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THE EFFECT OF ANESTHETICS AND HEAT TREATMENT ON DEFORMABILITY AND OSMOTIC FRAGILITY OF RED BLOOD CELLS

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Abstract—The effect of a number of anesthetics on osmotic fragility and deformability of human red blood cells was studied. These anesthetics all protected erythrocytes against osmotic lysis. An indication of separate mechanisms of action of these anesthetics emerged from the effects on cell deformability. Chlorpromazine and the lower alcohols caused a clear decrease of cell deformability, whereas alcohols with a chain-length exceeding C = 4 had no effect on deformability. This indicates that these two effects are not interrelated.

This conclusion was supported by the observation that heating of the cells to 48.8° for 4 min antagonized the effects of anesthetics on deformability, without modifying the protection against osmotic hemolysis.

Heating of the cells to 48.8° caused an irreversible modification of some membrane proteins, in particular spectrin and bands 7 and 8 protein. These proteins exhibited an irreversible loss of extractability at low ionic strength. Thus it seems likely that the influence of anesthetics on cell deformability is mediated via a direct or indirect effect on one or more of the heat-affected proteins, whereas the protection against osmotic hemolysis is caused by a different drug–membrane interaction.

A wide variety of chemically unrelated compounds, including both neutral and negatively and positively charged molecules, can act as anesthetics. Despite many studies on the molecular basis of anesthesia it is not yet clear whether there is only one single mechanism of anesthesia, or whether different classes of anesthetics act in different ways. Earlier studies of Seeman *et al.* seemed to indicate that there are no separate mechanisms of action of different classes of anesthetics [1]. This conclusion was reached a.o. from the mutual additive effects of various drugs. More recent studies of the same group revealed potentiation of some anesthetics by others, providing support for the possibility that different anesthetics may have different sites of membrane action [2, 3].

Anesthetics have been shown to produce many different effects on biological membranes, including Ca^{2+} displacement [4, 5], protection of red blood cells against osmotic hemolysis [1, 6], inhibition of cell fusion [7], changes in membrane fluidity [8], increased susceptibility to agglutination by plant lectins [9] and decreased deformability of erythrocytes [10]. Apart from the question of one single or different mechanisms of action of different anesthetics, a second problem of major significance for the elucidation of the process of anesthesia is whether these various effects are interrelated and caused by one specific interaction of the drug molecule with the biological membrane, or by interactions with different membrane constituents.

In this context the effects of several anesthetics on osmotic fragility and on red cell deformability were studied and compared to the effect of heat treatment of the cells. Such heat treatment has been reported to change the visco-elastic properties of the membrane [11, 12]. The results of this study are discussed in the present communication.

METHODS

Suspensions of washed red blood cells in buffered isotonic NaCl solutions were prepared as described previously [13]. Heating of red blood cells was done by submersion of glass tubes, containing a 40 per cent cell suspension, in a constant temperature water bath. The cell suspension was kept at the desired temperature during 4 minutes.

Osmotic fragility of the cells and protection against osmotic lysis by anesthetics was measured at a cell concentration of 2 per cent as described before [14].

Red cell deformability was studied by viscosity measurements on 88 per cent cell suspensions with a Brookfield LVT cone-plate viscometer, as described by Weed *et al.* [15]. Necessary pre-incubations were performed with 2 per cent cell suspensions, with subsequent concentration to a cell density of 88 per cent via centrifugation. As shown by Burton, the viscosity of an 88 per cent cell suspension is a direct parameter of cell deformability [16].

Ghosts were prepared according to Weed *et al.*, with several successive steps of gradual osmotic lysis [17]. In some experiments (see Results section) this procedure was preceded by one step of lysis via freezing in liquid nitrogen and thawing.

Spectrin elution from ghosts was performed as described by Bennett and Branton [18]. For electrophoretic analysis of membrane proteins, ghosts were dissolved in a solution containing 10 mM Tris, pH 8.0, 1 mM EDTA, 40 mM dithiothreitol, 7% sucrose and 1% sodium dodecyl sulphate at a protein

concentration of about 3 mg/ml. Polyacrylamide gel electrophoresis was performed as described by Fairbanks *et al.* [19]. Densitometric scans of Coomassie-blue stained gels were recorded on a Zeiss PMQ II spectrophotometer with scanning device. The obtained protein bands were numbered according to Steck [20].

RESULTS

The well-known protection of red blood cells against osmotic hemolysis by anesthetics is illustrated in Fig. 1. For all drugs used in the present study the concentration at which hemolysis is reduced to 50 per cent of the initial value ($C_{50\%}$) and the concentration for optimal protection (C_{opt}) was measured. The drug concentrations used in all further experiments were in the range $C_{50\%}$–C_{opt}.

The influence of a series of anesthetics on red blood cell deformability was measured by the red cell viscosity technique. Figure 2 shows the results with methanol and chlorpromazine at $C_{50\%}$. It appeared

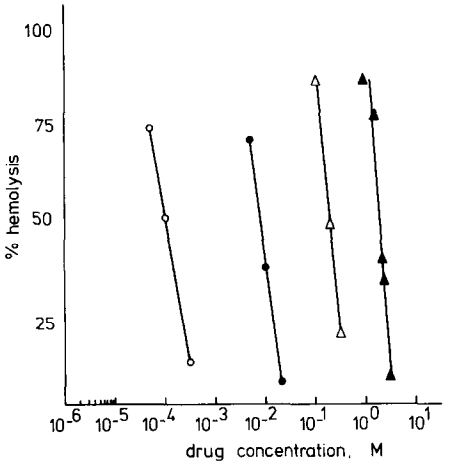

Fig. 1. Protection against osmotic hemolysis by anesthetics at 0.38%. Hemolysis in the abscence of drug was 70–80%. O—O chlorpromazine; ●—●, hexanol; △—△, butanol; ▲—▲, ethanol.

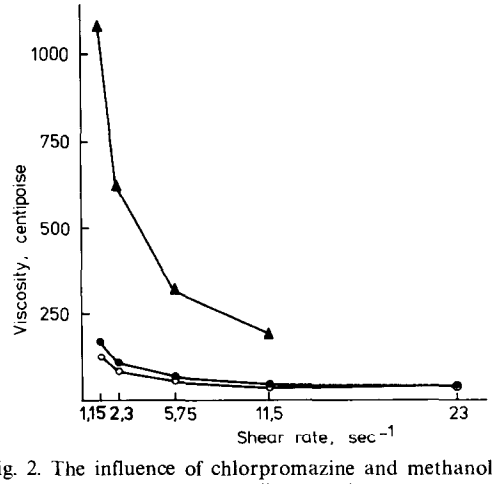

Fig. 2. The influence of chlorpromazine and methanol on the viscosity of an 88% red cell suspension. O—O, control; ●—●, 10^{-4} M chlorpromazine; ▲—▲, 2.7 M methanol.

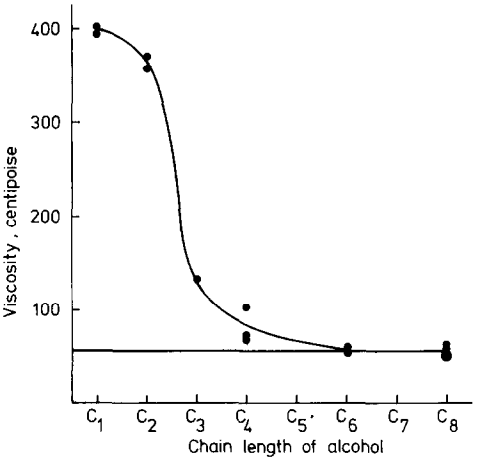

Fig. 3. The viscosity of an 88% red cell suspension measured at a shear rate of 5.75 s^{-1} in the presence of alcohols with different chain length at $C_{50\%}$. The straight line at about 50 cp is the viscosity as measured in the absence of anesthetics.

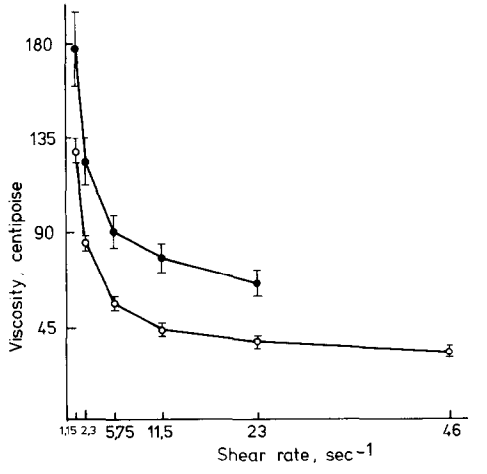

Fig. 4. The effect of heat-treatment (4 min at 48.8°) on the viscosity of an 88% red cell suspension. O—O, untreated cells; ●—●, heat-treated cells. The bars represent the SEM (n = 20 for the untreated cells and n = 14 for the heat-treated cells).

that the influence of alcohols on red cell deformability decreases, with increasing chain-length. In Fig. 3 the viscosity at a shear rate of 5.75 s^{-1} is plotted against the chain-length of a series of alcohols in the concentration range $C_{50\%}$–C_{opt}. At a chain-length higher than C = 4 the alcohols have no measurable effect on red cell deformability.

The drugs had similar although less pronounced effects on the deformability of red cell ghosts. Here again the influence of alcohols decreased with increasing chain-length.

Heating of intact red blood cells for 4 min at 48.8° causes a decrease of cell deformability (Fig. 4). This effect appeared to be irreversible: even 24 hr after heating of the cells the deformability was decreased as compared to untreated control cells. Addition of

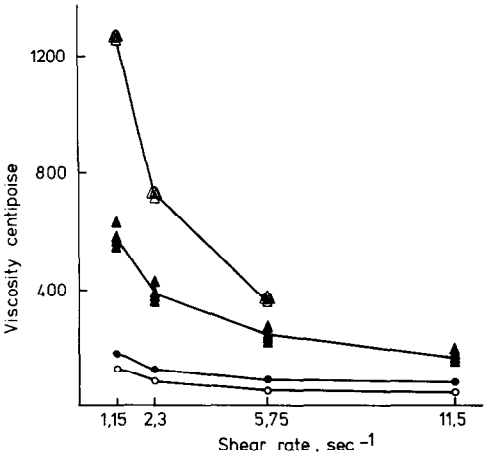

Fig. 5. The effect of 2.2 M ethanol on the deformability of normal and heat-treated red blood cells. O—O, untreated cells, control; ●—●, heat-treated cells; △—△, untreated cells in the presence of 2.2 M ethanol; ▲—▲, heat treated cells in the presence of 2.2 M ethanol.

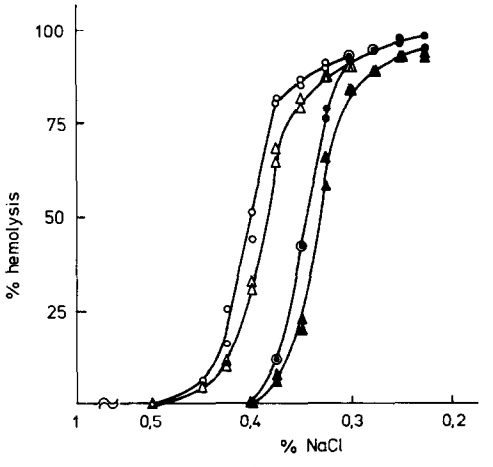

Fig. 6. Osmotic hemolysis as influenced by heat-treatment (4 min at 48.8°) and drug addition. O—O, untreated cells; ●—●, untreated cells in the presence of 2.2 M ethanol; △—△, heat-treated cells; ▲—▲, heat-treated cells in the presence of 2.2 M ethanol.

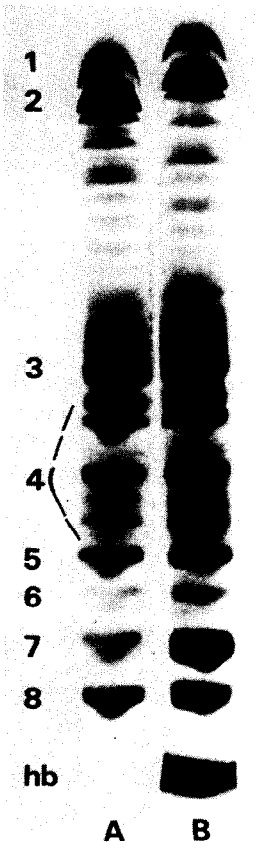

Fig. 7. SDS polyacrylamide gel electrophoresis of membrane proteins of untreated red blood cells (A) and of heat-treated red blood cells (B). The protein bands are numbered according to Steck.

chlorpromazine or one of the lower alcohols to pre-heated cells caused a further decrease of deformability, but the total effect was always much less than the effect of the anesthetic on normal cells (Fig. 5). In contrast, heating of ghosts had no effect on the deformability of the ghosts, nor did it modify the influence of anesthetics on deformability.

Heating of intact cells had no or only a very slight influence on the osmotic fragility of the cells and did not affect the protection of anesthetics against osmotic hemolysis (Fig. 6).

Further experiments were designed to clarify the effect of heating on erythrocytes. Ghosts, prepared from normal cells and from pre-heated cells were solubilized in SDS and subjected to polyacrylamide gel electrophoresis. The results are shown in Fig. 7. The most prominent effects of heating are increased retention of hemoglobin and a pronounced increase of the bands 7 and 8. Less pronounced but usually clearly visible are increases of some protein bands below the spectrin fraction and between bands 4.2 and 5. These effects are irreversible in the sense that no differences were noted when ghosts preparation was performed 24 hr after heating instead of immediately after heating.

It is known that when gels are overloaded with hemoglobin, dimers and tetramers of this protein appear in the electropherogram. The observed increases in the band 7 and band 8 regions cannot be attributed to hemoglobin polymers, however. Scan patterns at 400 nm of the gels, containing the proteins of ghosts obtained from heated cells, showed a peak at the hemoglobin position, but not at the bands 7 and 8 positions. Further, electropherograms of the ghosts after each successive step of the ghost preparation showed a progressive decrease of the hemoglobin band, with no concomitant decrease in the band 7 and band 8 region (Fig. 8).

Identical changes in the electropherogram were observed when packed cells, lysed by freezing in liquid nitrogen and thawing, were heated. In some experiments the membrane fraction was separated

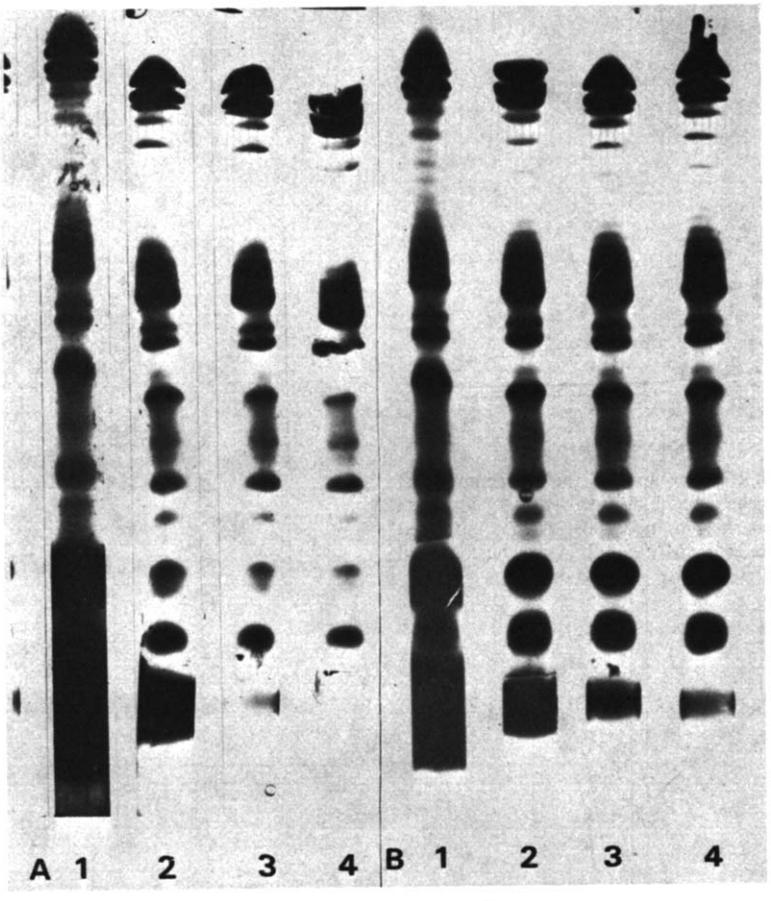

Fig. 8. SDS polyacrylamide gel electrophoresis of ghost proteins after the successive steps of ghost preparation. A 1–4; untreated cells; B 1–4: heat-treated cells.

from the hemolysate after freezing and thawing and converted to white ghosts by the usual procedure. If these ghosts were resuspended in the original hemolysate and heated, the same typical changes in the electropherogram could be observed. The effect was not inhibited by prior dialysis of the hemolysate against 30 mM KCl or by 30-fold dilution of the hemolysate. Upon further dilution the effect decreased gradually. No deviations from the normal pattern of the electropherograms were found, when normal ghosts were heated to 48.8° in hypotonic or isotonic buffered NaCl or KCl-solutions.

Spectrin can be eluted from normal ghosts in hypotonic solutions. Utilizing the extraction procedure of Bennett and Branton [18] the amount of spectrin eluted from normal ghosts increased, with decreasing salt concentration. Under identical experimental conditions spectrin was not eluted from ghosts, prepared from pre-heated red blood cells (Fig. 9). Band 7 protein from ghosts, prepared from pre-heated cells was also not extractable in hypotonic solutions, whereas band 8 protein appeared to be 50 per cent extractable. If ghosts prepared from normal cells were heated to 48.8° the spectrin was eluted with the same efficiency as from untreated ghosts.

The effect of pre-heating of intact cells on the extractability of spectrin from the corresponding ghosts has a very strict temperature dependence, as shown in Fig. 10. Pre-heating for 4 min up to 47° had

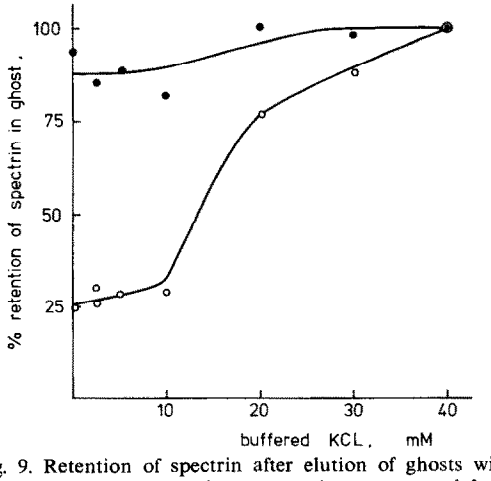

Fig. 9. Retention of spectrin after elution of ghosts with buffers of different molarity. ○—○, ghosts, prepared from untreated cells; ●—●, ghosts; prepared from heat-treated cells.

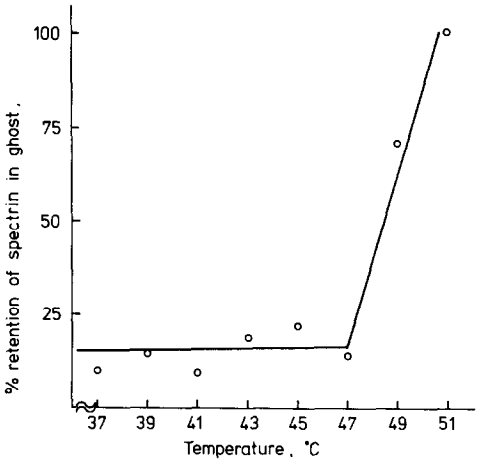

Fig. 10. The effect of heat-treatment of intact cells (4 min, at 37–51°) on the retention of spectrin in ghosts, after elution with 2.5 mM buffered KCl.

no effect, whereas pre-heating to 48.8° resulted in complete inhibition of spectrin elution.

If ghosts were prepared from normal red blood cells with one of the anesthetics present during the whole procedure, no deviations from the normal electropherogram band pattern were provoked. Similarly, the presence of one of the anesthetics did not influence the extractability of spectrin from normal ghosts.

DISCUSSION

Elevated temperatures affect the visco-elastic properties of the red cell membrane [11, 12, 21]. Rakow and Hochmuth showed that heating of red blood cells at 48.8° during a few minutes causes an irreversible decrease of elasticity of human red cell membranes, as measured with a parallel plate flow channel [11, 12]. The decreased cell deformability after heating described in this paper is in accordance with these observations. The irreversibility of these effects indicates a denaturating process in membrane proteins. Irreversible thermal membrane transitions at similar temperatures have been described utilizing scanning calorimetry and circular dichroism [22, 23]. The involvement of spectrin in a thermal transition centered at 49.5° was demonstrated by Brandts *et al.* [22].

The present study confirms the irreversible thermal transition in membrane proteins with a quite different technique. As shown, several proteins are affected. The most prominent changes concern band 7 and band 8 protein and spectrin (bands 1 and 2). The loss of extractability of spectrin upon heating to 48.8° is probably related to the thermal transition of spectrin around this temperature described by Brandts *et al.* [22]. This cannot be a simple transition in which only spectrin is involved, however. As shown, loss of extractability only occurs when intact cells, or ghosts in the presence of hemolysate are heated, but not when white ghosts in buffered salt solutions are similarly treated. It is tempting to speculate that band 7 and band 8 protein may be involved in the process. Not much is known about bands 7 and 8 proteins. Studies on ghosts prepared in isotonic medium by dielectric breakdown indicate that these proteins are real membrane proteins, partially eluted at low ionic strength during the usual ghost preparation procedures [24]. The possible interrelationship, between loss of extractability of these proteins and the loss of extractability of spectrin at still lower ionic strength caused by heat-treatment, will be the subject for further investigations.

It seems highly probable that there is a causal relationship between the heat-induced irreversible changes in these proteins and the observed decreased deformability. In this context it is important to stress that several independent lines of evidence indicate that spectrin is an important determinant of red blood cell deformability [25–27]. The fact that heating of red blood cells prior to addition of e.g. ethanol causes a partial block of the effect of the anesthetic on cell deformability (Fig. 5) indicates that this effect of anesthetics is achieved via a direct or indirect effect of the drug on one or more of the proteins, involved in the high temperature effect.

On the other hand, heating of the cells had no or only a slight influence on the osmotic fragility of the cells and did not modify the drug-induced protection against osmotic lysis (Fig. 6). This probably indicates that these two effects, protection against osmotic lysis and decreased cell deformability, are not directly interrelated and caused by different drug–membrane interactions. That drug-induced decreased cell deformability is actually a membrane effect is shown by the fact that effective drugs have similar effects on ghosts and on intact cells.

That the effects of anesthetics on osmotic fragility and on cell deformability are caused by different interactions is supported by another experimental observation. The utilized alcohols share the protective effect against osmotic hemolysis. The effect on cell deformability decreases, however, with increasing chain-length and has vanished at a chain-length of $C = 4$ and higher.

In a preceding paper it was shown that the effect on K^+ leakage from red blood cells was different for different anesthetics, indicating possible separate mechanisms of action of these drugs [28]. The results presented in this paper demonstrate a similar status with respect to the effect on cell deformability. These facts argue against the possibility of a uniform mode of action of the many different groups of anesthetics with regard to their various effects on biomembranes.

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