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QUANTITATIVE STUDIES ON LYSOLECITHIN MEDIATED HEMOLYSIS

BENZYLATED LYSOLECITHIN AS A PROBE TO STUDY EFFECTS OF TEMPERATURE AND RED CELL SPECIES ON THE HEMOLYTIC REACTION

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SUMMARY

The slow reacting hemolytic lysolecithin analog 1-octadecyl-2-benzyl-glycero-3-phosphorylcholine has been employed for a detailed study of the process of lysolecithin induced hemolysis. Using a radiolabelled analog we found that the different sensitivities of red cells from different species (chicken, man, cattle) are not paralleled by the binding affinities of lysolecithin. Moreover, lysophosphatide binding to the cells is reduced at low temperatures while its hemolytic activity increases. In contrast to continuous changes of lytic activity and binding between 0 and 37 °C, the velocity of the hemolytic reaction with human erythrocytes is extraordinarily fast at 10 °C. Experiments in sucrose containing buffer indicated principally different lysis mechanisms below and above 15 °C. We have further shown that cells loaded sublytically with the lysolecithin at 37 °C undergo spontaneous lysis upon cooling to 0 °C. The degree of lysis under these conditions, however, is diminished with increasing amounts of cell-bound lysolipid. Determinations of membrane microviscosities by means of fluorescence polarization revealed some qualitative relations between membrane fluidity and sensitivity to lysolecithin. The data are discussed on the basis of recent reports indicating that lysolecithin-distribution in mixed lipid phases may be heterogeneous depending on lipid composition and temperature.

INTRODUCTION

The process of lysolecithin-mediated lysis of red cells may, to some degree, serve as a model to eludicate the mode of action of lysophosphatides on cellular membranes. The theoretical comprehension of this interaction is fundamental for our understanding of some important biological activities of lysophosphatides, such as lysolecithin-mediated cell fusion [1] or the immune regulatory properties of synthetic lysophosphatides [2, 3].

Despite numerous efforts of several laboratories [4–9], the principal point of attack of lysolecithin in biological membranes remains controversial, and little is

known about the factors which render a cell more or less sensitive to lysolecithin lysis. In the present paper we have taken a new approach to study this phenomenon by using the slow reacting hemolytic lysolecithin analog *rac*-1-octadecyl-2-benzyl-glycero-3-phosphoryl-choline. This substance, which in the following will be simply called benzyl-lysolecithin, has been previously shown to be extremely useful for a detailed study of the hemolytic process [10] because of its retarded interaction with red cells, which apparently is due to an unusual aggregation status of this compound in aqueous solutions [11].

Our investigations were initiated mainly by two experimental results known from the literature, namely that red cells (a) of different species [6] and (b) at different temperatures [4, 7] exhibit characteristic differences in their sensitivity towards lysolecthin. Both effects have never been systematically studied, and many quantitative differences in the results of different authors may be due either to the use of different kinds of red cells or to different lysis temperatures. On the other hand, these effects are most certainly related to composition and properties of the membranes of these cells and their elucidation may thus contribute to our understanding of the hemolytic process as well as that of membrane structure in general.

MATERIALS AND METHODS

Benzyl-lysolecithin. Chemical synthesis and radiolabelling, as well as solution and storage conditions of 1-octadecyl-2-benzyl-glycerophosphorylcholine have been described before [10, 12].

Hemolysis and binding assays. Preparation of red cells as well as the determination of hemolytic activities and lysolecithin binding to cells were performed according to published procedures [10]. Standard conditions in all systems were: 1.2 ml of 0.5 % cell suspensions in 1.5 ml Eppendorf plastic centrifuge tubes. The suspensions contained 3.1, 5.5 and $9.4 \cdot 10^7$ cells/sample for chicken, human and bovine erythrocytes, respectively. All of them, upon complete lysis and centrifugation, gave an extinction of 1.0 ± 5 %, as determined in a Shimadzu UV 200 double beam photometer at 546 nm. 50 % lysis values (L_{50} dose) were determined graphically from dose vs. lysis curves after 4 h of incubation. A_{50} values, i.e. the amount of lysolipid adsorbed to the cells at 50% lysis, were extrapolated from simultaneous determinations of lysis and binding, using undiluted radiolabelled benzyl-lysolecithin at various concentrations close to the L_{50} dose. At up to 65 % hemolysis the red cell ghosts co-sedimented with the intact cells in an Eppendorf table centrifuge type 3200 at $8000 \times g$.

Fluorescence polarization. Measurement were carried out in a Hitachi-Perkin Elmer spectrophotometer model MPF 4 as described elsewhere [11], using diphenyl-hexatrien as a fluorescent probe. The measuring cell contained human or bovine red cell ghosts (60 μg protein) prepared according to Dodge [13] in 3 ml of 1 μM phospate buffer, pH 7.4, and 6 nmol of diphenyl-hexatrien. Excitation and emission wavelengths were 359 and 427 nm, respectively. Temperature shift was continuous at a rate of about 1 °C per min. Data of Fig. 5 represent the average of four experiments, two with increasing and two with decreasing temperature.

Buffers. Except where indicated otherwise, 0.01 M phosphate buffer, pH 7.2, in 0.85 % NaCl was used.

RESULTS AND DISCUSSION

Benzyl-lysolecithin, a slow reacting hemolytic substance, exhibits also an exceptionally strong variation in its hemolytic activity towards various types of red cells. As shown in Fig. 1a, the amounts of benzyl-lysolecithin that have to be added under standard conditions (see Methods, section 2) to produce 50 % hemolysis (L_{50} dose) of bovine, human or chicken erythrocytes at 22 °C vary from 4–13 and 25 nmol, respectively. From the data presented in Fig. 1b it is evident that binding equilibria at various lysophosphatide concentrations are identical for all three cell types. A rough calculation of the cell surfaces [14], moreover, revealed that in our assay all three cell suspensions represent about 100 cm^2 of cell surface per tube, indicating in fact that for the different cells different amounts of lysolecithin per surface area are needed to induce membrane disruption.

With respect to the temperature dependence of the hemolytic reaction, it is well known that, generally, lysolecithins are more active at 0 °C than at 37 °C [4, 7]. The two explanations for this phenomenon offered so far are that either lysolecithin binding is increased [4] or that lysolecithin metabolism is inhibited [7] at low temperature. Benzyl-lysolecithin proved to be particularly useful to investigate these questions since the temperature dependence of its lytic capacity is more pronounced than that of most other lysolecithin derivatives tested so far. Fig. 2 presents the L_{50} values of benzyl-lysolecithin at various temperatures for human, bovine and chicken erythrocytes. The most important result of this type of experiment is the finding that the different cells reveal a remarkable variability of the temperature dependence of their sensitivity to lysolecithin. Thus, the ratio of L_{50} at 30 °C to L_{50} at 0 °C is less than 2 for bovine but about 10 for chicken red cells, with human erythrocytes displaying an intermediate value of 3.5–4.0. Chicken and human cells, moreover, differ from bovine erythrocytes in that they exhibit a distinct minimum of their sensitivity to

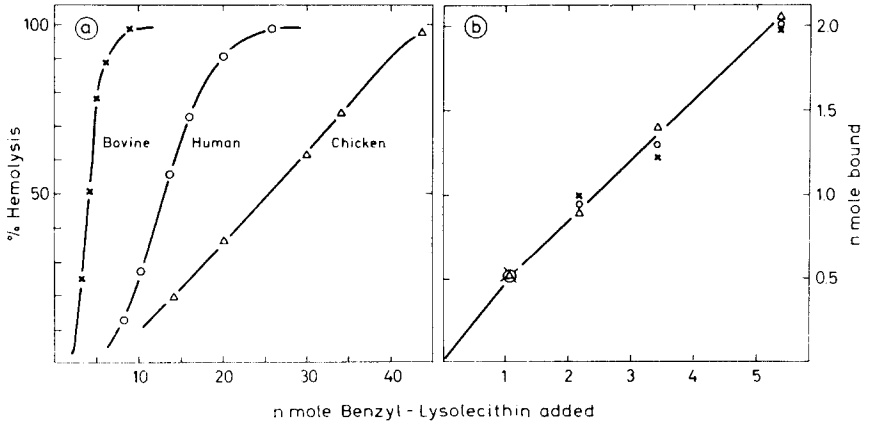

Fig. 1. Hemolytic activity and binding equilibria of benzyl-lysolecithin at 22 °C. ×—×, bovine; ○—○, human; △—△, chicken erythrocytes. All data obtained under standard conditions, i.e. after 4 h of incubation in 1.2 ml of 0.5 % cell suspensions, containing 3.1, 5.5 and 9.4 cells per sample for chicken, human and bovine erythrocytes, respectively. (b) gives binding data per total cell sediment.

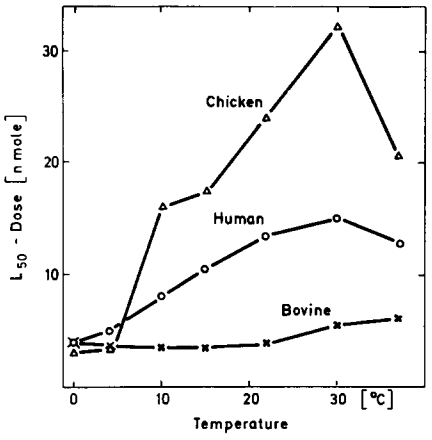

Fig. 2. Temperature dependence of L_{50} dose for benzyl-lysolecithin and chicken, human or bovine erythrocytes. L_{50} values were extrapolated from dose vs. lysis curves obtained at the respective temperatures after 4 h incubation under standard conditions (see Methods, section 2). L_{50} dose is given in nmol added per 1.2 ml cell suspension.

benzyl-lysolecithin, i.e. a maximum of their L_{50} values, at 30 °C. Resulting from these different effects of temperature on the three types of cells, the differences of their sensitivities to lysolecithin diminish constantly with decreasing temperature and are virtually abolished between 0 and 5 °C.

Binding studies at different temperatures revealed that in contrast to the suggestion of Collier [4] lysophosphatide binding is not enhanced but significantly

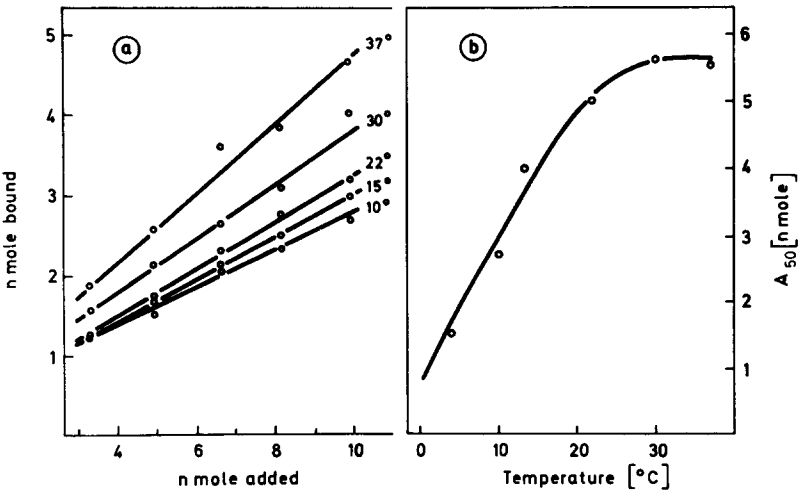

Fig. 3. Temperature dependence of binding of [^{14}C]-benzyl-lysolecithin to human erythrocytes. All data obtained under standard conditions as described under Methods, section 2. Binding is expressed in nmol bound per total cell sediment. (a) Binding equilibria after 4 h incubation with various lysolecithin concentrations, expressed in nmol per 1.2 ml. (b) A_{50} value represents amount of lysolipid adsorbed per total cell sediment at 50 % lysis under standard conditions.

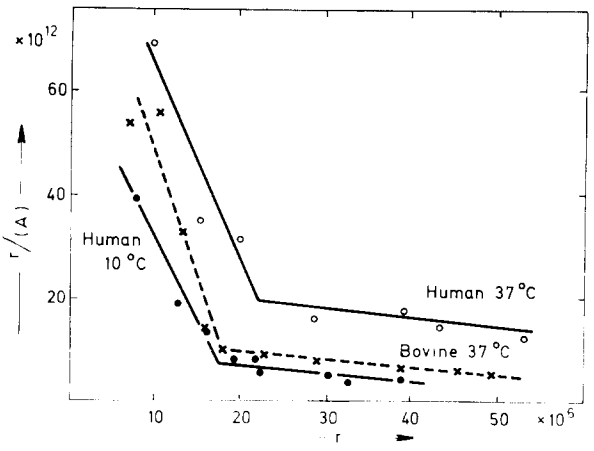

Fig. 4. Scatchard plots of binding equilibria of [^{14}C]benzyl-lysolecithin to human and bovine erythrocytes. *r*, bound benzyl-lysolecithin (molecules per cell); (A), free benzyl-lysolecithin (mol/l). Binding data were obtained after 4 h incubation with $2.1 \cdot 10^8$ human and $3.1 \cdot 10^8$ bovine erythrocytes in 1.2 ml of buffer. The slope of the resulting curves represents the equilibrium constants and is a measure of binding affinity [15]. All values are well below lytic concentrations.

diminished at lower temperatures (see Fig. 3a). From the slopes of the Scatchard plots [15] in Fig. 4 it becomes evident, moreover, that despite this reduced adsorption the affinity of binding is unchanged at low temperature and that even different cell types exhibit identical binding constants for the benzyl-lysolecithin. The fact that all plots are biphasic with two distinct equilibrium constants of about 1.7 and $37 \cdot 10^5$ (l/mol) is as yet unexplained. It may be of note, however, that similar results were obtained for lysolecithin binding to protein-free liposomes, excluding a major role of membrane proteins in this phenomenon.

Since the L_{50} values (Fig. 2) as well as the proportion of the added lysolipid that binds to the cells (Fig. 3a) both decrease with decreasing temperature it is not surprising that the amount of the lysin actually bound to the cells at 50 % lysis (A_{50} value) is reduced even more with falling temperature (see Fig. 3b). For human erythrocytes it is thus apparent that their membranes can take up about five times more of the lysolecithin at physiological temperature than at 0–4 °C, before losing their barrier properties for macromolecules. The fact that benzyl-lysolecithin is not degraded by red cell associated enzymes [10] also excludes the possibility that the high sensitivity of red cells to lysolipids at low temperature is due to an inactivation of lysolecithin-metabolizing enzymes [7]. It has thus to be concluded that at temperatures close to 0 °C, red cell membranes are in a physical state distinctly different from that at higher temperatures, one characteristic of this state being the fact that otherwise existing differences in lysolecithin sensitivity between different red cells are largely abolished.

The different reactivity of red cells with benzyl-lysolecithin at various temperatures is best demonstrated by comparing the rate of hemolysis between 0 and 37 °C. To compare hemolysis rates, however, one has to be aware of the fact that the velocity of the hemolytic reaction is extremely dependent up on the lysophosphatide

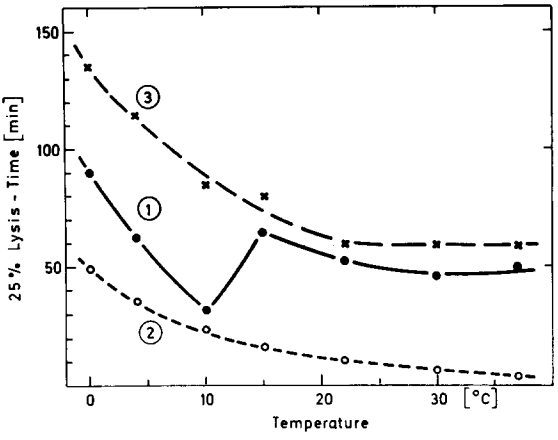

Fig. 5. 25 %-lysis time by benzyl-lysolecithin as a function of temperature. Lysolecithin concentrations were selected to give 80±2 % lysis in equilibrium (after 4 h) at all temperatures. 25 %-lysis times were extrapolated graphically from individual lysis kinetics at the indicated temperatures. (1) Human erythrocytes ($5.5 \cdot 10^7$ cells per 1.2 ml) in phosphate-buffered saline; (2) bovine erythrocytes ($9.1 \cdot 10^7$ cells per 1.2 ml) in phosphate-buffered saline; (3) human erythrocytes ($5.5 \cdot 10^7$ cells per 1.2 ml) in sucrose medium (50 mM KCl/95 mM NaCl/10 mM phosphate/27 mM sucrose, pH 7.2). Each value represents the average of at least two independent experiments.

concentration (at 37 °C, for instance, the 50 % lysis time of human red cells is 20, 40 or 110 min at benzyl-lysolecithin concentrations of 50, 20 or 14 nmol per 1.2 ml, respectively). From pilot experiments we therefore selected lysolecithin concentrations for each temperature which after 4 h resulted in 80±2 % hemolysis. Under these conditions we followed the lysis kinetics and plotted the time needed for 25 % lysis vs. temperature. Curves 1 and 2 in Fig. 5 show these data for human and bovine erythrocytes.

From these curves it is evident that in human red cells the onset of lysis is extraordinarily fast at 10 °C. At 4 °C as well as at 15 °C 25–30 min. more are needed for 25 % lysis than at 10 °C. The possibility that this striking discontinuity of the curve might be due to the reported [11] phase transition of benzyl-lysolecithin at 14 °C is ruled out by the absolutely continuous decrease of lysis times with increasing temperature in the case of bovine erythrocytes (curve 2, Fig. 5).

Data on the binding kinetics of benzyl-lysolecithin to human red cells at different temperatures have been published previously [11]. They revealed a rather constant binding rate between 0–15 °C and an increasing velocity of the reaction above this temperature. This excludes the possibility that the retarded lysis kinetics above 10 °C might be related to adsorption characteristics.

In this context it should be stressed again that the effect demonstrated in Fig. 5 is purely kinetic. The equilibrium is at all temperatures 80 % lysis and the temperature dependence of L_{50} or A_{50} values of benzyl-lysolecithin for human red cells shows no irregularities at or around 10 °C (see Figs. 2 and 3b). We are therefore faced with the fact that human erythrocyte membranes apparently undergo a significant structural change at about 10 °C.

This assumption is further confirmed by an experiment in which erythrocytes were not lysed in phosphate buffered saline but in a potassium-rich sucrose medium.

Under these conditions, a pure detergent lysis (i.e. the creation of "holes" big enough to allow the penetration of hemoglobin) should be unaffected as compared to the normal saline medium. If lysis induced by lysolecithin is primarily due to a loss of potassium and subsequent colloid osmotic swelling and rupture of cells, as has been discussed by others [16], hemolysis should be strongly reduced in a potassium- and sucrose-containing buffer. In fact, it is shown by curve 3 in Fig. 5 that above 15 °C the lysis kinetics in sucrose solution is very similar to that in saline. Below 15 °C, however, the results are completely different. While in saline a temperature shift from 15 to 10 °C reduces the 25 % lysis time from 65 to 34 min, the latter is increased in sucrose containing buffer to 80 min and is further increasing with falling temperature. Since binding of lysolecithin to red cells is apparently not influenced by the presence of sucrose (see Table I), one has to conclude that the mode of lysolecithin interaction with human red blood cells is definitely of different nature above and below 15 °C.

In trying to interpret the phenomena mentioned so far on a molecular basis, we have taken into consideration the parameter of "membrane fluidity" [17, 18]. We therefore determined the degree of fluorescence polarization (P) of diphenylhexatrien in red cell ghosts, which relates to membrane "fluidity" in that high P values correspond to a high restriction of the molecular mobility of the probe, i.e. to low "fluidity" and vice versa [18–20]. In fact, it may be seen from Fig. 6 not only that red cell ghosts exhibit the long-known decrease of P with increasing temperature [19], but in addition that the "fluidity" of the bovine erythrocyte membrane (curve 1) is significantly lower than that of human cells (curve 2). Moreover, the differences between the P values for the two types of membranes are smaller at 4 than at 40 °C. It thus appears that qualitatively there exists an inverse relationship between membrane fluidity and cellular sensitivity to lysolecithin (Fig. 2).

Quantitatively, however, the relation is less convincing. Especially the small effects of temperature on the lysolecithin sensitivity of bovine red cells, which have been demonstrated by the data in Fig. 2, are hardly explained by the data of Fig. 6 alone. On the other hand, we have previously demonstrated [22] by differential

TABLE I

BINDING OF [^{14}C]BENZYL-LYSOLECITHIN TO HUMAN RED CELLS IN THE ABSENCE AND PRESENCE OF SUCROSE

22 nmol (6000 cpm) of the benzyl-lysolecithin were added to 1.2 ml of the 0.5 % suspensions of human red cells at time zero. After 30 min incubation, samples were centrifuged and radioactivity in supernatant and sediment determined. As for the data of Fig. 5, the sucrose medium contained 50 mM KCl, 95 mM NaCl, 27 mM sucrose and 10 mM phosphate at pH 7.2.

Temperature (°C)	cpm bound / cpm free after 30 min incubation	
	In saline	In sucrose medium
37	1.37	1.38
30	0.81	0.79
22	0.36	0.38
15	0.29	0.26
10	0.27	0.33
4	0.17	0.14
0	0.10	0.07

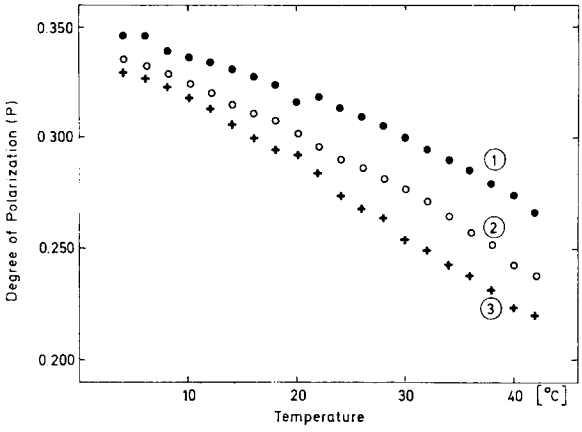

Fig. 6. Degree of fluorescence polarization (P) of diphenyl-hexatrien in erythrocyte ghosts. Measurements were carried out as described under Methods, section 3. The measuring cell contained 60 μg of red cell ghost protein (for human cells corresponding to about $5 \cdot 10^7$ ghosts) and 6 nmol diphenyl-hexatrien in 3 ml of 1 μM phosphate buffer, pH 7.4. (1) Temperature dependence of P in bovine; (2) in human erythrocyte ghosts. (3) P in human erythrocyte ghosts after addition of 10 nmol of benzyl-lysolecithin. Addition of only 5 nmol of the lysolipid resulted in a curve identical to (2).

scanning calorimetry that (a) lysophosphatides may be unevenly distributed between the various components of a mixed lipid phase and (b) relatively small amounts of lysolecithin may strongly affect the mixing behaviour of other lipids. We therefore feel that possibly the homogeneity of distribution of the lysolipid in the membrane lipid phase, which in turn will certainly be affected by the degree of "fluidity", may be more important in determining membrane sensitivity to lysolecithin lysis. A lower degree of homogeneity, i.e. a reduced miscibility of the lysophosphatide with other membrane lipids, would necessarily result in lysolecithin-rich areas on the surface and thus in an increased disruptive force of this detergent. The following experiments may be taken as an indication that such processes may indeed play a role in red cell lysis.

When human red cells are incubated with 20 nmol of benzyl-lysolecithin per $5.5 \cdot 10^7$ cells at 37 °C, hemolysis starts after a lag phase of about 25 min. (curve 2 in Fig. 7b), during which time the lysophosphatide is being constantly adsorbed to the cells (curve 1 in Fig. 7b and ref. 10). When the cells are transferred to 0 °C 6–7 min after the addition of the lysophosphatide, lysis begins instantanuously and is completed within the following 5–7 min (see Fig. 7b, curve 1). At that time about 4 nmol of the lysin are bound to the cells (Fig. 7a, curve 1), an amount which stays relatively constant during the process of cell lysis (Fig. 1a, curve 2). When both binding and lysis are performed at 0 °C (see Fig. 7c and d), the lytic process is in contrast extremely slow, and even after binding of 4 nmol of the lysolecithin to the cells exhibits a significant lag phase. We interpret these results by assuming that benzyl-lysolecithin is bound differently to membranes at 37 and 0 °C. The sudden lysis of lysolecithin-loaded cells upon cooling may be explained by a temperature-induced phase separation within the membrane, leading to lysolecithin-rich areas.

This effect of temperature is apparently dependent on the lysolecithin concentration in the membrane. As may be seen from Fig. 8 (curve 1), increasing time of preincubation at 37 °C, i.e. increasing lysolecithin content of the cells (see Fig. 7a),

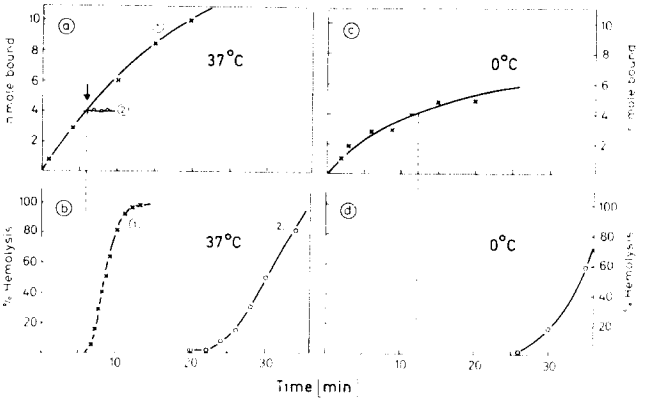

Fig. 7. Effect of cooling on human red cells, preloaded at 37 °C with benzyl-lysolecithin. All binding data are given in nmol bound per total cell sediment ($5.5 \cdot 10^7$ cells per 1.2 ml). (a) Adsorption kinetics of 20 nmol of benzyl-lysolecithin under standard conditions: curve 1 at 37 °C; curve 2 after transfer to 0 °C (arrow indicates time of temperature shift). (b) Lysis kinetics under conditions identical to (a). Curve 1, temperature shift from 37 to 0 °C after 6 min; curve 2, kinetics at 37 °C. (c) Adsorption kinetics of 100 nmol of benzyl-lysolecithin at 0 °C. (d) Lysis kinetics at 0 °C, conditions identical to (c).

leads to an increasing amount of lysis upon cooling only up to an adsorption period of about 8 min. After longer preincubation at 37 °C, the effect of temperature drop is reduced. This reduction is most pronounced after 15–20 min, which is shortly before lysis at 37 °C would start (curve 2, Fig. 8), corresponding to 8–10 nmol of cell-bound lysolipid. Considering our findings that lysolecithin analogs significantly increase the fluidity of human red cell ghosts (curve 3 in Fig. 6) and strongly affect the miscibility

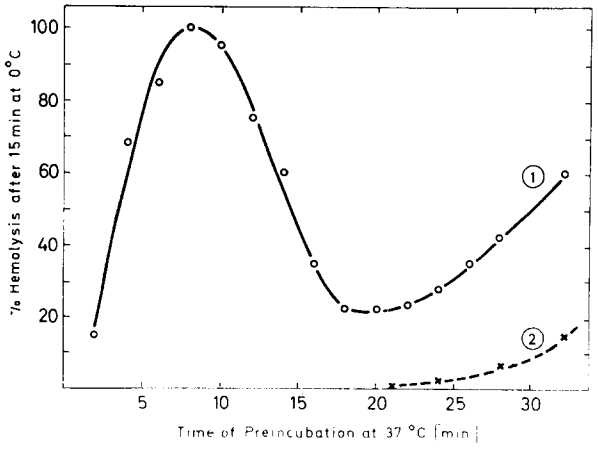

Fig. 8. Effect of cooling on human red cells, preloaded with varying amounts of benzyl-lysolecithin at 37 °C. Cells were mixed at time zero at 37 °C with 20 nmol of benzyl-lysolecithin under standard conditions. For corresponding binding curve see Fig. 7a, curve 1. After various times of preincubation at 37 °C samples were transferred to an icebath and centrifuged 10 min later. Curve 1 presents the amount of lysis under these conditions as a function of preincubation (adsorption) time. Curve 2 gives lysis data of control cells which were left at 37 °C with the same amount of lysolecithin.

of mixed lipid phases [22], this reduction of the temperature effect at high lysolecithin concentrations may be explained by an increased lipid miscibility and hence a reduction of temperature-induced phase separations.

Generalizing these assumptions one would expect that cellular sensitivity to lysolecithin is to a large extent controlled by the homogeneity of distribution of lysolecithin in the membrane. As mentioned above, this would easily explain the increasing hemolytic activity of lysolecithin with decreasing temperature. Moreover, the reported discontinuous change in susceptibility of human red cells to benzyllysolecithin at 15 °C (Fig. 5) can be understood on the same basis, since Tanaka and Ohnishi very recently [23] demonstrated a chain reorientation of the phosphatidylcholine moiety in human erythrocytes at about 18 °C.

NOTE ADDED IN PROOF (*received Sept. 27, 1976*).

Additional data have been published, supporting the notion of a lipid state transition in human erythrocyte membranes at about 15 °C: Bieri, V. G. and Wallach, D. F. H. (1976) Biochim. Biophys. Acta 443, 198–205.

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REFERENCES

1 Lucy, J. A. (1970) Nature 227, 815–817
2 Westphal, O., Fischer, H. and Munder, P. G. (1970) Proceedings of the 8th International Congr. Biochemistry, Switzerland, p. 319
3 Langer, W., Munder, P. G., Weltzien, H. U. and Westphal, O. (1973) Z. Immunitätsforsch. 145, 52
4 Collier, H. B. (1952) J. Gen. Physiol. 35, 617–628
5 Jung, F. (1959) Acta Biol. Med. Germ. 2, 481–495
6 Gottfried, E. L. and Rapport, M. M. (1963) J. Lipid Res. 4, 57–62
7 Munder, P. G., Ferber, E. and Fischer, H. (1965) Z. Naturforsch. 20b, 1048–1061
8 Arnold, D. and Weltzien, H. U. (1968) Z. Naturforsch. 23b, 675–683
9 Reman, F. C., Demel, R. A., De Gier, J., van Deenen, L. L. M., Eibl, H. and Westphal, O. (1969) Chem. Phys. Lipids 3, 221–233
10 Weltzien, H. U. (1973) Biochim. Biophys. Acta 311, 6–14
11 Weltzien, H. U., Arnold, B., Blume, A. and Kalkoff, H. G. (1976) Chem. Phys. Lipids 16 (3), 267–275
12 Weltzien, H. U., Arnold, B. and Westphal, O. (1973) Liebigs Ann. Chem. 1973, 1439–1444
13 Dodge, T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130
14 Weltzien, H. U. (1975) Exptl. Cell Res. 92, 111–121
15 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
16 Lawrence, A. J., Moores, G. R. and Steele, J. (1974) Europ. J. Biochem. 48, 277–286
17 Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731
18 Petit, V. A. and Edidin, M. (1974) Science 184, 1183–1185
19 Shinitzky, M., Dianoux, A. C., Gitler, C. and Weber, G. (1971) Biochemistry 10, 2106–2113
20 Rudy, B. and Gitler, C. (1972) Biochim. Biophys. Acta 288, 231–236
21 Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603–615
22 Blume, A., Arnold, B. and Weltzien, H. U. (1976) FEBS Lett. 61, 199–202
23 Tanaka, K. and Ohnishi, S. (1976) Biochim. Biophys. Acta 426, 218–231