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Quantitative Analysis of Hemolytic Action of Lysophosphatidylcholines *in Vitro*: Effect of Acyl Chain Structure

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Hemolysis of rabbit erythrocytes by several lysophosphatidylcholines (lysoPCs) containing C_{12}—C_{18} saturated and *cis* unsaturated acyl chains was investigated *in vitro*. The degree of hemolysis was dependent on the erythrocyte concentration used. Based on this dependency, two determinants for the drugs' hemolytic activity, namely, their affinity for the cell membrane and their intrinsic hemolytic activity, were analyzed separately to clarify the relationship between these factors and the acyl chain structure. Elongation of the acyl chain in saturated lysoPCs was found to greatly increase the affinity and slightly increase the intrinsic activity. In contrast, the introduction of a *cis* double bond into the acyl chain was found to slightly decrease the affinity and to significantly decrease the intrinsic activity. The affinity and the intrinsic hemolytic activity are discussed in relation to critical micellar concentration and bilayer solubilization ability, respectively.

Keywords——erythrocyte; hemolysis; lysophosphatidylcholine; acyl chain; affinity; intrinsic activity; structure–activity relationship; critical micellar concentration; bilayer solubilization

The investigation of hemolysis induced by drugs is of great importance in pharmaceutical sciences not only to evaluate their actions on membranes but also to develop injectable dosage forms of these hemolytic drugs. In recent years, lysophospholipid-type compounds, such as platelet activating factors, have been extensively studied for possible medical applications.[1] The hemolytic activities of lysoPCs and their analogs have been studied to clarify structure–activity relationships.[2] Lysophosphatides are known to hemolyze erythrocytes in two steps, *i.e.* binding of the hemolysin to the membrane and following membrane perturbation. Accordingly, it is important to examine the effect of drug structure on each step separately. However, previous studies on lysoPC-induced hemolysis[2b,c] have delt only with the apparent structure–activity relationship without any consideration of the separate contributions of these two steps to the hemolytic activity. In the case of ether-deoxy type lysoPC analogs,[2a] on the other hand, the hemolytic activity was quantitatively analyzed using ^{14}C-labelled

compounds, although data on unsaturated hemolysin were not obtained.

Thus, to elucidate the effect of the acyl chain structure (chain length and unsaturation) on the hemolytic activity, we quantitatively investigated *in vitro* hemolysis by a series of lysoPCs using the cell concentration variation method,[3] which is experimentally easier than the radioisotope method.

Experimental

Materials——L-α-LysoPCs (lauroyl, myristoyl, palmitoyl, stearoyl, and oleoyl), L-α-egg yolk phosphatidylcholine (egg PC), and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co., Ltd. 1-Anilinonaphthalene-8-sulfonic acid ammonium salt (ANS) was supplied by Wako Pure Chemical Industries Ltd.

Preparation of Erythrocyte Suspension——Defibrinated rabbit blood prepared according to the procedures in JP XI was centrifuged and the supernatant was discarded. The cells were washed three times with PBS (pH = 7.4) and were then resuspended in PBS. The hematocrit values, measured by the Winterobe method,[4] of the suspensions were adjusted to 50% with PBS, corresponding to a cell count of 6.9×10^9/ml.

Hemolysis Measurement——Preincubated (37 °C, 10 min) lysoPC PBS solutions[5] were mixed with small aliquots of the cell suspensions. The mixtures were incubated for 30 min at 37 °C with shaking and then centrifuged to separate unhemolyzed cells. Hemolysis was complete within 1—2 min. The absorbance of the diluted supernatants was measured at 540 nm (A_{sample}) and the percentage hemolysis ($H\%$) of each sample was calculated using the following equation:

$$H\% = (A_{sample} - A_0)/(A_{100} - A_0) \times 100 \quad (1)$$

where A_{100} and A_0 are the absorbances of 100 and 0% hemolyzed cells, respectively, *i.e.* cells incubated in 10-fold diluted PBS or PBS. (LysoPCs were found not to affect the absorption spectrum of released hemoglobin.)

Determination of Critical Micellar Concentration (cmc)——The cmc values of the lysoPCs in PBS at 37 °C were determined by the fluorescence titration method using ANS.[6]

Solubilization of Egg PC Bilayer by LysoPCs——Aliquots of lysoPCs $CHCl_3$ solutions were mixed in test tubes with an egg PC $CHCl_3$ solution. After the solvent had been evaporated, the residual films were dried under vacuum overnight. Nitrogen-bubbled PBS was then poured onto the films. The final concentrations of egg PC and lysoPCs were 100 μM and 0—100 μM, respectively. The sealed tubes were incubated at 37 °C for 2 d. The solubilization was monitored by measuring the absorbances at 600 nm of the vortexed solutions.

Results and Discussion

Lysophosphatides as well as surfactants hemolyze erythrocytes in two steps.[2d,3] The first step is binding of the hemolysin to the membrane. This is followed by membrane perturbation, which results in a permeability change, leading to osmotic rupture of the cell. Consequently, the two determinants for hemolysis are 1) a drug's affinity to the membrane and 2) its membrane-perturbing activity or "intrinsic hemolytic activity."[3] The latter can be expressed as the inverse of the intramembrane drug concentration required to cause hemolysis. That is, if a very small amount of membrane-bound drug causes hemolysis, the intrinsic hemolytic activity of the drug is very high. The relationship between these hemolytic factors and the acyl chain structure will be discussed.

Apparent Hemolytic Activity

Figure 1 shows the dose–response curves for hemolysis induced by various lysoPCs at a cell concentration of 1%. The hemolytic activity depends on the acyl chain structure in the order of stearoyl > palmitoyl > myristoyl > oleoyl > lauroyl, as reported previously.[2b,c] The elongation of the saturated acyl chain enhances, while the introduction of a *cis* double bond reduces, the hemolytic activity. However, the structure–activity relationship in Fig. 1 as well as in the previous reports[2b,c] was an apparent one, because hemolytic dose–response curves reflect both the membrane binding and intrinsic hemolytic activities and only the former depends on the cell concentration. For this reason, cell concentration-independent factors should be compared.

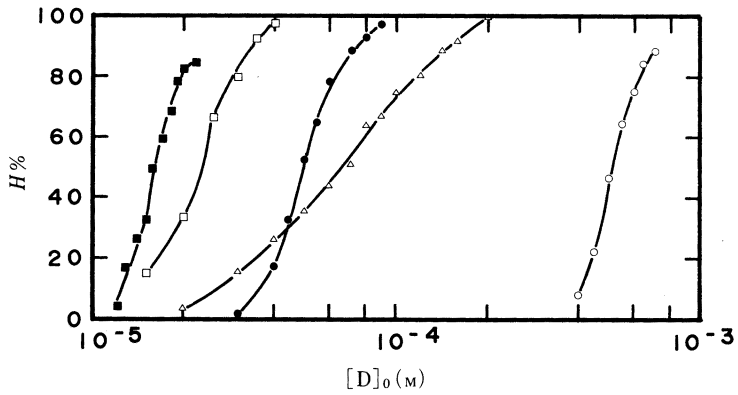

Fig. 1. Dose–Response Curves for Hemolysis Induced by Various LysoPCs at a Cell Concentration of 1%

$[D]_0$, drug (lysoPC) concentration; H%, % hemolysis according to Eq. 1.
○, lauroyl lysoPC; ●, myristoyl lysoPC; □, palmitoyl lysoPC; ■, stearoyl lysoPC; △, oleoyl lysoPC.

Estimation of Free and Intramembrane LysoPC Concentrations

Evaluation of the membrane affinity and the intrinsic hemolytic activity of lysoPCs requires consideration of free and intramembrane lysoPC concentrations, which can be determined by the cell concentration variation method.[3)]

The concentration of lysoPC required for hemolysis was found to depend on the cell concentration as in surfactant-induced hemolysis.[7)] For example, Fig. 2 shows the dose–response curves for myristoyl lysoPC-induced hemolysis at cell concentrations of 0.5—2.0%. An increase in the cell concentration shifts the curve to the right. This suggests binding of the hemolysin to the erythrocyte membrane. The amount bound to the membrane can be estimated using a material balance equation:

$$[D]_0 = [D]_f + [D]_m(V_m/V) \quad (2)$$

where [D] is the drug concentration at a given % hemolysis and subscripts 0, f, and m represent total, free, and intramembrane, respectively. V_m and V are the membrane volume (packed cell volume) and suspension volume, respectively; *i.e.* V_m/V is the membrane volume ratio (hematocrit value divided by 100). Equation 2 is essentially the same as Thron's equation[3)] which is based on cell count: cell volume used in Eq. 2 can be determined more easily. When the drug concentration required to cause a given % hemolysis, $[D]_0$, is plotted against the erythrocyte concentration, V_m/V, the expected linear relation will enable us to evaluate $[D]_f$ and $[D]_m$. Figure 3 shows plots of $[D]_0$ *vs.* V_m/V for myristoyl lysoPC at 10—90% hemolysis. The correlation coefficients of linear regression were greater than 0.990. The $[D]_f$ and $[D]_m$ values at various values of % hemolysis were obtained from the intercepts and the slopes, respectively.

Free Drug Concentration and cmc

Though the necessity of lysolipid micelles for lysis has been debated, a definite conclusion has not yet been reached.[2d)] A comparison of the free drug concentration at which 50% of the cells are hemolyzed (determined above, $[D]_f^{50}$) with the cmc (Table I) will give some information relevant to this debate. Except for myristoyl lysoPC, the $[D]_f^{50}$ values are slightly higher than the corresponding cmc values, suggesting the presence of lysoPC micelles in the hemolysis concentration ranges. If some hydrophobic or amphiphilic membrane components are solubilized by the lysoPC micelles, the cmcs will decrease.[8)] Thus, the micelles may also exist in the case of myristoyl lysoPC-induced hemolysis.

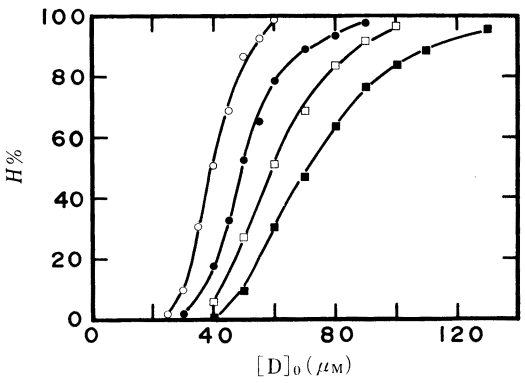

Fig. 2. Dose–Response Curves for Myristoyl LysoPC-Induced Hemolysis at Various Cell Concentrations

Cell concentration: ○, 0.5%; ●, 1.0%; □, 1.5%; ■, 2.0%.

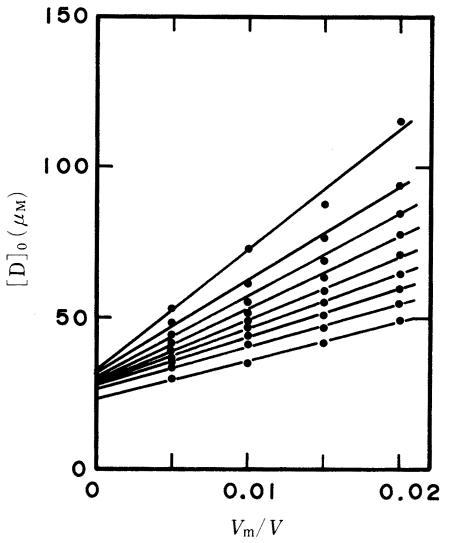

Fig. 3. Determination of Free and Intramembrane Drug Concentrations at Various Values of % Hemolysis

V_m/V, cell volume ratio.
Hemolysis % values are, from the bottom line to the top line, 10, 20, 30, 40, 50, 60, 70, 80, 90%, respectively. Refer to Eq. 2.

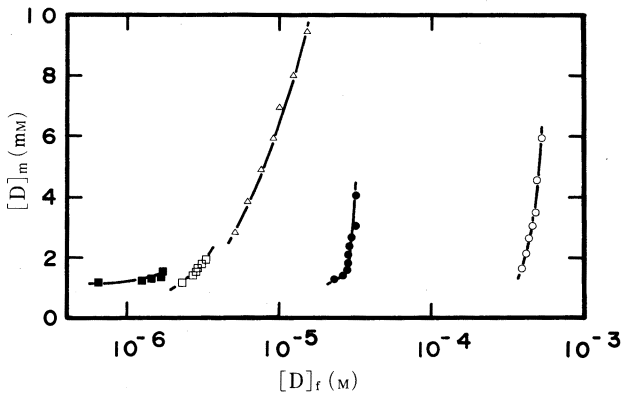

Fig. 4. Adsorption Isotherms for Cell Binding of Various LysoPCs at 37 °C

$[D]_f$, free drug concentration; $[D]_m$, intramembrane drug concentration. The symbols are the same as in Fig. 1.

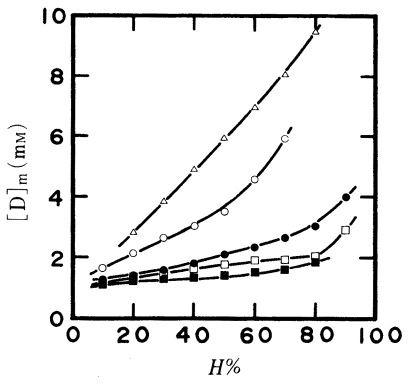

Fig. 5. Relationship between % Hemolysis and Intramembrane Drug Concentration for Various LysoPCs

The symbols are the same as in Fig. 1.

Membrane Affinity

The binding isotherms at 37 °C in the hemolysis concentration ranges for lysoPCs are drawn in Fig. 4 based on the $[D]_f$ and $[D]_m$ values. These isotherms can be explained in terms of neither the Langmuir equation[9] nor the Freundlich equation,[9] as in the case of surfactant-induced hemolysis.[7] Marked changes of the membrane structure in the hemolysis concentration range and/or cooperative binding are considered to be the reasons for the complicated adsorption behavior. Instead of thermodynamic binding constants, apparent partition coefficient K_{50}, defined as $[D]_m^{50}$ divided by $[D]_f^{50}$ can be used as a measure of the

TABLE I. Various Parameters for LysoPC-Induced Hemolysis[a]

LysoPC	cmc (M)	$[D]_f^{50}$ (M)	$[D]_m^{50}$ (M)	K_{50}	$[D]_m^{50}$ (molec./cell)	% Bound[b]
Lauroyl	4.7×10^{-4}	4.8×10^{-4}	3.6×10^{-3}	7.5×10^{0}	1.6×10^{8}	7
Myristoyl	4.8×10^{-5}	3.2×10^{-5}	2.1×10^{-3}	6.6×10^{1}	9.0×10^{7}	40
Palmitoyl	2.9×10^{-6}	3.2×10^{-6}	1.8×10^{-3}	5.8×10^{2}	8.1×10^{7}	85
Stearoyl	2.5×10^{-7}	1.0×10^{-6}	1.1×10^{-3}	1.3×10^{3}	4.8×10^{7}	93
Oleoyl	2.0×10^{-6}	8.2×10^{-6}	5.9×10^{-3}	7.4×10^{2}	2.6×10^{8}	88

a) The listed values except for the cmc values are the averages for 2—3 experiments. *b*) Percent bound at a cell concentration of 1%.

affinity of the lysoPCs to the membrane.[3] The K_{50} values are summarized in Table I.

Intrinsic Hemolytic Activity

The intramembrane drug concentration, $[D]_m$, is plotted against the % hemolysis in Fig. 5. The degree of hemolysis depends on the amount of drug bound to the erythrocyte. The curves can be interpreted as the distribution of the resistance of the cells to lysis. That is, individual cells have different threshold $[D]_m$ values for hemolysis. The distribution of resistance may be related to the cell age as in chlorpromazine-induced hemolysis.[10] Since the intramembrane drug concentration necessary for hemolysis depends on the resistance of the cells, it is reasonable that the intrinsic hemolytic activity, which should be characteristic for the drug and the erythrocytes used, is defined as the inverse of the intramembrane drug concentration at 50% hemolysis ($[D]_m^{50}$). The $[D]_m^{50}$ values are given in Table I.

The $[D]_m^{50}$ values expressed as lysoPC molecules per cell are also listed in Table I. These values are approximately equal to those reported for ether-deoxy lysoPCs (9.5×10^7, 8.1×10^7, 6.2×10^7, and 7.1×10^7 molecules/cell for lauroyl, myristoyl, palmitoyl, and stearoyl lysoPCs, respectively), directly determined using ^{14}C-labeled compounds.[2d] This result shows the validity of our analysis method. The $[D]_m^{50}$ values are comparable to the phospholipid content of the erythrocytes. Rabbit erythrocytes have been reported to contain 3.0 mg of phospholipid per ml of packed cells,[11] corresponding to 3.8 mM on the assumption that the phospholipids have an average molecular weight of 800. Thus, large amounts of lysoPCs, comparable to the amount of phospholipid, must bind to the cell for hemolysis.

Structure–Activity Relationship

The relationship between the two determinants for the hemolytic activity of lysoPC (the membrane affinity and the intrinsic hemolytic activity) and the acyl chain structure of the lysoPC are discussed below.

For saturated lysoPCs (C_{12}—C_{16}), the membrane affinity, expressed as K_{50}, increases by a factor of *ca.* 9 with the addition of two methylene groups to the acyl chain (Fig. 6a). The slope of the linear portion is considered to be related to the free energy of transfer of a methylene group from the aqueous phase to the "membrane phase."

A similar phenomenon was observed in the free energy change for micellization. LysoPCs are known to form micelles in aqueous solutions.[6] The cmc values in PBS at 37 °C were determined by a fluorometric method.[6] The plot of $-\log$ cmc *vs.* acyl chain carbon number (Fig. 6b) for the saturated lysoPCs (C_{12}—C_{18}) shows a linear relationship as reported previously under other experimental conditions.[6b] The slope is proportional to the micellization energy per methylene group, *i.e.* the free energy of transfer of a methylene group from the aqueous environment to the hydrophobic "micellar phase."[8]

The presence of a *cis* double bond in the acyl chain was found to reduce the membrane

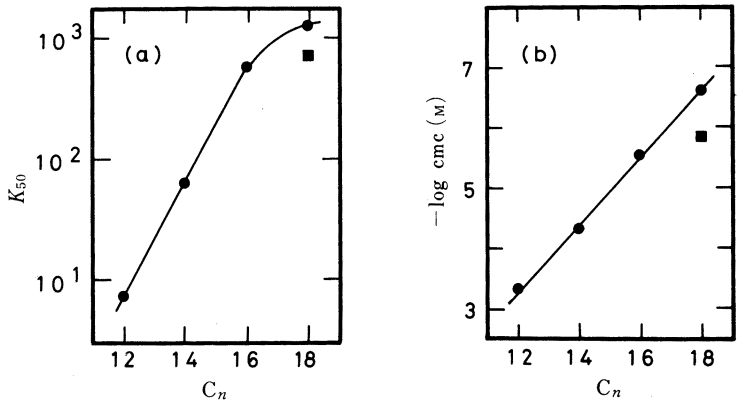

Fig. 6. Dependence of Affinity and of cmc on Acyl Chain Structure of LysoPC

Circles, saturated lysoPC; squares, *cis* unsaturated lysoPC; C_n, carbon number of lysoPC acyl chain.

(a) K_{50}, apparent partition coefficient of lysoPC to the cell at 50% hemolysis. Each point represents the average of 2—3 experiments.

(b) cmc values were determined by a fluorescence titration with ANS.[6]

affinity as well as the micellization ability (*i.e.* increased the cmc) of lysoPCs, reflecting the hydrophilic character of the double bond. It is known from solubility and micellization studies that the introduction of a double bond is equivalent to the removal of 1 to 1.5 carbon atoms from a saturated chain.[8]

The similarity of the membrane affinity to the micellization ability suggests the importance of hydrophobic interactions in the binding of lysoPCs to the erythrocyte membrane. However, as reported for deoxy-lysoPC,[2a] the membrane affinity of stearoyl lysoPC is less than the value predicted from the linear relationship (Fig. 6a). The reason may be that most membrane-constructing phospholipids contain C_{16}—C_{18} fatty acyl chains.[4]

For saturated lysoPCs the intrinsic hemolytic activity ($1/[D]_m^{50}$) was found to increase with the number of carbon atoms in the acyl chain (Fig. 7a). Namely, lysoPC containing an acyl chain similar to that of membrane phospholipid shows greater membrane-perturbing activity. However, the increase in membrane perturbation per methylene group is not as marked as the increase in membrane affinity. In contrast, the presence of a *cis* unsaturated bond in the acyl chain reduces the intrinsic hemolytic activity by a factor of 5.

The intrinsic hemolytic activity is closely related to the mechanism of the membrane perturbation. The membrane lipid region is the most plausible candidate for a point of action.[2d] Accordingly, the solubilization of lipid bilayers by lysoPCs was examined. The presence of increasing amounts of lysoPCs in lipid bilayers is known to cause the solubilization of the membranes (bilayer to mixed micelle transition).[12] The lysoPC to egg PC molar ratios at which the solubilization occurred are plotted in Fig. 7b. For saturated lysoPCs the bilayer solubilization ability is related to the intrinsic hemolytic activity. However, in spite of its low intrinsic hemolytic activity the solubilization ability of oleoyl lysoPC is even higher than that of stearoyl lysoPC. Reman *et al.*[2c] reported that the bilayer membrane of lipids extracted from bovine erythrocytes is destroyed by oleoyl lysoPC as effectively as by stearoyl lysoPC.

In the interaction of plasma membrane and liposome with various types of long-chain alkanoic acids, the *cis* unsaturated alkanoic acids are preferentially partitioned to the liquid crystalline phase, while the saturated and *trans* unsaturated ones to the gel phase.[13] If the erythrocyte membrane consists of membrane proteins and lipid microdomains, it is plausible that the two types of lysoPCs, the saturated and the *cis* unsaturated, may be preferentially

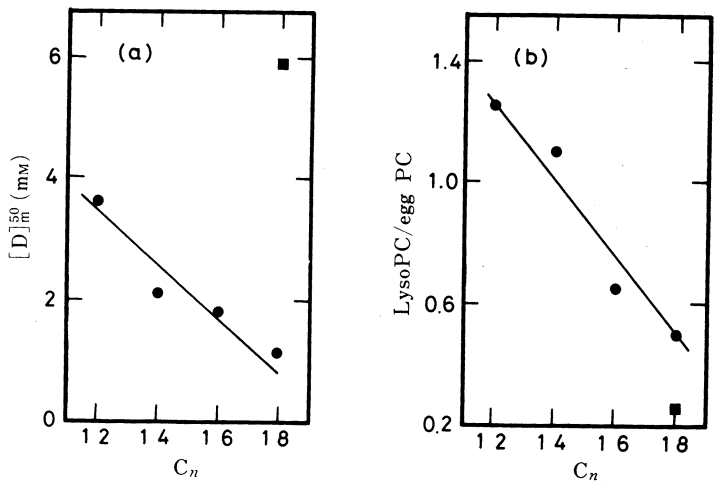

Fig. 7. Dependence of Intrinsic Hemolytic Activity and of Bilayer Solubilization Ability on Acyl Chain Structure of LysoPC

Circles, saturated lysoPC; squares, *cis* unsaturated lysoPC; C_n, carbon number of lysoPC acyl chain.

(a) $[D]_m^{50}$, intramembrane lysoPC concentration at 50% hemolysis (inverse of intrinsic hemolytic activity). Each point represents the average of 2—3 experiments.

(b) LysoPC to egg PC molar ratio at the solubilization of the egg PC bilayer.

partitioned to the gel-like and liquid crystalline-like domains, respectively. This assumption can be supported by the fact that such a difference between the saturated and the *cis* unsaturated lysoPCs was not observed in the solubilization of the bilayers of extracted lipids[2c] and egg PC as mentioned above. In the case of the alkanoic acids,[13] no difference in membrane fluidizing effects, determined by measuring the fluorescence polarization change of diphenylhexatriene, has been also reported in the homogeneous liquid crystalline bilayer of egg PC. The detailed mechanism of the membrane perturbation by these two kinds of lysoPCs is not obvious at the present stage. However, the perturbing ability of stearoyl lysoPC to the gel phase seems to be more effective than that of oleoyl lysoPC to the liquid crystalline phase. The importance of lipid domains or phase boundaries for the permeability change by lysoPC has been suggested.[2d]

The apparent hemolytic data (Fig. 1) will be reconsidered on the basis of the above quantitative analysis. The percentages of the lysoPCs bound at a cell concentration of 1%, calculated from the $[D]_f^{50}$ and $[D]_m^{50}$ values, are listed in Table I. In the case of palmitoyl, stearoyl, and oleoyl lysoPCs, *ca.* 90% of the hemolysin was bound to the cell because of their high affinity, so that the difference in hemolytic concentration results practically from the intrinsic activity difference. On the other hand, in the case of myristoyl and lauroyl lysoPCs, the contribution of the affinity to the hemolytic concentration predominates over that of the intrinsic activity.

Thus, after analyzing the cell concentration dependency of the hemolytic dose–response curves the hemolytic activity of the lysoPCs can be described in terms of two hemolytic determinants, the membrane affinity and the intrinsic hemolytic activity. Structure–activity relationship studies such as the present report based on these essential factors are a useful guide to the understanding of the membrane-perturbing action of this type of drugs and to the development of injectables containing them.

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