BBA 75301

MEMBRANE EXPANSION OF ERYTHROCYTE GHOSTS BY TRANQUILIZERS AND ANESTHETICS

P. SEEMAN, W. O. KWANT AND T. SAUKS

Department of Pharmacology, University of Toronto, Toronto 5, Ontario (Canada)

(Received January 27th, 1969)

SUMMARY

1. The ghosts of erythrocytes, hemolyzed in 0.3% NaCl (pH 7) and in a final hematocrit of 0.002%, appeared to be spherical under phase contrast microscopy. From photographic measurements of the diameter, the mean area of the erythrocytes was found to be 134.8 μ^2.

2. The spherical ghosts were monitored in a Coulter counter and mean cell volume computer. The mean cell volume was found to be between 140 and 152 μ^3, and the corresponding membrane areas were between 130 and 138 μ^2.

3. When the cells were hemolyzed in the presence of low concentrations of drugs ("ghost-formation" method), the mean cell volume value of the ghosts 2 h later was larger than control.

4. When low concentrations of the drugs were added to the prehemolyzed, spherical ghosts, the cells immediately became irregular and cup shaped. The mean cell volume at this moment fell, and this may be explained by the sensitivity of the Coulter counter to cell shape. Over the next 2 h the cells re-sphered spontaneously in the presence of the drug. The final mean cell volume was greater than control. (This procedure is referred to as the "sealed-ghost-expansion" method.)

5. The ghost-formation method and the sealed-ghost-expansion method gave similar results quantitatively. By these methods it was found that the erythrocyte ghost membrane area expanded by 1.3–1.6% for the alcohol anesthetics and by 1.5–2.7% for chlorpromazine at drug concentrations which are known to anesthetize nerves and also to inhibit hypotonic hemolysis by 50%.

6. At high but sublytic concentrations of these drugs, the maximum amount of membrane area expansion observed was around 5%.

INTRODUCTION

There is an extensive literature on the ability of drugs and chemical compounds to penetrate, expand and modify the properties of lipid, protein and lipoprotein monolayers spread at the air and water interface[1–12]. The effects of drugs on the electrical properties of artificial ultrathin lipid and lipoprotein films[13–15] formed between two aqueous phases have also been studied[16–20]. A third model membrane system, that of spherulites or myelin figures, has been used to study the effects of narcotics, steroids and other drugs[21,22].

It would be desirable to confirm directly whether or not some of the interesting drug effects described for the above three membrane model systems also occur in biological membranes. SHANES[23] and SKOU[24], for example, have predicted an expansion of biomembranes in the presence of anesthetics, on the basis that such an expansion does occur in a lipid monolayer at the air–water interface.

The new techniques described in this paper demonstrate that it is possible to monitor small changes in the surface area of erythrocyte ghost membranes. Such techniques can be readily applied to biomembranes of any type provided the membranes can be disrupted and converted into vesicular form; it is known that broken membranes spontaneously assume a vesicle shape[25–30].

METHODS

Preparation of the stock erythrocyte suspension

A 1.5-ml sample of fresh venous blood drawn from a fasting volunteer was heparinized with about 50 units/ml blood. The sample was centrifuged at 1500 × *g* for 15 min and the plasma and buffy coat removed. A stock erythrocyte suspension of about $1.8 \cdot 10^6$ cells/ml was prepared using 154 mM NaCl in 10 mM sodium phosphate buffer (pH 7).

The ghost-formation method

An aliquot of 0.5 ml of the suspension of intact erythrocytes was added to a Coulter plastic vial (Coulter Electronics, Hialeah, Fla.) which contained 10 ml of a dilute drug solution made up in 0.3 % NaCl in 10 mM sodium phosphate buffer (pH 7). The final suspension was immediately mixed thoroughly by inversion but without frothing or bubbling. 2 h later the mean cell volume of the erythrocyte ghosts was monitored by means of a Model F Coulter counter and an mean cell volume computer (Coulter Electronics, Hialeah, Fla.). The Coulter probe had an aperture of 100 μ in diameter, the aperture setting was 16, the attenuation was 0.707, the threshold setting on both the counter and computer was 10 units, and the sample volume was 0.5 ml. The time taken by the Coulter counter to pull 0.5 ml through the 100-μ aperture was 14 sec; the mean cell volume computer required 7 sec for one determination. Approx. 20000 cells were averaged during these 7 sec, while 40000 cells were counted over the entire 14-sec period. The mean cell volume computer, which has a replicate reproducibility of better than 0.6% (ref. 31) was calibrated by determining the mean cell volume of the erythrocytes using a Clay–Adams microhematocrit centrifuge and non-heparinized microhematocrit tubes (MSE, Measuring and Scientific Equipment, Great Britain), measuring 75 mm × 0.8 mm internal diameter[32,33]. The microhematocrit tubes were centrifuged at 12500 rev./min (15500 × *g*) until complete translucency of the packed cell column occurred (KOEPPE's[34] criterion) and until the hematocrit value was constant with no further packing of the cells; this required 3 min of centrifugation. When calibrating the mean cell volume computer by calculating the mean cell volume from the microhematocrit and the Coulter cell count, allowance was made for about 2% extracellular space in the packed cell column[35]. The mean cell volume reading was taken at least three or four times for each sample, and in most cases a large number of samples (between 6 and 20) were prepared for each drug concentration.

Under these experimental conditions the shape of the erythrocyte ghost was spherical by phase-contrast microscopy. It was, therefore, possible to calculate the surface area of the ghost membrane and any changes in this membrane area brought about by the drugs.

The sealed-ghost-expansion method

An aliquot of 0.5 ml of the stock suspension of intact erythrocytes was added to 10 ml of 0.3% NaCl (51.5 mM) in 10 mM sodium phosphate buffer (pH 7), which did not contain any drug. During the subsequent 1.5–2.5 h the ghosts completed their spontaneous processes of membrane sealing and cell reswelling[32,36]. The control mean cell volume of the ghost suspension was monitored only once; since the Coulter counter sample required 0.5 ml, there remained 10 ml in the vial after this control measurement. The control step just mentioned was not entirely necessary and in later experiments was omitted. A small aliquot (less than 0.2 ml) of concentrated drug solution was then added, and the contents of the vial inverted three times gently. The suspension remained for an additional 2 h at room temperature. During this time the ghost slowly resumed its initial spherical shape. The mean cell volume of the ghosts was then monitored. The radius and the surface area of the membrane of the spherical ghost were thus computed. To facilitate rapid conversion of volume readings into membrane area, a volume–area table was constructed using an IBM 7094 computer.

MATERIALS

1-Pentanol and 1-nonanol were purchased from Fisher Scientific Co. Benzyl alcohol was obtained from British Drug Houses. Chlorpromazine hydrochloride (Thorazine, Largactil) was a gift from Smith Kline and French, Philadelphia, and Rhone–Poulenc, Montreal.

RESULTS

The area of the normal erythrocyte ghost and the osmotic swelling properties of the erythrocyte using the Coulter counter

The data in Fig. 1 show the mean cell volumes of the erythrocytes at various NaCl concentrations. It can be seen that the mean cell volume increased at lower NaCl concentrations until a maximum volume of around 153 μ^3 was reached at about 0.45% NaCl. The mean cell volume values in Fig. 1 were taken in the Coulter counter about 1 h after the addition of the intact erythrocytes to the hypotonic solutions. Although erythrocytes became ghosts at around 0.45% NaCl, a further reduction in NaCl concentration did not lead to an increase or a decrease in the mean cell volume. At very low NaCl concentration (below 0.2% NaCl) the apparent ghost mean cell volume was reduced. Most if not all of this drop in apparent mean cell volume is artifactual and results from the lowered conductivity of the medium. The addition of some NaCl to raise the final NaCl concentration to above 0.2% raises the mean cell volume back to the 150 μ^3 region.

The data in Fig. 1 indicate that the mean cell volume of the erythrocyte ghosts was the same at all NaCl concentrations between 0.4% and 0.2% NaCl. The

concentration of 0.3% NaCl, therefore, was chosen as the standard concentration for preparing ghosts. In Fig. 1 the mean cell volume at 0.3% NaCl was 152.5 μ^3; the area of such a mean cell is 138.04 μ^2.

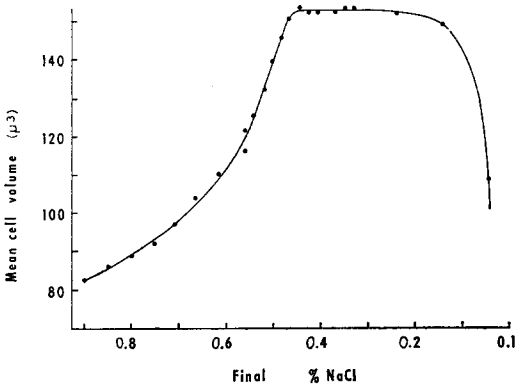

Fig. 1. Mean cell volumes of erythrocytes in hypotonic solutions at 20° and 10 mM sodium phosphate buffer (pH 7). The intact erythrocytes become ghosts at around 0.45% NaCl. Below 0.15% NaCl there is an apparent drop in ghost mean cell volume; this is artifactual, resulting from low conductivity of the solution, and is removed by the addition of NaCl to 0.2% or more, restoring the mean cell volume to 150 μ^3.

Membrane area and cell shape as seen in phase-contrast microscopy

Fig. 2a is a phase-contrast photograph of ghosts of erythrocytes hemolysed in 0.3% NaCl. The shapes of the cells are spherical. When the cells were suspended in a drop hanging from a glass cover slip, small pseudopods or spikes of the order of 0.3 μ in length and width were seen extending from the cell. These pseudopods were artifacts brought about by the proximity of the glass surface (see ref. 34 for a discussion on this matter). The appearance of pseudopods was markedly reduced when siliconized slides and coverslips were used. The pseudopods were not seen at all using plastic coverslips.

The diameters of a large number of spherical ghosts of the sort shown in Fig. 2a were measured. A Zeiss stage micrometer marked at 10-μ intervals was used for calibration. Two diameters, perpendicular to one another, were measured for each cell, and then averaged. For a total number of 281 cells measured, the mean diameter was 6.54 $\pm$ 0.41 μ (mean $\pm$ S.D.). The area of a spherical ghost with this diameter is 134.27 μ^2. The mean of the areas of these cells is 134.83 μ^2. This value of 134–135 μ^2 is about the same as that found by the Coulter counter and mean cell volume computer for ghosts in 0.3% NaCl.

Measurement of membrane area expansion, using the ghost-formation method

The ghost-formation method, as outlined in METHODS, is depicted in Fig. 3a. The erythrocytes hemolyze osmotically in 0.3% NaCl, seal and reswell, all in the presence of the drug, before the mean cell volume is monitored by the Coulter counter and computer.

The effect of 1-nonanol on the membrane area of the ghost, as determined by the ghost-formation method, is shown in Fig. 4. The mean cell volume and membrane

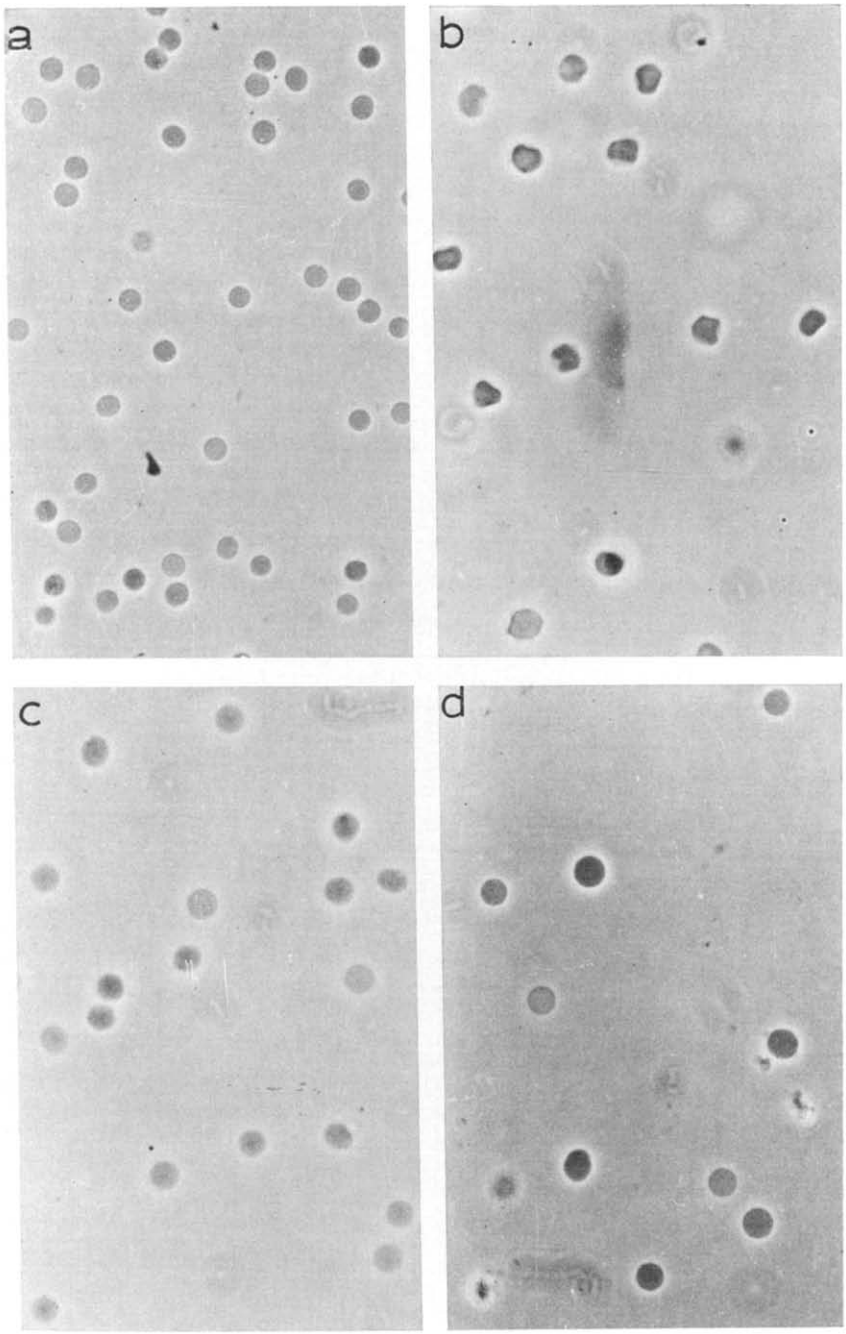

Fig. 2. Phase-contrast appearance of erythrocyte ghosts between siliconized glass surfaces 0.2 mm apart. The magnification scale can be calculated on the basis that the spherical ghost in each photograph is about 6.5 μ in diameter. (a) Spherical appearance of ghosts of cells hemolyzed in 0.3% NaCl. Cells average 6.54 μ in diameter. b, c, and d are phase-contrast photographs taken after the addition of $1.6 \cdot 10^{-2}$ M benzyl alcohol. (b) Irregularly shaped ghosts at 30 min. (c) Prolate spheroid-shaped and spherical ghosts at 1 h. (d) Re-sphered ghosts at 3 h.

area of the ghosts at zero or low drug concentration was 148.5 μ^3 and 135.61 μ^2, respectively. The mean cell volume and area rise to a maximum of 159.5 μ^3 and 142.23 μ^2 at $5 \cdot 10^{-4}$ M nonanol. Drug concentrations higher than this are directly and nonspecifically lytic (see ref. 37).

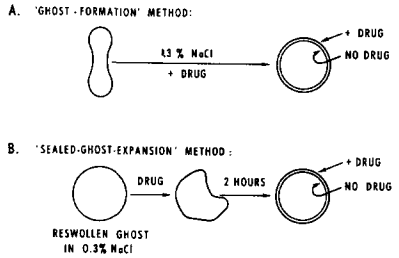

Fig. 3. Methods for studying ghost membrane expansion by drugs. The ghost-formation method is when the intact erythrocytes are hemolyzed in hypotonic solution in the presence of the drug. The sealed-ghost-expansion method is when the drug is added to preformed, spherical ghosts. In both methods, 2 h after the addition of the drug the cells are again spherical, and they are then monitored by the Coulter counter and mean cell volume computer.

The concentration of 1-nonanol which inhibits hypotonic hemolysis by 50% is about 10^{-4} M (ref. 33). At this concentration the area was found to be increased by 2.2 μ^2 or 1.62% (see Fig. 4).

The ghost-formation results in Fig. 5 (top line) show the effect of 1-pentanol on the mean cell volume and area of the ghosts. The concentration of 1-pentanol which inhibits hypotonic hemolysis by 50 % is $2.4 \cdot 10^{-2}$ M (refs. 38, 39). At this concentration it can be seen from the results in Fig. 5 (top) that the membrane area expanded by 1.35 %.

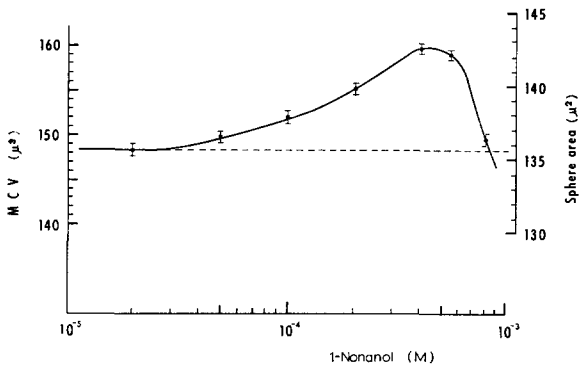

Fig. 4. The expansion of erythrocyte ghost membranes by 1-nonanol. These data were obtained by the ghost-formation method. At 10^{-4} M 1-nonanol the expansion of the spherical ghosts is about 1.6% in area. MCV, mean cell volume.

Effect of drugs when added to prehemolyzed, spherical ghosts

When the anesthetics or tranquilizers were added to the preformed spherical ghosts, the cells immediately became irregular in shape, depending on the final concentration. Figs. 2b and 2c, for example, illustrate the irregularly shaped and prolate spheroid-shaped cells that occurred after $1.6 \cdot 10^{-2}$ M benzyl alcohol was added.

Over the subsequent 1.5–2.5 h the cells spontaneously reverted to their original spherical shape, despite the continued presence of the drug; this is shown in Fig. 2d.

Chlorpromazine·HCl also produced such changes in shape, but they were less pronounced. Fig. 6a. illustrates that there was very little distortion of the ghosts by $5 \cdot 10^{-6}$ M, a concentration which is protective to erythrocytes[40].

High concentrations of chlorpromazine elicited cup shapes and irregular forms (see Fig. 6b) which subsequently reverted to the spherical form (Fig. 6c). Very high or lytic concentrations of chlorpromazine produced irregularly shaped cells which remained that way indefinitely (Fig. 6d).

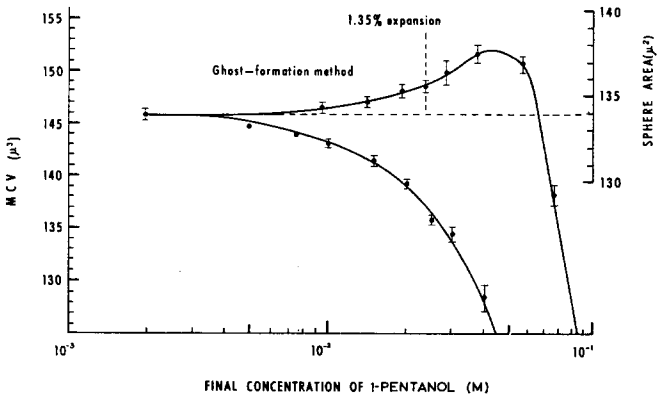

Fig. 5. The top curve shows the volumes of spherical ghosts formed from intact cells in the presence of 1-pentanol (ghost-formation method). The bottom curve shows that the addition of 1-pentanol to preformed spherical ghosts immediately leads to an apparent drop in the mean cell volume (ghost-flattening method; see text). MCV, mean cell volume.

Membrane area expansion, using the sealed-ghost-expansion method

The photographs in Figs. 2 and 6 illustrate that after the anesthetics and tranquilizers were added to spherical ghosts, the cells became transiently misshapen. When the mean cell volume of these misshapen ghosts was measured immediately or a few moments after the addition of the drug, then it was observed that the mean cell volume was lower than the control value for the cells. This is shown in the results plotted in the lower curves in Fig. 5 for 1-pentanol and Fig. 7 for chlorpromazine. This drop in mean cell volume immediately upon the addition of drug was not accompanied by any changes in the total cell count. The drug could not have increased the osmotic pressure of the extracellular fluid. This is because the drug concentration was too low (10^{-5}–10^{-4} M for chlorpromazine and 1-nonanol) and also because the lower aliphatic alcohols equilibrate completely across the erythrocyte membrane in less than 60 sec (ref. 41). (The transmembrane equilibration of the closely related fatty acids is less than 1 sec (ref. 42).)

It is possible that this immediate drop in mean cell volume was associated in some way with the shape of the cell even though the actual volume may not have been changed by the drug[43–47].

As already shown in Figs. 2c and 6c, the cup-shaped and misshapen cells resphered within 2–3 h following addition of the drug. The mean cell volume values taken at this time were all greater than control. The expanded membrane areas

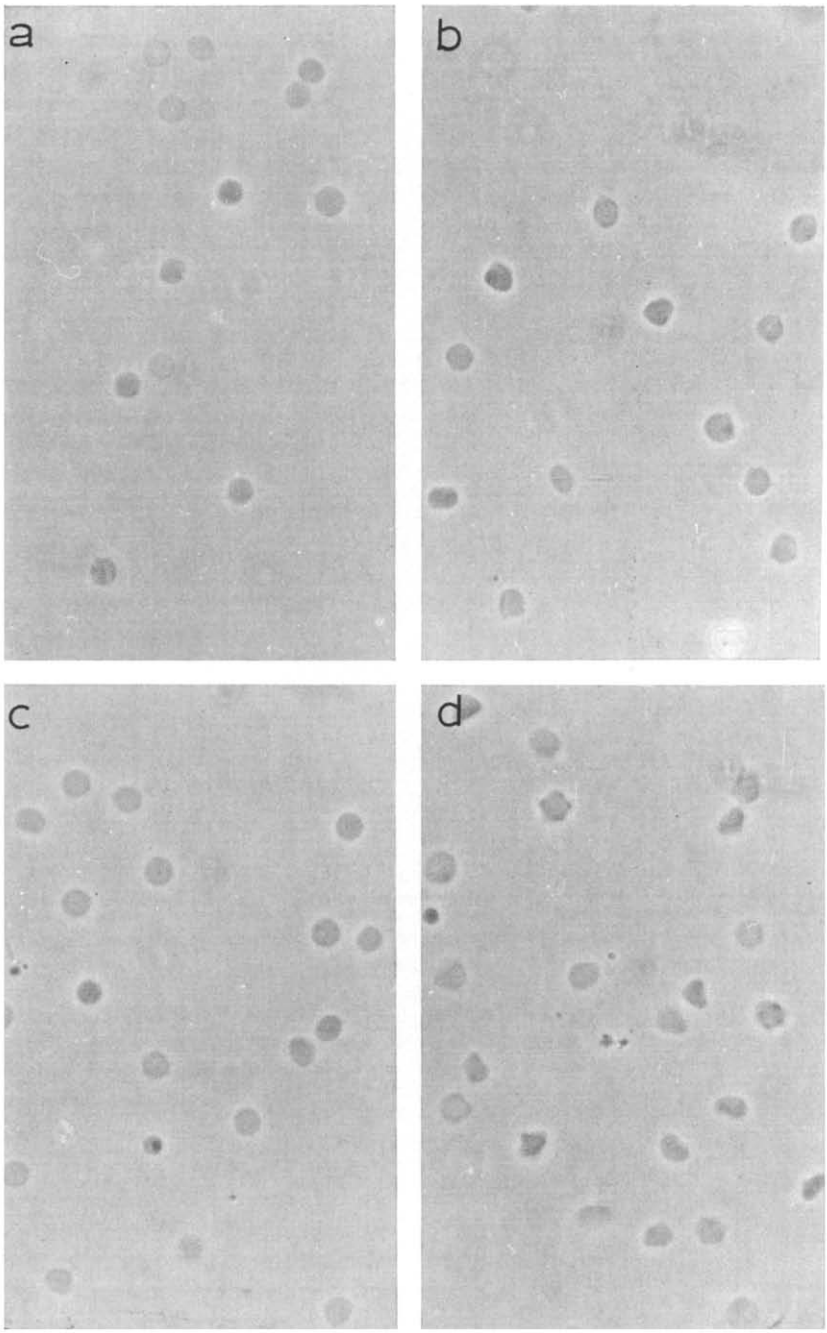

Fig. 6. Phase-contrast appearance of erythrocyte ghosts after the addition of chlorpromazine·HCl to preformed spherical ghosts in 0.3% NaCl. All photographs are the same magnification; spherical ghosts measure about 6.5 μ in diameter. (a) 30 min after the addition of $5 \cdot 10^{-6}$ M drug. Very little effect on shape. Some prolate spheroid cells. (b) 30 min after adding $2 \cdot 10^{-5}$ M chlorpromazine. The cells are irregular. (c) 1.5 h after adding $2 \cdot 10^{-5}$ M chlorpromazine. The cells have now virtually all re-sphered (compare with b). (d) After the addition of $4 \cdot 10^{-5}$ M chlorpromazine, the cells are highly irregular in shape and remain this way. This concentration of chlorpromazine is lytic to cells.

could be calculated and the procedure is conveniently referred to as the "sealed-ghost-expansion" method (see Fig. 3b) to distinguish it from the "ghost-formation" method.

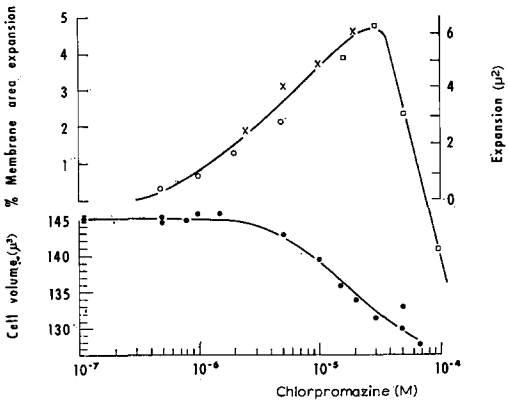

Fig. 7. Expansion of the erythrocyte ghost membrane by chlorpromazine·HCl, using the sealed-ghost-expansion method. The ghost volumes immediately after the addition of chlorpromazine are smaller than control (bottom curve; see also Fig. 5, bottom curve), but 2 h later these cells are greater than control (top curve). Chlorpromazine concentrations above $4 \cdot 10^{-5}$ M are lytic to the membrane.

Results for chlorpromazine, using the sealed-ghost-expansion method, are shown in Fig. 7. The values for the control membrane area in these experiments were between 130 and 133 μ^2. The data in Fig. 7 represented by the square points are results for chlorpromazine using the ghost-formation method. It can be seen that the results are in agreement.

The concentration of chlorpromazine which inhibits hypotonic hemolysis (of a very dilute suspension of erythrocytes) by 50% is between $2 \cdot 10^{-6}$ and $5 \cdot 10^{-6}$ M (see refs. 40 and 48). From Fig. 7 it can be seen that at these chlorpromazine concentrations the membrane area of the ghosts expanded by 1.5–2.7%.

DISCUSSION

The amount of membrane area expansion at different concentrations of anesthetic

The main experimental finding in this study is that the erythrocyte ghost membrane area expanded by 1.35–2.7% at anesthetic concentrations which are known to inhibit hypotonic hemolysis by 50%. These anesthetic concentrations are similar to those which anesthetize nerves[38,39]. It was found in an earlier study[33] that the membrane area of the intact erythrocyte expanded by around 3–3.5% at the same concentrations of these drugs.

Anesthetics might expand the membrane area by: (1) dissolving right into the membrane and occupying bulk space[38,39,49,50] without thickening the membrane[51]; (2) an induced secondary extension of the membrane following the adsorption of the anesthetic "adsorption–extension" hypothesis of SCHNEIDER[52]; (3) displacing membrane-bound components of the membrane, such as Ca^{2+}, which normally keep the membrane condensed (see ref. 33 for references); (4) inducing conformational changes

in the membrane proteins[53,54]; (5) changing the state of hydration of the membrane[55–57].

It is possible that the nerve cell membrane may also be expanded in the presence of these anesthetics.

The shape of the erythrocyte ghost

PONDER[34] has stated that in general an erythrocyte, immediately after hypotonic hemolysis, spontaneously reverts to the original shape of the cell. More recently RAND AND BURTON[58,59], using phase microscopy, found that human erythrocytes become "normal and biconcave ghosts" after hemolysis induced by sucking portions of the intact cell into a micropipette. WHITTAM AND AGER[60], on the other hand, found that the ghosts of erythrocytes hemolyzed in water appeared to be spherical under the phase-contrast microscope. The observations in the present paper also indicate that the ghost is spherical. Firstly, the cells appeared spherical by phase contrast, and secondly, the mean cell volume of the cells in 51.5 mM NaCl was about 150 μ^3. It is possible that an important factor in explaining the apparent differences in results between workers is that the ratio of cells to hypotonic solution was different. With extensive cell washing or in very low hematocrits the amount of membrane-bound albumin may be exceedingly small; FURCHGOTT AND PONDER[61] have claimed that albumin is important in controlling cell shape.

Comparison of the methods for measuring membrane area expansion

Although the ghost-formation method and the sealed-ghost-expansion method give the same results quantitatively, the latter is better for theoretical reasons. Unfavorable features in the ghost-formation method are that the drug may act on and modify some of the hemolytic events such as membrane-stretching rate[62,32], membrane disruption[63,64], membrane sealing and cell reswelling[32].

The area of the normal erythrocyte membrane

As already mentioned in connection with Figs. 1, 4, 5 and 7, the areas of the normal erythrocyte ghosts varied between 130 and 138 μ^2, the most frequent value being 134 μ^2. The value obtained from the measurements of the phase contrast photographs was 134–135 μ^2. These values compare with the value of 138 μ^2 found by CANHAM AND BURTON[67].

The values for membrane area reported by WESTERMAN *et al.*[68] and also HOUCHIN *et al.*[69] were based on a formula by PONDER[34]. PONDER considered the erythrocyte to be a spheroid with the dimples made convex instead of the normal concave appearance. The shape of this cell is an oblate spheroid[70], and the appropriate formula that PONDER should have used is:

$$\text{Area} = 2\pi a^2 + \frac{\pi b^2}{e} \ln\left(\frac{1+e}{1-e}\right)$$

where $e = (a^2 - b^2)^{1/2} / a$, a is the major semi-axis and b is the minor semi-axis, and where $b = 0.67 \times$ thickness of cell (see ref. 71). (This formula for the oblate spheroid may be found in ref. 72.) Using this formula, the data of WESTERMAN *et al.*[68] result in a value of 134.8 μ^2, and those of HOUCHIN *et al.*[69] result in a value of 125 μ^2. Considering all these values, it appears that the most appropriate value for the membrane area of the normal erythrocyte is around 134 μ^2.

A possible method of "ghost flattening" for the determination of drug-induced membrane expansion

It is interesting to calculate the drug-induced area expansion from the shape-factor point of view. As shown in Fig. 2c, after the addition of the drug to spherical ghosts, the cells became prolate spheroid in shape. The cell then has a major axis (= $2a$) and a minor axis (= $2b$) of rotation, where $2b/2a$ is the aspect ratio[46]. As pointed out by ANDERSON *et al.*[45] and by GREGG AND STEIDLEY[46], the Coulter counter recording system is sensitive to the shape of the particle. Prolate and oblate spheroids having the same volume as spheres will actually be recorded as smaller apparent volumes.

An example of how this ghost flattening may be carried out is shown as follows. Consider the results of Fig. 5, bottom curve, where after the addition of 1-pentanol the cells were immediately recorded as having a smaller apparent volume. The cells at this moment were observed to be mostly prolate spheroids. The drop in apparent volume must be a result of the change in shape, since the cells do not immediately shrink in volume, as indicated from microhematocrit studies[32]. Furthermore there is no reason for the cells to shrink, since there is no osmotic gradient established for any appreciable length of time. 1-[^{14}C]Pentanol equilibrates virtually instantaneously across the cell membrane; unpublished studies (using rapid reaction kinetics) in this laboratory have shown that this alcohol has a half-time of equilibration of the order of a second or so. Moreover, since concentrations of 1-nonanol and chlorpromazine ranging down to $5 \cdot 10^{-7}$ M also elicit this apparent drop in mean cell volume, it cannot be a result of an osmotic gradient.

From Eqn. 13 of GREGG AND STEIDLEY[46], therefore, the aspect ratio of the prolate spheroid can be worked out. The aspect ratio, s, is defined as the diameter/thickness ratio or $2b/2a$ and is obtained from the following equation:

$$\frac{R}{R_0} = \frac{1 + \frac{1.1s}{2}}{1 + \frac{1.1s_0}{2}}$$

where R is Coulter counter resistance of the drug-affected cell and R_0 is the resistance of the control ghost. The aspect ratio of the spherical control ghost, s_0, is equal to 1. Hence,

$$s = \frac{b}{a} = \frac{2\left(1.55 \frac{\text{MCV}}{\text{MCV}_0} - 1\right)}{1.1}$$

where the mean cell volume (MCV) is an index of R.

The concentration of 1-pentanol which inhibits hypotonic hemolysis by 50 % is $2.4 \cdot 10^{-2}$ M (ref. 39). At this concentration the apparent MCV is 136.7 μ^3 (Fig. 5). The aspect ratio works out to be $b/a = 0.826$, using the MCV of 136.7 μ^3 and the MCV_0 of 145.7 μ^3. Since it is known that these drugs do not immediately affect the volume of the cells[32], the real volume of the drug-affected ghost is actually still 145.7 μ^3. The volume of a prolate spheroid is $4\pi ab^2$ and so $ab^2 = 34.70$ μ^2; subsequently $a = 3.72$ μ and $b = 3.072$ μ. The surface of a prolate spheroid is:

$$2\pi b^2 + \frac{2\pi a^2 b}{(a^2 - b^2)^{1/2}} \sin^{-1} \frac{(a^2 - b^2)^{1/2}}{a}$$

and this works out to be 135.65 μ^2. Since the surface area of the control ghost sphere is 134.0 μ^2, this represents an expansion of 1.23% in area.

This value of 1.23% expansion, calculated from shape-factor considerations, and using the data of Fig. 5, bottom curve, compares with the value of 1.35% obtained by the ghost-formation method, using the data of Fig. 5, top curve. The close agreement between the results of these grossly different approaches is probably fortuitous. The chief drawback in relying on the ghost-flattening approach is that not all the cells are prolate spheroids at the time of mean cell volume measurement. If improved, however, this method may be useful in studying effects of drugs on a variety of cellular and subcellular organelles.

ACKNOWLEDGMENTS

This work was supported by Grants ME-2875 and MA-2951 of the Medical Research Council of Canada.

The authors are grateful to Dr. L. Endrenyi for his assistance in programming for the IBM 7094.

REFERENCES

1 R. A. DEMEL AND L. L. M. VAN DEENEN, *Chem. Phys. Lipids*, 1 (1966) 68.
2 J. H. SCHULMAN, in G. H. BOURNE, *Cytology and Cell Physiology*, Clarendon Press, Oxford, 1951, p. 119.
3 J. T. DAVIES AND E. K. RIDEAL, *Interfacial Phenomena*, Academic Press, New York, 1961, p. 226.
4 J. C. SKOU, *Acta Pharmacol. Toxicol.*, 10 (1954) 325.
5 J. C. SKOU, *Biochim. Biophys. Acta*, 30 (1958) 625.
6 A. M. SHANES, *Pharmacol. Rev.*, 10 (1958) 59.
7 G. ZOGRAFI AND D. E. AUSLANDER, *J. Pharm. Sci.*, 54 (1965) 1313.
8 J. A. CLEMENTS AND K. M. WILSON, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1008.
9 J. A. CLEMENTS AND K. M. WILSON, *Intern. Anesthetics Clin.*, 1 (1963) 969.
10 A. W. CUTHBERT, *Pharmacol. Rev.*, 19 (1967) 59.
11 R. A. DEMEL, S. C. KINSKY AND L. L. M. VAN DEENEN, *J. Biol. Chem.*, 240 (1965) 2749.
12 L. L. M. VAN DEENEN AND R. A. DEMEL, *Biochim. Biophys. Acta*, 94 (1965) 314.
13 T. HANAI, D. A. HAYDON AND J. TAYLOR, *Proc. Roy. Soc. London, Ser. A*, 281 (1964) 377.
14 A. H. MADDY, C. HUANG AND T. E. THOMPSON, *Federation Proc.*, 25 (1966) 933.
15 P. MUELLER, D. O. RUDIN, H. TI TIEN AND W. C. WESTCOTT, *Nature*, 194, (1962) 979.
16 W. D. SEUFERT, *Nature*, 207 (1965) 174.
17 P. MUELLER AND D. O. RUDIN, *Nature*, 213 (1967) 603.
18 J. DEL CASTILLO, A. RODRIGUEZ, C. A. ROMERO AND V. SANCHEZ, *Science*, 153 (1966) 185.
19 P. MUELLER AND D. O. RUDIN, *Biochem. Biophys. Res. Commun.*, 26 (1967) 398.
20 S. C. KINSKY, S. A. LUSE AND L. L. M. VAN DEENEN, *Federation Proc.*, 25 (1966) 1503.
21 A. D. BANGHAM, M. M. STANDISH AND N. MILLER, *Nature*, 208 (1965) 1295.
22 A. D. BANGHAM, M. M. STANDISH AND G. WEISSMANN, *J. Mol. Biol.*, 13 (1965) 253.
23 A. M. SHANES, *Proc. 22nd Intern. Congr. Physiol.*, 1 (1962) 93.
24 J. C. SKOU, *J. Pharm. Pharmacol.*, 13 (1961) 204.
25 E. G. GRAY AND V. P. WHITTAKER, *J. Anat. London*, 96 (1962) 79.
26 E. DE ROBERTIS, P. A. DE IRALDI, G. R. DE L. ARNAIZ AND L. SALGANICOFF, *J. Neurochem.*, 9 (1962) 23.
27 V. P. WHITTAKER, I. A. MICHAELSON AND R. J. KIRKLAND, *Biochem. J.*, 90 (1964) 293.
28 R. M. MARCHBANKS, *Biochem. J.*, 104 (1967) 148.
29 D. M. MILLER AND C. L. HANNAY, *Can. J. Physiol. Pharmacol.*, 43 (1965) 675.
30 V. T. MARCHESI AND G. E. PALADE, *J. Cell Biol.*, 35 (1967) 385.
31 P. G. HATTERSLEY AND D. RAGUSA, *Am. J. Clin. Pathol.*, 37 (1967) 229.
32 P. SEEMAN, T. SAUKS, W. ARGENT AND W. O. KWANT, *Biochim. Biophys. Acta*, 183 (1969) 476.
33 P. SEEMAN, W. O. KWANT, T. SAUKS AND W. ARGENT, *Biochim. Biophys. Acta*, 183 (1969) 490.

34 H. KOEPPE, in E. PONDER, *Hemolysis and Related Phenomena*, Grune and Stratton, New York, 1948, p. 56.
35 L. J. BEILIN, G. J. KNIGHT, A. D. MUNRO-FAURE AND J. ANDERSON, *J. Gen. Physiol.*, 50 (1966) 61.
36 R. I. WEED AND A. J. BOWDLER, *J. Clin. Invest.*, 45 (1966) 1137.
37 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1767.
38 P. SEEMAN, *Intern. Rev. Neurobiol.*, 9 (1966) 145.
39 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1632.
40 P. SEEMAN AND J. WEINSTEIN, *Biochem. Pharmacol.*, 15 (1966) 1737.
41 E. HUTCHINSON, *Arch. Biochem.*, 38 (1952) 35.
42 J. W. GREEN, *J. Cellular Comp. Physiol.*, 33 (1949) 247.
43 J. VAN STEVENINCK, W. K. GJÖSUND AND H. L. BOOIJ, *Biochem. Pharmacol.*, 16 (1967) 837.
44 W. O. KWANT AND J. VAN STEVENINCK, *Biochem. Pharmacol.*, 17 (1968) 2215.
45 E. C. ANDERSON, D. F. PETERSEN AND R. A. TOBEY, *Biophys. J.*, 7 (1967) 975.
46 E. C. GREGG AND K. D. STEIDLEY, *Biophys. J.*, 5 (1965) 393.
47 R. I. WEED AND A. J. BOWDLER, *Blood*, 29 (1967) 297.
48 P. SEEMAN AND W. O. KWANT, *Biochim. Biophys. Acta*, BBA 75302.
49 L. J. MULLINS, *Chem. Rev.*, 54 (1954) 289.
50 D. F. SEARS AND E. L. FULLER, *Resp. Physiol.*, 5 (1968) 175.
51 P. SEEMAN, *Biochem. Pharmacol.*, 15 (1966) 1753.
52 H. SCHNEIDER, *Federation Proc.*, 27 (1968) 912.
53 D. BALASUBRAMANIAN AND D. B. WETLAUFER, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 762.
54 J. METCALFE, P. SEEMAN AND A. S. V. BURGEN, *Mol. Pharmacol.*, 4 (1968) 87.
55 J. B. FINEAN, R. COLEMAN, W. G. GREEN AND A. R. LIMBRICK, *J. Cell Sci.*, 1 (1966) 287.
56 C. D. MITCHELL AND D. J. HANAHAN, *Biochemistry*, 5 (1966) 51.
57 M. BARCLAY, R. K. BARCLAY, E. S. ESSNER, V. P. SKIPSKI AND O. TEREBUS-KEKISH, *Science*, 156 (1967) 665.
58 R. P. RAND AND A. C. BURTON, *Biophys. J.*, 4 (1964) 115.
59 R. P. RAND AND A. C. BURTON, *J. Cellular Comp. Physiol.*, 61 (1963) 245.
60 R. WHITTAM AND M. E. AGER, *Biochem. J.*, 93 (1964) 337.
61 R. F. FURCHGOTT AND E. PONDER, *J. Exptl. Biol.*, 17 (1940) 117.
62 A. KATCHALSKY, O. KEDEM, C. KLIBANSKY AND A. DE VRIES, in A. L. COPLEY AND G. STAINSBY, *Flow Properties of Blood and Other Biological Systems*, Pergamon Press, New York, 1960, p. 155.
63 P. SEEMAN, *J. Cell Biol.*, 32 (1967) 55.
64 R. F. BAKER, *Federation Proc.*, 26 (1967) 1785.
65 J. F. HOFFMAN, M. EDEN, J. S. BARR, JR., AND R. H. S. BEDELL, *J. Cellular Comp. Physiol.*, 51 (1958) 405.
66 G. M. GUEST AND M. WING, *J. Clin. Invest.*, 21 (1942) 257.
67 P. B. CANHAM AND A. C. BURTON, *Circulation Res.*, 22 (1968) 405.
68 M. P. WESTERMAN, L. E. PIERCE AND W. N. JENSEN, *J. Lab. Clin. Med.*, 57 (1961) 819.
69 D. N. HOUCHIN, J. I. MUNN AND B. J. PARNELL, *Blood*, 13 (1958) 1185.
70 R. P. RAND, *Federation Proc.*, 26 (1967) 1780.
71 E. PONDER, *Quart. J. Exptl. Physiol.*, 14 (1924) 37.
72 R. C. WEAST, *Handbook of Chemistry and Physics*, The Chemical Rubber Co., Cleveland, 1967, p. A258.