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QUANTITATIVE STUDIES ON LYSOLECITHIN-MEDIATED HEMOLYSIS

USE OF ETHER-DEOXY LYSOLECITHIN ANALOGS WITH VARYING ALIPHATIC CHAIN-LENGTHS

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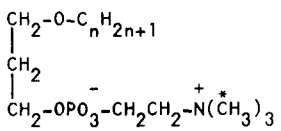
Summary

The process of lysolecithin-mediated hemolysis has been investigated by use of various ether-deoxy lysolecithin analogs (1-alkyl-propanediol-3-phosphoryl-choline) with alkyl residues of 10—22 carbon atoms. Hemolytic activities were defined either as molar amounts to be added for 50% lysis (L_{50}) or as cell-bound amounts at 50% lysis (A_{50}). It was found, that in contrast to L_{50}, A_{50} values are independent of experimental conditions. Moreover, L_{50} values primarily reflect the binding affinities, while A_{50} values give more accurate information on the actual membrane-disturbing potential. The strongest hemolytic C_{16}-lysolecithin analog required $2 \cdot 10^7$ or $5 \cdot 10^7$ molecules bound per cell for 50% lysis at 0 or 37°C, respectively, corresponding to about 10 or 25% of the total membrane phospholipids. Evidence is presented, indicating that (a) lysophosphatides bind to cells below their critical micelle concentration, (b) micelles themselves are not generally necessary for cell lysis.

Red cells of different species (man and cattle) as well as at varying temperatures exhibit significantly different sensitivities in terms of L_{50} and A_{50} values. These differences, however, depend on the degree of hydrophobicity of the lysolecithins and disappear in the case of lysolipids having C_{10} or C_{12} aliphatic residues. The data are in agreement with our hypothesis that cellular sensitivity to lysolecithin lysis may be determined by the degree of segregation of lysolecithin-rich areas within the membrane lipid phase.

Introduction

A detailed understanding of the molecular processes involved in lysolecithin-induced red cell lysis may be helpful in the elucidation of the mode of interaction of lysolipids and detergents in general with biological membranes. As has



n = 10, 12, 14, 16, 18, 22

Fig. 1. Chemical structure of ether-deoxy lysolecithins, i.e. 1-alkyl-propanediol-3-phosphorylcholine. * Indicates position of ^{14}C-label in radiolabelled compounds [6].

been pointed out previously [1—3], synthetic analogs of lysolecithin offer a number of advantages in such investigations as compared to lysolecithin isolated from natural sources: (a) their chemical structure is well defined and can be altered at will, (b) depending on their structure, they may be fully resistant to metabolism by red cell-associated enzymes [4,5], and (c) they can easily be labelled with radioactive isotopes [6]. For the present study we used a homologous series of so-called ether-deoxy lysolecithins (Fig. 1), which are phosphorylcholine esters of propanediol-(1,3)-monoethers, and have been first described in 1966 [1,7].

We have used these substances of greatly varying hydrophobicity to investigate lysophosphatide binding, hemolytic activity, effects of temperature and cell species as well as critical micelle concentration and micellar size in relation to the length of the aliphatic chain in the lysolecithin analogs. Some of these data have been presented earlier in a preliminary report [8].

Materials and Methods

Lysophosphatides. Chemical synthesis and radiolabelling of ether-deoxy lysolecithin analogs (Fig. 1) have been described before [1,6]. The C_{10}—C_{16} analogs are readily soluble in water or buffers, whereas the C_{18} and C_{22} derivatives had to be sonicated for 30 s before use. The C_{16} analog is in all its physicochemical properties, including behaviour on thin-layer plates, surface activity and micelle formation, highly similar to lysolecithin isolated from natural sources.

Hemolysis and binding assays. Binding of ^{14}C-labelled lysophosphatides and lysis experiments were performed according to published procedures [3,9]. Standard conditions were as follows: $9.4 \cdot 10^7$ or $5.5 \cdot 10^7$ cells (for bovine or human erythrocytes, respectively) were suspended in 1.2 ml of 0.1 M phosphate buffer in 0.85% NaCl at pH 7.2. This corresponds in both cases to about 90 cm^2 of cell surface per sample [10].

Critical micelle concentration and micellar size. Critical micelle concentrations were determined by the dye inclusion method with methylorange [11], as well as by light scattering, as described before [12]. Micellar size was extrapolated from Zimm [12,13] plots of light scattering data. Scattering intensities were strongly angle dependent for C_{18} and C_{22} analogs, but not for the shorter chain derivatives. All data given for 37° C.

Results

Hemolytic activities and binding characteristics at 37° C

It is well established by the work of Reman et al. [2] and ourselves [1] that the hemolytic activity of lysolecithin as well as of its 2-deoxy analogs is optimal for aliphatic chain-lengths of 16—18 carbon atoms (see curve 1 in Fig. 2a). The amount of the C_{10} analog necessary for 50% lysis is about 250 times higher than that of C_{16}, and also the L_{50} value of the very long chain C_{22} analog is 4—5 times higher than that of the hexadecyl derivative. To decide, whether these differences in the lytic activities might be explained on the basis of different binding affinities of these substances to red cells, we have labelled all of them with ^{14}C in the choline methyl groups [6] and determined binding equilibria at 37° C at various lipid concentrations. From Scatchard plots [14] of these data we have calculated the equilibrium constants K_c, which are plotted versus lysolipid chain-length in Fig. 2b. It is immediately evident that the K_c values behave exactly inversely to the L_{50} values, i.e. in parallel to hemolytic activities, in that the optimal affinity to red cells is found for the C_{16} analog.

This finding allows a rather simple explanation of the afore-mentioned differences in lytic activity in that apparently most of the strongly lytic agents

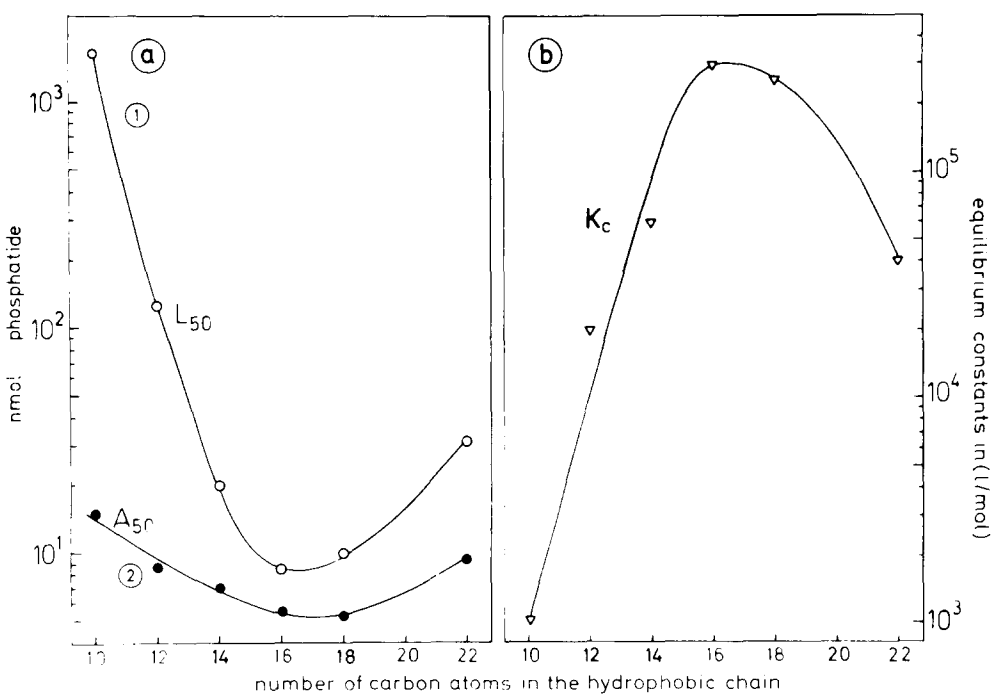


Fig. 2. Hemolytic activities and equilibrium constants of ether-deoxy lysolecithins for human erythrocytes at 37° C. $5.5 \cdot 10^7$ cells per 1.2 ml. L_{50} = amount of lysolecithin (nmol) added per sample for 50% hemolysis. Determined from dose vs. lysis curves after 4 h incubation. A_{50} = cell bound amount (nmol per total cell sediment) of ^{14}C-labelled lysolecithin at 50% lysis. Determined from binding vs. lysis curves after 4 h incubation. K_c = Equilibrium constants of binding. Calculated from Scatchard plots [14] of binding equilibria of ^{14}C-labelled lysolecithins at varying concentrations.

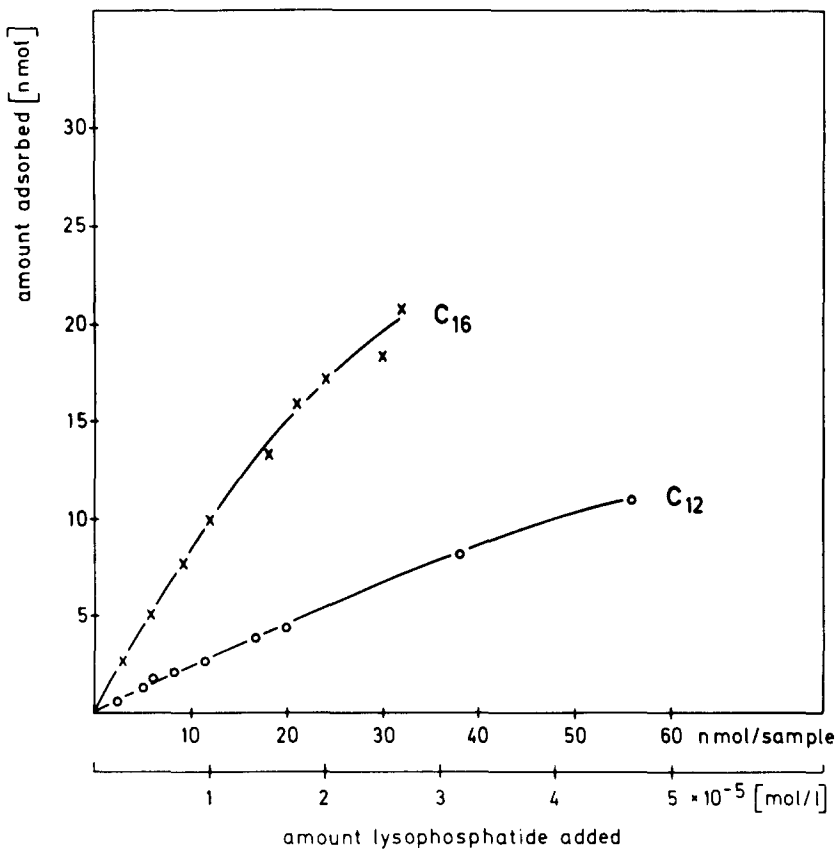


Fig. 3. Binding of ^{14}C-labelled C_{12} and C_{16} ether-deoxy lysolecithins to human erythrocytes at 37° C. Cell count ($2.2 \cdot 10^8$ cells/1.2 ml) differs from standard conditions (see Materials and Methods and Fig.2). Note that all concentrations of the C_{12} derivative are well below its critical micelle concentration of $2 \cdot 10^{-4}$ M (see Table I).

is adsorbed to the cell surface, whereas of the derivatives with shorter or longer chains only a small proportion will find its way to the cellular membrane (see also Fig. 3).

To determine the actual "membrane-disturbing activity" of our phosphatides, we have therefore previously introduced the so-called A_{50} value [10], i.e. the amount of lysolipid actually adsorbed to the cells at 50% lysis. These values show much less variation for the different lysolecithins (see curve 2 in Fig. 2a), but they also exhibit a significant minimum for C_{16}. This indicates that also when lytic activity is expressed in terms of the number of molecules that have to be bound to produce lysis, the hexadecyl derivative is the most active one of this series of lysolecithin analogs. C_{16} requires about $5 \cdot 10^7$ molecules bound per cell to produce 50% hemolysis with human erythrocytes at 37° C. Assuming a surface pressure of some 30 dynes/cm in the erythrocyte membrane, as proposed by Demel et al. [15], at which pressure the C_{16} analog occupies an area of 45 Å^2 per molecule (own unpublished data), this amounts to a total area of $23 \cdot 10^8$ Å^2 per cell. Taking a value of $163 \cdot 10^8$ Å^2 [10] for the surface of the

human erythrocyte, C_{16} covers about 8—14% of that surface at 50% lysis, depending on whether it is included in one or both halves of the membrane double layer.

Effects of cell concentration on the lytic reaction

The importance of defining hemolytic activities by the determination of cell-bound (A_{50}) rather than added (L_{50}) amounts of lysolecithin is underlined by the following experiment: L_{50} and A_{50} values for C_{12} and C_{16} analogs were determined at cell concentrations varying from $2 \cdot 10^7$ to $25 \cdot 10^7$ cells/ml. As shown in Fig. 4a, only the L_{50} values for C_{16} are related to cell density by a linear function, whereas the curve for C_{12} is bent and flattens towards higher cell concentrations. Hence, L_{50} values allow no absolute determination of lytic amounts per cell for short chain lysolecithins. In contrast to that it may be seen from Fig. 4b that the A_{50} values for both substances are in a perfectly linear relation to cell concentration, allowing accurate determinations of the numbers of molecules per cell necessary for lysis, independent of experimental conditions.

The different behaviour of long- and short-chain lysolipids in these experiments may be explained by the data in Fig. 4c. Here we have plotted the partition quotient of the two ^{14}C-labelled lysolecithins between cell sediment and supernatant at a constant relation of added lysolecithin per cell (1.5 nmol per 10^7 cells) as a function of cell density. It is clear from these data that in contrast to C_{16}, this quotient is strongly dependent on cell density for C_{12}. We assume that this is due to the fact that C_{16} is always employed above, while C_{12} is used below its critical micelle concentration (see Table I). The concentration of free molecules of lysolecithin will thus be constant under all conditions for the C_{16}, while it is increasing with lysolecithin and cell concentration for the C_{12} derivative.

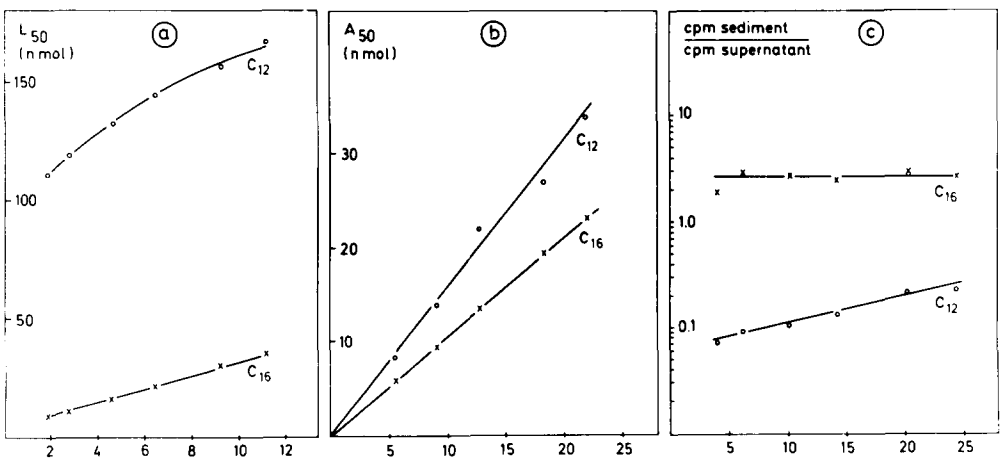


Fig. 4. Effects of cell concentration on hemolysis and binding of C_{12} and C_{16} ether-deoxy lysolecithins at 37° C. (a) L_{50} values and (b) A_{50} values in relation to cell concentration. (c) Partition quotient of ^{14}C-labelled lysolecithins between cell sediment and supernatant at a constant ratio of 1.5 nmol of lysophosphatide per 10^7 cells as a function of cell concentration.

TABLE I

CRITICAL MICELLE CONCENTRATION AND MICELLAR SIZE OF ETHER-DEOXY LYSOLECITHINS

Values for critical micelle concentrations are averages from determinations of spectral shift of methylorange at 484 nm [11,12] and of light scattering data [12]. Micellar weights were determined from Zimm plots [12,13] of light scattering intensities at varying lipid conditions.

	Number of carbon atoms per chain					
	10	12	14	16	18	22
Critical micelle concentration (mol/l)	$2 \cdot 10^{-3}$	$2 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	10^{-5}–10^{-6}	10^{-5}–10^{-6}	$<10^{-6}$
Micellar weight	15 000	30 000	46 000	68 000	$2.5 \cdot 10^{6}$	8×10^{9}
Molecules/micelle	40	70	100	140	4 900	14×10^{6}

Critical micelle concentration, micelle structure and hemolysis

To relate the hemolytic activities of the various lysophosphatides to their physical properties in aqueous solution, we have determined the critical micelle concentrations as well as the micellar sizes of all substances. These data are summarized in Table I. The micelle concentrations represent the average of data obtained by the method of dye-inclusion [11] and by light scattering under a 90° angle [12]. The apparent micellar weight was determined from Zimm plots [13] of light scattering intensities at various lipid concentrations. The scattering intensities were independent of the scattering angle for the C_{10}—C_{16} lysolipids, but exhibited increasing angle dependence for the C_{18} and C_{22} analogs, respectively. In fact, the value for C_{22} represents data obtained 1 h after sonication, while after prolonged standing at 37° C this substance tends to aggregate to even larger particles.

The critical micelle concentrations obtained were reproducible and accurate within a limit of some 20% for the C_{10}—C_{14} compounds. With longer chain lysolipids, however, the resulting curves were without distinct breaks and thus only rough estimates of the micelle concentrations were possible (see Fig. 5). It is not clear at present, whether this is due to the presence of different types of micelles or simply to insensitivity of the detection methods at such very low lipid concentrations.

In comparison with the data of Fig. 2 it is apparent, that the increasing L_{50} values in the order $C_{16} \rightarrow C_{10}$ as well as the decrease in binding affinities can be related to the increase in critical micelle concentrations from about $5 \cdot 10^{-5}$ M for C_{16} to $2 \cdot 10^{-3}$ M for C_{10}. This means, that it is mainly the degree of hydrophobicity, which determines L_{50} values in this homologous series of detergents. The micellar size does not seem to be of great importance in this respect, since it changes only by a factor of 4.5 from the C_{10} to the C_{16} analog, whereas the hemolytically similar compounds with C_{16} and C_{18} chains differ in the size of their aggregates by a factor of 37. On the other hand, this sudden increase of micellar size for lysolecithins with more than 16 carbons per aliphatic chain may explain the somewhat unexpected decrease of lytic activity and binding affinity for long-chain derivatives. Assuming that lysolecithins adsorb to cellu-

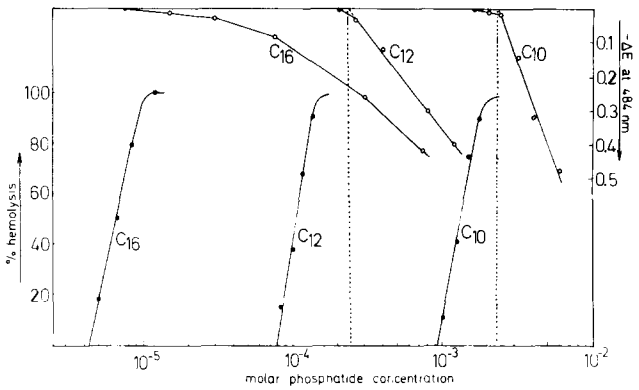


Fig. 5. Comparison of critical micells concentrations of deoxy lysolecithins with their hemolytic activities towards human erythrocytes at 37° C. Upper part: changes of extinction of 20 nM methylorange solution in 20 nM Tris buffer, pH 7.2, at 484 nm in relation to lysolecithin concentration. Lower part: Degree of hemolysis under standard conditions (see Materials and Methods) in relation to lysolecithin concentration. Dotted lines indicate critical micelle concentrations.

lar surfaces as single molecules, micelles of increasing size (i.e. apparently of greater micellar stability) in connection with lower critical micelle concentration would increasingly compete with the cellular membrane for lysolecithin molecules and thus lead to lower apparent binding affinities. This, however, brings up the questions of whether lysolecithin binds to cells in the form of micelles or of single molecules, and whether micelles are required for lysis, or not.

We have collected some data which may substantiate our previous notion [12] that the presence of micelles is not a necessary prerequisite for lysolecithin-mediated cell lysis, and that binding of lysolipids to the cells is controlled by the equilibrium between a monomolecular solution and molecules embedded in the cellular membrane. The fact that single molecules of lysolecithin do adsorb to cells is clearly proven by the binding curve in Fig. 3, which shows binding of the C_{12} lysolipid to red cells at less than 1/10 of its critical micelle concentration (Table I). Moreover, for the C_{10} and C_{12} lysolecithins hemolysis begins at 1/2 to 1/3 of their critical micelle concentrations (Fig. 5). Finally, we have followed the adsorption and lysis kinetics of mixed micelles of C_{16} and C_{22} lysolecithins in which either one of the two constituents had been labelled with ^{14}C. Fig. 6a shows the binding data, Fig. 6b the hemolysis curves of these experiments. An amount of 10 nmol C_{16} alone leads to very rapid binding and hemolysis (about 65%). Equilibrium is reached in both cases within 1—2 min (see curves 1 and 1^x). The adsorption kinetics of 20 nmol of the pure ^{14}C-labelled C_{22} lipid (which does not cause lysis at 37° C) is represented by curve 3. When 10 nmol of C_{16} and 20 nmol of C_{22} are co-sonicated, mixed micelles are formed as indicated by the strongly retarded hemolysis (curve 2^x in Fig. 6b). Binding of C_{22} is not significantly altered under these conditions (curve 2b), whereas C_{16} binds much slower than in its free form (curve 2a vs. 1).

To evaluate the significance of curve 2a, we included the experiment represented by curve 4 in Fig. 6b. In this case, where 20 nmol C_{16} analog were mixed with 20 nmol of C_{22}, it is apparent that part of C_{16} derivative is not

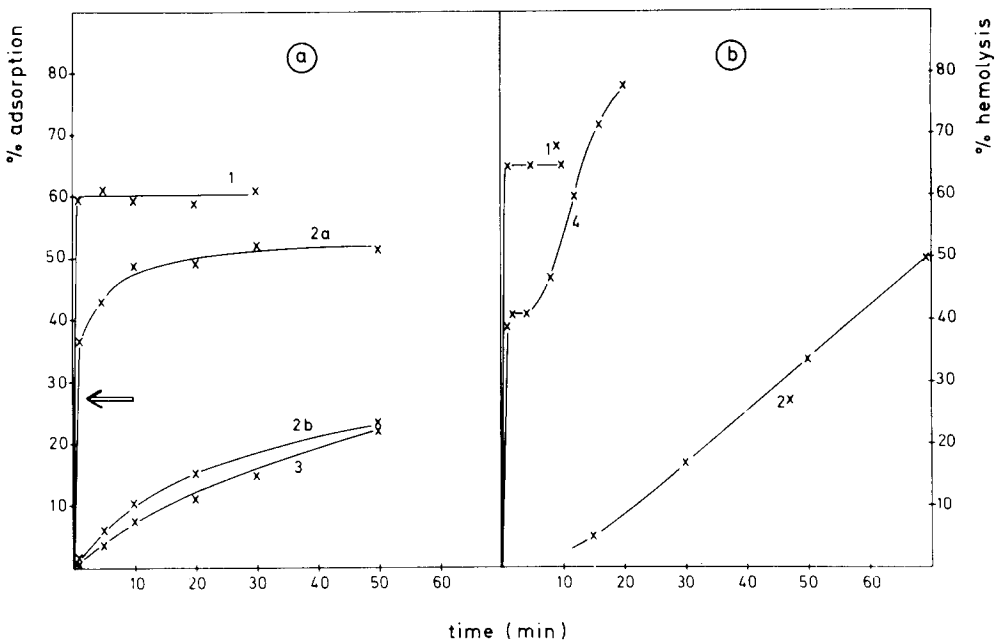


Fig. 6. Binding and lysis kinetics of mixtures C_{16} and C_{22} ether-deoxy lysolecithins to human erythrocytes at 37°C. Lipid concentration per $5.5 \cdot 10^7$ cells in 1.2 ml were for curves 1 and 1^x: 10 nmol ^{14}C-labelled C_{16}; curves 2a and 2^x: 10 nmol ^{14}C-labelled C_{16} + 20 nmol C_{22}; curve 2b: 10 nmol C_{16} + 20 nmol ^{14}C-labelled C_{22}; curve 3: 20 nmol ^{14}C-labelled C_{22} and curve 4: 20 nmol ^{14}C-labelled C_{16} and 20 nmol C_{22}. 8-fold amounts of these values were dispersed in 9.2 ml of buffer, sonicated three times for 15 s with 1-min interruptions without cooling, using a Branson sonifier model G 7061 F (amplification 4), equipped with a microtip. Solutions were then placed into a 37°C water bath and mixed after 5 min with 400 µl of a red cell suspension of $1.1 \cdot 10^9$ cells/ml. Samples were drawn after various intervals and adsorption and lysis determined as described under Materials and Methods.

included into the mixed micelles, leading to a fast primary hemolytic reaction, (corresponding to lysis by about 8 nmol C_{16}), followed by a retarded secondary process. From simultaneous binding experiments it was found, that during the first 1—3 min only 30%, i.e. 6 nmol, of the added 20 nmol of C_{16} were adsorbed to cells. Assuming that also in the 1 : 2 mixtures of the two lipids, depicted by curves 2a and 2b, about 30—40% of the C_{16} would be free, this could lead maximally to an immediate binding of some 25—30% (see arrow) of the added ^{14}C-labelled C_{16} analog. Probably, with the excess of long-chain lysolipid the percentage of free C_{16} will be even lower. Under these conditions, however, it is clearly evident that the two constituents of the mixed micelles bind to erythrocytes with different kinetics (curves 2a and 2b). Hence, we conclude that the micelles do not bind to the cells and that the two lysolecithin derivatives are released from them at different rates.

Effects of temperature and cell type on hemolysis

The ether-deoxy lysolecithins with C_{16} or C_{18} side chains exhibit a pronounced temperature dependence of their hemolytic activity as has been described earlier for natural lysolecithin and various synthetic analogs [1,4,16]. Thus, L_{50} for the C_{16} analog is reduced at 0°C from 8 to 3.2 nmol. Accordingly, the

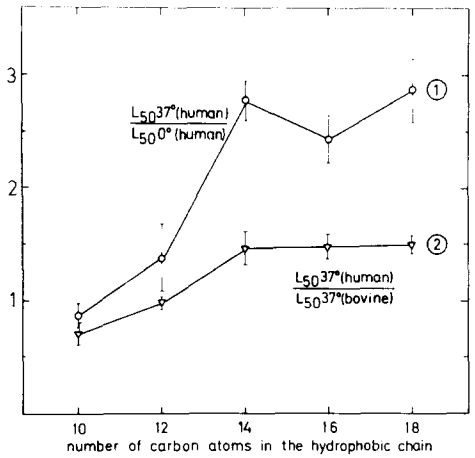


Fig. 7. Relation of L_{50} values for human erythrocytes at 37 and 0°C or for human and bovine red cells at 37°C as a function of lysolecithin chain-length. L_{50} values for individual lysolecithins were determined under standard conditions (see Materials and Methods) from dose vs. lysis curves. Curve 1: relation of L_{50} at 37°C to L_{50} at 0°C for human red cells; curve 2: relation of L_{50} for human to L_{50} for bovine erythrocytes, both at 37°C.

number of cell-bound molecules causing 50% lysis at 0°C drops to about $2 \cdot 10^7$ molecules/cell, which is in the order of only 10 mol % of the total cell phospholipid content or less than 10% of the cell surface.

The temperature dependence of lytic activity, however, largely depends on the chain-length of the lysolipid. In Fig. 7 we plotted in curve 1 the relation of L_{50} values for human red cells at 37°C over the L_{50} values at 0°C as a function of the number of carbon atoms in the aliphatic chain. As mentioned above, this relation is between 2 and 3 for the C_{16}—C_{18} analogs. The value for C_{14} was also found in this range. For C_{12} and C_{10}, on the other hand, this quotient is close to 1, i.e. for these substances there is hardly any difference between their hemolytic activities at 37 and 0°C. The assay system thus reveals a principal difference in the mode of interaction of long- and short-chain lysolecithins with cellular membranes.

Interestingly, a very similar result is obtained, when the quotient of L_{50} for human over L_{50} for bovine erythrocytes (both at 37°C) is compared for the various deoxy lysolecithins (see curve 2, Fig. 7). Like natural and for instance benzyl lysolecithin [9], C_{14}—C_{18} deoxy lysolecithins show a significantly higher lytic activity (i.e. lower L_{50} values) against bovine than human erythrocytes. This difference, however, is completely abolished for the C_{12} analog, and for C_{10} even a reverse relation has been found. No differences in binding to human or bovine red cells have been found for any of the ether-deoxy lysolecithins employed in this study, which is in full agreement with our findings for benzyl-lysolecithin [9].

Discussion

The process of lysolecithin-mediated hemolysis is still not well understood. The present study contributes some new experimental results to three particu-

larly interesting questions: (1) what are the properties required for a strongly hemolytic molecule, (2) what determines the sensitivity of different red cells to a given hemolytic substance, and (3) what is the role of temperature in the hemolytic reaction.

We have used a homologous series of ether-deoxy lysolecithin analogs (see Fig. 1), which in contrast to "natural" lysolecithin are not metabolized by red cell-associated enzymes [3]. Their hemolytic properties, however, are well comparable to the natural compounds with corresponding aliphatic chain-length [1,3].

The use of ^{14}C-labelled derivatives enabled us to define hemolytic activity in addition to the commonly used L_{50} dose [1,16] by what we call the A_{50} dose, i.e. the molar amount of cell-bound lysolecithin necessary for 50% lysis [9]. A_{50} thus allows one to determine the number of molecules bound per cell that cause membrane disruption, while L_{50} was shown to be simply a reflection of binding equilibrium constants (K_c). Nevertheless, also the A_{50} values vary with the chemical structure and like L_{50} they, too, exhibit a minimum for the C_{16} analog. These findings in connection with our data on micellar sizes (Table I) imply, that a strongly hemolytic lysophosphatide should possess the maximal degree of hydrophobicity which still allows the formation of small micelles (micellar weight below 10^5 daltons).

Our results further indicate that lysolecithins bind to cells far below their critical micelle concentration (see Fig. 3 and Table I), that micelles are apparently not required for hemolysis (Fig. 5) and that micelles themselves probably do not bind to cellular surfaces (Fig. 6). On the other hand, adsorption kinetics are strongly affected by the micellar structure (Fig. 6). In agreement with previous observations [12] we therefore conclude that the rate-determining step in lysolecithin adsorption to cells is the release of single molecules from micellar aggregates.

Finally, we have found parallels between the reactivity of red cells from different species and the sensitivity of one type of erythrocytes at various temperatures towards the homologous series of deoxy lysolecithins (Fig. 7). The hemolytic activities of the C_{14}, C_{16} and C_{18} derivatives are always greater at low than at high temperature and always greater towards bovine than human erythrocytes. In the case of C_{12}, however, these differences are abolished, and for the C_{10} analog even inverted in both systems. Moreover, the differences in lysolecithin sensitivity between different types of red cells are largely reduced at low temperatures [9]. According to previously published data [9] it is further apparent that membranes of human erythrocytes are of lower microviscosity (i.e. higher lipid mobility) than those isolated from bovine red cells, and that in both membranes microviscosity increases with falling temperature [9].

It thus appears that bovine erythrocytes react to lysolecithin like human red cells at comparatively reduced temperature, and that cellular sensitivity to lysophosphatides is in some way related to the packing and physical state of lipid hydrocarbon chains in the membrane. As we have argued previously [9,17] the physical state of membrane lipids may, in a temperature- and composition-dependent way, interfere with the homogeneity of lysolecithin distribution in the lipid phase, with a heterogenetic distribution leading necessarily to lysolecithin-enriched areas in the membrane. It is resonable to assume, however, that

lysolecithins of various chain-length per se possess distinctly different miscibilities with other lipids, especially with respect to the effects of temperature. In fact, lysolecithins with aliphatic chains in the "physiological" range of 14—18 carbons exhibit a distinctly different temperature dependence from those with extremely short side chains. This is in full agreement with our hypothesis that cellular sensitivity to lysolecithin is to a great part determined by the distribution of lysolecithin, and thus probably by the homogeneity of the distribution of other membrane lipids.

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