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STRUCTURE- AND CONFIGURATION-DEPENDENT EFFECTS OF C18 UNSATURATED FATTY ACIDS ON THE CHICKEN AND SHEEP ERYTHROCYTE MEMBRANE *

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High concentrations of unsaturated fatty acids are known to cause hemolysis. At low concentrations, however, unsaturated *cis* fatty acids have been found to protect erythrocytes against hypotonic hemolysis. In the present experiments we examined the effect of oleic (18:1), linoleic (18:2), linolenic (18:3), and elaidic (18:1) acid on the osmotic fragility of chicken and sheep erythrocytes, which markedly differ in their resistance to osmotic rupture. The results are summarized as follows: (A) The phenomenon of stabilization was observed in both species alike. (B) Interaction of cells with the fatty acids under isotonic conditions led to a persistent stabilization, i.e., the cells remained more resistant against osmolysis even after several washings. (C) Oleic and elaidic acid protected against osmotic rupture with a high degree of specificity. Linoleic and linolenic acid were much less protective. Thus, this effect appears to be specific for one double bond. (D) Contrary to the unsaturated fatty acids with *cis* configuration, elaidic acid with the *trans* configuration showed no biphasic behaviour, and even at the highest concentrations applied no hemolysis was observed.

Introduction

The influence of substances on the osmotic resistance of cells is a parameter for their interaction with the plasma membrane. It is well known that a number of lipophilic compounds stabilize red blood cells against hypotonic hemolysis [1]. Lipid soluble anesthetics and phenothiazine tranquilizers were found to show such effects, while water soluble drugs were not effective [2,3]. In other studies some free fatty acids were shown to reduce hypotonic hemolysis of human erythrocytes [4,5]. Unsaturated fatty acids have been shown to exhibit a biphasic behaviour in this respect, i.e. they stabilize erythrocytes at low concentration ranges, but promote hemolysis at higher concentrations [6]. This was demonstrated for the three C18 fatty acids of the *cis* configuration, oleic acid, linoleic acid and linolenic acid.

The present study was performed with chicken and sheep erythrocytes, which show different degrees of resistance against osmotic rupture. This prompted us to use these two cell types to examine how general the effects of unsaturated fatty acids on the erythrocyte membrane are, and to what extent these substances can be used as a probe to look for possible differences between the membrane of the nucleated avian erythrocyte and that of unnucleated sheep red blood cells. In addition to the unsaturated fatty acids of the *cis* configura-

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tion we included also elaidic acid with the *trans* configuration to investigate the question of a structure- or configuration-dependent specificity in the effects of free fatty acids on the membrane. Finally, we addressed ourselves to the question of optimal conditions for interaction of fatty acids with the membrane, and whether alterations of the membrane by unsaturated free fatty acids are reversible phenomena.

Materials and Methods

Erythrocyte preparations. Heparinized blood was drawn from an adult male sheep and from adult male and female chickens of the White Leghorn strain. The animals were raised in our own breeding facilities. After three washings with Hank's solution and removal of the buffy coat both suspensions were adjusted to a 50 : 50 (v/v) solution.

Determination of osmotic resistance. The osmotic fragility of chicken and sheep erythrocytes was assayed as follows: 50 μl of the respective erythrocyte stock solution were mixed with 5 ml of a 20 mM phosphate buffer, pH 7.2, that contained different NaCl concentrations ranging from 0.85% to 0.02%. After a 30 min incubation at room temperature the samples were centrifuged (5 min at 2000 rpm) and the absorbance at 540 nm was determined.

Treatment with fatty acids. Hypotonic conditions. The effect of fatty acids on the osmotic fragility of erythrocytes was determined in a similar fashion as described by Raz and Livne [6]. The tested free fatty acids, dissolved in methanol, were mixed with the buffered NaCl solution just prior to addition of the red cells. Up to 15 μl methanol were added, which did not affect the osmotic fragility of the erythrocytes. A 50 μl aliquot of the erythrocyte stock suspension was mixed with 5 ml of a 20 mM sodium phosphate buffer, pH 7.2. Various concentrations of each fatty acid were mixed with two different NaCl concentrations that had been found to cause different degrees of hemolysis. After incubation for 30 min at room temperature the probes were treated and measured as described above.

Isotonic conditions. To assay the interaction of fatty acids with the erythrocyte membrane under isotonic conditions and to determine the persistence of the effects, red blood cells were incubated with various fatty acid concentrations in phosphate-buffered saline (pH 7.2) for one hour at room temperature and washed three times by centrifugation (2000 rpm) with the same buffer. These cells were then subjected to analysis of osmotic resistance as described above.

Reagents. All fatty acids were obtained from Sigma. Phosphate-buffered saline (pH 7.2): 6.78 g NaCl, 1.42 g Na_2HPO_4 and 0.4 g KH_2PO_4 in 1 l distilled water.

Results

Osmotic resistance of sheep and chicken erythrocytes

Erythrocytes from sheep and chicken exposed to buffered NaCl solutions of gradually decreasing concentrations were found to differ markedly in their resistance to hypoosmotic shock as depicted in Fig. 1. Whereas sheep erythrocytes exhibited 50% lysis at 0.40% NaCl, chicken red blood cells were not lysed until 0.22% buffered NaCl solution. We also observed the higher osmostic resistance of erythrocytes from female chickens, as reported by others [7].

Effects of cis and trans C18 fatty acids on hypoosmotic lysis

The effects of unsaturated fatty acids were determined at two different degrees of hemolysis. According to the results depicted in Fig. 1, for chicken erythrocytes buffers with 0.14 and 0.18%

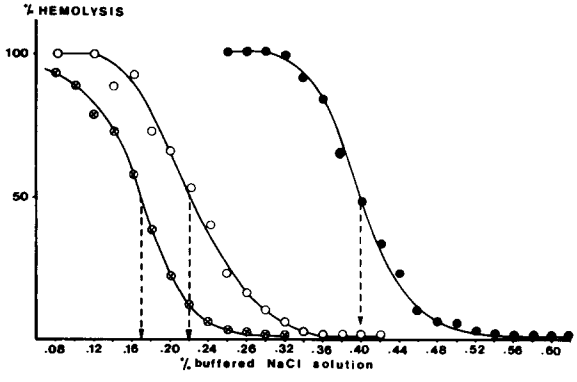

Fig. 1. Osmotic resistance of erythrocytes from male sheep (●), and male (○) and female (⊗) chicken determined by measuring the absorbance of hemoglobin at 540 nm; 100% corresponds to values obtained with distilled water.

NaCl, and for sheep erythrocytes 0.40 and 0.44% NaCl were used. Chicken (Fig. 2) as well as sheep erythrocytes (Fig. 3) were stabilized by all unsaturated fatty acids tested in a certain concentration range. All *cis* fatty acids showed biphasic behaviour causing complete lysis at higher concentrations. Surprisingly, elaidic acid (*trans* configuration) did not promote lysis even at the highest concentrations used, but continued to stabilize.

A dependence of the stabilizing effect on the number of double bonds was observed. The saturated stearinic acid did not show any protecting effect, nor cause hemolysis at high concentrations (data not shown). Thus, at least one double bond (oleic and elaidic acid) is the prerequisite for the stabilization effect. Increasing the number of double bonds, however, does not lead to an amplification of the effect, but rather to a decrease in the extent of stabilization, as suggested by the data with chicken erythrocytes (Fig. 2 and Table I). As elaidic acid was found to be very effective in protecting against osmotic rupture, the effect of stabilization appears to be independent of configuration, but resulting from a structure with one double bond in either *cis* or *trans* configuration.

Fig. 4 represents a quantitative comparison of the stabilizing effect of elaidic and oleic acid over the whole range of NaCl concentrations. In contrast to *cis* configurated fatty acids causing hemolysis at high concentrations, the protecting effect of elaidic acid observed at $3 \cdot 10^{-5}$ M is further amplified by increasing the concentration to $3 \cdot 10^{-4}$ M.

Isotonic treatment of erythrocytes with fatty acids and persistence of the stabilizing effect

The experiments so far were done with hypotonic solutions, to which the different concentrations of fatty acids were admixed immediately before addition of the red blood cells. Thus, the

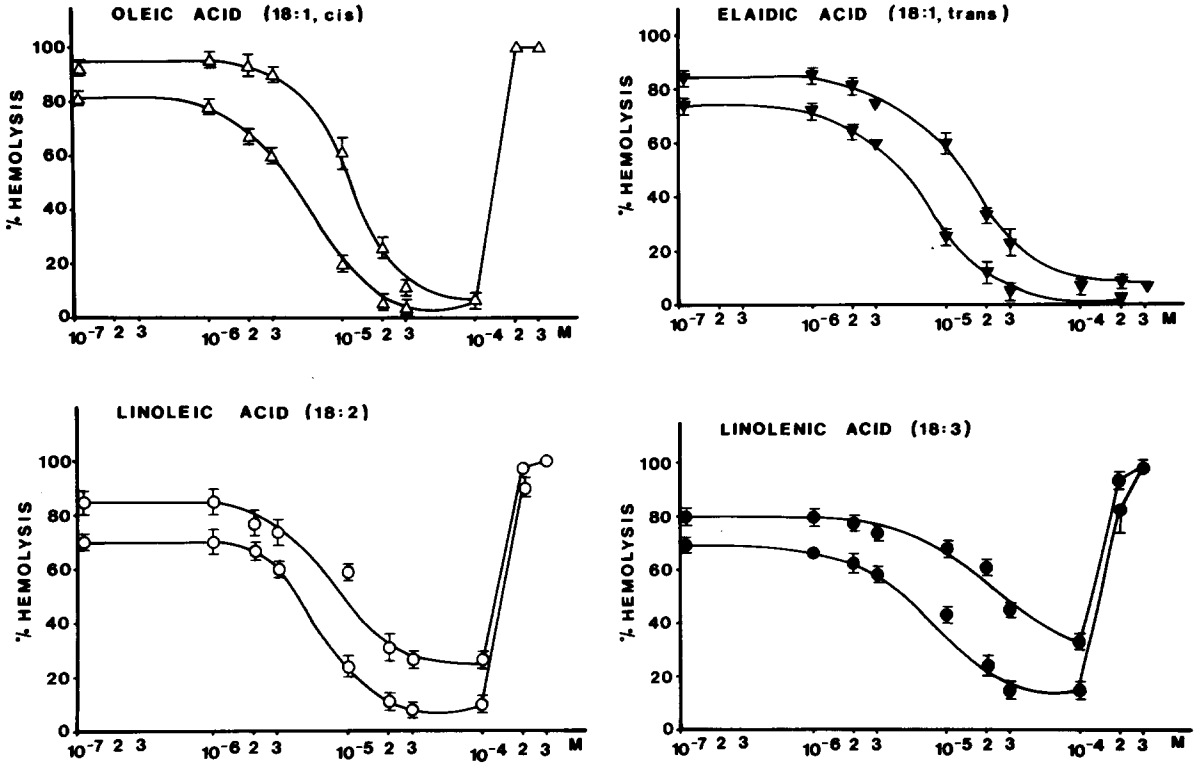

Fig. 2. Effects of hypotonic treatment with unsaturated C18 fatty acids on the osmotic resistance of chicken erythrocytes at 0.14 (upper curves) and 0.18 (lower curves) % NaCl. Erythrocytes were added to various concentrations of the respective fatty acid in the buffered hypotonic NaCl solution.

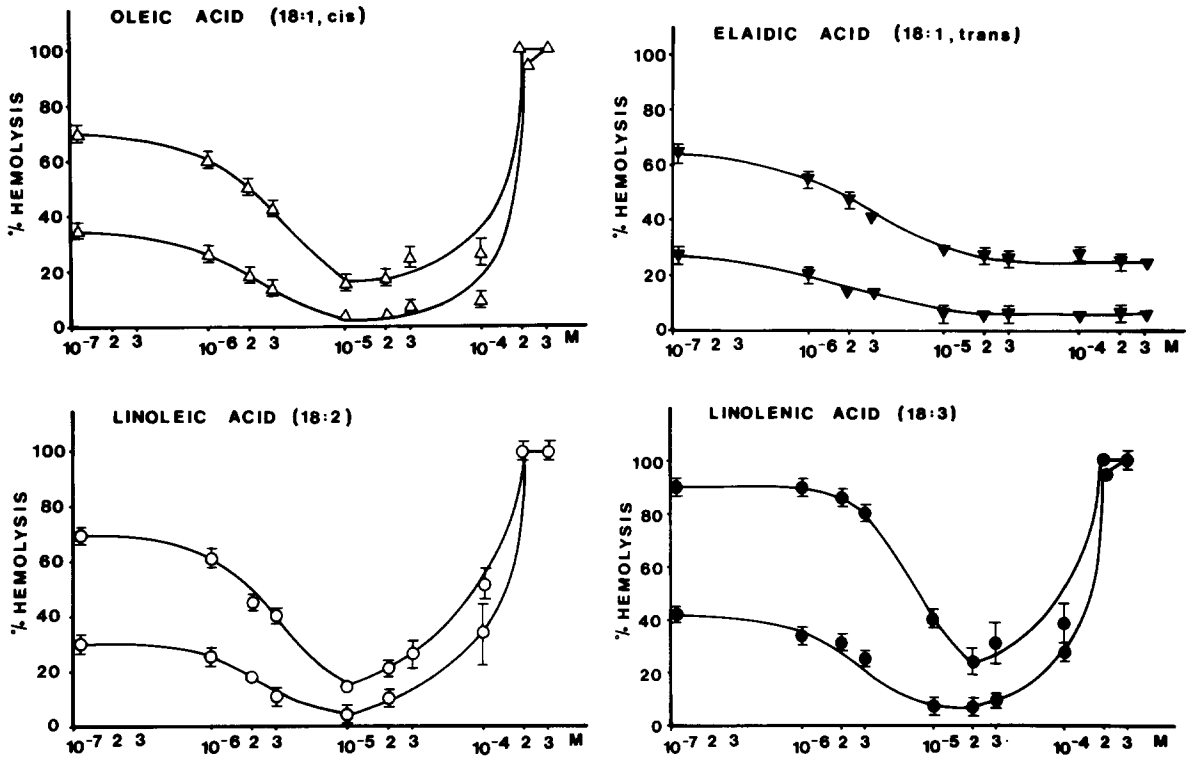

Fig. 3. Effects of hypotonic treatment with unsaturated C18 fatty acids on the osmotic resistance of sheep erythrocytes at 0.40 (upper curves) and 0.44 (lower curves) % NaCl. Experimental setup as described for Fig. 2.

interaction of fatty acids with the erythrocyte membrane took place under hypotonic conditions which might cause structural alterations of the membrane favouring the binding of fatty acids. Alternatively, the interaction might occur at a specific binding site, and would not require the stress of hypotonic condition. In an attempt to answer this question we modified the experimental

TABLE I

PERCENT INHIBITION OF OSMOLYSIS OF CHICKEN ERYTHROCYTES BY HYPOTONIC TREATMENT WITH UNSATURATED C18 FATTY ACIDS

NaCl concn. (%)	% Hemolysis		Fatty acid	% Inhibition	
	Expt. 1	Expt. 2		Expt. 1	Expt. 2
0.14	90	94	Oleic	93.7	93.4
0.18	80	80		97.5	97.5
0.14	83	85	Elaidic	90.5	91.7
0.18	75	72		94.7	97.3
0.14	90	80	Linoleic	73.0	70.0
0.18	75	78		86.7	85.1
0.14	77	80	Linolenic	56.3	60.0
0.18	70	68		80.0	78.0

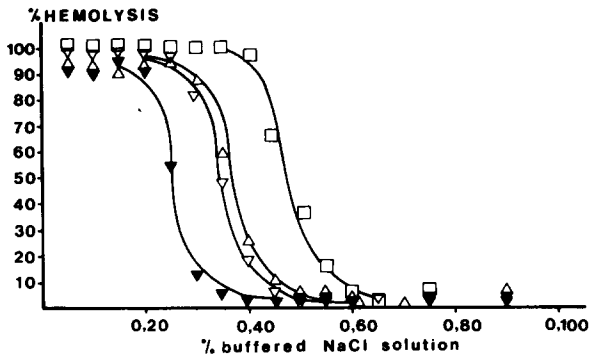

Fig. 4. Quantitative comparison of the stabilizing effect of high concentrations of oleic (△, $3 \cdot 10^{-5}$ M) and elaidic (▽, $3 \cdot 10^{-5}$ M; ▼ $3 \cdot 10^{-4}$ M) acid. Note the increased resistance induced by $3 \cdot 10^{-4}$ M elaidic acid. (□, control, without fatty acid.)

protocol by incubating erythrocytes at isotonic conditions with different concentrations of unsaturated fatty acids. After washing three times, the treated cells were tested in the osmolysis assay as described. As shown in Figs. 5 and 6 treatment of both kinds of erythrocytes with fatty acids at isotonic conditions resulted in a protection similar to that observed in the previous experiments, and this effect persisted over three cycles of washing. The same structural specificity was observed under this experimental rationale, yet the suggested specificity for one double bond appeared clearly expressed for both species: The strongest effect was exhibited by oleic and elaidic acid, and the stabilization was hardly detectable with linolenic

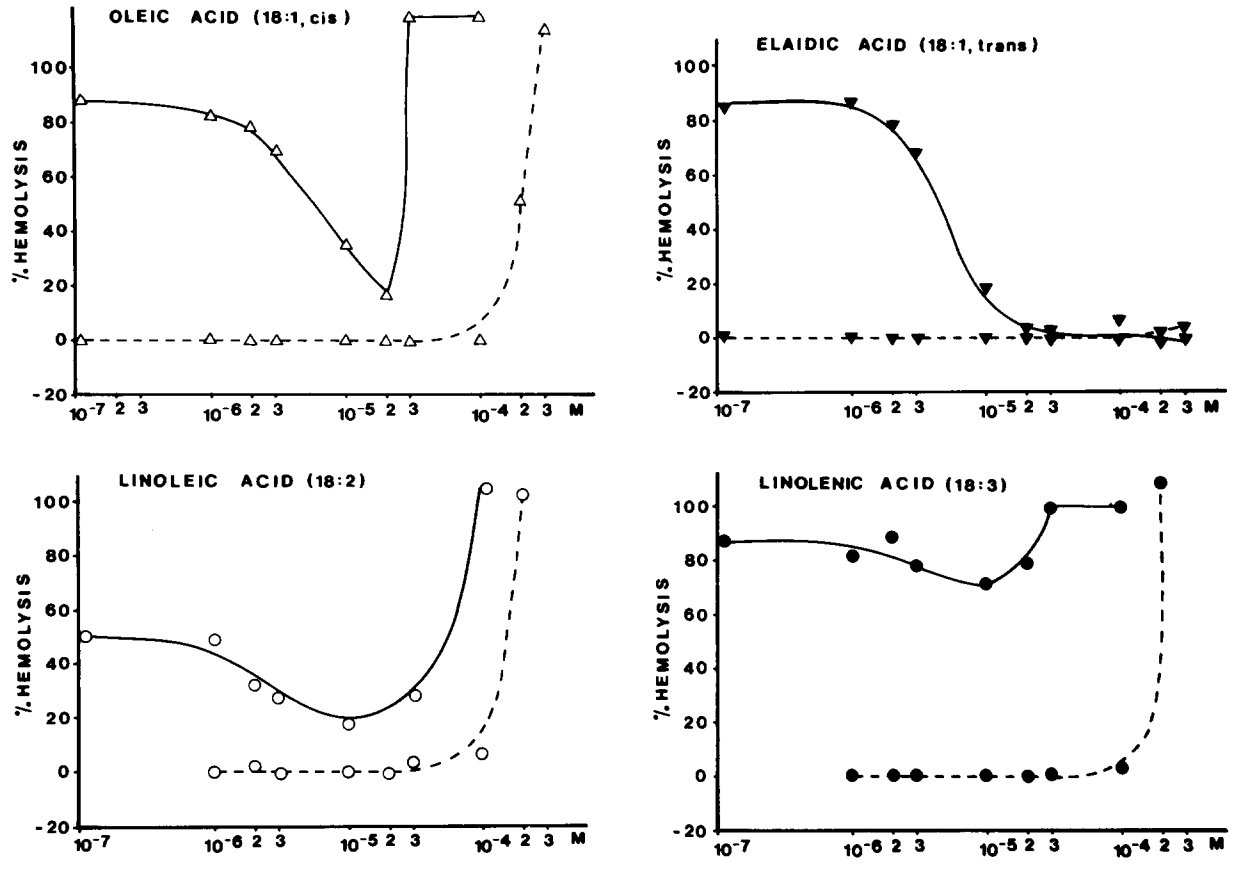

Fig. 5. Effects of isotonic treatment with unsaturated C18 fatty acids on the osmotic resistance of chicken erythrocytes. Erythrocytes were incubated with various concentrations of the respective fatty acid in isotonic buffered saline. After 3 washings with phosphate-buffered saline (pH 7.2) they were analysed for osmotic lysis in buffered 0.14% NaCl solution. The dotted lines represent the hemolytic effect of fatty acids without hypoosmotic shock.

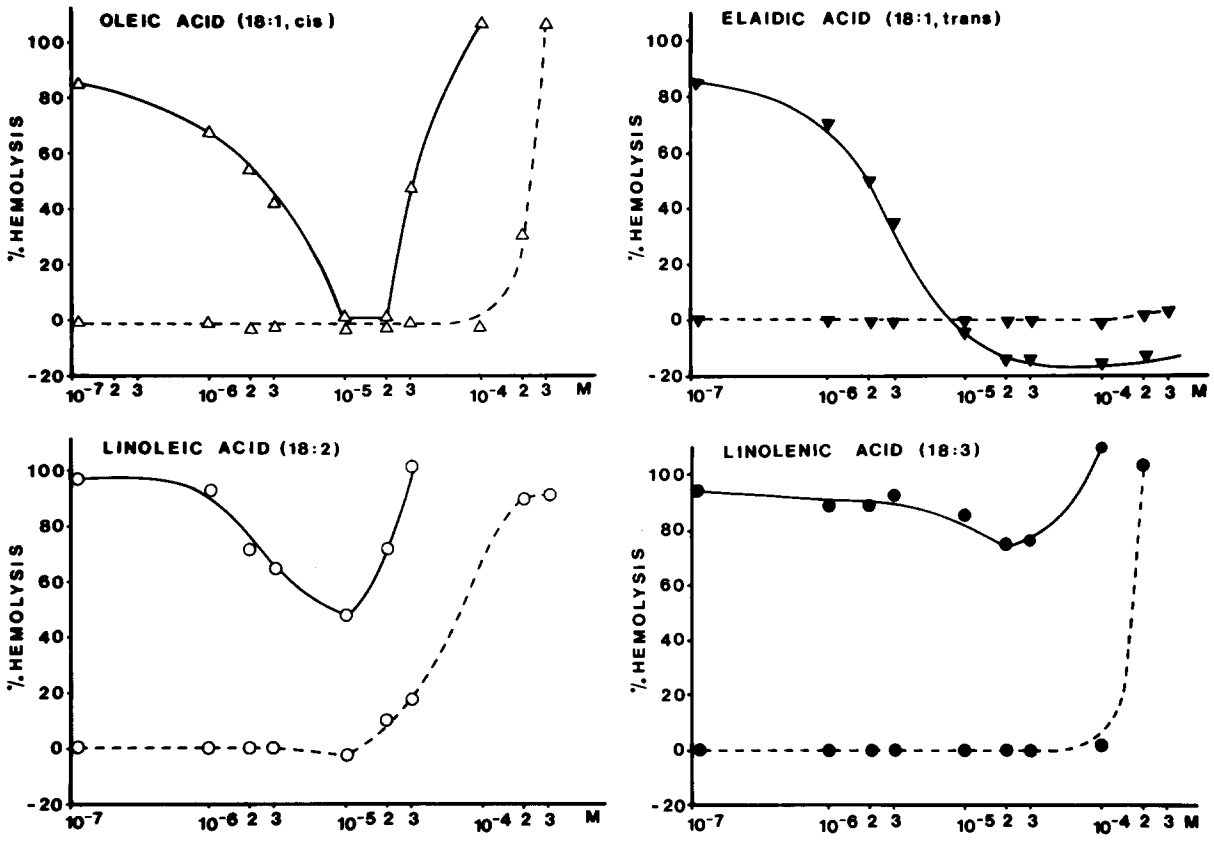

Fig. 6. Effects of isotonic treatment with unsaturated C18 fatty acids on osmotic resistance of sheep erythrocytes at 0.40% NaCl. Experimental details as described for Fig. 5.

acid. Linoleic acid (18 : 2) showed a medium level of protection. Thus, the interaction of unsaturated fatty acids with the erythrocyte membrane does not depend on the distortion the membrane may suffer under hypotonic conditions.

Discussion

Very little is known about the mechanism of binding and the exact physiological role(s) of free fatty acids in the cell membrane. Considering, however, the numerous reports about effects of these compounds on membrane structure and functions, one can assume that this role is manifold.

In the present study, we examined exclusively fatty acids with 18 carbon atoms in a direct experimental approach, i.e. effect on osmolysis, and focussed our attention on the question of structure and configuration specificity.

The following main observations could be made: (1) The protecting effect of unsaturated C18 fatty acids was seen both with chicken and sheep erythrocytes. Considering the phylogenetic distance between these two species one can assume that this effect is a general phenomenon. (2) The binding of unsaturated fatty acids to the membrane is not a weak and reversible one, but rather quite a stable interaction, that persists over several cycles of centrifugation and washing. This result is in contrast to earlier studies by others [6] describing a rapidly reversible stabilizing effect of unsaturated fatty acids on human erythrocytes. (3) A finding to be stressed is the fact that the interaction between unsaturated fatty acids and erythrocytes took place and led to protection against

osmotic rupture with a high degree of specificity for one double bond under isotonic conditions, but with a low degree of this specificity under hypotonic conditions. Previous similar studies were performed under hypotonic conditions, i.e. erythrocytes were added to a hypotonic buffered solution of fatty acids [6]. Based on their results these authors proposed a mechanism of stabilization according to which the interaction would be enabled due to moieties of the membrane bilayer exposed only under the distortion caused by hypotonic stress. (4) No specificity as to configuration was observed as both *cis* (oleic) and *trans* (elaidic) C18 : 1 fatty acids exhibited equal stabilization effects. Interestingly, in contrast to the *cis* fatty acids tested, high concentrations of elaidic acid never caused hemolysis, but continued to stabilize the membrane.

Hydrophobic substances like long-chain fatty acids could be expected to bind to both the lipid and to the protein components of biological membranes.

The mechanism of binding of fatty acids to the lipids of membranes was extensively analysed in a recent study [8]. Although interaction with the lipid bilayer is a good possibility for all unsaturated fatty acids, we feel that due to the specificity of stabilization exhibited by the C18 : 1 fatty acids, an interaction with the protein moiety of the membrane appears to be a more likely alternative.

There are other reports in agreement with our hypothesis of a specific protein binding site for oleic or elaidic acid. Wetzker et al. [9] demonstrated recently that oleic acid and calmodulin competitively interact with the calmodulin binding part of the membrane bound Ca^{2+}-ATPase. Numerous other effects, however, on the $(Na^{+}+K^{+})$-ATPase and Mg^{2+}-ATPase of the erythrocyte membrane by long-chain fatty acids do not seem to be specific for unsaturated fatty acids [10,11].

Finally, it should be mentioned that there are reports about the stabilizing effect of unsaturated fatty acids on erythrocytes to occur in vivo. Red blood cell membranes from rats raised on a diet with low content of essential fatty acids were studied by Ehrström et al. [12]. Although no changes in membrane fluidity as monitored by spin-label motion were found, the diet caused an increased osmotic sensitivity of erythrocytes deficient in essential fatty acids. Thus, these data would be in line with our direct proof of the protecting effect of unsaturated fatty acids under physiological conditions.

Our studies suggest a specific binding site for fatty acids containing one double bond in the erythrocyte membrane of chicken and sheep. Experiments are presently carried on to determine the nature of this binding structure, and whether this specificity is influenced by the chain length of the fatty acid molecule.

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