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DIFFERENTIAL EFFECTS OF LIPIDS ON THE OSMOTIC FRAGILITY OF HAMSTER ERYTHROCYTES

AVINOAM LIVNE, PIETER J. C. KUIPER* AND NAOMI MEYERSTEIN
Division of Life Sciences, Negev Institute for Arid Zone Research, Beer Sheva (Israel)
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SUMMARY

1. There was no difference in osmotic potential between erythrocytes of control and heat-exposed hamster, thus the increased red cell osmotic fragility of the heat-exposed animal is related to membrane properties of the erythrocytes.

2. Linolenoyl sorbitol at 4 μg/ml ($9 \cdot 10^{-6}$ M) increased the osmotic stability of the erythrocytes of both animal groups in a parallel fashion. This lipid did not modify the frequency distribution of hemolysis of the erythrocytes.

3. At 65 % hemolysis, added linolenoyl and stearoyl sorbitol similarly protected both groups of erythrocytes. At 95 % hemolysis, specific interactions were apparent: linolenoyl sorbitol (at 6 μg/ml) induced almost full protection against osmotic hemolysis of both groups of erythrocytes. Stearoyl sorbitol (at 6 μg/ml) was only partially effective, and the protection afforded to the erythrocytes from heat-exposed animals was particularly limited.

4. Linolenoyl sorbitol expanded the erythrocyte membrane at hypotonic media more effectively than stearoyl sorbitol.

5. The data support the hypothesis that the unsaturated acyl groups of the membrane lipids contribute to higher osmotic stability of the erythrocyte.

INTRODUCTION

The osmotic fragility of erythrocytes of the hamster is increased following a prolonged exposure of the animal to an elevated ambient temperature[1]. The increase in osmotic fragility is correlated with changes in lipids and fatty acids of the red blood cells[2]. These changes include a profound decrease in the content of the linolenoyl conjugate of the phospholipids and a concomitant increase of the palmitoyl conjugate in the erythrocytes of the heat-exposed hamster. We therefore postulated that the linolenoyl group contributes to higher stability of the erythrocytes against hemolysis in hypotonic solutions. If this hypothesis is valid, it is expected that added lipids, rich in linolenic acid, should confer on the erythrocytes an increased resistance to hemolysis.

A wide variety of surface-active compounds have been shown to decrease the

* Laboratory for Plant Physiological Research, Agricultural University, Wageningen, The Netherlands.

osmotic fragility of erythrocytes[3–11]. Of particular interest for the present study are the reports concerning the effects of lipids and fatty acids on osmotic fragility of erythrocytes. SEEMAN[11] showed that stearic acid, lysolecithin and lecithin (both palmitoyl conjugates) at 10^{-5}–10^{-4} M decrease the osmotic fragility of human red blood cells in a diminishing order of effectiveness. EHRLY *et al.*[4] reported that fatty acids (sodium salts) reduce hemolysis of human red blood cells in hypotonic solutions. The undissociated fatty acids appeared to be ineffective in their system. VAN ECK (personal communication) found that monogalactosyl diglyceride and phosphatidyl choline, both rich in linolenoyl content (about 70 % of the total fatty acid conjugates) stabilized human red blood cells against hypotonic hemolysis.

Clearly, various lipids reduce the osmotic fragility of erythrocytes. However, there are no reports relating the effectiveness of lipids in reducing the osmotic fragility to their fatty acid composition. It became of interest to compare the stabilizing effects of lipids which vary in the degree of unsaturation of the fatty acids. Red blood cells from control and heat-exposed hamsters are particularly suitable for this comparison, since the fatty acid composition of the erythrocytes of the hamster is modified by heat exposure of the animal[2].

METHODS

Adult male hamsters (*Mesocricetus auratus*) were divided into two groups. One was kept at 20–23° (control), while the other was maintained at 35 ± 1° (heat-exposed). The animals were amply provided with rat chow, succulent vegetables and water. The period of heat treatment lasted at least 1 month, as required for the expression of hematological changes in heat-acclimated hamsters[1].

Preparation of erythrocyte suspension

Blood was collected from decapitated animals into heparin-containing tubes (40 units/ml blood) and centrifuged for 10 min at 700 × *g*. The supernatant and the upper layer of the cell suspension (about 5 % of the blood volume) were removed by aspiration. The cells were washed three times by suspension in 6 vol. of 150 mM NaCl, pH 7.1, and subsequent centrifugation. Formed elements of blood other than erythrocytes were essentially removed by this procedure. The final preparation of washed red blood cells contained less than one leucocyte per 10^4 erythrocytes. A stock of 30 % cell suspension, containing about $4.25 \cdot 10^9$ cells per ml and 150 mg hemoglobin per ml was prepared for measurements of osmotic properties.

Osmotic potential of the erythrocyte suspensions

Stock suspensions of individual animals (about 3 ml) were hemolyzed by freezing and thawing. The osmolality of the hemolyzates was determined by a Fiske osmometer (Fiske Association, Inc., Uxbridge). The osmotic potential of the packed erythrocytes was calculated by correcting for the osmolality of the saline medium.

Measurement of osmotic fragility

An aliquot of 20 μl of the stock suspension (about $8.5 \cdot 10^7$ cells) was pipetted into 5 ml of 2 mM sodium phosphate, pH 7.2, containing NaCl at various concentrations. Lipids were mixed with the buffered NaCl solutions just prior to the

addition of the red cell media. Ethanol was added to each tube to a final volume of 10 μl, since the stock solutions of the lipids contained ethanol. Immediately after the addition of the cells, the contents of each tube was thoroughly mixed. After 15 min at 18–19° the cells were centrifuged at 2000 $\times$ *g* for 5 min. The supernatant was removed and the percent hemolysis determined by measuring the absorbance of hemoglobin in the supernatant with a Beckman DU spectrophotometer (at 543 nm) or a Klett colorimeter (filter No. 54). The percent hemolysis, determined by these instruments, was identical.

Measurement of relative cell volume

For these measurements, a 40 % stock suspension of washed erythrocytes was prepared in a medium of 155 mM NaCl and 2 mM sodium phosphate buffer, pH 7.2. A volume of the suspension was diluted rapidly with 1.5 vol. of the buffer solution containing varying concentrations of NaCl and, where indicated, the acyl sorbitol. Hematocrit and the percent hemolysis were determined following 15 min incubation at 20°. Microhematocrit tubes were used for hematocrit determination. Following centrifugation, the supernatant was diluted 25-fold in water prior to calorimetric determination of hemolysis. The volume of the cells was corrected for hemolysis as described by GUEST AND WING[12].

Hemoglobin determinations

Hemoglobin of the stock solution was determined according to the method of DRABKIN AND AUSTIN[13].

MATERIALS

Linolenoyl sorbitol preparation lot No. 3259 was kindly supplied by the Selney Co., New York. A portion of this preparation was hydrogenated, using palladium oxide on charcoal (10 %) as a catalyst, to yield a saturated preparation. The fatty acid composition of both preparations was determined as described[14], and it is presented in Table I. It can be seen that the preparations are not homogeneous, but for simplicity they are referred to according to their major fatty acid component. Aliquots of the stearoyl sorbitol, kept in chloroform at $-18°$, were evaporated under a stream of N_2 and further exposed to reduced pressure (20 mm Hg) for 30 min to dispose of residual chloroform. Stock solutions of the lipids were daily prepared in 67 % ethanol.

RESULTS AND DISCUSSION

Effect of linolenoyl sorbitol preparation

Linolenoyl sorbitol preparation (Table I) was found effective in reducing the osmotic fragility of hamster red blood cells and therefore the following studies were performed with this preparation.

Fig. 1 demonstrates that erythrocytes from control hamsters hemolyze at a lower NaCl concentration than erythrocytes from heat-exposed animals, as already reported[1]. This was demonstrated in over 30 experiments. This difference in the osmotic fragility could be due to a lower osmotic potential in the control erythrocytes.

TABLE I

FATTY ACID COMPOSITION OF THE PREPARATIONS OF STEAROYL SORBITOL AND LINOLENOYL SORBITOL AS PERCENTAGE OF TOTAL FATTY ACIDS

Fatty acid	*Stearoyl sorbitol*	*Linolenoyl sorbitol*
Palmitic acid	12.3	6.5
Stearic acid	86.6	0
Oleic acid	0	25.7
Linoleic acid*	0	18.1
Linolenic acid	0.8	49.0

* 1/3 of this fraction may be hexadecatrienoic acid.

Actually, the osmotic potential in the control erythrocytes was slightly higher, but the difference was not significant. Taking into consideration the standard error (about 4.5 mosmoles) the difference in osmotic fragility could be attributed to a difference in osmotic potential as well as to a difference in membrane properties (Table II). Fig. 1 also shows that addition of linolenoyl sorbitol at 4 μg/ml (about $9 \cdot 10^{-6}$ M) increases the osmotic stability of the erythrocytes of both animal groups. The shift of the hemolysis curves to a lower NaCl concentration occurred in a parallel fashion.

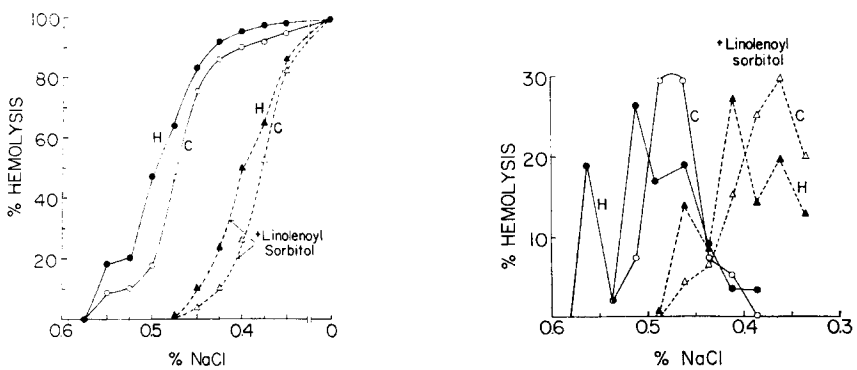


Fig. 1. Red cell osmotic fragility of control (C) and heat-exposed (H) hamsters. The effect of the addition of 4 μg/ml linolenoyl sorbitol is presented by dashed lines. The standard error is in the range of 0.2–1.5 %.

Fig. 2. Frequency distribution of red cell osmotic fragility of control and heat-exposed hamsters. The data from Fig. 1 are expressed as hemolytic increments. Symbols as in Fig. 1.

For the erythrocytes of the two groups of animals the shift amounted to about 0.1 % (or 17 mM) NaCl. Fig. 2 presents the frequency distribution of the percentage hemolysis derived from the curves of Fig. 1. While the control population appears to be homogeneous with respect to hemolysis, the red cell population from the heat-exposed animals is heterogeneous in agreement with earlier data[1]. Addition of linolenoyl sorbitol does not modify the patterns of the frequency distribution of hemolysis.

TABLE II

OSMOTIC POTENTIAL OF ERYTHROCYTES FROM CONTROL AND HEAT-EXPOSED HAMSTERS

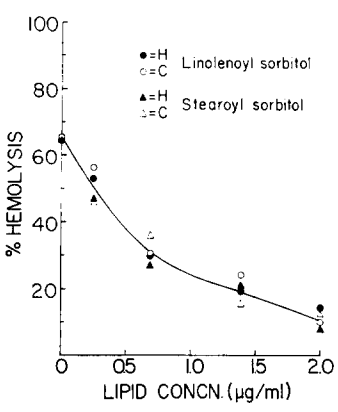
	Osmotic potential mosmoles (mean ± S.E.)	*Significance (t test)*
Control (13)*	297.7 ± 4.3	
Heat exposed (13)*	287.0 ± 4.6	N.S. ($P > 0.1$)

* Number of animals.

Comparison of linolenoyl sorbitol and stearoyl sorbitol preparations

The effect of various concentrations of linolenoyl sorbitol and stearoyl sorbitol on the osmotic fragility of erythrocytes from control and heat-exposed animals was tested at two levels of hemolysis.

Fig. 3 represents data for 65 % hemolysis at the absence of any added lipid. This level of hemolysis is on the linear portion of the osmotic fragility curve (Fig. 1) and is commonly used for the comparison of added chemicals[11,15]. The two lipids similarly protected both groups of erythrocytes, within the range of concentrations tested (up to 2 μg/ml).



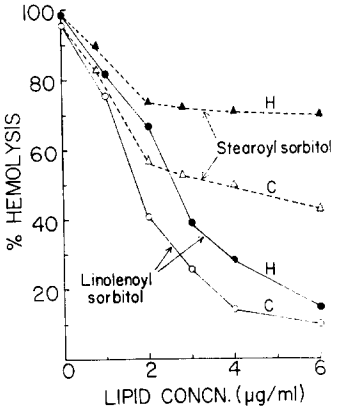


Fig. 3. Effect of linolenoyl sorbitol and stearyol sorbitol preparations on the red cell osmotic fragility of control (C) and heat-exposed (H) hamsters. NaCl concentration: 0.4 %.

Fig. 4. Effect of linolenoyl sorbitol and stearyol sorbitol preparation on the red cell osmotic fragility of control (C) and heat-exposed (H) hamsters. NaCl concentration: 0.455 % for C and 0.485 % for H.

The requirement for a protecting agent may be more demanding at lower NaCl concentration and higher level of hemolysis. By suspending the untreated erythrocytes from control and heat-exposed animals in a medium of 0.455 and 0.485 % NaCl, respectively, hemolysis was increased to about 95 %. Thus, specific differences between the lipids and between the groups of erythrocytes could be explored. Fig. 4 shows that, indeed, at this higher level of hemolysis a higher concentration of the added lipids was required to afford protection to the erythrocytes. For a given concentration of

either lipid preparation, the osmotic stability of the control erythrocytes was greater than the stability of the erythrocytes of the heat-exposed animals. Striking differences between linolenoyl sorbitol and stearoyl sorbitol were apparent at concentrations higher than 2 μg/ml. At 6 μg/ml, linolenoyl sorbitol induces almost full protection in both groups of erythrocytes. However, stearoyl sorbitol was much less effective at the higher concentrations, and only partial protection was afforded by this preparation, particularly for the erythrocytes from the heat-exposed hamsters. Consequently, control erythrocytes and erythrocytes from heat-exposed animals differed more in osmotic stability in presence of stearoyl sorbitol than in presence of linolenoyl sorbitol.

It is concluded that the protecting materials may have two effects: a specific effect manifested at high hemolysis level (Fig. 4) and a non-specific effect found at a lower level of hemolysis (Fig. 3). The non-specific effect is in agreement with earlier reports[11,15].

The interactions between the lipids and the erythrocytes shown in Fig. 4 are of interest in relation to the observed changes in osmotic fragility and lipid composition of the erythrocytes due to heat exposure of the animals[2]. The present data support the hypothesis[2] that the unsaturated acyl groups of the lipids contribute to higher osmotic stability of the erythrocytes.

Membrane expansion of erythrocytes by acyl sorbitols

The anti-hemolytic effect of some anesthetics was related to an increase in the critical hemolytic volume of the erythrocytes[10,16,17]. Thus, it was of interest to study the effect of the acyl sorbitol preparations on the critical cell volume of hamster erythrocytes. Greater quantities of the lipids than described above were added in this experiment, since a more concentrated cell suspension had to be used for the hematocrit determination.

Fig. 5 presents the relative cell volume under varying medium tonicity. The control erythrocytes attained a larger critical hemolytic volume than the erythrocytes from the heat exposed animals. Since the average volume of hamster erythrocyte is 73.5 μm^3 at isotonic media for both animal groups[1], the calculated surface areas of the prehemolytic spheres are 123.1 μm^2 for control and 118,6 μm^2 for the heat-exposed

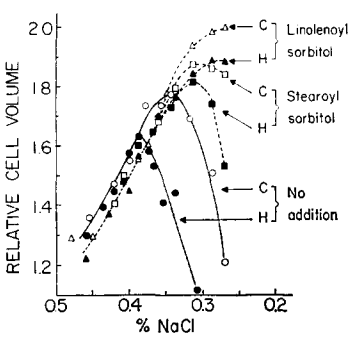


Fig. 5. Relative cell volume of erythrocytes from control (C) and heat-exposed (H) hamsters. The effect of the addition of the acyl sorbitols (120 μg/ml) is presented by dashed lines. The concentration of NaCl given in the abscissa was corrected for the volume of the added cells. The cell volume at isotonic media was taken as 1.0 and the relative cell volume was calculated according to GUEST AND WING[12].

hamsters. This difference correlates with the difference in osmotic fragility between the two erythrocyte groups[1] (Fig. 1).

The added lipids did not affect the volume of the red blood cells at isotonic NaCl solution. As can be seen from Fig. 5 the two acyl sorbitols clearly increased the critical hemolytic volume of both erythrocyte groups. The increase in area of the pre-hemolytic spheres due to the added linolenoyl sorbitol was 9.2–9.4 % (11.6 and 11.1 μm^2 in control erythrocytes and erythrocytes from heat exposed hamsters, respectively). The corresponding increase in area caused by stearoyl sorbitol was 5.0–5.2 %. Thus, linolenoyl sorbitol appears to be more effective in expanding the erythrocyte membrane at hypotonic concentration.

Several possible mechanisms were proposed by SEEMAN *et al.*[17] for the expansion of the membrane area of human erythrocytes by anti-hemolytic substances: (1) incorporation of the added molecules into the membrane; (2) adsorption–extension theory[18]; (3) displacement of a component which keeps the membrane in a condensed state; (4) conformational changes in proteins. The mechanism of the membrane expansion of the hamster erythrocytes by the acyl sorbitols is apparently due to the incorporation of these lipids into the membrane. The following observations support this conclusion: While the membrane stabilization by tranquilizers was readily reversible[10], we observed (unpublished data) that erythrocytes treated with the acyl sorbitols retained the increased osmotic stability even after several isotonic washings. Following the addition of tritium-labeled stearoyl sorbitol, a major portion of the label was still associated with the cells after several cycles of washing with isotonic solutions. Furthermore, over 95 % of the incorporated label was found in the ghosts of these cells following lysis in hypotonic medium.

These observations support the first mechanism listed above, but they do not exclude the possible involvement of the other mechanisms.

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