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Effect of Some Lysosomotropic Substances on Erythrocyte Membrane

H. Kleszczyńska, J. Łuczyński*, S. Witek*, S. Przestalski

Department of Physics and Biophysics , Agricultural University, Norwida 25, 50-375 Wrocław, Poland
* Department of Chemistry, Technical University of Wrocław, Wrocław, Poland

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Abstract

The effect of N,N-dimethylaminoethyl esters of fatty acids (DM-n) on pig red blood cell hemolysis and erythrocyte membrane fluidity has been investigated. In the hemolytic experiments the hemolytic activity of the compounds studied was determined, and was found to increase with alkyl chain length and followed the sequence: DM-15 > DM-13 > DM-11 > DMDA > DM-9. The fluorimetric studies were done using the fluorescent probe TMA-DPH, which allowed us to calculate polarization coefficient P and hence determine relative changes in membrane fluidity induced by the lysosomotropic substances. The compounds of highest hemolytic activity, which have fifteen- and thirteen-carbon-atom alkyl chain (DM-15 and DM-13), significantly affect the erythrocyte membrane fluidity.

Keywords: Lysosomotropic substances, aminoesters of fatty acids, erythrocyte membrane, hemolytic activity, fluidity

Introduction

The term lysosomotropic substances introduced by de Duve et al. [8] denotes weak basic amines which can pass through biological membranes and can be concentrated several hundred times in lysosomes of mammalian cells. The pK_a values of such lysosomotropic amines lie in the range 5 to 9. If such amines bear a long hydrophobic chain, they become detergents upon protonation inside the lysosomes and interact with the membranes causing a change in its structure and properties; at higher concentrations, disintegration of the membrane can be expected [2]. Release of lysosomal enzymes into cytoplasm could degrade cellular structure and result in cell death [16]. The biological activity of lysosomotropic compounds depends on their basicity and molecular structure [6]. Application to tumour cells control was demonstrated because of lower (about 0.5 unit) pH in tumour cells than that in normal tissues [20]. Some lysosomotropic substances can be applied as anti-MDR drugs [7, 11].

A group of 2-dimethylaminoethyl esters of fatty acids (DM-n) was synthesized in our laboratory as soft (i.e. easily degradable) lysosomotropic substances. The compounds DM-n inhibit the growth of yeast *Saccharomyces cerevisiae* [14] and yeast-like organisms [1]. The inhibition of yeast plasma membrane H^+-ATPase by DM-n compounds was also found [21].

The aim of the studies undertaken was to determine biological activity of the selected lisosomotropic substances with reference to their molecular structure. Therefore, their effect on fluidity of the erythrocyte membrane, as well as hemolytic activity, were investigated. The erythrocyte membrane was chosen as a relatively simple and very useful model of the biological membrane, although erythrocytes have no lysosomes and vacuoles.

Materials and Methods

Chemicals

A series of 2-dimethylaminoethyl esters of saturated fatty acids (structure shown in Fig.1) was synthesized in our laboratory by acylation of 2-dimethylaminoethanol with alkanoyl chlorides and subsequent transformation into their hydrochlorides by passing gaseous HCl through ethereal solution of free aminoester. The DM-n compounds were obtained [1] as white crystals, soluble in water and having high purity (>99%).

N,N-dimethyldodecylamine hydrochloride (DMDA) was prepared from free amine (Aldrich) and gaseous HCl in ethereal solution, m.p. (178-180) 0°C.

The fluorescent probe, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was from Molecular Probes (Eugene, OR, USA).

Hemolytic Studies

The experiments were conducted on fresh, heparinized pig blood. For washing the erythrocytes and in the experiments performed we used an isotonic phosphate solution of pH 7.4 (131mM NaCl, 1.79mM KCl, 0.86mM $MgCl_2$, 11.79mM Na_2HPO_4 $2H_2O$, 1.80mM $Na_2H_2PO_4$ H_2O). Upon removal from plasma, the erythrocytes were washed four times in phosphate buffer and then incubated in the same solution but containing proper amounts of the compounds studied. The modification was conducted at 37°C for 0.5 h, each sample containing 10 ml of erythrocyte suspension of 2%, 4%, 6% and 8% hematocrit, stirred continuously. After modification 1 ml samples were taken, centrifuged and the supernatant assayed for hemoglobin content using a spectrophotometer (Spekol 11,Carl Zeiss, Jena) at 540 nm wavelength [2, 9]. Hemoglobin concentration in the supernatant, expressed as percentage of hemoglobin concentration in the supernatant of totally hemolyzed cells, was assumed as the measure of the extent of hemolysis.

Fluorimetric Studies

Erythrocytes were prepared for modification as in the hemolytic experiments. The modification with the substances studied lasted for 0.5 h at 37°C and 2% hematocrit. After the end of modification the modifier was removed and 5 ml samples were prepared for fluorimetric investigations, the hematocrit being 0.05%. The modified erythrocytes in the samples were labelled with the fluorescent probe TMA-DPH at 10^{-6} M concentration. The measurements were performed with a SFM 25 spectrofluorimeter (KONTRON). On the basis of fluorescence intensity measurements the polarization coefficient was calculated according to the formula [4, 15]:

$$P = \frac{I_{II} - GI_{\perp}}{I_{II} + GI_{\perp}}$$

where

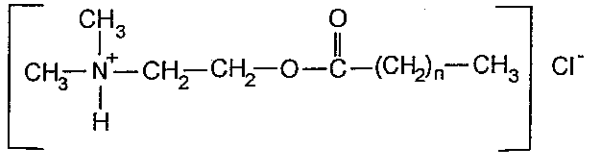
I_{II} – intensity of fluorescence emitted in direction parallel to the polarization plane of the exciting light,

$I_{\perp}$ – intensity of fluorescence emitted in direction perpendicular to the polarization plane of the exciting light,

G – diffraction constant, dependent on wavelength.

Results

The studies performed are concerned with the lysosomotropic compounds shown in Fig. 1; a DMDA compound was included as a standard one. The effect of those substances on red blood cell hemolysis and erythrocyte memb-



n = 8 (DM-9)
n = 10 (DM-11)
n = 12 (DM-13)
n = 14 (DM-15)

Fig. 1. The structure of lysosomotropic substances.

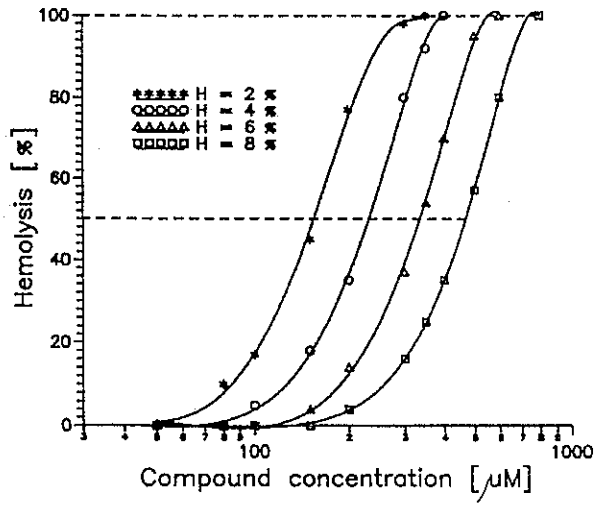


Fig. 2. Relation between percent hemolysis and concentration of compound DM-11 for four hematocrits at pH 7.4 and 37°C.

rane fluidity was investigated. In the hemolytic experiments the hemolytic activity of the compounds and also concentration ranges of their hemolytic activity for four hematocrits were determined. The hemolytic concentrations were found from the relationship between percentage of hemolyzed cells and modifier concentration for the following concentrations of erythrocytes in the solution: 2%, 4%, 6% and 8%. Such relations, in the form of sigmoidal curves, are shown in Fig. 2 for a compound denoted as DM-11. The four hemolytic curves seen in the figure correspond to four different hematocrits. It is known [13] that concentration of the compound that causes hemolysis increases linearly with the number of erythrocytes in the suspension, and thus with increased hematocrit the curves we get are shifted towards higher concentrations. The results of the studies can be presented as linear relations between compound and cell concentrations for 50% and 100% cell hemolysis (as shown in Fig. 2). For 50% hemolysis and all the compounds the dependence is shown in Fig. 3A, and for 100% hemolysis in Fig. 3B. From the plots one can read the compound concentrations that cause 50% and 100% hemolysis for any hematocrit. The bar graphs of Figs. 4 (A, B) represent compound concentrations which cause 50% (Fig. 4A) and 100% hemolysis (Fig. 4B) at unit hematocrit. On the basis of the results shown in Figs. 4A and 4B, the sequence of hemolytic activity of the permeatoxins studied was found to be: DM-15 > DM-13 > DM-11 > DMDA > DM-9. The compound DM-15 was found to be the most active, causing 50% and 100% hemolysis at lowest concentrations compared to the rest of the lysosomotropic substances.

The fluorimetric studies allowed to calculate polarization coefficients for the erythrocyte membrane modified with the compounds studied using the fluorescent probe TMA-DPH. The results represented in Figure 5 indicate that compounds DM-15 and DM-13 cause a decrease of the polarization coefficient in the concentration range 10^{-6} – 10^{-5}M, with respect to unmodified cells. In addition, the polarization coefficient decreases, on the whole, with increasing compound concentration. The greatest changes are induced by DM-15 compound. The decrease in polarization coefficient of erythrocyte membrane induced by compounds DM-15 and DM-13 indicates that the red blood cell fluidity increases in the concentration range studied.

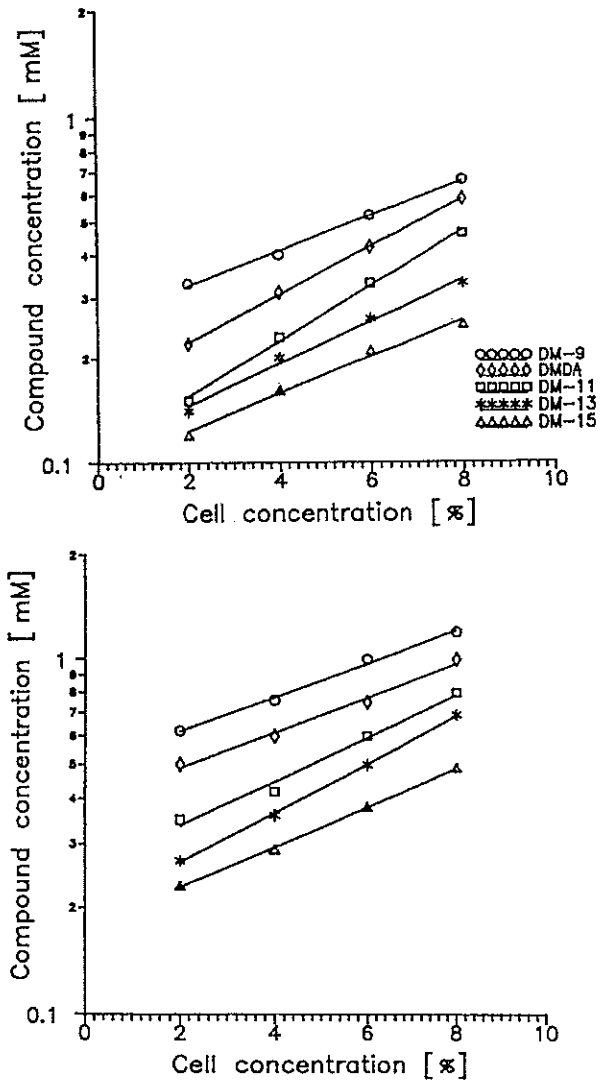


Fig. 3. Relation between cell concentration and compound concentration that causes 50% hemolysis (A) and 100% hemolysis (B), at pH 7.4 and 37°C.

Discussion

Studies have been carried out on the effect of selected lysosomotropic substances (DM-n) on red blood cell hemolysis. The purpose of the investigations was to determine the hemolytic activity of the compounds, which is known to be in direct correlation with biological activity. Various biologically active substances were tested for biological activity on the basis of hemolytic investigations [3, 12, 13, 17, 18].

The lysosomotropic compounds used in this study constitute a homologous series of hydrochlorides, which have identical polar heads but different alkyl chain lengths in the ester moiety (Fig. 1); the compound DMDA has a long alkyl substituent instead of ester fragment in DM-11 compound, which is closely similar in structure. The compounds can exist in a solution in the charged protonated form being in equilibrium with a free amine depending on pH of the milieu. The compounds DM-n have pK_a value about 6.4 for all series members, determined in 90% methanol (Witek et al., date unpublished) so in experimental conditions the charged form dominates.

The concentrations of the compounds that induce 50% and 100% hemolysis at 1% hematocrit (Figs. 4 A and B), found in hemolytic experiments, have been assumed as a measure of their hemolytic activity. The investigation results show that this activity is closely connected with the alkyl chain length of the compound studied. The most active proved to be the hydrochloride DM-15, which caused 50% and 100% hemolysis at lowest concentrations (0.11 and 0.21 mM) compared to the other compounds; DM-15 has the longest alkyl substituent in the ester fragment. The hemolytic activity of the other compounds decreases with decreasing length of their alkyl chains and follows the sequence: DM-15 > DM-13 > DM-11 > DMDA > DM-9. It seems that the length of long hydrophobic substituent plays an essential role; the structure of the substituent has a small influence. A similar relation between hemolytic activity and alkyl chain length has been found for various biologi-

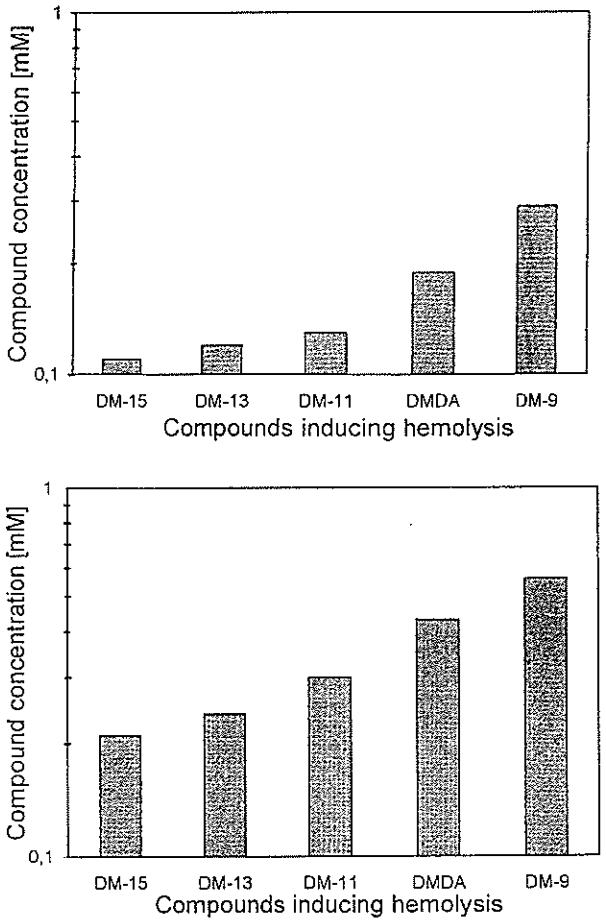


Fig. 4. Compound concentrations causing 50% hemolysis (A) and 100% hemolysis, at 1% hematocrite, pH 7.4 and 37°C.

cally active substances [10, 12, 13]. A similar correlation was also found for yeast plasma membrane H^+ - ATPase inhibitory activity of the DM-n compounds [21].

The molecular mechanism of the interaction between lysosomotropic substances and the biological membrane is not exactly known. Numerous studies have, however, shown that hydrophobicity of the compound plays an important role here. If lysosomotropic substances are cationic, they interact electrostatically with negatively charged

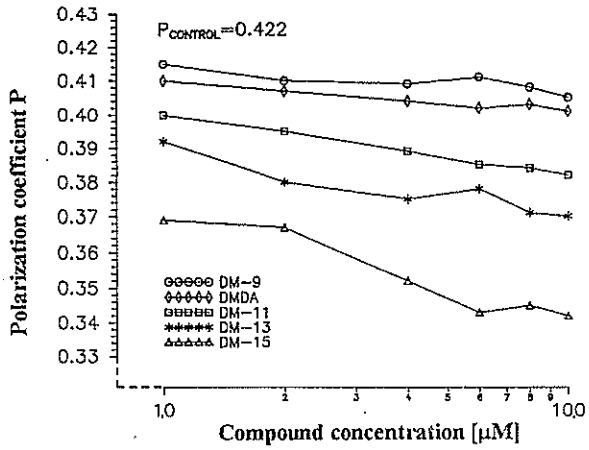


Fig. 5. Polarization coefficient versus concentration of DM compounds.

membrane components in the hydrophilic region. The interaction between the polar heads of the erythrocyte membrane and the charged heads of the compounds favour incorporation of the alkyl chains into the membrane, a process governed by hydrophobic interaction which increases with increasing alkyl chain length. Thus, first the membrane catches the hydrochloride molecules from its surroundings, which after remaining for some time in the polar region incorporates into the membrane hydrophobic core, the incorporation depth depending on the molecule hydrophobicity. The quickly progressing hemolysis in the presence of hydrochlorides indicates that the substances are present mostly in the external layer of the erythrocyte membrane, as suggested by Deuticke [5] and Sheetz and Singer [19]. The membrane changes its properties as a result of structural alterations, which at high enough modifier concentrations leads to cell lysis.

The fluorimetric studies showed that lysosomotropic substances are present in the membrane even when their concentration in the erythrocyte suspension is low and does not cause hemolysis, though it alters membrane fluidity. Compounds DM-15 and DM-13 due to their hydrophobic properties may penetrate membrane and easily affect its fluidity (Fig. 5). A fluidity change may induce disturbance in the membrane transport function and, with increasing modifier concentration, may lead to destabilization resulting in cell lysis.

Acknowledgments

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