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Hypertonic Cryohemolysis of Human Red Blood Cells

T.M.A.R. Dubbelman, A.W. de Bruijne, K. Christianse, and J. van Steveninck

Sylvius Laboratories, Laboratory for Medical Chemistry, Wassenaarseweg 72, Leiden, The Netherlands

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Summary. Hypertonic cryohemolysis of human erythrocytes is caused by incubation of the cells in hypertonic medium at a temperature of 20–50 °C (stage 1), with subsequent cooling to 0 °C (stage 2). In 0.86 M sucrose hemolysis increases, with increasing stage 1 temperature, whereas in 1 M NaCl cryohemolysis has a temperature optimum at a stage 1 temperature of about 30 °C.

Cryohemolysis is inhibited by preceding ATP depletion of the cells and by preincubation of the cells in hypertonic medium at 0 °C. In general, anesthetics inhibit cryohemolysis strongly. Only in 1 M NaCl at stage 1 temperatures in the range of 40–50 °C is cryohemolysis stimulated by these drugs, if present during the entire incubation period. This effect is abolished, however, when the anesthetic is added after prior incubation of the cells at 40–50 °C in 1 M NaCl.

Ghost-bound ANS fluorescence indicates complicated conformation changes in the membrane structure during the various experimental stages leading to cryohemolysis.

Some of the experimental results can be considered as examples of molecular hysteresis, thus indicating several different metastable structures of the membrane, under various experimental conditions.

The described results support the working hypothesis of Green and Jung that the experimental procedure results in membrane protein damage, preventing normal adaptation of the membrane during cooling.

Cold-induced hemolysis in hypertonic medium has been described by many authors (Lovelock 1953, 1954, 1955, 1957; Morris, 1975; Meryman, 1974; Meryman, Williams & Douglas, 1977; Green & Jung, 1977; Jung & Green, 1978; Zade-Oppen, 1968). When erythrocytes are incubated in a hypertonic medium at a temperature above 20 °C with subsequent cooling to a temperature below 20 °C, hemolysis takes place.

The mechanism at the molecular level of this type of hemolysis is still unclear. It seems likely that both the volume change, induced by hypertonicity, and the temperature shift are involved in the process. Lovelock (1955) ascribed hemolysis by thermal shock to a mechanical failure of the cell membrane, leading to loss of phospholipids and choles-

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terol from the membrane. Morris (1975) found a correlation between loss of membrane lipids in hypertonic medium and the small amount of hemolysis before cooling, but no correlation between lipid loss and lysis by subsequent cooling. Green and Jung (1977) found no significant differences between cholesterol and phospholipid loss in hypertonic *vs.* isotonic media and thus no correlation between hemolysis by thermal shock and loss of membrane lipids.

The exact role of cell shrinkage in the process of thermal shock is also unclear. The volume reductions in 1 M NaCl and in 2 M NaCl are identical and maximal (about 52%), but cold-induced hemolysis is much more extensive in 1 M NaCl. Further, in hypertonic media of equal osmolality the degree of cold-induced hemolysis is dependent on the solute anions present in the medium. Based on these observations, Morris (1975) suggested that cellular shrinkage is not the critical factor for the induction of thermal shock.

A working hypothesis, taking into account both cell shrinkage and the lowering of temperature in the process of thermal shock, was postulated by Green and Jung. These authors suppose that a phase transition of membrane lipids will take place during cooling of the cells and that the normal membrane has to undergo some accommodation to this phase transition and the resulting decreased fluidity. Based on their experimental results, these authors further suggested, as a working hypothesis, that the cell shrinkage, caused by hypertonicity, might perturb the actin-spectrin system of the cells, thus interfering with this necessary accommodation of the membrane during cooling (Green & Jung, 1977; Jung & Green, 1978).

In previous investigations we studied the effects of heating and of anesthetics on red blood cell membranes, especially with reference to the possible interaction of these drugs with spectrin (De Bruijne & Van Steveninck, 1978; Van Gastel, Van Steveninck & De Bruijne, 1973; Dubbelman, De Bruijne & Van Steveninck, 1977). In the present study we extended these investigations to the phenomenon of hypertonic cryohemolysis, in an attempt to test the working hypothesis of Jung and Green. The results, presented in this communication, support the suggestion that perturbation of the spectrin conformation may be involved in this phenomenon.

Materials and Methods

Freshly drawn heparinized human blood was centrifuged and the erythrocytes were washed three times in buffered isotonic NaCl solution. Unless otherwise stated, hypertonic

cryohemolysis was evoked by adding 0.2 ml of a 50% cell suspension in isotonic NaCl to 5 ml of a hypertonic solution, preincubated at the desired temperature (20–50 °C). The hypertonic solution was either 0.86 M sucrose, solved in isotonic NaCl, or 1 or 2 M NaCl. After incubation for 10 min at this temperature (stage 1) the suspension was incubated another 10 min in melting ice (stage 2). The 10-min incubation periods were chosen for practical reasons. Hemolysis is time-dependent both with respect to stage 1 and stage 2 incubation periods, with a fast component completed within 10 min and a very much slower second component over at least 3 hr. Hemolysis was measured as described by Green and Jung (1977).

ATP-depletion in red blood cells was brought about by incubation during 24 hr at 37 °C, essentially as described by Bennett (1977).

Ghosts were prepared by gradual osmotic lysis as described by Weed, Reed and Berg (1963). Electrophoretic analysis of membrane proteins was performed according to Fairbanks, Steck and Wallach (1971). The obtained protein bands were numbered according to Steck (1974). Spectrin was eluted from ghosts as described by Bennett and Branton (1977).

Measurements of ghost-bound 1-anilino-8-naphthalene sulfonate (ANS) fluorescence were performed as described by Fortes and Hoffman (1971), utilizing an Aminco Bowman spectrophotofluorometer with constant-temperature cell compartment.

Results

Cryohemolysis in 0.86 M sucrose and in 1 M NaCl at different stage 1 temperatures is shown in Fig. 1. These experiments confirm the results of Green and Jung (1977) in hypertonic sucrose solution. These authors did not study the influence of stage 1 temperature on cryohemolysis in 1 M NaCl solution, however. The results shown in Fig. 1 reveal a fundamental difference between the two systems. Whereas hemolysis in 0.86 M sucrose progressively increased with increasing temperature of stage 1 incubation, it showed a maximum at about 30 °C in 1 M NaCl. These experiments were repeated with many different blood samples with always similar results. Mean values ($\pm$SD) of cryohemolysis at different stage 1 incubation temperatures are given in Table 1.

When red blood cells were depleted of ATP by incubation at 37 °C during 24 hr in an isotonic medium, they became more resistent to cryohemolysis (Fig. 2). Even if this preincubation was continued for 48 or 72 hr, cryohemolysis remained at this low level.

The effect of preincubation at 0 °C in hypertonic medium prior to stage 1 incubation was studied. 0.2 ml of the cell suspension in isotonic medium was added to 5 ml 1-M NaCl or to 5 ml 0.86-M sucrose at 0 °C. The resulting suspension was incubated at 0 °C for 10 min. Subsequently the suspension was heated to stage 1 incubation temperature (20–50 °C) and from there on the usual experimental protocol was followed. The

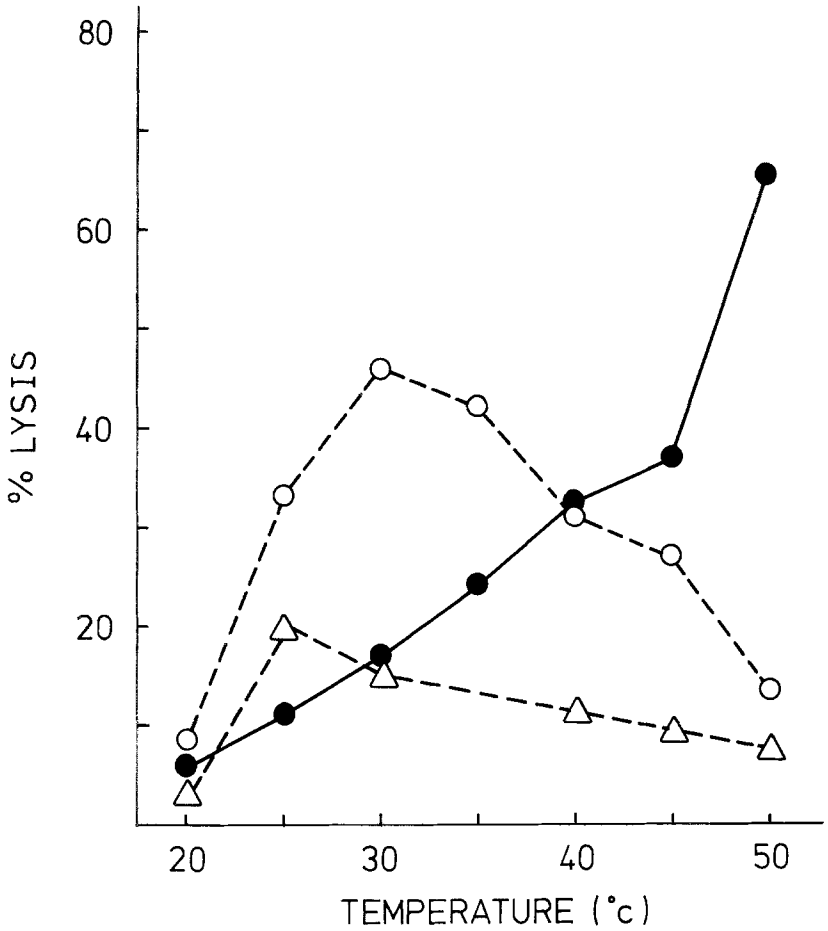

Fig. 1. Hypertonic cryohemolysis in 0.86 M sucrose (●—●), 1 M NaCl (○—○) and 2 M NaCl (△—△) as a function of stage 1 incubation temperature

Table 1. The influence of stage 1 incubation temperature on cryohemolysis in 1 M NaCl[a]

Stage 1 temperature	% lysis, ±S.D.	*n*
25	33.2±9.1	31
30	45.8±7.0	35
40	32.9±8.3	31
50	13.7±3.7	32

[a] All measurements were done in triplicate; *n* is the number of different blood samples.

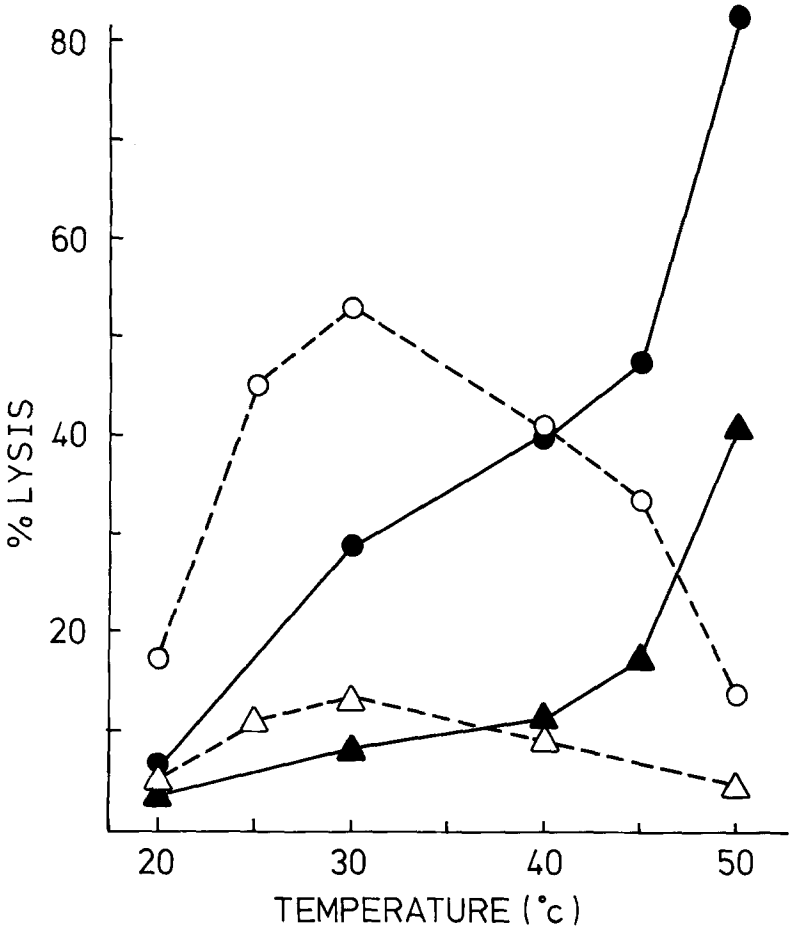

Fig. 2. The influence of ATP-depletion on hypertonic cryohemolysis. ○—○: fresh cells, 1 M NaCl; △—△: ATP-depleted cells, 1 M NaCl; ●—●: fresh cells, 0.86 M sucrose; ▲—▲: ATP-depleted cells, 0.86 M sucrose

results are shown in Fig. 3. Apparently preincubation at 0 °C in hypertonic medium inhibits cryohemolysis almost completely. There was no significant effect of increasing the stage 1 incubation to 15 or 20 min after preincubation at 0 °C.

In further experiments the effects of anesthetics (as defined by Seeman, 1972) on cryohemolysis was studied. Hexanol inhibited cryohemolysis at stage 1 temperature of 25–40 °C. At higher temperature the inhibition decreased in 0.86 M sucrose, whereas in 1 M NaCl cryohemolysis was even stimulated (Fig. 4). Similar results were obtained with ethanol

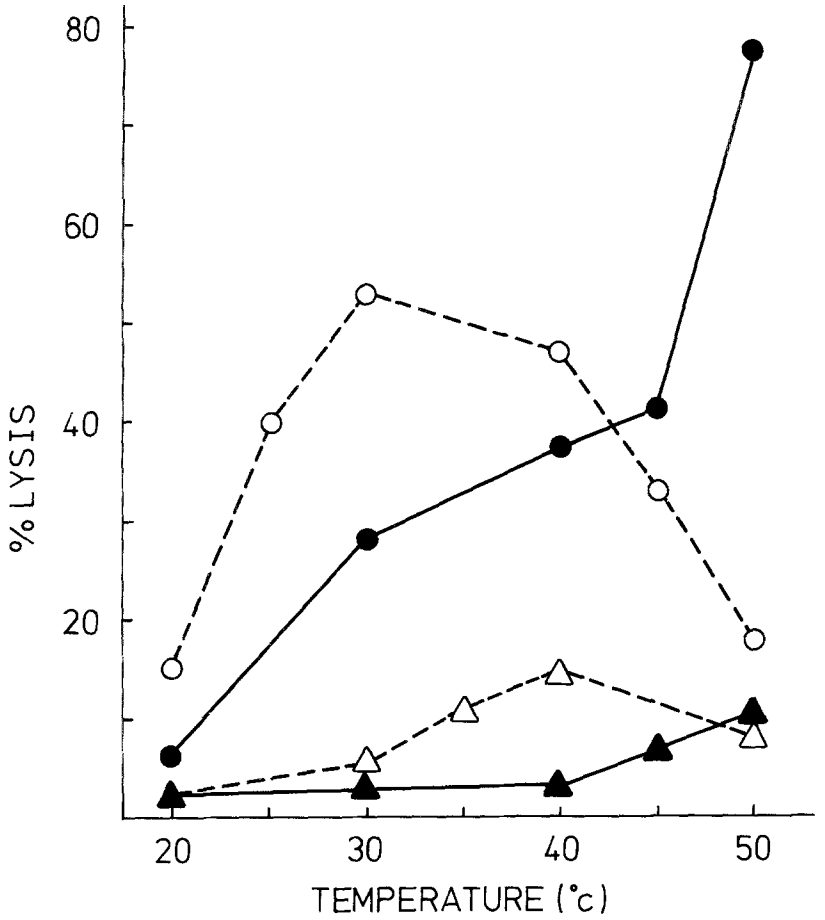

Fig. 3. The influence of preincubation at 0 °C in hypertonic medium on cryohemolysis. ○—○: lysis in 1 M NaCl, standard procedure; △—△: lysis in 1 M NaCl after preincubation in 1 M NaCl at 0 °C; ●—●: lysis in 0.86 M sucrose, standard procedure; ▲—▲: lysis in 0.86 M sucrose after preincubation in 0.86 M sucrose at 0 °C

(2.4 M) and chlorpromazine (0.12 mM). In these experiments the anesthetic was present in the hypertonic medium from the beginning. In another set of experiments stage 1 incubation was done for 10 min without anesthetics. Then the drug was added to the medium and stage 1 incubation was continued for another 2 min prior to cooling. This had no significant influence on the effect of anesthetics in 0.86 M sucrose, but in 1 M NaCl the stimulating effect of anesthetics at temperatures over 40 °C was abolished (Fig. 5).

The membrane proteins of ghosts, prepared from red blood cells subjected to the usual procedure of cryohemolysis (with stage 1 tempera-

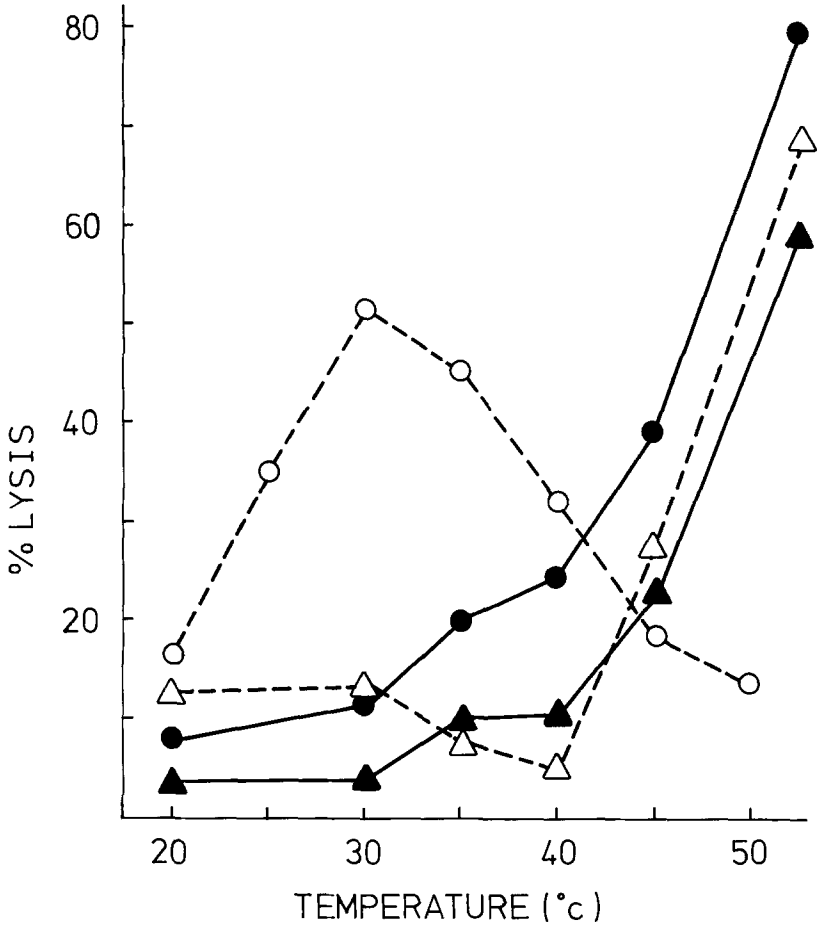

Fig. 4. The influence of hexanol (15 mM) on cryohemolysis. ○—○: lysis in 1 M NaCl, control; △—△: lysis in 1 M NaCl, 15 mM hexanol added; ●—●: lysis in 0.86 M sucrose, control; ▲—▲: lysis in 0.86 M sucrose, 15 mM hexanol added

tures of 20, 30, 40 and 50 °C) was studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein pattern showed no abnormalities with stage 1 temperatures of 20–40 °C. At a stage 1 temperature of 50 °C, however, there is a pronounced increase of band 7 protein as compared to ghosts prepared from cells incubated at room temperature in isotonic medium (Fig. 6*a, c* and *d*). In ghosts prepared from cells heated to 50 °C in isotonic medium both band 7 and band 8 proteins were strongly increased (Fig. 6*b*), as described in a previous paper (De Bruijne & Van Steveninck, 1978).

About 80% of the spectrin is eluted from normal ghosts at low

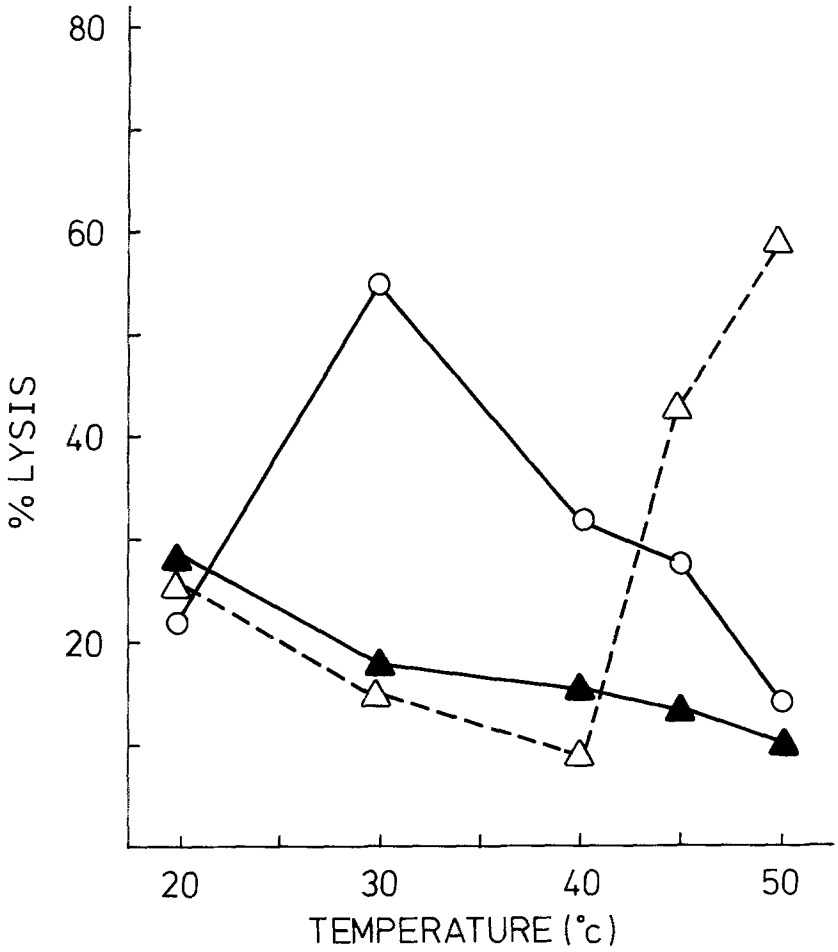

Fig. 5. The influence of hexanol (15 mM) on cryohemolysis in 1 M NaCl. ○—○: control; △—△: 15 mM hexanol present during stage 1 incubation; ▲—▲: 15 mM hexanol added after 10 min stage 1 incubation followed by an additional stage 1 incubation period of 2 min

ionic strength, following the procedure of Bennett and Branton (1977). The extractability of spectrin is altered, when the ghosts were prepared from cells that have been heated to 50 °C. It appeared that the medium in which the red blood cells were heated has a strong influence on the extractability of spectrin. Heating of the cells to 50 °C in isotonic NaCl, 1 M NaCl and 0.86 M sucrose, resulted in an extractability of spectrin of 0, 30, and 75%, respectively.

Phase transitions and lipid-protein interactions in biological membranes have been studied with many different techniques, including fluo-

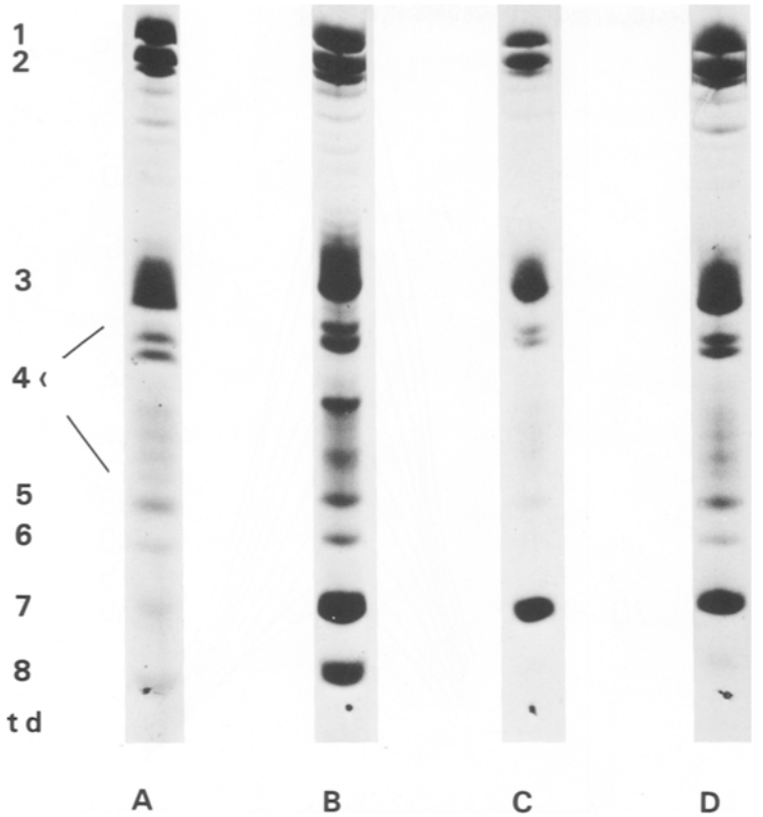

Fig. 6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of membrane proteins. (*A*): control, normal ghosts; (*B*): ghosts prepared from red blood cells incubated at 50 °C in isotonic NaCl; (*C*): ghosts prepared from red blood cells incubated at 50 °C in 1 M NaCl; (*D*): ghosts prepared from red blood cells incubated at 50 °C in 0.86 M sucrose

rescent probes (Aithal, Kalra & Brodie, 1974; Rubalcava, De Munoz & Gitler, 1969; Brocklehurst *et al.*, 1970; Freedman & Radda, 1969; Fortes & Hoffman, 1971; Wallach *et al.*, 1970). Therefore we studied the influence of temperature and medium tonicity on ghost-bound ANS fluorescence. At all tonicities fluorescence appeared to be strongly temperature-dependent and to decrease with increasing temperature (Fig. 7). Fluorescence was also dependent on the KCl concentration in the medium, increasing with increasing tonicity. From a double-reciprocal plot showing the relationship between relative fluorescence and ghost concentration (Fig. 8), it could be deduced that the enhanced fluorescence at high KCl concentrations is due to both an increase in the quantum yield of membrane-bound ANS molecules and to increased binding of

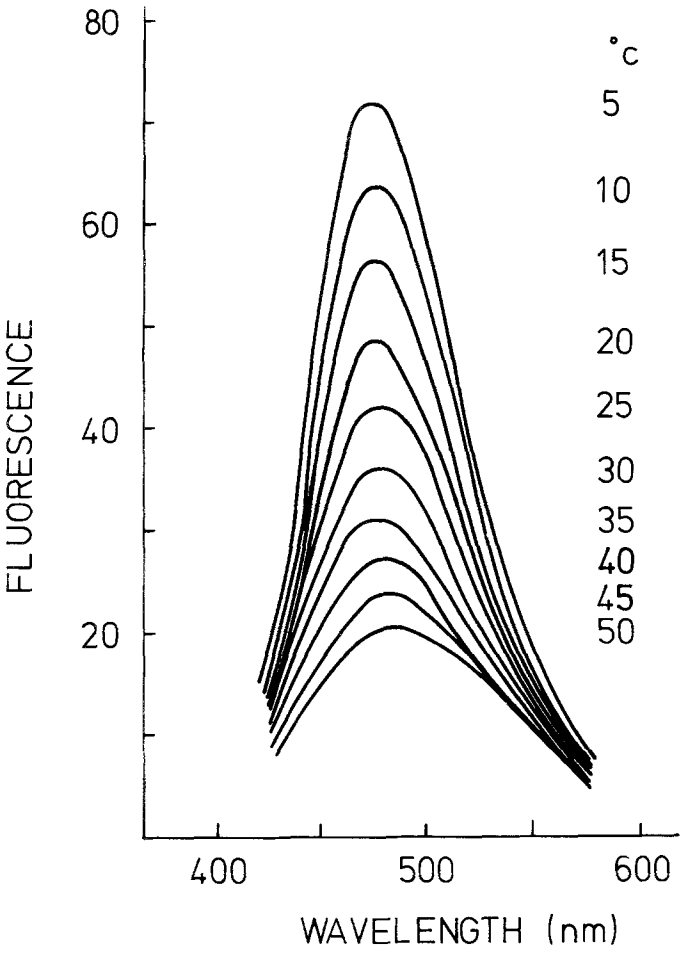

Fig. 7. Emission spectra of ghost-bound ANS measured at different temperatures. Excitation wavelength: 380 nm

ANS molecules to the ghosts. Similar studies at varying temperatures showed that increased fluorescence at lower temperatures is only due to increased quantum yield.

A peculiar effect was observed when the ghosts were exposed to experimental conditions, similar to those of the cryohemolysis protocol, in the presence of ANS. The ghosts were incubated (either in 0.1, 1, or 2 M KCl) for 10 min at a stage 1 temperature of 0, 24, 30, 40 or 50 °C, cooled to 0 °C (stage 2), incubated for another 10 min, and subsequent brought rapidly to 22 °C. At this temperature relative ANS fluorescence was measured as a function of time, using the sample that had been kept at 0 °C throughout both stage 1 and stage 2 incubation as

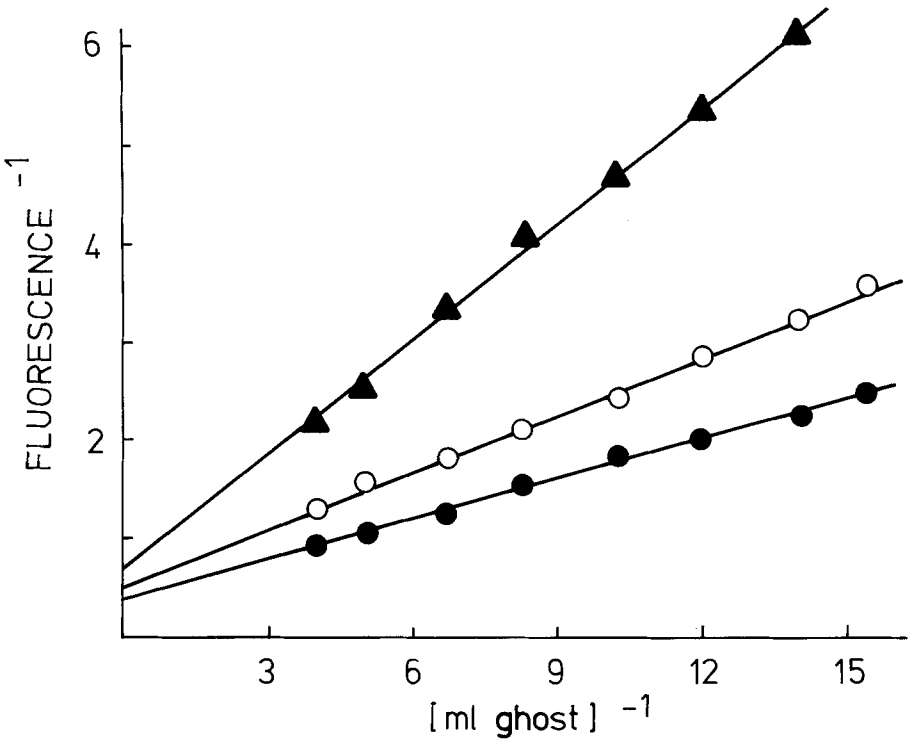

Fig. 8. The binding of ANS to ghosts as a function of the ghost concentration. ▲—▲: 0.1 M KCl; ●—●: 1 M KCl; ○—○: 2 M KCl

Table 2. Relative fluorescence of ghost-bound ANS in the course of time, in relationship to stage 1 incubation temperature and medium composition[a]

Stage 1 temperature (°C)	Medium	2 min	10 min	20 min	30 min	40 min
0	0.1 M KCl	1.00	1.00	1.00	1.00	1.00
24	„ „ „	1.01	1.00	0.99	0.99	0.99
30	„ „ „	1.03	1.01	0.99	1.01	1.00
40	„ „ „	1.10	1.07	1.06	1.06	1.05
50	„ „ „	1.48	1.43	1.42	1.42	1.41
0	1 M KCl	1.00	1.00	1.00	1.00	1.00
24	„ „ „	1.06	1.02	1.01	1.00	1.00
30	„ „ „	1.06	1.04	1.03	1.00	1.00
40	„ „ „	1.12	1.06	1.05	1.03	1.02
50	„ „ „	1.35	1.23	1.21	1.21	1.20
0	2 M KCl	1.00	1.00	1.00	1.00	1.00
24	„ „ „	1.08	1.04	1.02	1.03	1.01
30	„ „ „	1.09	1.04	1.01	1.01	1.00
40	„ „ „	1.14	1.06	1.05	1.04	1.03
50	„ „ „	1.25	1.15	1.16	1.14	1.11

[a] Fluorescence of the samples kept at 0 °C during stage 1 incubation is arbitrarily put at 1.00.

a reference. The results are shown in Table 2. Although relative fluorescence at higher temperatures *per se* is decreased (Fig. 7), a stage 1 incubation at higher temperatures with subsequent cooling results in a stronger relative fluorescence as compared to the reference, a phenomenon that is only slowly and, at stage 1 temperatures of 40–50 °C, partly not reversible.

Discussion

The basic observations on cold-induced hypertonic hemolysis were confirmed and extended by the present investigations. Jung and Green postulated, based on their experimental results, the following working hypothesis. During stage 1 incubation cell shrinkage, caused by the hypertonic incubation, may produce folding of the membrane with localized changes in surface force. This severe cell shrinkage may perturb membrane proteins, presumably the actin-spectrin system. During subsequent cooling the membrane lipids may undergo a phase transition, to which the membrane has to accommodate. The assumed alterations in the membrane protein framework during stage 1 incubation could interfere with such accommodation. Some of the observations described in the present paper support this view.

The effect of pH on hypertonic cryohemolysis in hypertonic sucrose is quite different from the pH effect in hypertonic NaCl (Jung & Green, 1978). The present studies showed some further differences, for instance, the influence of stage 1 incubation temperature. Whereas cryohemolysis increased with increasing stage 1 incubation temperatures in the 0.86 M sucrose system, it exhibited a clear optimum at about 30 °C in hypertonic NaCl, decreasing both at lower and at higher temperatures (Fig. 1). The lower hemolysis with 2 M NaCl as compared to 1 M NaCl is in accordance with observations of Green and Jung (1977). Both the pH and the stage 1 temperature effects indicate a basic difference between the mechanisms in the two systems and point to the involvement of proteins in the process. Conformation changes in membrane proteins in the physiological temperature range have been described with varying techniques and appear to be temperature, pH, and ionic strength dependent (Bull & Breese, 1973; Bieri & Wallach, 1975; Jackson *et al.*, 1973; Brandts *et al.*, 1977; Verma & Wallach, 1976). In several of these studies it could be shown that spectrin was involved in these structural transitions. At 50 °C an irreversible change in spectrin takes place. This change *per se* does not cause hemolysis. The much lower hypertonic cryohemolysis in hypertonic NaCl at high stage 1 temperatures suggests that perturbation of the spectrin network is involved in hypertonic cryohemolysis:

the final effect is apparently partially blocked by the structural transition of the spectrin network at high temperatures.

Some further experimental results are in accordance with this possibility. After heating intact cells to 50 °C, the electrophoretic pattern of the membrane proteins had changed (Fig. 6). Heating in isotonic medium resulted in a pronounced increase of band 7 and band 8 polypeptides, as described before (De Bruijne & Van Steveninck, 1978). Heating in hypertonic medium resulted in a similar increase of band 7 protein, but not of band 8 protein. Although not much is known about the functions of band 7 and 8 proteins, they probably represent real membrane proteins, as pointed out by Saleemuddin, Zimmermann and Schneeweiss (1977).

With the procedure adopted in the present studies, about 80% of the spectrin is extracted from normal ghosts in hypotonic medium. Heating in isotonic medium results in a complete loss of extractability, as described before (De Bruijne & Van Steveninck, 1978). Heating in hypertonic NaCl leads to a partial loss of extractability, whereas heating in hypertonic sucrose solution has no significant influence on spectrin elution, as described in *Results*. Here again the influence of medium composition on the perturbation of protein structure is obvious. Although these effects are only observed after heating to about 50 °C, it is quite well possible that similar, but completely reversible, changes in membrane proteins take place at lower incubation temperatures, as will be discussed below.

The effect of ATP-depletion on cryohemolysis (Fig. 2) could be explained along similar lines of reasoning. The influence of ATP-depletion on the properties of spectrin was described by Lux, John and Ukena (1978).

The influence of anesthetics (including a wide variety of chemically unrelated compounds) on cryohemolysis is remarkable. These drugs protect red blood cells against osmotic hemolysis (Kwant & Van Steveninck, 1968) and cause a decreased deformability of erythrocytes (Van Gastel *et al.*, 1973; De Bruijne & Van Steveninck, 1978). Experimental evidence indicates, that the latter effect is caused by an interaction of the drug with spectrin (De Bruijne & Van Steveninck, 1978). In this context it was interesting to study the effects of anesthetics on cryohemolysis. As shown in Figs. 4 and 5, the general tendency is a strong protection by anesthetics against cryohemolysis, except at temperatures over 40 °C, where cryohemolysis in hypertonic NaCl is stimulated by these drugs. This augmentation of cryohemolysis is blocked, however, if the anesthetic is added after preincubation at 45 or 50 °C (Fig. 5). This effect resembles

the effect of heat treatment on drug-induced decreased deformability of the cells. Heating of red blood cells to 50 °C causes a small decrease of deformability, when measured subsequently at 25 °C. Anesthetics cause a much more pronounced decrease of deformability, but this drug-induced effect is strongly inhibited by preceding heat-treatment of the cells (De Bruijne & Van Steveninck, 1978). This similarity may very well reflect the involvement of spectrin not only in control of cell deformability but also in the mechanism of cryohemolysis.

The experiments on ghost-bound ANS fluorescence seem to reflect slowly reversible and partly irreversible changes of the membrane structure during the different stages of the experimental protocol. Rubalcava *et al.* (1969) described increased binding of ANS to ghosts when the NaCl concentration was raised from 0.01 to 0.15 M, without increase of quantum yield. They ascribed this increased binding to salt-induced changes in the membrane. The results presented in this paper are in accordance with this interpretation but show that at much higher salt concentrations in the medium the quantum yield also increases (Fig.8) and that increased fluorescence at decreased temperature (Fig. 7) is completely due to increased quantum yield. Based on these observations the following predictions could be made.

After stage 1 incubation, subsequent cooling should lead to increased ANS fluorescence. If, during stage 1 incubation, a major change in membrane structure would have taken place, it could be anticipated that the final fluorescence might be decreased. As shown in Table 2, however, the results are quite different. As compared to the control, stage 1 incubation at higher temperatures leads to increased ANS fluorescence. This increased fluorescence is slowly reversible in the course of time at stage 1 temperatures up to 40 °C but is, at least partially, irreversible at a stage 1 temperature of 50 °C, especially at relatively low ionic strength of the medium. These phenomena certainly reflect complex structural changes during the experimental protocol of cryohemolysis, in accordance with the working hypothesis.

Finally, some of the experimental observations can be considered from the viewpoint of molecular hysteresis. Green and Jung made the following observation: Incubation of red cells in 0.86 M sucrose at 37 °C (stage 1) followed by cooling to 0 °C and eventually subsequent dilution of the medium to isotonicity leads to hemolysis. However, incubation in 0.86 M sucrose at 37 °C, followed by restoration of isotonicity at that temperature with subsequent cooling to 0 °C does not result in hemolysis. A more or less similar observation is described in this paper. According to the adopted standard procedure, red blood cells in isotonic NaCl

at 0 °C are introduced in a large volume of hypertonic medium at 20–50 °C (stage 1). Subsequent cooling to 0 °C results in cryohemolysis. If, however, the cells are first introduced in the hypertonic medium at 0 °C (preincubation), then heated to 20–50 °C (stage 1), and finally cooled to 0 °C (stage 2), cryohemolysis is strongly inhibited (Fig. 3). In both examples the red blood cells were subjected to exactly the same experimental conditions but in a different sequence, and in both cases the final results were quite different, indicating that some or other form of molecular hysteresis is involved in the process.

As described by Neumann (1973) molecular hysteresis indicates the existence of more than one metastable structure in the biological object, under control of experimental conditions like pH, ionic strength, temperature, etc. Further studies from this point of view may be important for the elucidation of the phenomenon of hypertonic cryohemolysis.

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