

STUDIES ON THE LYSIS OF RED CELLS AND BIMOLECULAR LIPID LEAFLETS BY SYNTHETIC LYSOLECITHINS, LECITHINS AND STRUCTURAL ANALOGS

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The results obtained in this study show a great influence of the acyl chain on the lytic behavior of lysolecithins and lecithins

1 The lysolecithins studied, ranged in acyl chain length from 10 to 18 carbon atoms

The palmitoyl- and stearylderivatives were found to be the most active, whereas the shortchain compounds like decanoyl-lysolecithin showed no activity at all

The possible influence of the glycerolic hydroxylgroup was studied by means of 8 different desoxy-lysolecithins, varying in chain length from 12 to 26 carbon atoms

This structural modification, however, turned out to be negligible The highest activity was also found for compounds with 16 and 18 carbon atoms, while the shorter and longer chain ones (lauroyl C12, myristoyl C14, arachidoyl C20) were less active, or not active at all (behenoyl C26, cerotoyl C22)

Introduction of double bonds in the acyl chain also rendered the compounds less active (oleoyl-lysolecithin, linoleoyl-lysolecithin)

2 Structural variations in the desoxycompounds, like extension of the alkanediol skeleton of the molecule or increase of the distance between the phosphate group and the quaternary ammonium moiety had virtually no effect

3 It was observed that lecithins with a chain length from 8 to 12 carbon atoms (per acyl chain) are capable of lysing red cells

The lecithin with highest activity was found to be di-undecyloyl-lecithin which was almost as active as the most potent lysolecithin

Shortening or lengthening of the acyl chains diminished the lytic activity, just as in the case of the lysolecithins and the desoxy-lysolecithins

Therefore it is concluded that lysolecithins, desoxy-lysolecithins and lecithins reveal maximum lytic activity at a distinct chain length

4 It appears that the action of lysolecithins, desoxy-lysolecithins and lecithins toward red cells and lipid bilayers shows reasonable similarity with the exception of some unsaturated compounds This might indicate that the interaction of the lytic agents with the lipid-constituents of the membrane plays an important role in the process

The occurrence of small quantities of lysolecithin in the lipoprotein complexes of living cells is generally accepted now¹⁾, and in the blood plasma fairly high concentrations were demonstrated²⁾ Extensive studies showed that mono-acyl phosphoglycerides can play an important role as intermediates in phospholipid metabolism^{3 1)} Even in the metabolically rather inactive erythrocyte various pathways for the conversion of lyso-compounds were demonstrated⁵⁾

Lysolecithin is well-known for its highly lytic properties. Relatively low concentrations will cause complete disorganization and disruption of membrane structures. Because of these potent properties it might well be possible that the physiologically occurring and enzymatically controlled amounts influence the organization of lipid molecules in the interface. This idea is supported by recent findings that the resistance of bimolecular lipid membranes is significantly lowered by introduction of lysolecithin in the film forming solution⁶⁾. The properties mentioned above make it interesting to elucidate the chemical and physical characteristics that rule the behaviour of lysolecithin. Therefore a series of chemically well-defined lysolecithins with different fatty acid chains was prepared. In this paper we report the lytic activity of these compounds towards beef erythrocytes.

In addition, homologous series of lecithins and desoxy-lysolecithins were tested. Part of these results were already reported in a preliminary note published in 1967⁷⁾.

Until recently most experiments were carried out with poorly defined lysophosphoglycerides. Gottfried and Rapport⁸⁾ investigated the hemolytic

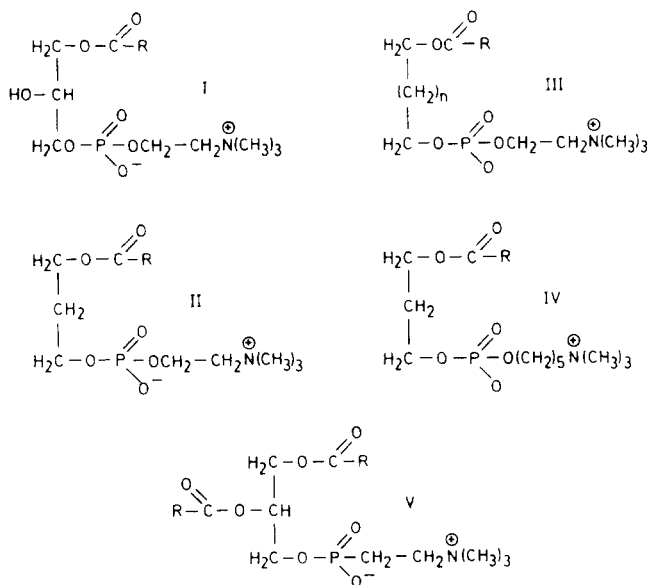


Fig. 1. Structural formulas of the compounds used.

- I 1-acyl-lysolecithin
- II 1-acyl-desoxy-lysolecithin
- III 1-acyl-(α - ω)-alkanediol-phosphorylcholine
- IV 1-acyl-propanediol-3-phosphoryl-5-trimethyl-amino-pentane-1-ol
- V 1,2-diacyl-lecithins

action of a series of lysophosphoglycerides and found that the nature of the linkage of the hydrocarbon chain to glycerol does not influence hemolytic activity

Recently, Arnold and Weltzien⁹⁾ published results on a number of choline phospholipids. Qualitatively their results are in agreement with the results in our previous communication and with those presented in this paper. However, some quantitative differences can be noted, which could be related to the fact that they used human red cells instead of beef erythrocytes.

Materials and methods

The following lysolecithins (fig 1, formula I) were obtained by hydrolysis of synthetic lecithins with snake venom (*Crotalus adamanteus*), phospholipase A (EC 3.1.1.4)¹⁰⁾

1-stearoyl	- <i>sn</i> -glycero-3-phosphorylcholine	I ^a
1-palmitoyl	- <i>sn</i> -glycero-3-phosphorylcholine	I ^b
1-myristoyl	- <i>sn</i> -glycero-3-phosphorylcholine	I ^c
1-undecyloyl	- <i>sn</i> -glycero-3-phosphorylcholine	I ^d
1-caprinoyl	- <i>sn</i> -glycero-3-phosphorylcholine	I ^e
1-oleoyl	- <i>sn</i> -glycero-3-phosphorylcholine	I ^f
1-linoleoyl	- <i>sn</i> -glycero-3-phosphorylcholine	I ^g

The following lysolecithin analogs (fig 1, formula II) were synthesized according to methods published elsewhere¹¹⁾

1-cerotoyl-	propanediol-3-phosphorylcholine	II ^a
1-behenoyl-	propanediol-3-phosphorylcholine	II ^b
1-arachidoyl-	propanediol-3-phosphorylcholine	II ^c
1-stearoyl-	propanediol-3-phosphorylcholine	II ^d
1-palmitoyl-	propanediol-3-phosphorylcholine	II ^e
1-myristoyl-	propanediol-3-phosphorylcholine	II ^f
1-lauroyl-	propanediol-3-phosphorylcholine	II ^g

Synthetic ω ω' alkanediol-phosphorylcholines (fig 1, formula III)

1-palmitoyl-butanediol	-4 phosphorylcholine	III ^a
1-palmitoyl-pentanedol	-5 phosphorylcholine	III ^b
1-palmitoyl-hexanedol	-6 phosphorylcholine	III ^c

Synthetic propane diol-3-phosphoryl-trimethyl amino-alkane diol (fig 1, formula IV)

1-palmitoyl propanediol-3-phosphoryl-5-trimethyl-amino-pentane-1-ol	IV ^a
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The following lecithins were synthesized according to the methods of Bear and Buchnea¹²), de Haas and van Deenen¹³) and Cubero Robles and de Jongh¹⁴) (fig. 1 formula V)

1,2-di-heptanoyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^a
1,2-di-octanoyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^b
1,2-di-nonanoyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^c
1,2-di-decanoyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^d
1,2-di-undecyloyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^e
1,2-di-lauroyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^f
1,2-di-myristoyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^g
1,2-di-linoleoyl	- <i>sn</i> -glycero-3-phosphorylcholine	V ^h
1-oleoyl-2-butyryl	- <i>sn</i> -glycero-3-phosphorylcholine	V ⁱ

Known amounts of the compounds were dispersed in 0.9% saline solutions (sometimes we had to sonicate in order to obtain stable lysin dispersions)

Beef erythrocytes were washed 3 times in 10 volumes 0.9% saline and then resuspended in 0.9% saline to give suspensions with a 50% hematocrit. The lysis test was carried out by adding a small volume of the lysin solution to 5 ml of 0.9% saline in the cuvette of a Vitatron spectrophotometer after which the transmission was adjusted at 100%. Subsequently, 50 μ l of the erythrocyte suspension was added which caused the transmission to drop to zero, thereafter the change in transmission at 625 m μ as a function of time was recorded. The time required to cause 50% change in transmission at various concentrations of the lytic agent is recorded.

Bilayer experiments were carried out as described by van Zutphen *et al*¹⁵), in the type of cell developed Thompson¹⁶)

The temperature in all these experiments was 20°C, unless stated otherwise.

Results

The lytic properties of some lysolecithins are compared in fig. 2. Stearoyl-lysolecithin (I^a) was found to be the most potent compound. Hemolysis starts at a minimum concentration of about 5×10^{-6} mole/l. From thereon the time needed for 50% hemolysis decreases very rapidly with increasing concentration, but at a concentration of 10^{-5} mole/l the process operates at a maximum velocity. Apparently a certain "lag time" is needed before hemolysis can start. Shorter chain analogs like palmitoyl-lysolecithin (I^b) and myristoyl-lysolecithin (I^c) require higher minimum concentrations to lyse red cells, but show a much shorter "lag-time" than stearoyl-lysolecithin. A further shortening of the chain length leads to a rapid decrease of lytic

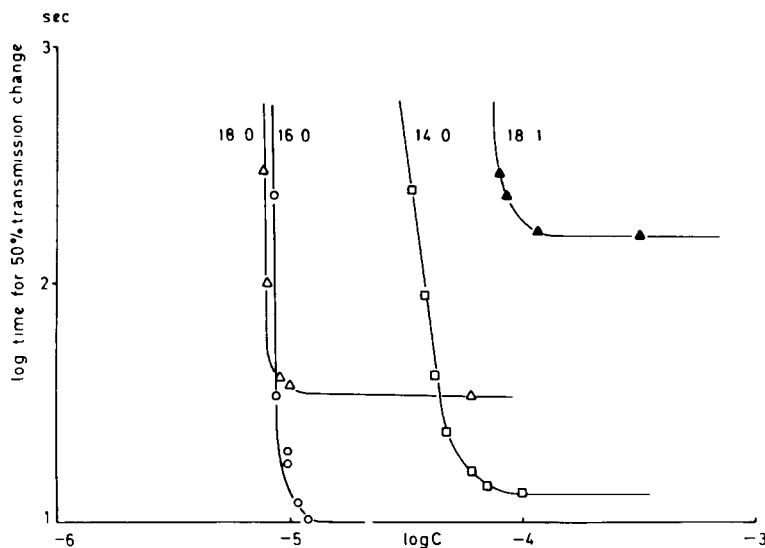


Fig 2 Lytic action on beef erythrocytes of (stearoyl) lysolecithin I^a, (palmitoyl)lysolecithin I^b, (myristoyl)lysolecithin I^c and (oleoyl)lysolecithin I^f

activity Undecyloyl-lysolecithin (I^d) caused 50% lysis at a concentration of 1.8×10^{-3} mole/l in 30 min, while caprinoyl-lysolecithin (I^e) was completely inactive at concentrations up to 1.5×10^{-3} mole/l. A loss of activity as brought about by a decrease in chain length, can also be observed by introducing double bonds (*viz* 1-oleoyl-lysolecithin) in the acyl chain. Introduction of 1 double bond diminishes the activity already considerably (compare fig 2) and a second one renders the compound almost inactive. The linoleoyl-lysolecithin (I^f) caused 50% lysis in 1 hr at a concentration of 10^{-3} mol/l.

In addition to variations in the paraffin chain, also compounds with modifications in the polar region of the molecule were tested to find out the influence of this part of the molecule on the hemolytic activity. In fig 3, the data for a number of desoxy-lysolecithins are represented. When we compare the lytic activities of these compounds with those of the corresponding lysolecithins (fig 2) it is obvious that the elimination of the hydroxyl group leads to a slight increase of activity. Likewise, in this series of desoxy-compounds the stearoyl derivative exhibits the highest activity. Shortening of the chain length increases the active minimum concentration, whereas the lag time is reduced. Increase of the acyl chain length results in a drastic change in the hemolytic process. The arachidoyl compound showed, at all concentrations tested, a very long "lag phase" (fig 2, II^c).

With behenoyl-desoxy-lysolecithin activity could only be found at elevated

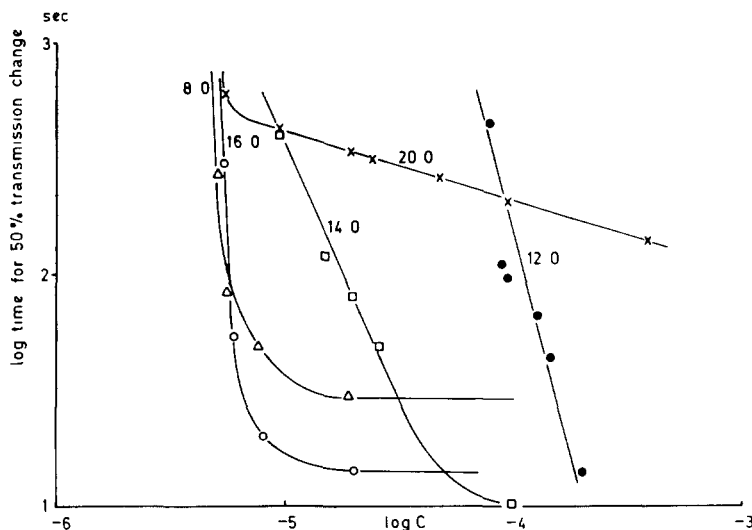


Fig 3 Lytic action on beef erythrocytes of (arachidoyl)desoxy-lysocithin II^a, (stearoyl)desoxy-lysocithin II^d, (palmitoyl)desoxy-lysocithin II^c, (myristoyl)desoxy-lysocithin II^f and (lauroyl)desoxy-lysocithin II^e

temperatures, and even then hemolysis proceeded very slowly (50% hemolysis in 28 min at a concentration of 2.2×10^{-4} mol/l and a temperature of 37°C) The cerotoyl derivative was completely devoid of any activity, up to concentrations of 3×10^{-4} mol/l and a temperature of 40°C A modification of the polar part of the molecule was brought about by the extension of the diol skeleton from propanediol to butanediol, pentanediol or hexanediol The results obtained with these compounds are given in fig 4 It is clear that the lytic activity is hardly influenced by this modification

Increasing the distance between phosphate group and the quaternary ammonium moiety slightly effects the lytic activity This can be concluded by comparing the lytic activity of 1-palmitoyl propanediol-3-phosphoryl-5-trimethyl-amino-pentane-1-ol (see fig 4, IV^a) with 1-palmitoyl-desoxy-lysocithin (II^c)

Until recently, lecithins were not considered to be lytic agents, but this study once more demonstrates that some lecithins can be just as lytic as some of the most potent lysocithins This is demonstrated in fig 5, which represents the lytic activities of a number of homologous lecithins with two identical acyl chains When we compare the most active compound in this series, the 1,2-di-undecyloyl-lecithin, (V^e) with those of the lysocithin and desoxy-lysocithin series, it is obvious that the minimum concentration of lecithin required is in the same order as that of the lysocompounds (fig 2)

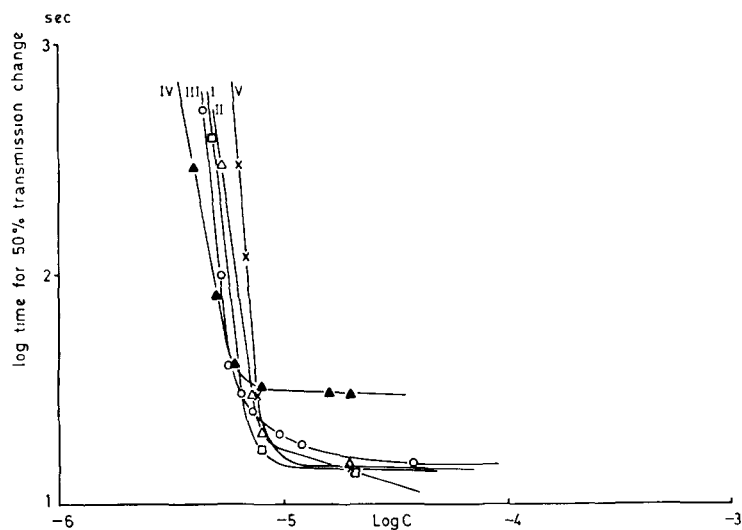


Fig 4 Lytic action on beef erythrocytes of

(1-palmitoyl)propanediol-3-phosphorylcholine II^c ,

(1-palmitoyl)butanediol-4-phosphorylcholine III^a ,

(1-palmitoyl)pentanediol-5-phosphorylcholine III^b ,

(1-palmitoyl)hexanediol-6-phosphorylcholine III^c ,

and (1-palmitoyl)propanediol-3-phosphoryl-5-trimethyl-amino-pentane-1-ol (IV^a)

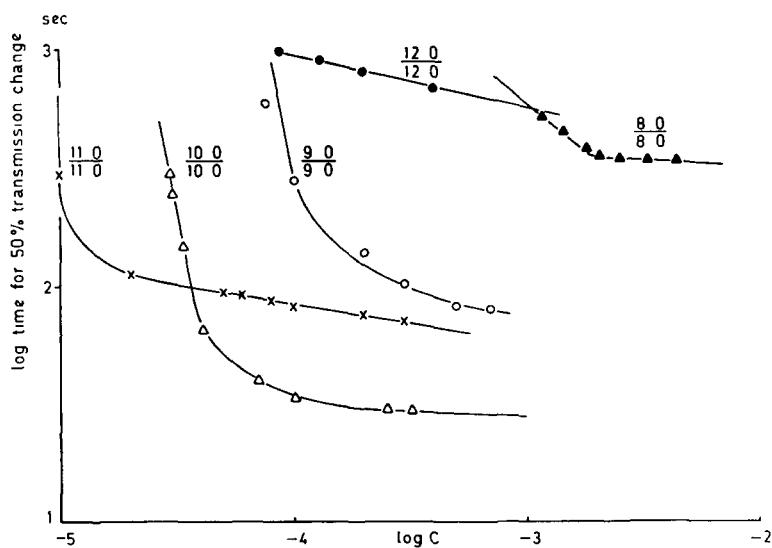


Fig 5 Lytic action on beef erythrocytes of di-octanoyl-lecithin V^b , di-nonanoyl-lecithin V^c , di-decanoyl-lecithin V^d , di-undecyloyl-lecithin V^e , and di-lauroyl-lecithin V^f

former was inactive at room temperature. Raising the temperature to 37°C initiated lysis by this compound at a concentration of 0.13×10^{-3} mol/l after 15 min.

Fig. 6 shows the results obtained so far with bimolecular lipid leaflets generated from total lipid extracted from beef erythrocytes. Just as in the case of the hemolysis experiments the stearyl-lysolecithin (I^a) turned out to be the most lytic compound. Shortening of the chain length shows the same effect as in the hemolysis experiment, that is to say a loss of activity. Unsaturation of the acyl chain also gives a loss of activity, but by far not so dramatic as in the lysis tests (compare oleoyl-lysolecithin I^f and linoleoyl-lysolecithin I^g).

Another remarkable thing is the behaviour of the di-octanoyl-lecithin V^b and the di-linoleoyl-lecithin V^h. Both were rather inactive towards erythrocytes but showed much more potency towards lipid bilayers.

Discussion

The process of hemolysis by lytic agents has been a challenge to many investigators. Nevertheless the mechanism is still not very well understood. On the basis of studies by Ponder¹⁷⁾, Rideal¹⁸⁾, Jung¹⁹⁾ and many others it seems likely that hemolysis by compounds like lysolecithin can be considered to be built up from at least 5 consecutive reactions. Starting with an adsorption to the membrane (step 1) the lysin molecules subsequently penetrate into lipoprotein complex (step 2) where they induce a change in the molecular organization (step 3).

This leads to a radical change in permeability of the membrane, and a disturbance of the osmotic equilibrium (step 4). Finally hemoglobin leaks out (step 5). Step 1 and 5 are considered to proceed very fast.

The results presented in this paper are summarised in fig. 7. Plotting the concentration of lytic agent required to cause a 50% transmission change in 10 minutes versus the number of carbon atoms in the acyl chain, a minimum concentration for both lysolecithins and desoxy-lysolecithins is found in the range from 16 to 18 carbon atoms.

It is striking that Ponder¹⁷⁾ working with alkyl-sulfonates and Hooghwinkel²⁰⁾, using alkyltrimethyl-ammoniumbromides, found the C₁₆ homologue to be the most active. The free hydroxyl group in the lysolecithin molecule hardly influences hemolysis.

For the lecithin the minimum concentration is reached in the case of di-undecyloyl-lecithin. This compound is virtually as active as lysolecithin or desoxy-lysolecithin derivatives with stearic or palmitic acid. With respect to the lytic properties of these phospholipids, the ability to form micelles or aggregates with a low micellar weight should be underlined.

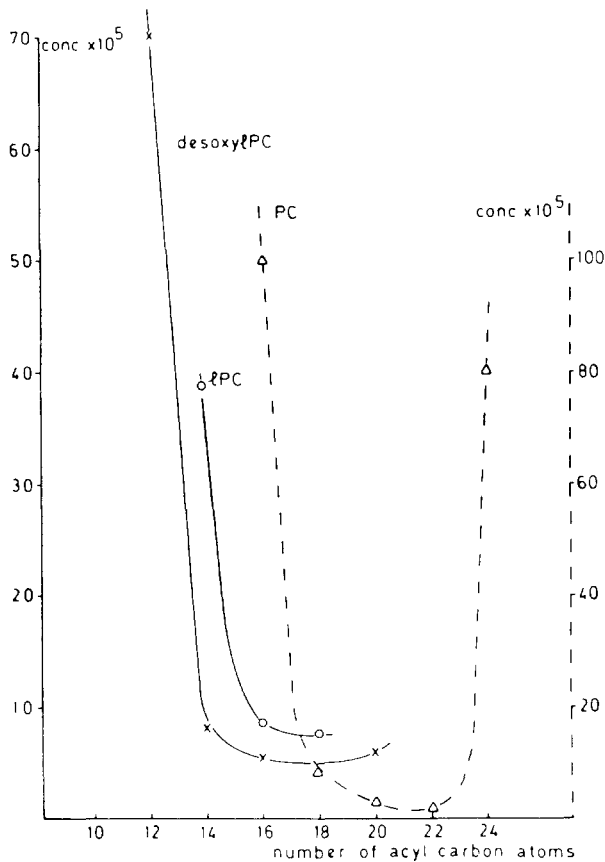


Fig 7 The dependence of lytic activity on the acylchain length for

- I lysolecithins
- II desoxy-lysolecithins
- III lecithins (note the difference in concentration scales)

According to data from Jung¹⁹) the bend in the curve, when concentration is plotted against time for 50% hemolysis, (see fig 2 of this paper), corresponds with the critical micellar concentration (CMC) of lysolecithin. Below this concentration the rate of hemolysis is very much dependent on the concentration of the lytic agent, this in contrast to concentrations above the CMC.

In the presence of lytic agents, the molecular organization can be thought to change in several ways. It is generally assumed that lysolecithin interacts primarily with the lipid constituents of the membrane. Support for this idea can be found in fig 6. This figure represents the influence of several lyso-

lecithins and lecithins on the stability of bimolecular lipid membranes, generated from total lipid of beef erythrocytes. The curves show reasonable resemblance with the ones obtained in hemolysis experiments, but one should be careful not to oversimplify, because proteins will also play an important role. It is well known for example that in the bloodplasma lysolecithin is predominantly bound to albumin, and not associated with lipoproteins.

If we confine ourselves to an attack on the lipid part, two suggestions can be made.

a) The lysolecithin forms a complex with cholesterol by means of which this lipid is deprived of its structural function.

Some authors even suggest a solubilization and release of this lipid from the membrane structure. This idea is supported by observations that an increase in chain length of desoxy-lysolecithins increased the lytic activity as well as the solubilization of crystalline cholesterol²¹⁾.

In this respect it is also relevant to mention incubations of erythrocytes with lecithin dispersions, which result in a loss of cholesterol from the membrane²²⁾. If this loss exceeds 30% of the total cholesterol content of the membrane, a hemolysis of the red cell occurs. The small amounts of lysolecithin causing lysis are however, incapable to release such quantities of cholesterol from the membrane by an exchange reaction.

b) The lysin molecules are considered to act as wedges²³⁾, which destroy the natural orientation of the lipid molecules, when they penetrate. Support for this view is given by the electronmicroscopy studies of Bangham and Horne²¹⁾.

They showed that the bimolecular lamellar structure of a lecithin mesophase can be changed into a micro-emulsion on addition of lysolecithin to the lecithin dispersion.

The differences in lag time between stearoyl- and palmitoyl-lysolecithin as well as between lysolecithins and lecithins are probably caused by kinetic differences in the hemolysis process as well as in differences in distribution between cell surface and surrounding medium. Also size and stability of the micelles will be of great importance.

It is a fact that the physical properties of lysolecithin and analogs are highly dependent on the chemical identity of the paraffin chain. Stearoyl- and oleoyl-lysolecithin form stable monolayers with high collapse pressures whereas palmitoyl-, myristoyl-, and linoleoyl-lysolecithin mono-layers show lower collapse pressures. Several investigators²⁵⁾ tried, mostly without success, to correlate lytic potency of various compounds with their surface activity. The molecular areas of the lysolecithin as determined from spreading experiments at the air/water interface, are independent of the fatty acid chain, but primarily ruled by the size of the zwitterionic head-

group. When lysolecithins with lytic activity are solved in water, they form spherical micelles²⁶⁾, just like soaps. This in contrast to the lytically inactive lecithins like dimyristoyl-lecithin and higher homologues. Dispersions of these compounds in water consist of spontaneously formed big aggregates²⁷⁾ in which the lipids are orientated in concentric bimolecular shells (so termed liposomes). It is obvious that the tendency of the phospholipid molecules to orientate themselves in several distinct structures²⁸⁾ will be of great importance for their ability to cause a reorientation in the organized biological interface. Studies on molecular arrangements of lipid dispersions in water of both lytic and non-lytic lecithins are in progress now. Investigations on critical micellar concentration and micellar weight are also being carried out as they might contribute to a better understanding of the kinetics of the hemolytic process.

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