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DIFFERENTIAL EFFECTS OF LIPIDS ON THE OSMOTIC FRAGILITY OF ERYTHROCYTES

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SUMMARY

Stearic, oleic, linoleic and linolenic acids, the methyl esters of these acids, as well as their hydroxy analogs were tested for their potency to stabilize human erythrocytes against hypotonic hemolysis. The erythrocyte stabilization was affected by the polarity and the degree of unsaturation of the added compound and by the level of hemolysis employed. The stabilization afforded by the unsaturated fatty acids decreased with increasing number of double bonds while the opposite was true for the alcohol series. The interaction of both the polar and the hydrophobic portions of a stabilizing compound with the erythrocyte membrane contributes to the overall stabilization effect. To account for the differential effects of the lipids, is it proposed that some modifications of the membrane are being revealed on excessive swelling of the erythrocytes in a hypotonic medium.

INTRODUCTION

Stabilization of red blood cells against hypotonic hemolysis by various substances has been extensively investigated, particularly as a model system for the study of the mode of action of various drugs[1,2]. Indeed, a correlation has been established between the antihemolytic activity of anesthetics as well as of phenothiazine tranquillizers and their clinical potencies[3–5]. Lipid solubility of the added compounds appears to be a critical requirement for their stabilization effect. Roth and Seeman[2] concluded that all lipid-soluble anesthetics protect red cells from hypotonic hemolysis, while water-soluble drugs are not effective.

The antihemolytic activity of a series of phenol anesthetics correlated with the partition coefficient in octanol–water[6]. Furthermore, the erythrocyte stabilization potency of alcohol anesthetics increased with the increasing number of the methylene groups in the alcohol molecule[7]. A hydrophobic interaction between the erythrocyte membrane and the stabilizing anesthetics was inferred from these studies[6,7].

How general is the requirement for the hydrophobic nature of the stabilizing compounds? Ehrly *et al.*[8] observed that sodium salts of fatty acids reduced erythro-

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cyte hemolysis in hypotonic solutions, while the undissociated acids were ineffective. This observation prompted us to investigate the role of the polar group in the interaction of lipids with erythrocyte membrane.

METHODS

Heparinized human (adult male) venous blood was washed as described[9]. A stock of 50% cell suspension was prepared for measurement of the osmotic properties. For determination of osmotic fragility, a 20-μl aliquot of the stock suspension was rapidly mixed with 5 ml of 2 mM sodium phosphate solution, pH 7.2, containing NaCl of varying concentrations. The tested lipids, dissolved in methanol, were mixed with the buffered NaCl solutions just prior to addition of the red cells. Up to 15 μl of methanol were added, which did not affect the osmotic fragility of the erythrocytes. After remaining for 10 min at room temperature (20–21 °C), the suspensions were centrifuged at 2000 $\times g$ for 5 min. The percent hemolysis was determined by measuring the absorbance of hemoglobin in the supernatant at 540 nm. Each experiment was repeated 5–6 times with essentially identical results.

Measurement of the relative cell volume was as described[10]. The volume of the red cells in hypotonic media was corrected for hemolysis according to Guest and Wing[11].

All chemicals were obtained from Sigma-Israel, Ramat-Gan. The fatty acids, esters and alcohols appeared as single spots on thin-layer chromatography with silicic acid.

RESULTS

C_{18} compounds, differing with respect to the degree of unsaturation and the polar head group, were tested for their potency to stabilize erythrocytes against hypotonic hemolysis. Fig. 1 represents the stabilizing effect of oleic, linoleic and linolenic acids as well as of the alcohol analogs and the methyl esters of these acids. The measurements presented were conducted in a 68–70 mM NaCl media, an osmolality leading to 65% hemolysis without added lipids. This level of hemolysis is on the linear portion of the osmotic fragility curve and is commonly used for comparison of effects of added chemicals[11,12]. All lipids produced the commonly found biphasic effect, increasing stabilization at low concentration and decreasing stabilization at high concentrations[3]. The acids were most effective in conferring maximum stabilization, while the methyl esters of the acids appeared to be least effective.

The requirement for a protecting agent may be more demanding at lower NaCl concentration and higher level of hemolysis. By suspending the erythrocytes in a medium of 60–62 mM NaCl, hemolysis was increased to about 95%. Thus, specific differences between the various lipids may be explored[10]. Fig. 2 shows that, indeed, at this high level of hemolysis the acids do stablilize erythrocytes against hemolysis while the alcohols and the methyl esters do not at all. This distinct difference does not apply to stearic acid (Fig. 3), as the stabilizing effect of this saturated fatty acid is rather low.

The different stabilizing effect by the acid and alcohol forms, as shown above,

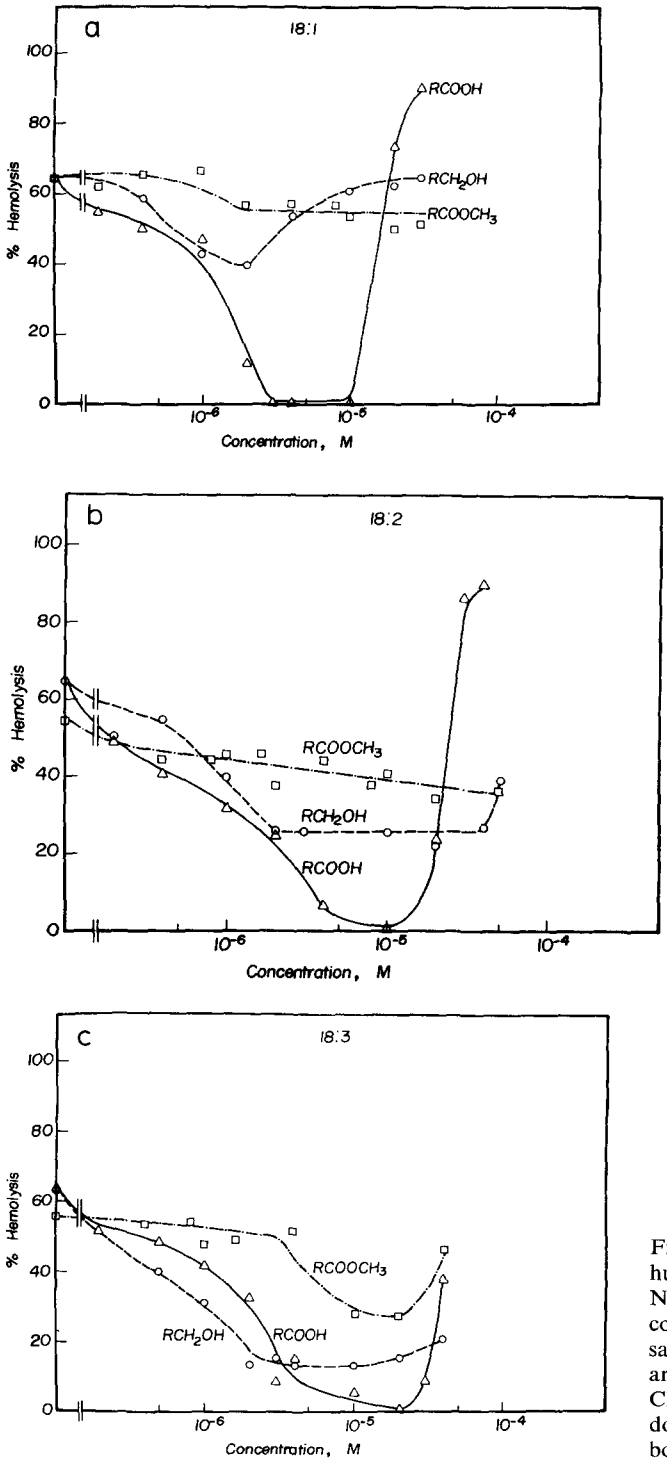

Fig. 1. Osmotic fragility of human erythrocytes at 69 mM NaCl, as affected by varying concentrations of C_{18} unsaturated fatty acids, alcohols and fatty acid methyl esters. Chains containing (a) one double bond, (b) two double bonds, (c) three double bonds.

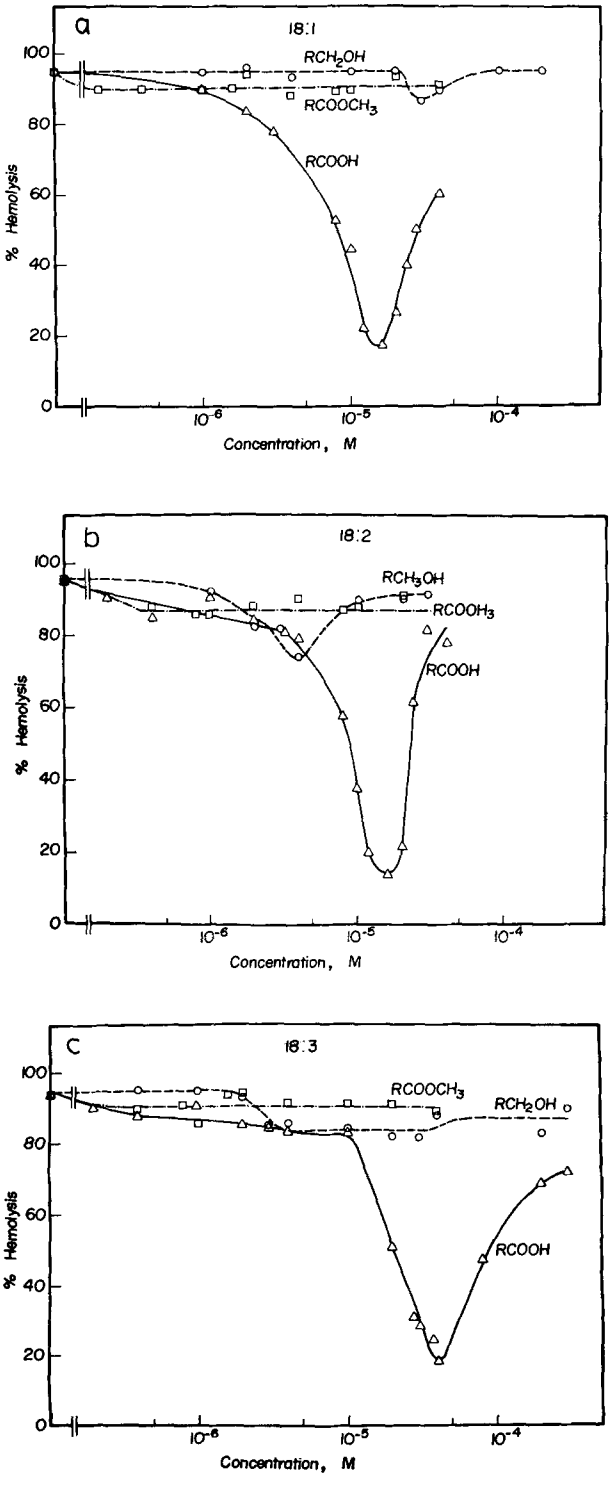

Fig. 2. Osmotic fragility of human erythrocytes at 69 mM NaCl, as affected by varying concentrations of C_{18} unsaturated fatty acids, alcohols and fatty acid methyl esters. Chains containing (a) one double bond, (b) two double bonds, (c) three double bonds.

could be either a particular case or, alternatively, a general phenomenon. It was therefore of interest to compare the acid and alcohol forms of other compounds. Vitamin A appears to be suitable for such a comparison: both Vitamin A acid (retinoic acid) and vitamin A alcohol (retinol) stabilize rabbit erythrocytes[13] and

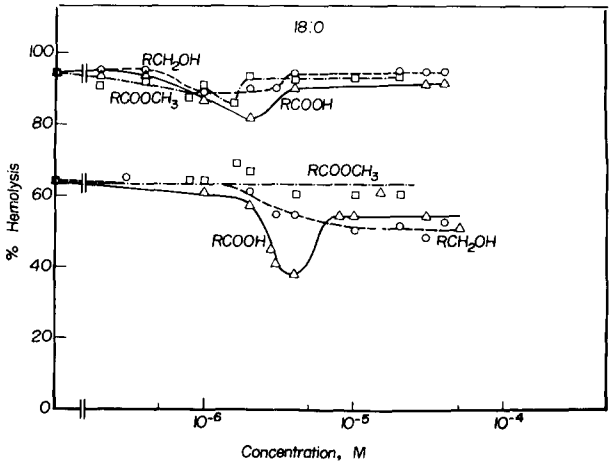

Fig. 3. Effect of stearic acid, stearyl alcohol and stearic acid methyl ester on the osmotic fragility of human erythrocyte at 65% and 96% hemolysis.

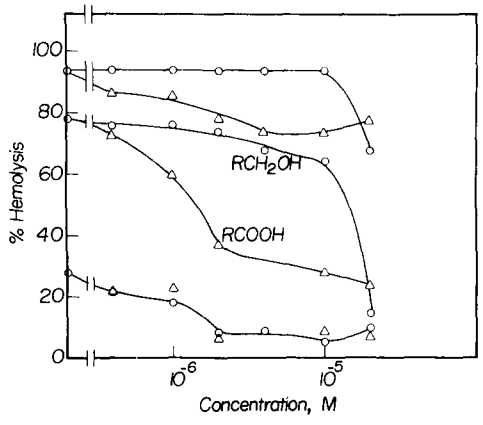

Fig. 4. The effect of vitamin A acid (△) and vitamin A alcohol (○) on the osmotic fragility of human erythrocytes at the levels of 24, 76 and 96% hemolysis.

retinol stabilized human erythrocytes at low level of hemolysis[14]. Fig. 4 compares the stabilizing effects of vitamin A acid and alcohol at three levels of hemolysis: 24, 78 and 96%. While the acid and the alcohol exert similar stabilizing effects at the low level of hemolysis, vitamin A acid is clearly more effective than vitamin A alcohol at the higher levels of hemolysis.

The reduced osmotic fragility of erythrocytes by anesthetics, expressed as an elevated critical hemolytic volume, was related to an expansion of the membrane area in hypotonic media[15]. The effect of linolenic acid and linolenyl alcohol was

therefore measured. Neither linolenic acid nor linolenyl alcohol affected the erythrocyte volume in isotonic medium, but both the acid and the alcohol increased the critical hemolytic volume of the erythrocyte. Linolenic acid was found to be more effective than linolenyl alcohol in expanding the erythrocyte membrane in hypotonic NaCl solution.

Similarly to other stabilizing compounds[3,16], the protective effect of the various lipids tested in the present work, including vitamin A, is reversible: it can be washed away following a single cycle of washing and centrifugation.

DISCUSSION

Numerous different compounds stabilize erythrocytes against hypotonic hemolysis within certain concentration ranges, but enhance hemolysis at higher concentrations. On this basis, Seeman[14] concluded that the stabilizing compounds are nonspecific hemolysins. Although the stabilizing effect is not a unique feature of a single group of chemicals, our data indicate a great measure of specificity within a group of lipids. This specificity is related to the number of double bonds of the hydrophobic chain, the polarity of the molecule and the level of hemolysis at which the compound was added.

To illustrate the relationship between the degree of unsaturation of the hydrocarbon chain and the stabilization potency, relevant data from Figs 1 and 3 are compared in Fig. 5. Alcohols with increasing number of double bonds are accordingly more potent stabilizers. However, the opposite is true for the fatty acids[8], with the obvious exception of stearic acid. These observations support the hypothesis[10,17] that the unsaturated bonds of the lipids contribute to higher osmotic stability of

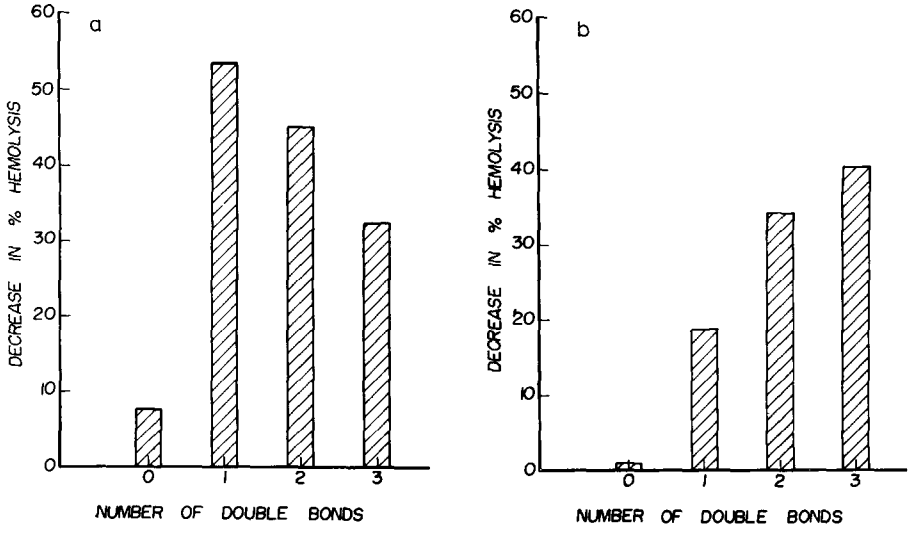

Fig. 5. Decrease in percent hemolysis increment by $2 \cdot 10^{-6}$ M fatty acids (a) and by $1.5 \cdot 10^{-6}$ M fatty alcohols (b); calculated from the data given in Figs 1 and 3. Hemolysis of control erythrocytes: 65%.

erythrocytes. Yet, the data also indicate that the degree of unsaturation is but one of the factors determining the overall stabilizing effect.

A distinctly higher concentration of added lipids was required, at the higher level of hemolysis, to afford maximum stabilization (compare Figs 1 and 2). To account for this increased concentration of the stabilizer required at high hemolysis level, it may be assumed that additional stabilization sites[15] are being unmasked in the erythrocyte membrane with increasing cell volume. The modification revealed with the more hypotonic medium is not only quantitative, but also qualitative: the proposed additional sites interact preferentially with only some of the lipids (Fig. 2, ref. 10) while at lower swelling level the differential lipid effect is less distinct (Fig. 1).

The stabilizing effect involves a hydrophobic interaction of the stabilizer with the erythrocyte membrane[6,7]. However, the stabilizing capacity of stearic acid and of fatty acids methyl esters is clearly inferior to the lesser hydrophobic compounds. Esterification of stearic acid with a polyol[10] results in a more effective stabilizer than the saturated acid itself. Thus, the antihemolytic activity cannot solely be predicted from values of partition coefficient in oil–water or in octanol–water systems. The antihemolytic activities also depend on the limiting aqueous

TABLE I

OCTANOL–WATER PARTITION COEFFICIENTS[19] EXPRESSED AS LOG *P*

Acid	*Log P*	*Methyl ester*	*Log P*	*Alcohol*	*Log P*
Stearic acid	5.43	Methyl ester	5.93	Stearyl alcohol	4.68
Oleic acid	5.36	Methyl ester	5.86	Oleyl alcohol	4.61
Linoleic acid	5.08	Methyl ester	5.58	Linoleyl alcohol	4.33
Linolenic acid	4.80	Methyl ester	5.30	Linolenyl alcohol	4.05

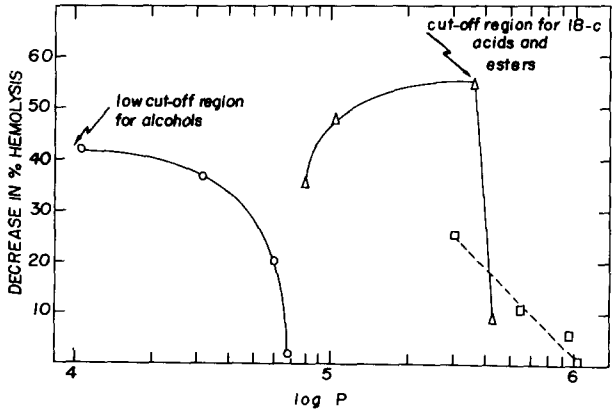

Fig. 6. Decrease in percent hemolysis increment by $2 \cdot 10^{-6}$ M fatty acids, $1.5 \cdot 10^{-6}$ M fatty alcohols and $1 \cdot 10^{-5}$ M fatty acid methyl esters (Figs 1 and 3) as related to the octanol–water partition coefficients of these compounds (Table I, based on ref. 19.), expressed as log *P*.

solubilities of these compounds. The low solubilities will tend to cause the phenomenon of cut-off[18] wherein the compounds mostly exist as aggregates in suspension rather than in the molecular solution. Very few free molecules thus reach the membrane to stop hemolysis. The existence of such aggregates is possible under the conditions of the present experiments since the compounds were added to the aqueous solution immediately before adding the erythrocytes. By graphing the percent decrease in hemolysis (Fig. 5) *versus* the octanol–water partition coefficients of these compounds[19] (see Table I and Fig. 6), it is apparent that a cut-off effect occurs with compounds of very high partition coefficient. The cut-off point for the alcohols may occur at a lower partition coefficient than for the acids and esters.

It may be concluded that the interaction of both the polar group and the hydrophobic portion of a stabilizing compound with the erythrocyte membrane contribute to the overall stabilization effect.

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