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ANESTHETICS EXPAND ERYTHROCYTE MEMBRANES WITHOUT CAUSING LOSS OF K^+

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SUMMARY

1. The volumes of erythrocyte ghosts, spherical in shape, were monitored in an electronic cell detector having a 100-channel pulse-height analyzer. Anesthetics which were either neutral (alcohols) or positively or negatively charged (phenothiazines and barbiturates) all increased the volumes of the spherical ghosts. At concentrations of these compounds which cause local anesthesia as well as antihemolysis, the anesthetics expanded the membrane area by 2–6 %. This was seen for the entire population of different sized ghosts.

2. At concentrations which were pharmacologically anesthetic and anti-hemolytic, all three classes of anesthetics had no effect on the pre-lytic leakage of K^+ from the cell. This excluded pre-lytic leakage of K^+ as a possible explanation of the anti-hemolytic effect of anesthetics. High and sublytic concentrations of the anesthetics, however, greatly increased the pre-lytic leakage of K^+ from the normal value of 18 % to over 50 %.

INTRODUCTION

Lipid-soluble anesthetics protect human erythrocytes from osmotic hemolysis[1,2]. This protection can be explained by the fact that anesthetics expand the membrane area of the erythrocyte[3–6], such that the cell can then hemolyse at a higher critical hemolytic volume, V_c. It was necessary to examine whether membrane expansion occurred for cells of different sizes.

It is known that approximately 10–15 % of the intracellular K^+ leaks prelytically from the erythrocyte in hypotonic solution[7–9] before the cell bursts[10] and loses hemoglobin. When hypotonic hemolysis is carried out slowly, however, the prelytic leak of K^+ increases to over 20 %, and hemoglobin release occurs at a lower salt concentration[7,11]. A rapid leakage of potassium also occurs in glucose or sucrose solutions and this leads to a reduced osmotic fragility of the cells[12]. Since it is known that sublytic concentrations of various drugs also cause prelytic leakage of K^+ (ref. 13), there was the possibility that anesthetics lowered the osmotic fragility of the erythrocyte by increasing this prelytic leakage. The present experiments indicate that anesthetics expand and protect erythrocyte membranes without causing a prelytic loss of K^+.

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METHODS

Preparation of the stock suspension of erythrocytes

A sample of fresh venous human blood (pre-prandial) was heparinized (50 I.U./ml), and centrifuged and washed 3 times with 0.9 % NaCl in 15 mM Tris–HCl buffer (pH 7) to remove the plasma and the buffy coat. The cells were finally resuspended in the washing solution at about $1.8 \cdot 10^6$ cells per ml.

Multi-channel analysis of erythrocyte ghost volumes

An aliquot of 0.5 ml of the suspension of intact erythrocytes was added to 10 ml of dilute drug solution made up in 0.3 % NaCl in 15 mM Tris–HCl, pH 7, and the tubes (Falcon sterile disposable and dust-free plastic tubes, 17 mm × 100 mm) were immediately mixed vigorously with a vortex mixer. The solutions had all been previously filtered through a millipore filter (0.45 μm diameter pores) in order to remove all dust. It is known that approximately 2 h after this step of osmotic hemolysis, the erythrocyte ghosts become spherical and grossly sealed[6,10]; at this time, therefore, the ghosts were sized by a specially constructed electronic cell detector with a 100-channel pulse-height analyzer[14,15]. All steps were carried out at room temperature (22 ± 1°). The concentration of 0.3 % NaCl is the lowest that can be used in order to maintain electrical conductivity, yet still ensure that all erythrocytes hemolyse[6].

Erythrocyte protection by anesthetics; measurement of prelytic leakage of K^+

The effect of various anesthetics on the osmotic fragility of erythrocytes was tested using solutions of different osmolarity. The procedure was to put 1 ml of drug solution (drug dissolved in 0.9 % NaCl in 15 mM Tris–HCl buffer, pH 7) into a 12 mm × 75 mm test tube, and then add 200 μl of the washed erythrocyte suspension (6 % hematocrit). After mixing and leaving the tube for 5 min at room temperature, a final aliquot of 1.5 ml of drug solution was rapidly injected into the test tube; this final aliquot contained drug at the same concentration as existed in the 5-min incubation step, but contained different concentrations of NaCl ranging from 0.9 down to 0.3 % NaCl in 15 mM Tris–HCl buffer, pH 7. After remaining at room temperature for a further 10 min, the tubes were centrifuged at 1500 × g for 2 min, and the supernatants were immediately measured for the amount of hemoglobin and K^+ released from the cells. Hemoglobin was determined from the absorbance of the solution at 543 nm in a Zeiss spectrophotometer. The concentration of potassium in the supernatant was determined by flame photometry using an EEL (England) flame photometer.

RESULTS

Uniform expansion of ghosts by positive, neutral and negative anesthetics

The spherical erythrocyte ghosts, as monitored by the electronic cell volume detector, consisted of a bell-shaped distribution of cell volumes which was symmetrically shifted up-channel in the presence of positive, neutral or negative anesthetics, as shown in Fig. 1.

The volume mode of the control cells was at about channel number 31, corresponding to approximately 145 μm^3 for volume, and to about 134 μm^2 (*cf.* refs. 6, 8, 16). Chlorpromazine at $2 \cdot 10^{-5}$ M symmetrically shifted the population of ghosts by about

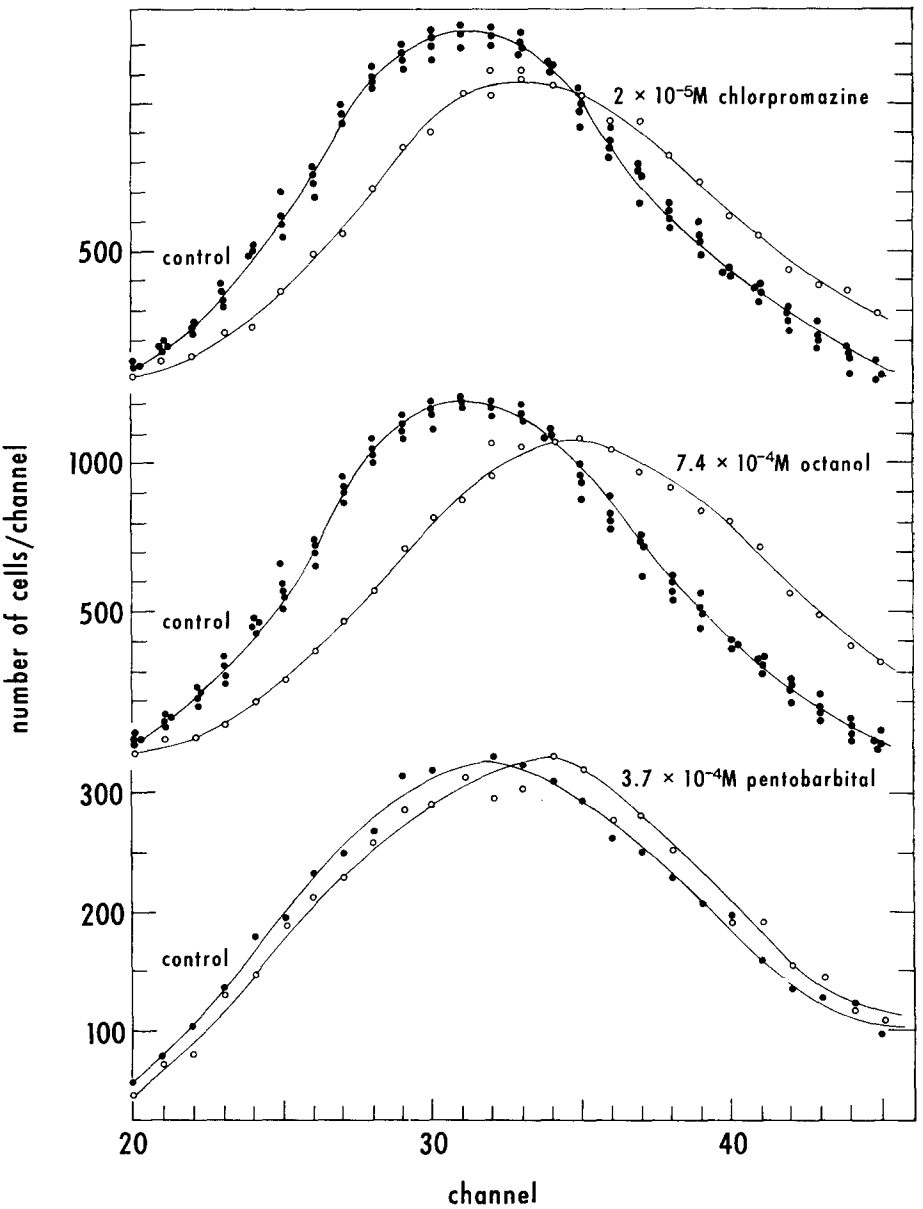

Fig. 1. Expansion of erythrocyte ghost membranes by positive (chlorpromazine), neutral (octanol) and negative (pentobarbital) anesthetics. The anesthetics were added to fresh erythrocyte ghosts and, after allowing time for the cells to re-sphere, the ghost volumes were monitored by an electronic cell detector connected to a 100-channel pulse-height analyzer. For the ghosts without drug the volume-mode was at about channel 31 which correspond to approximately 145 μm^3 (volume) and 134 μm^2 (area). In the presence of the drugs the entire population of cell volumes shifted up-channel, indicating membrane area expansions of from 2–6%.

2 channels, the volume mode increasing to approximately 155 μm^3 (area = 140 μm^2). The chlorpromazine-induced membrane area expansion thus amounts to about 4.5 % at $2 \cdot 10^{-5}$ M in agreement with earlier work where different techniques were used[6].

Similar calculations for the effect of $7.4 \cdot 10^{-4}$ M octanol in Fig. 1 indicate that the membrane area expansion was of the order of 6 %. The local anesthetic concentration for octanol is lower than that used in Fig. 1 (around $2 \cdot 10^{-4}$ M (ref. 17)), and it is known that the cell membranes expand by about 2–3 % at local anesthetic concentrations[6]. At the bottom of Fig. 1, data for $3.7 \cdot 10^{-4}$ M sodium pentobarbital are presented, where the membrane area expansion amounted to 1.9 %; the local anesthetic concentration for sodium pentobarbital is at about $3.4 \cdot 10^{-4}$ M (unpublished results).

In general, the up-channel shift was proportional to the anesthetic concentration. This is shown in Fig. 2, where the membrane area expansion for 55 mM butanol was about 3 % and that for 110 mM butanol was about 5 %. This multi-channel analyzer technique, however, is not convenient for precise dose-response curves, since the over-all accuracy and speed is less than the mean-cell-volume technique[6]; the principal advantage of the present method is to demonstrate symmetrical expansion of the different-sized cells.

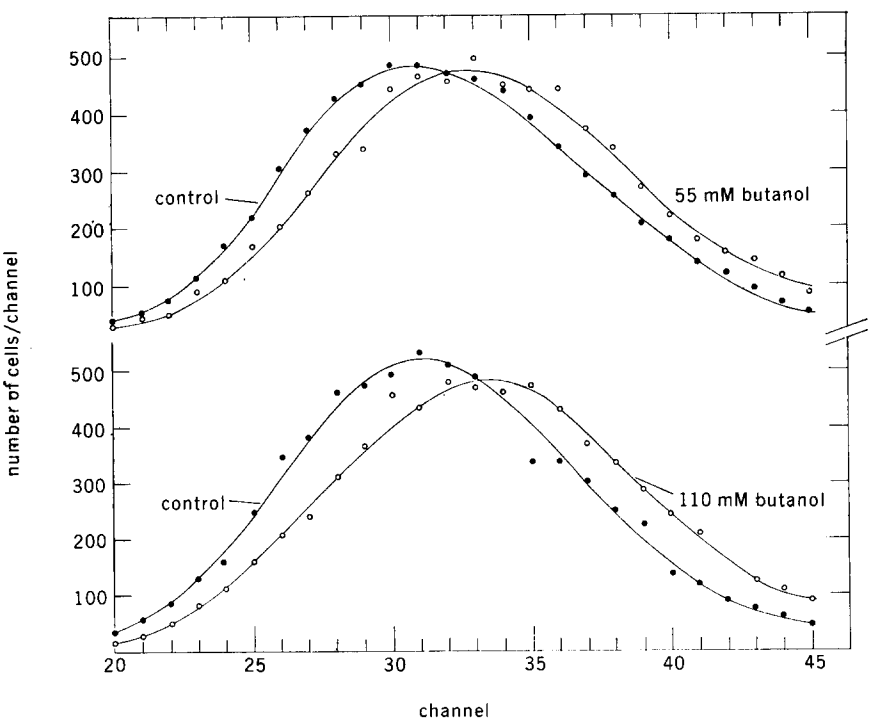

Fig. 2. Showing that the up-channel shift in ghost volumes was proportional to the concentration of the anesthetic. The membrane area expansion for 55 and 110 mM butanol was about 3 and 5%, respectively (Procedure same as in Fig. 1).

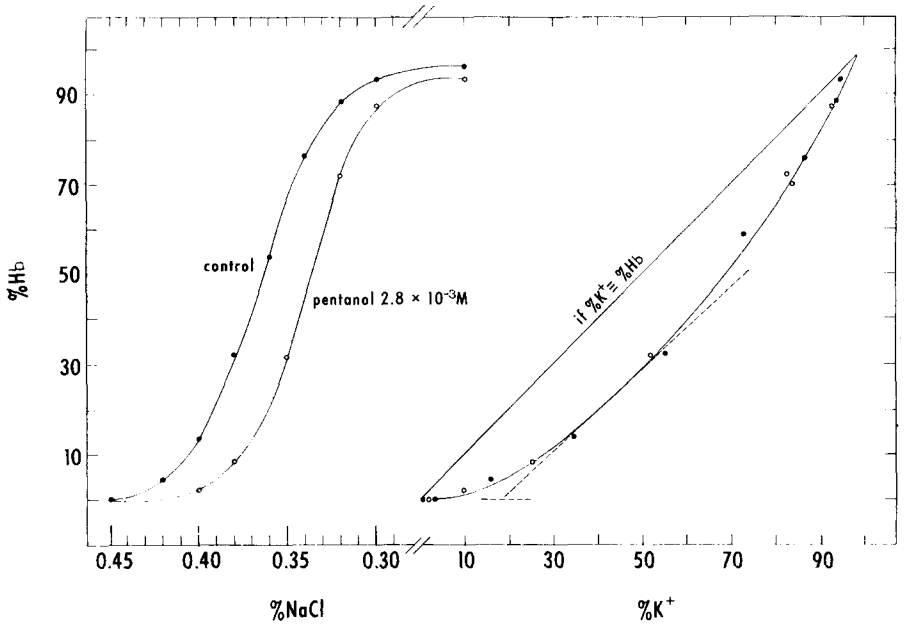

Fig. 3. In the presence of 2.8 mM pentanol which reduced the osmotic fragility of human erythrocytes by 0.03% NaCl (left-hand side of figure), there was no effect on the pre-lytic leakage of K^+ from the cells (right-hand side of figure). The open circles represent values for pentanol, the closed ones for no drug. The extrapolated pre-lytic leakage of K^+ at 0% hemoglobin release (%Hb) was about 18% in the presence or absence of the anesthetic.

Erythrocyte protection by anesthetics; negligible role of prelytic K^+ leak

At concentrations which are pharmacologically anesthetic, the drugs did not increase the prelytic leak of K^+. This is shown in Fig. 3 (right side) for a neutral anesthetic (pentanol) and in Fig. 4 for a negative anesthetic (sodium pentobarbital). The absence of prelytic K^+ leak in the presence of a positive anesthetic (chlorpromazine) has already been published by KWANT AND VAN STEVENINCK[18]. The amount of prelytic K^+ leakage can be derived from a graph of the % hemoglobin released *versus* the % K^+ released, as outlined by KWANT AND VAN STEVENINCK[18] and SEEMAN *et al.*[7]. The straight line (at 45°) connecting 0 % release to 100 % release (in both Figs. 3 and 4) is the relation expected for a parallel release of hemoglobin and K^+ during hemolysis. It has been shown by many other workers (see ref. 18 for further references), however, that there is always a prelytic release of K^+ in hypotonic media. The % K^+ lost, therefore, will be disproportionately more than the % hemoglobin lost. Drawing a tangent to the curve of % K^+ lost–hemoglobin lost and extrapolating to 0 % hemoglobin release, it can be seen that the amount of prelytic leakage of K^+ was about 18 % in these experiments. Although the anesthetics did not alter the prelytic K^+ leakage, they reduced the osmotic fragility of the erythrocytes, as shown in Fig. 3 for 2.8 mM pentanol.

Although no prelytic K^+ leakage occurred at drug concentrations in the anesthetic region, it was observed that very high drug concentrations which were just sublytic greatly increased the prelytic K^+ leakage. This is shown in Fig. 5 for butanol. Butanol causes local anesthesia at approximately 60 mM (ref. 19) and protects 50 % of an ery-

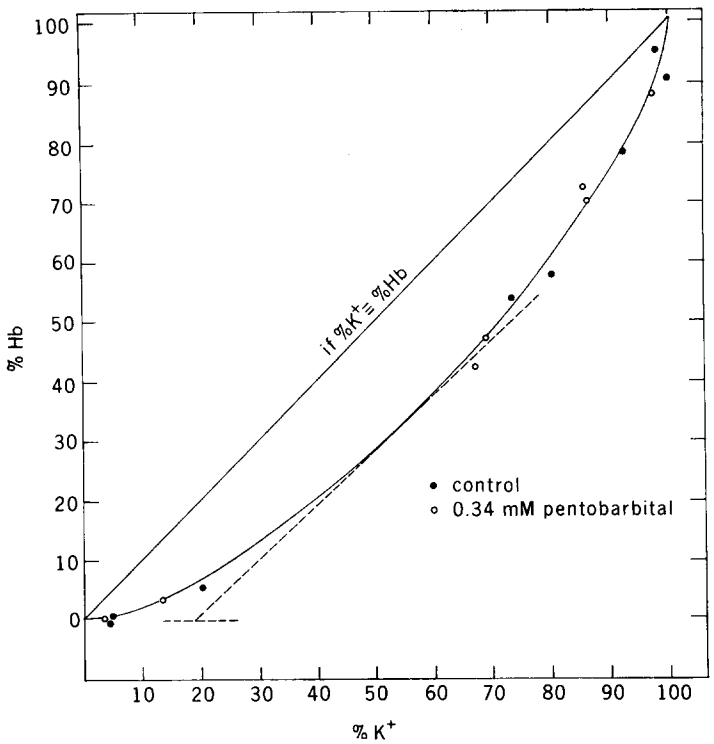

Fig. 4. Demonstrating that the negatively charged anesthetic, sodium pentobarbital, has no effect on the pre-lytic leakage of K^+ at concentrations ($3.4 \cdot 10^{-4}$ M) which cause anti-hemolysis and local anesthesia. At 0% hemoglobin release (%Hb) there was about 18% K^+ release whether or not the anesthetic was present.

throcyte population at about 42 mM (unpublished results). As shown in Fig. 5, much higher butanol concentrations, up to 130 or 350 mM, did not increase the prelytic leakage of K^+. At the sublytic concentration of 400 mM, however, it can be seen (bottom of Fig. 5) that butanol produced a large prelytic K^+ leakage of 40–60 %, depending on the length of the pre-incubation period (normally 5 min, see METHODS).

The data presented in Fig. 5 reveal that the most sensitive part of the % K^+– % Hb curve which indicates whether or not a prelytic leakage of K^+ has occurred is at the lower levels of hemolysis, between 0 and 10 %. Since it was possible that a very small prelytic leakage of K^+ could occur in this region, experiments were done where many points were taken between 0 and 10 % hemolysis. This is shown in Fig. 6 for a 2.8 mM pentanol and 3.5 mM heptanol. No increased prelytic leakage of K^+ occurred. In fact, it can be seen in Fig. 6 that in this particular experiment the amount of prelytic K^+ leakage was somewhat reduced by 3.5 mM heptanol.

DISCUSSION

The results indicate that the spherical volumes of the entire population of different-sized ghosts were all increased by positive, neutral and negative anesthetics. The drug concentrations at which the ghost area expansion occurred did not have any

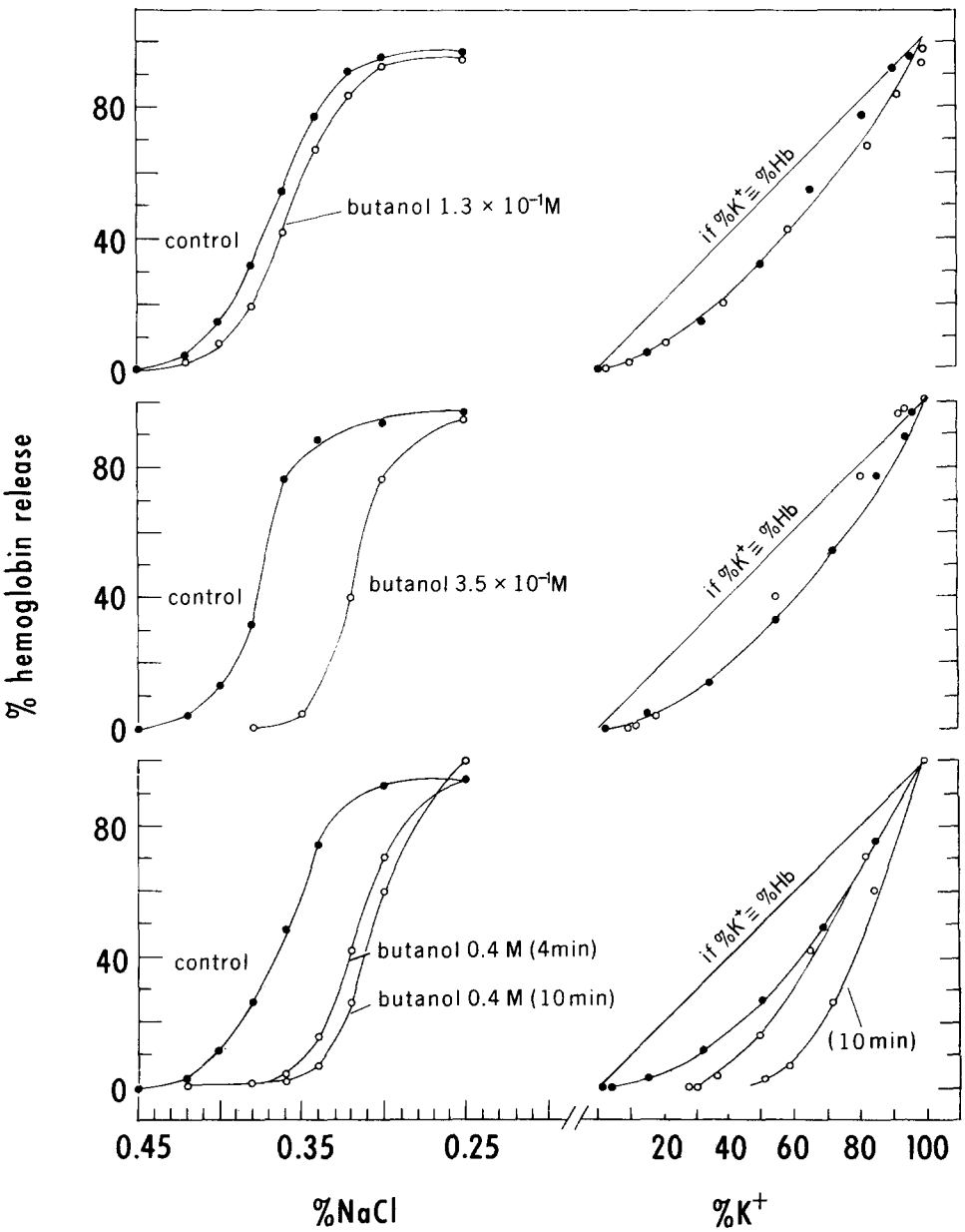

Fig. 5. Showing the distinction between the concentration range for anti-hemolysis and that for pre-lysis. The top and middle graphs indicate that butanol in the anti-hemolytic range of 0.35 M or less lowers the osmotic fragility of erythrocytes but has no effect on the pre-lytic leakage of K^+, as revealed by identical % K^+–% Hb curves in the presence and absence of anesthetic. The pre-lytic range, however, occurs at butanol concentrations higher than 0.35 M, as shown in the bottom graphs where 0.4 M butanol not only shifted the osmotic fragility but also greatly increased the pre-lytic leakage of K^+ to around 50% K^+ loss or more at low values for hemoglobin release.

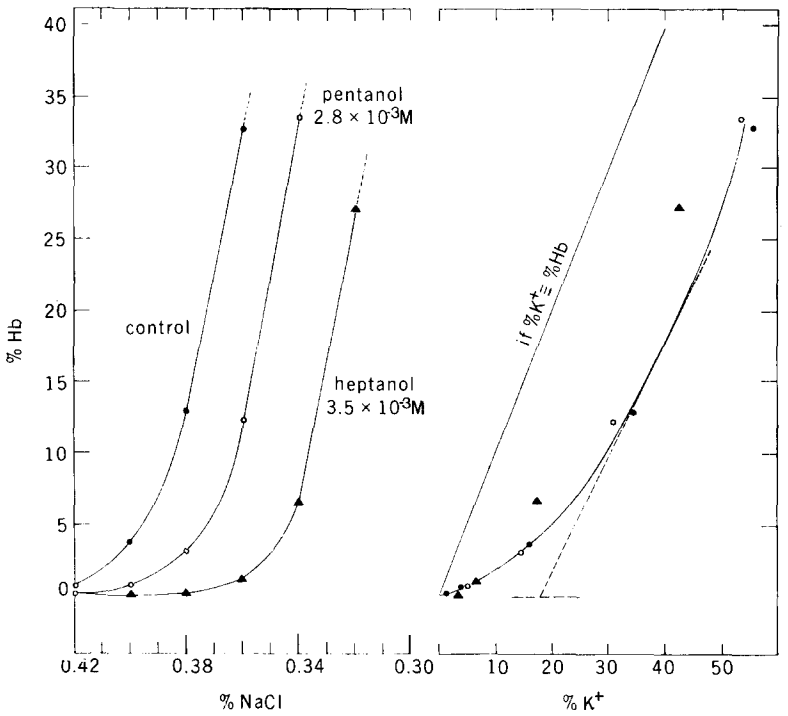

Fig. 6. A detailed study of the action of pentanol and heptanol at very low levels of osmotic hemolysis. At concentrations which were effective in reducing hypotonic hemolysis the two alcohols did not alter the pre-lytic leakage of K^+ from the cells; the tangent to the % K^+–% Hb release curve extrapolated to 18% in both the absence and presence of the anesthetics.

effect on the prelytic leakage of K^+ from intact erythrocytes. Subhemolytic drug concentrations increased the prelytic K^+ leakage.

The anti-hemolytic and membrane-expanding properties of anesthetics have been discussed previously[6,7,19–22]. The purpose of the present experiments was to examine whether the different-sized cells all became expanded by the anesthetics. While it is true that all cells expand, it is not yet clear whether all the different-sized cells expand precisely to the same extent on an absolute or relative basis. This uncertainty stems from the fact that the volume-distributions before and after the drug are not exactly parallel (see Fig. 1). A second difficulty is that it is impossible to be sure that the electronic cell detector is correctly calibrated on an absolute basis. A third point is that a shift upward of two channel sizes for a small cell in channel number 24, for example, represents an increase in surface area of about 3.5 %; a two-channel shift for a cell in channel number 39, however, only represents a membrane area expansion of 3.2 %, and it is not known whether the accuracy of the present electronic system is adequate to state that the 0.3 % difference is significant.

The fact that butanol did not increase the prelytic K^+ leakage until the concentration exceeded 0.35 M agrees with the results of PARPART AND GREEN[22] who worked with rabbit erythrocytes.

It is interesting to note that the negative anesthetics, such as sodium pentobarbital, expanded the membranes to approximately the same extent as neutral and

positive anesthetics (see RESULTS), particularly since it is known that in isotonic solution the negative molecules crenate intact erythrocytes while the neutral and positive molecules induce cup-shaped erythrocytes[23].

One of the mechanisms of drug-induced membrane expansion might be that the anesthetic inhibits a contractile membrane-associated ATPase. It has recently been shown, however, by LANDMARK AND ØYE[24] that the phenothiazine local anesthetics have anti-hemolytic effects at concentrations which only slightly inhibit the membrane's (Na^+–K^+)-ATPase.

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