amount of hemolysis, the anesthetic increased the critical hemolytic relative volume from 1.73 to 1.84. Since the size of the intact cell in isotonic solution was 82.5 μ^3, the critical hemolytic volume went up from 142.73 μ^3 to 151.9 μ^3. The corresponding areas for these two prelytic spheres are 132.08 μ^2 and 137.61 μ^2. The anesthetic, therefore, has increased the area of the membrane by 5.53 μ^2 or 4.18%.

It would be desirable to know the increase in V_c at a variety of drug concentrations. The data in Fig. 6 shows the increase for only one concentration. Since a single determination of the critical hemolytic volume required considerable time for the large number of microhematocrit and hemoglobin determinations, it was difficult to test various concentrations of a drug on the same erythrocytes on the same day. Utilizing the normal osmotic fragility curve (Fig. 1) and the drug-induced shift in osmotic fragility (Figs. 1 and 2), it was possible to work out the amount of membrane area expansion (brought about by the drug) as follows:

The experimental points in Fig. 7 show the relative cell volumes in hypotonic NaCl solutions. The data represented by circles were obtained using Van Allen hematocrit tubes for a cell suspension of 0.3% (v/v); the crosses in Fig. 7 were obtained using the Coulter counter and mean cell volume computer for an erythrocyte concentration of 0.002%. It can be seen that there is agreement between the two sets of data. Only the Van Allen data are plotted in the right-hand lytic limb of the curve. It is not possible to obtain the osmotic fragility of the cells (*i.e.* the right-hand part of the curve in Fig. 6) using the Coulter counter, since this instrument counts and sizes ghosts as well as intact cells [27,29,33].

The critical hemolytic volume, V_c, is at the apex where the osmotic swelling curve joins the osmotic fragility curve. In general in dilute suspensions (1% cells or less) the V_c was around 1.8. In Fig. 7 the V_c is 1.825. Since the volume of the erythrocytes in isotonic solution was usually 82.5 μ^3, the volume of the prehemolytic sphere was 150.56 μ^3 with an area of 136.86 μ^2.

The drug-induced shifts in the osmotic fragility curve are also drawn in Fig. 7. The magnitude of these shifts have been obtained form the data of Fig. 2; 50% anti-hemolysis, for example, is associated with a reduction in fragility of 0.03 g/100 ml NaCl.

The apices of these shifted swelling–fragility curves represent the drug-induced increases in the critical hemolytic volume, V_c. The absolute and relative area increments associated with these higher V_c values are shown in Fig. 8. These results of Fig. 8 indicate that 50% anti-hemolysis is associated with approx. 3.5% expansion of membrane area.

Using the data of Fig. 8 the explicit dose–response relation for any particular drug can be worked out. The membrane area expansion of intact erythrocytes by 1-pentanol is shown in Fig. 9.

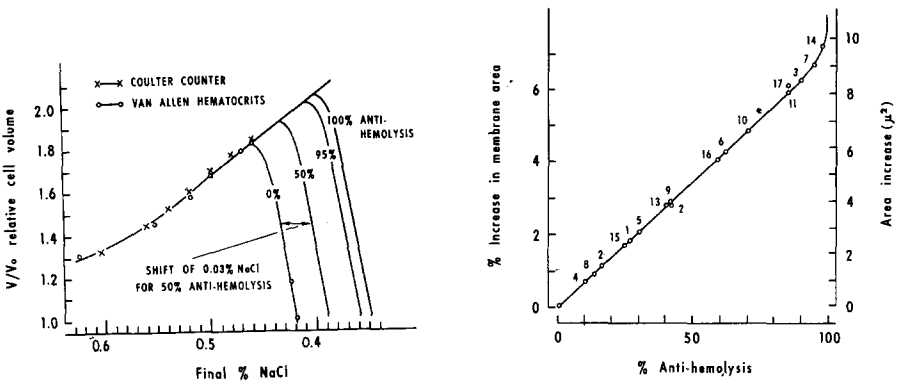

Fig. 7. A plot of how the drug-induced increase in the critical hemolytic volume, V_c, could be worked out, knowing the drug-induced shift in osmotic fragility and the normal osmotic swelling curve of erythrocytes. The ordinate represents the cell volumes in hypotonic solution relative to the volume in 0.9% NaCl. O—O, data obtained using the Van Allen hematocrit tubes; ×—×, those using the Coulter counter and mean cell volume computer. The drug-induced shifts in osmotic fragility are drawn at the far right for 0 (*i.e.* control in the absence of drug), 50, 95 and 100% cell protection or anti-hemolysis. The apex (or maximum ordinate) of each curve shows the approximate V_c for each degree of anti-hemolysis.

Fig. 8. The increase in membrane area at different degrees of drug-induced anti-hemolysis. The values have been calculated according to the procedure shown in Fig. 7. The number besides each dot stands for a different alcohol anesthetic at a different concentration (see Fig. 2).

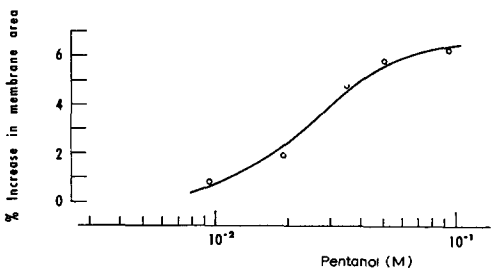

Fig. 9. The dose–response curve for pentanol. The values for the increase in erythrocyte membrane area at different pentanol concentrations have been worked out by the procedure of Fig. 7.

DISCUSSION

Membrane area expansion

The results confirm earlier findings[1–15] that anesthetics protect erythrocytes against hypotonic hemolysis and indicate (1) that the protection occurs over a wide range of hemolyzing rates, (2) that an anesthetic concentration which reduces hemolysis by 50% also expands the membrane area of the intact erythrocyte by about 3.5%, and (3) that the maximum amount of expansion before the membrane lyses is of the order of 7%.

The membrane expansion that occurs increases the surface area/volume ratio of

the cell. According to the results of others[34–38], erythrocytes with higher area/volume ratios should be associated with a lower osmotic fragility. It appears that the membrane area expansion brought about by the anesthetics may account for the reduced osmotic fragility *in vitro*.

Mechanism of membrane area expansion by drugs

There are several possible mechanisms which may explain the drug-induced expansion of the membrane area of the intact erythrocytes.

(1) Perhaps the simplest mechanism would be that of a volume increase in the membrane as a result of the "burying" of anesthetic molecules right into the membrane. MULLINS[39] has hypothesized that anesthetics dissolve within the membrane, and SEARS[40] has shown that gases can expand liquid hydrocarbons. Current studies underway in this laboratory at present, wherein the uptake of radioactive anesthetics by pure membranes is being examined, indicate that around one-third of the observed membrane expansion can be accounted for by the bulk volume of the molecules.

(2) A second possible mechanism for membrane expansion would be that of the "adsorption–extension" hypothesis of SCHNEIDER[41].

(3) A third possibility is that the anesthetic can displace some membrane-associated component which normally keeps the membrane in a condensed state. A good example of this would be membrane-associated Ca^{2+} (ref. 42). It is well known that Ca^{2+} condenses lipid monolayers spread on a Langmuir trough[43,44]. It is also known that anesthetics can displace Ca^{2+} which are bound to lipids[45].

(4) Anesthetics are also known to produce conformational changes in proteins[46]. It is possible, therefore, that the anesthetics may distort and expand the membrane proteins, either directly or by affecting the state of protein hydration.

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