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Cold-Induced Hemolysis in a Hypertonic Milieu

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Summary. Suspension of human erythrocytes at 37 °C in an environment made hypertonic by increasing concentrations of sodium chloride and sucrose was followed by hemolysis when the temperature was lowered to 0 °C. Two distinct stages were involved in this hemolytic phenomenon, the first being incubation with hypertonic solute at some temperature above 20 °C with an increasing effect up to 45 °C, and the second stage consisting of lowering the temperature below 15 °C with increasing hemolysis down to 0 °C. The rate of cooling was not an important factor, but the presence of ions reduced the extent of cold-induced hemolysis in hypertonic sucrose. No significant release of membrane phospholipid and cholesterol accompanied this hemolysis. The solubilization of membrane protein components was investigated, with some differences appearing on sodium dodecyl sulfate polyacrylamide gel electrophoresis between hypertonic and isotonic supernatants. Spectrin could not be identified in solubilized form. Correlation of the temperatures of note in these studies with results from the literature on other biological effects of temperature-induced phase transitions in membrane lipids strongly points to the conclusion that such transitions are involved in the mechanism of cold-induced hypertonic hemolysis. It is postulated that the hypertonic milieu has resulted in membrane-protein alteration damage which prevents normal adaption to the new physical state of the membrane lipids during cooling.

In 1954, hemolysis due to "thermal shock", the result of placing cells in hypertonic salt solutions followed by sudden chilling to 0 °C, was first mentioned (Lovelock, 1954). Certain membrane lipids were recovered in the supernatant, and the investigator felt that this loss of components was responsible for the hemolytic effect observed. The present study was intended to characterize various aspects of this intriguing yet poorly understood phenomenon, and to gain some insight into its mechanism at the molecular level.

It is becoming increasingly clear that temperature-induced phase transitions in lipid bilayer structures are of importance in relation to membrane integrity and may also be associated with many membrane-related functions, such as enzyme activities (Zakim & Vessey, 1975) and molecular permeation (Linden, Wright, McConnell & Fox, 1973). In contrast

to the well-delineated mechanisms underlying hypotonic hemolysis (Ponder, 1948), however, the nature of the lytic effect of hypertonicity is not immediately clear. The present studies indicate that both hypertonicity and phase separation of lipids are simultaneously required for the hemolytic effect. The studies also indicate that this lytic effect of hypertonicity is neither associated with appreciable lipid loss, nor the result of high ionic strength.

Materials and Methods

Freshly obtained human erythrocytes of unselected blood types were washed three times in 0.85% sodium chloride. The final concentration of cells in the indicated buffer or hypertonic solutions was 2% v/v. Unless otherwise indicated, sodium phosphate-buffered saline is 0.02 M with respect to phosphate, and 0.15 M with respect to sodium chloride at pH 7.4.

The phenomenon to be described is brought about in two discrete and necessary phases in temporal sequence, which will be referred to as stage 1 and stage 2, respectively. At the end of the indicated stage 1 and 2 incubations, the cells were centrifuged at room temperature for 10 min at 2,000 rpm and the supernatant read at 543 nM in a Gilford spectrophotometer. The per cent lysis was calculated by relating the absorbance of the supernatant to a figure for 100% lysis obtained by totally lysing the appropriate control in each case. Temperatures were controlled with a digital thermometer, in some cases controlling both the bath temperatures and the temperature in the vessel. Controls were included in which the same conditions were duplicated, but without cooling in stage 2. In most cases, the amount of lysis without cooling was negligible.

For studies of the solubilized components in the supernatant, the proteins were measured directly with the Folin reaction (Lowry, Rosebrough, Farr & Randall, 1951) and the lipids determined after chloroform-methanol extraction of the supernatant (Folch, Ascoli, Lees, Meath & LeBaron, 1951).

Lipid phosphorus was measured in the washed organic phase (Svanborg & Svennerholm, 1961). Phospholipid classes were studied using thin-layer chromatography on silica gel (Mangold & Schmid, 1964), which was also used to estimate the cholesterol content of the supernatant. Supernatant proteins were also studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fairbanks, Steck & Wallach, 1971). Chemicals used were reagent grade. Triton X-100 was obtained from Rohm and Haas, Philadelphia, Pennsylvania, and colchicine and saponin were obtained from Sigma, St. Louis, Missouri.

Results

Cold-Induced Hemolysis in Hypertonic Solution

Suspensions of human erythrocytes in a hypertonic environment provided by increasing concentrations of sodium chloride resulted in negligible hemolysis at 37 °C incubation (first stage), but when the temperature was lowered to 0 °C (second stage), significant hemolysis took place (Fig. 1). Similar cold-induced hemolysis was also observed with hyper-

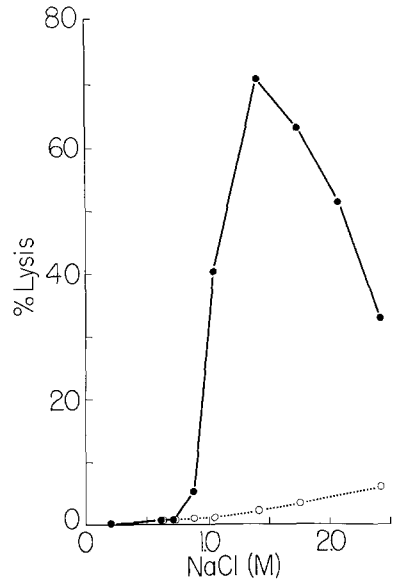

Fig. 1. Cold-induced hemolysis of human erythrocytes in hypertonic NaCl solutions. Two per cent erythrocytes, in phosphate-buffered saline, pH 7.4, containing varying concentrations of NaCl, were incubated at 37 °C (stage 1 incubation) for 10 min, then at 0 °C (stage 2 incubation) for another 10 min. Hemolysis with cold incubation (solid line) and without a cold stage (dotted line)

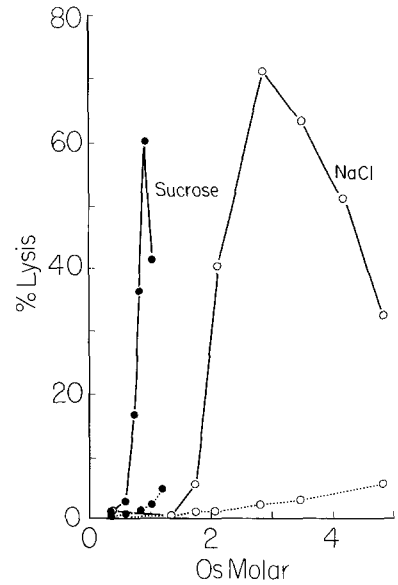

Fig. 2. Cold-induced hemolysis in hypertonic solutions of sucrose and NaCl as a function of osmolarity. Experimental procedure was the same as Fig. 1. ●, using sucrose in phosphate-buffered saline and ○, using NaCl in phosphate buffer

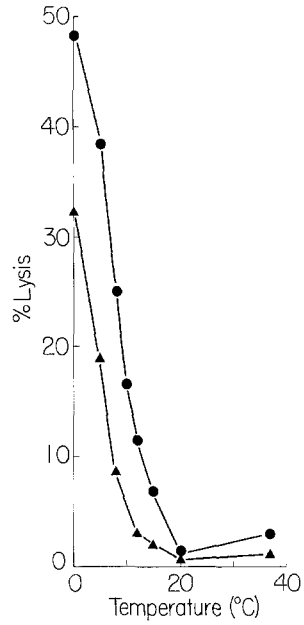

Fig. 3. Cold-induced hemolysis in hypertonic solutions of sucrose, as a function of the temperature during stage 2 incubation. Erythrocyte suspensions (2% v/v in phosphate-buffered saline, pH 7.4) contained 0.98 M (●) and 0.86 M (▲) sucrose, and were incubated at 37 °C for 10 min (stage 1) followed by 10 min (stage 2) at the specified temperatures

tonic sucrose (Fig. 2). On comparing the sucrose and sodium chloride on an osmolar basis (Fig. 2), it was found that sucrose produced a comparable effect at lower tonicity. In both cases, a slight fall was noted with the highest concentrations studied. Morphological observations of erythrocytes by phase microscopy in the presence of hypertonic sodium chloride and sucrose during the temperature fall to 0 °C revealed severely crenated cells but no evidence of spherocyte formation. This makes the possibility that hemolysis somehow was preceded by swelling of the erythrocytes extremely unlikely.

The Effects of Temperature at Each Stage of Cold-Induced Hypertonic Hemolysis

Hemolysis was greatly affected when the temperature of the stage 2 incubation was varied with stage 1 temperature held constant at 37 °C (Fig. 3). It was found that the stage 2 temperature had to be 15 °C or below for significant hemolysis to result. Increasing hemolysis occurred as the temperature was lowered below 15 °C.

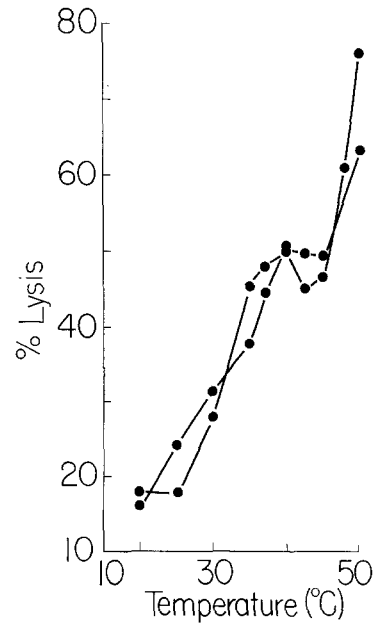

Fig. 4. Cold-induced hemolysis in hypertonic sucrose solution, as a function of the temperature of stage 1 incubation. Erythrocyte suspensions (2%) in phosphate-buffered saline, pH 7.4, containing 0.86 M sucrose were incubated at the indicated temperatures for 10 min, then at 0 °C for 10 min (stage 2). Two independent experiments are shown

Cold-induced hypertonic hemolysis was also affected as the temperature of stage 1 was varied between 20 and 50 °C with the conditions of stage 2 held constant (Fig. 4). As the temperature was increased, there was a progressive increase in the magnitude of hemolysis, with a shoulder appearing at 40 °C. Where the cells were never exposed to hypertonic solution above 15 °C, no lysis occurred after cold stage 2 incubation.

Effects of Incubation Time on Cold-Induced Hypertonic Hemolysis

Cold-induced hypertonic hemolysis was significantly affected as the time of incubation in stage 2 was increased (Fig. 5), and the previously adopted arbitrary time of 10 min corresponded to approximately 70–90% of the maximum with an incubation of an hour. The shape of the time course appears to indicate population heterogeneity with only a part of the total population affected, resulting in hemolysis. Hemolysis was negligible when the stage 1 incubation was omitted (Fig. 5).

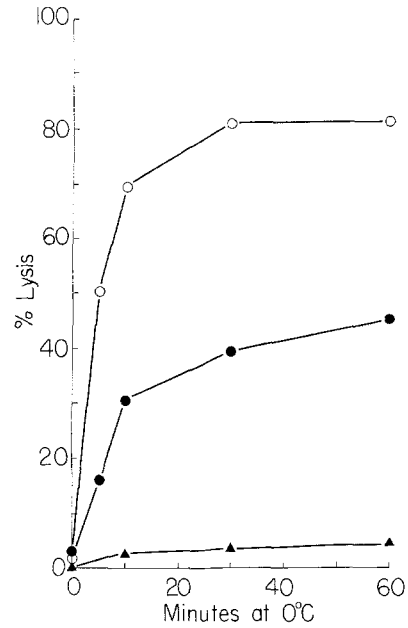

Fig. 5. Effect of stage 2 incubation time on cold-induced hypertonic hemolysis. Erythrocyte were suspended (2% v/v) in phosphate-buffered saline, pH 7.4, containing 0.86 M sucros and subjected to the stage 2 incubation at 0 °C for indicated time intervals. Closed circle indicate phosphate-buffered saline. Open circles represent results after a 10-min stage incubation at 37 °C, and triangles without such incubation. Open symbols have salin omitted

The effect of incubation time during stage 1 was also significan (Fig. 6). Again it can be seen that the 10-min stage 1 incubation perioc at 37 °C corresponds approximately to 70% of the maximum achievec at 60 min with the slope of the curve much less after 10 min. Withou the stage 2 cold incubation there was no significant hemolysis for u to 60 min (Fig. 6).

Effect of Rate of Cooling on Hemolysis

In order to study the effect of rate of cooling as a factor in "therma shock", the following experiment was carried out. Samples were incu bated in the presence of 0.98 M sucrose in phosphate-buffered salin at 37 °C. One set of samples was cooled to 10 °C as rapidly as possibl (less than 2 min), and a second set was cooled at the rate of 1 °C pe min. There was essentially no difference in the extent of hemolysis, indi cating that the rapidity of temperature fall does not seem to be a crucia factor in the production of hemolysis.

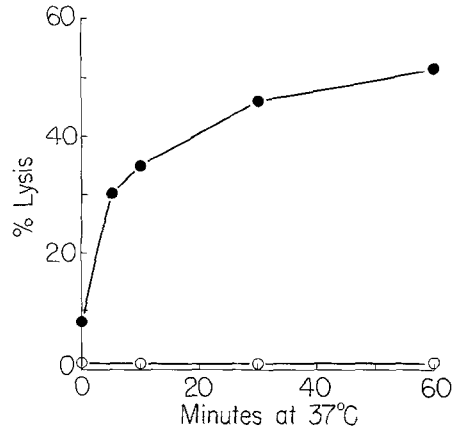

Fig. 6. Effect of stage 1 incubation time on cold-induced hypertonic hemolysis. Erythrocytes (2% v/v) were suspended in phosphate-buffered saline containing 0.98 M sucrose, then subjected to a 37 °C stage 1 incubation for the indicated time interval. Hemolysis was measured with (●) and without (○) a 10-min stage 2 incubation at 0 °C

Ionic Effects on Cold-Induced, Hypertonic Hemolysis

Since the studies outlined in Fig. 2 indicated a substantial difference between the extent of cold-induced hemolysis in sucrose and in sodium chloride at any particular osmolar concentration, the ionic effect could have an important role. The effects of ionic environment on cold-induced hypertonic hemolysis were studied using 0.86 M sucrose in distilled water, in 0.02 M phosphate buffer, and in phosphate buffer with 150 mM sodium chloride (Fig. 7). The ions seemed to exert some protective effect on the extent of hemolysis. In the absence of any added ions, the extent of hemolysis rose to 100% at approximately 12 °C. In the presence of phosphate buffer, the maximum extent of hemolysis was 65%, and with sodium chloride as well, slightly over 30%. This reduction in hemolysis is much greater than could be accounted for by the slight fall noted at the highest osmolarities (Fig. 2). In each case the extent of hemolysis was greatly increased as the stage 2 temperature was lowered.

The shape of the time course of lysis with and without the added ions was found to be similar (data not shown). Similar but not identical degrees of cold-induced hemolysis were also produced in hypertonic solutions of other ions, such as KCl, Na_2SO_4 (data not shown) and $CaCl_2$ (Fig. 8). With KCl and Na_2SO_4, omission of stage 2 cold incubation resulted in only minimal hemolysis. With $CaCl_2$, significant hemolysis was observed without the stage 2 incubation (Fig. 8).

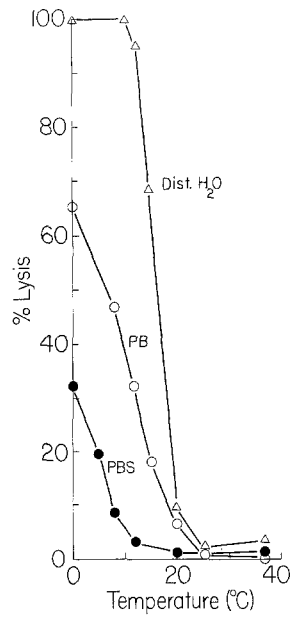

Fig. 7. Effect of ionic strength on cold-induced hypertonic hemolysis. Erythrocytes (2% v/v were suspended in 0.86 M sucrose in distilled water (△), in phosphate buffer (20 mM) (○) and in phosphate-buffered saline (●). Each of these was subjected to a 10-min stage 1 incubation at 37 °C followed by a 10-min stage 2 incubation at each temperature indicated

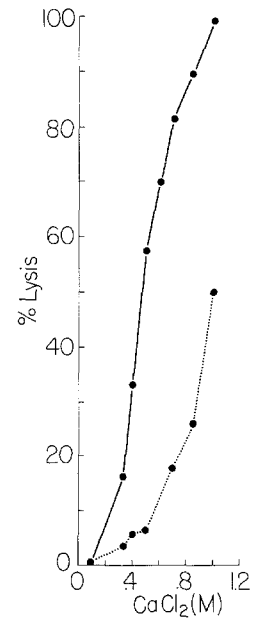

Fig. 8. Cold-induced hemolysis in hypertonic $CaCl_2$ solution. Experimental procedure are the same as those of Fig. 1. Hemolysis with a cold stage 2 incubation (solid line) and without (dotted lines)

Requirement for Hypertonicity in Each Stage

It was shown that stage 1 required hypertonic solutions at temperatures above 15–20 °C for significant lysis to occur. When cells were incubated at 37 °C in isotonic solution, the supernatant removed, the cells chilled to 0 °C and added to chilled 6% NaCl, no lysis occurred.

Requirement for the hypertonic state at stage 2 was demonstrated in the following way. Identical samples of cells were incubated with 6.0% NaCl in phosphate buffer at 37 °C for 10 min (stage 1). A fourfold excess of isotonic phosphate buffer was added gradually to one set to reduce the hypertonicity and the samples were then incubated for 10 min at 0 °C (stage 2). The second set of samples was cooled to 0 °C for 10 min (stage 2), then a fourfold excess of phosphate buffer was added for volume correction. Essentially no lysis was observed in the first set but profound lysis was seen, as expected, in the second, indicating a requirement for hypertonicity in stage 2, as well as stage 1.

Effects of Detergents, Sulfhydryl and Other Reagents on Cold-Induced Hemolysis

To assess the specificity of hypertonicity in producing cold-induced lysis, the following experiments were devised. Detergents were added to the cells and the nonbound fraction was then removed by washing, followed by cold exposure. The highest concentrations of detergents which resulted in little or no lysis at room temperature were employed. Using 0.005% Triton X-100 there was minimal lysis at 37 °C, but at 0 °C hemolysis of approximately 5% was observed. Similarly, when 0.01% saponin was used, minimal lysis occurred at 37 °C, whereas 7% lysis was found at 0 °C. This indicates a slight effect of cold with these amphipaths, but only in concentrations which are borderline lytic in the warm. At lower concentrations, there was no cold-induced lysis. Of the sulfhydryl reagents checked, only mercuric chloride in a concentration of 0.1 mM had a significant effect, giving 33% lysis. N-ethyl-maleimide and iodoacetamide had no effect up to a concentration of 1 mM even at 37 °C. Other membrane-interacting reagents, including calcium chloride (4×10^{-3} M), colchicine (10^{-6} M), and ouabain (10^{-6} M), were added in stage 1 and allowed to remain throughout stage 2, but had no effect on cold-induced hypertonic hemolysis in sucrose.

Effect of Cold on Hypotonic Lysis

The effects of cold were also observed in a situation where partia hemolysis was caused by hypotonicity. Dilutions of isotonic phosphate buffered saline in distilled water were made, incubated at 37 °C fo 10 min, and the effect of a 10-min 0 °C incubation was measured a each dilution. There was a small effect of cold increasing the exten of hemolysis at most by 10–20%.

Solubilization of Membrane Components During Cold-Induced Hypertonic Hemolysis

After incubation in hypertonic media in each stage, the supernatant were extracted for phospholipid and cholesterol. The controls consiste of similar incubation in an isotonic environment. Release of a smal amount of phospholipid was found, which was essentially the same a the case of isotonic sodium chloride. No appreciable increase was note after cold stage incubation versus that observed without such exposur (Table 1). Thin-layer chromatography of these phospholipids showed n qualitative or obvious quantitative differences. Neither was there a signifi cant difference in the small amounts of cholesterol observed betwee hypertonic and isotonic supernatants (data not shown). Studies of solubil ized protein revealed small increases in protein with hypertonic as op posed to isotonic exposure, but this could be explained by hemolysi itself.

Table 1. Loss of phospholipids during cold-induced hypertonic hemolysis

NaCl (%)	Temperature (°C)		Phospholipid released (% total)			
	Stage 1	Stage 2	Exp. I	Exp. II	Exp. III	Exp. IV
1.0	37		0.17	4.25	1.08	0.58
6.0	37		0.58	4.58	2.58	2.08
1.0	37	0	0.17	2.42	0.92	0.92
6.0	37	0	0.08	2.17	2.17	2.84

Erythrocytes (2% v/v) were suspended in phosphate-buffered saline, pH 7.4, containin 1.0 and 6.0% NaCl, incubated at 37 °C for 10 min (stage 1). One set was reincubate at 0 °C for 10 min (stage 2). The stage 2 incubation was omitted for the other set. Th cell suspension was centrifuged (90,000 × *g*) and the supernatant extracted with chloroform methanol. Aliquots equivalent to 4 ml of packed cells (or 12 mg phospholipid) were assaye for lipid phosphorus (P × 25). Results of five independent experiments are shown. Eac value represents the mean of two to three determinations.

Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of the supernatant showed membrane proteins, since such bands were not found in lysates centrifuged free of membranes. However, these bands could not be immediately identified as any of the known membrane peptides. Spectrin could not be identified in the supernatants. Some alteration could have taken place. On the other hand, there was no difference in the pattern obtained after stage 1 incubation between the isotonic and hypertonic samples. Differences were seen between the samples after stage 2 incubation. For example, a new small molecular weight band appeared. These differences may or may not be significant and are the subject of continuing study.

Discussion

The basic observation here is the finding of significant cell lysis when the temperature of erythrocytes is brought from a level above 20 °C to some point below this while constantly in a hypertonic environment. Two well-recognized causes of hemolysis are chemical assault on the membrane by hemolysins and direct osmotic swelling due to metabolic or transport abnormalities (Ponder, 1948). Common to both of these forms of hemolysis is swelling of the cells from a disk to sphere (Teitel, 1965). The membrane is evidently incapable of being stretched beyond a certain limit and eventually ruptures (LaCelle, 1970). Physical causes of hemolysis, such as radiation, are also known to be essentially due to this mechanism (Cook, 1956). Cold-induced hypertonic hemolysis described here is not associated with any pre-lytic swelling and no apparent stretching of the membrane occurs. A mechanism other than simple membrane stretching has to be postulated to explain this form of hemolysis.

It is well established that lipid structures in the natural membrane undergo phase transitions as the temperature is lowered (Stein, Tourtellotte, Reinert, McElhaney & Rader, 1969; Oldfield & Chapman, 1972). An important consequence of these thermal transitions is thought to be lateral phase separation of membrane components (Shimshick & McConnell, 1973). At least two critical temperatures define this event, the higher one corresponding to the beginning and the lower one to the completion of this process (Linden *et al.*, 1973). Significant correlation has been demonstrated between phase separations and biological functions in natural membranes. The permeability of dipalmitoylphosphatidyl

choline liposomes to Na^+ and sucrose is enhanced at 41 °C, the temperature at which this phospholipid undergoes phase transition (Papahadjopoulos, Jacobson, Nir & Isac, 1973). Zakim and Vessey (1975) showed that a phase transition of membrane lipids took place at 16 °C and this change in membrane fluidity altered the substrate specificity of a membrane-bound liver microsomal enzyme. The values of these critical temperatures are known to vary depending on such factors as fatty acid chain length and degree of saturation. In the present studies the characteristic temperature lies between 15 and 20 °C below which the cells must be exposed at stage 2 for hemolysis to occur, and this may correspond to the completion of lateral phase separation of membrane lipids. The finding of increasing hemolysis as the temperature of stage 2 is lowered from 15 to 0 °C suggests the possibility of another phase separation within this region. The 41 °C point separates the temperature effect on hemolysis of stage 1 incubation into two distinct zones. It is quite possible that this temperature corresponds to the beginning of the lateral phase separation. The nature of the temperature effect above 41 °C may be quite different and could be due to protein alterations.

No hemolysis is seen with cold exposure alone. This indicates a central role for hypertonicity in the production of this hemolysis. Where hemolysis resulted from other mechanisms, detergents, sulfhydryl reagents and hypotonic exposure, the effect of temperature was never striking. Hypertonicity itself, although necessary, is not sufficient to produce hemolysis. However, high salt concentrations at 25 and 37° have been shown (Passow, 1969) to result in increased red cell membrane permeability to Na^+ and K^+. One can speculate that the normal membrane has to undergo some accomodation during cooling to the normal phase transitions of the lipids and resultant decreased fluidity. Alterations in the membrane protein framework (Wallach, 1975) could have occurred due to hypertonicity, interfering with such accommodation. Experiments showing that lysis does not result when the hypertonicity of the medium is reduced to isotonicity by dilution prior to stage 2 indicates that whatever membrane changes occur during this phase, they are in part or wholly reversible.

Cold-induced hypertonic lysis is not associated with any significant loss of phospholipid, cholesterol or protein from the membrane during stage 1. Changes in protein solubilized in stage 2 are difficult to assess because of the possible effects of the high salt concentration on the protein released by the hemolysis itself. No correlation was observed in the present studies between the lytic effect of hypertonicity and cold

and the loss of membrane lipid components, contrary to the findings of Lovelock (1954). Spectrin does not appear in significant amounts in the supernatant, at least in recognizable form. Whether changes have occurred in this membrane protein not resulting in solubilization is currently being pursued. One possibility is that spectrin has been degraded to a smaller molecular size, preventing its ready localization on SDS gel electrophoresis.

Preliminary studies on fresh erythrocytes stratified according to the *in vivo* age (cell density) indicate a much greater susceptibility to this form of lysis on the part of old compared to young (less dense) cells. This difference in susceptibility could well be at the cell membrane level and might be the manifestation of an important aspect of cell membrane integrity.

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