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EFFECTS OF TEMPERATURE AND BOVINE SERUM ALBUMIN ON LYSIS OF ERYTHROCYTES INDUCED BY DILAUROYLGLYCEROPHOSPHOCHOLINE AND DIDECANOYLGLYCEROPHOSPHOCHOLINE

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Summary

The effects of the incubation temperature and bovine serum albumin on hemolysis induced by short-chain phosphatidylcholine were examined. The rate of hemolysis of human, monkey, rabbit, and rat erythrocytes by dilauroylglycerophosphocholine showed biphasic temperature-dependence: hemolysis was rapid at 5—10°C and above 40°C, but slow at around 25°C. In contrast, the rate of lysis of cow, calf, sheep, pig, cat, and dog erythrocytes did not show biphasic temperature-dependence, but increased progressively with increase in the incubation temperature. Bovine serum albumin increased the hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine or didecanoylglycerophosphocholine: it shortened the lag time of lysis and reduced the amount of phosphatidylcholine required for lysis. A shift-down of the incubation temperature from 40 to below 10°C also shortened the lag time of lysis of human erythrocytes induced by dilauroylglycerophosphocholine and reduced the amount of phosphatidylcholine required for lysis.

Gottfried and Rapport [1] studied the hemolytic actions of a series of lysophospholipids on human, rabbit and sheep erythrocytes at 37°C and found that the nature of the linkage of the hydrocarbon chain to glycerol did not influence their hemolytic actions. Reman et al. [2] showed that the acyl chain had a great influence on the lytic actions of phosphatidylcholine, lysophosphatidylcholine and deoxylysophosphatidylcholine on beef erythrocytes, using a series of chemically well-defined derivatives with different fatty acid chains. Among the phosphatidylcholines with chain lengths of 8 to 12 carbon atoms (per acyl

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chain) that they examined, diundecanoylglycerophosphocholine had the highest activity and dilauroylglycerophosphocholine the least. Arnold and Weltzien [3] also investigated the lytic activities of the homologous series of lysophosphatidylcholine, modified lysophosphatidylcholine and short-chain phosphatidylcholine on human erythrocytes; they found that didecanoylglycerophosphocholine was lytic but dilauroyl- and dipelargonoylglycerophosphocholine were not. It is interesting that among the various phospholipids tested only didecanoylglycerophosphocholine caused more rapid lysis of human erythrocytes at 10 than at 37°C.

We are interested in the relation between the structure of the membranes of erythrocytes and the lytic effects of various phospholipids. We studied the lysis of human erythrocytes by dilauroylglycerophosphocholine at various temperatures and found that the rate of lysis showed a biphasic dependence on temperature. We also studied the effects of bovine serum albumin and shift-down of the incubation temperature on the lysis of human red cells by dilauroylglycerophosphocholine. Preliminary results of these studies have been published [4].

Materials and Methods

Buffer. Veronal-buffered saline free of Ca^{2+} and Mg^{2+} was used throughout. It contained 0.375 g of sodium 5,5-diethylbarbiturate, 0.575 g of 5,5-diethylbarbituric acid, 8.5 g of sodium chloride per l of distilled water, pH 7.5.

Erythrocytes. The human erythrocytes used were from freshly drawn, heparinized blood of healthy donors. The blood was centrifuged at 300 × *g* for 5 min and the plasma and buffy coat were discarded. The precipitated cells were then washed three times with veronal buffered saline and used for experiments within 24 h. Erythrocytes of other animals were obtained in a similar way.

Lipids. Didecanoyl- and dilauroylglycerophosphocholines were obtained from Serdary Research Laboratories Inc., Ontario, Canada and were used without further purification. Vesicles of didecanoyl-, and dilauroylglycerophosphocholine were prepared by suspending the dried sample of lipid in veronal buffered saline and sonicating the suspensions in a bath-type sonicator for 30 min at room temperature.

Bovine serum albumin (crystalline) and fatty acid-free bovine albumin were obtained from Armour Pharmaceutical Co., U.S.A. and Nutritional Biochemicals Co., Cleveland, Ohio, respectively.

Measurement of hemolysis. Hemolysis was measured using cells labeled with radioactive chromate by the method of Inoue et al. [5]. Veronal buffered saline solution containing an appropriate amount of phosphatidylcholine (0.4 ml) was preincubated for 10 min at the required temperature and the reaction was started by adding 0.1 ml of ^{51}Cr-labeled erythrocyte suspension ($5 \cdot 10^7$ cells/ml) which had been kept in ice-water. The reaction mixture was gently shaken occasionally. After incubation the mixture was centrifuged at 200 × *g* for 5 min and 250 µl of the supernatant was carefully removed for counting in an auto-gamma counter (Aloka Auto-well Gamma Counter). The percentage hemolysis was then calculated as follows:

$$\frac{\text{counts in 250 } \mu\text{l of the supernatant} \times 2}{\text{counts in } 2.5 \cdot 10^6 \text{ erythrocytes}} \times 100$$

Results

Effects of temperature on hemolysis of human and cow erythrocytes by dilauroylglycerophosphocholine. When human erythrocytes were incubated with excess dilauroylglycerophosphocholine (21 nmol/ml) at various temperatures for 90 min, the rate of hemolysis was found to depend on the incubation temperature. The rate of lysis was very high above 40°C, but with decrease in temperature the rate decreased to a minimum at 30—25°C, and then increased again to a maximum at around 5—10°C, as reported in the previous paper [4]. The amount of phosphatidylcholine required for lysis of human erythrocytes was least at 10°C, and more at 45, 40 and 0°C, in this order. In contrast, the rate of lysis of cow erythrocytes by excess dilauroylglycerophosphocholine (17 nmol/ml) did not show a biphasic dependence on temperature: it was slow at 0—10°C and rapid above 15°C.

Effects of temperature on hemolysis of erythrocytes from various animals by dilauroylglycerophosphocholine. The sensitivities of erythrocytes of various animals to dilauroylglycerophosphocholine were examined at temperatures between 0 and 40°C and classified into two types on the basis of the results. Monkey, rabbit and rat erythrocytes were of the human type, their hemolysis being rapid at 10 and 40°C but slow at 0 and 25°C (Fig. 1A). The lysis of chicken erythrocytes, was slightly different but it was also more rapid at 0 and 40°C than at 10 or 25°C. In contrast, the lysis of calf, sheep, pig, dog, and cat erythrocytes, like that of cow erythrocytes (Fig. 1B) increased with increase in the incubation temperature.

Effect of temperature on hemolysis of human erythrocytes by didecanoylglycerophosphocholine. Hemolysis of humun erythrocytes by didecanoylglyc-

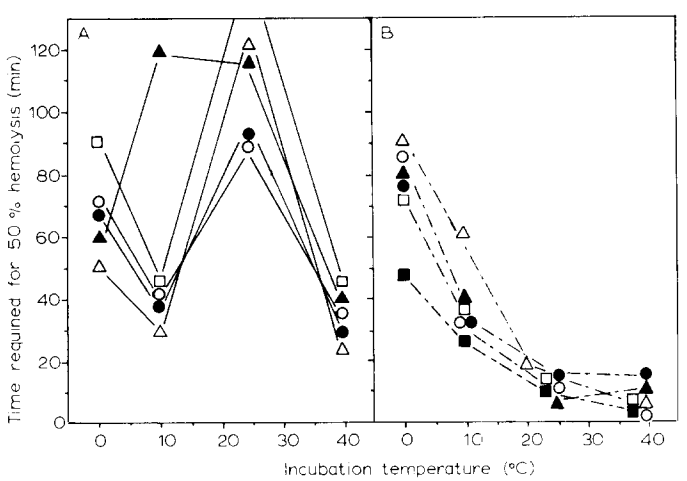

Fig. 1. Effect of temperature on the rate of hemolysis of erythrocytes of various animals by dilauroylglycerophosphocholine. (A) Human (□); monkey (○), rabbit (●), rat (△) and chicken (▲) erythrocytes were incubated with 21 nmol/ml of dilauroylglycerophosphocholine for various periods at different temperatures. (B) Cow (△), sheep (○), dog (●), calf (□), pig (■) and cat (▲) erythrocytes were treated with dilauroylglycerophosphocholine as described in A. The times required for 50% hemolysis are plotted against the incubation temperatures.

erophosphocholine also showed a biphasic change with temperature, being faster at 10 and 40°C than at 25°C.

Effect of bovine serum albumin on hemolysis of human erythrocytes by dilauroyl- and didecanoylglycerophosphocholine. The lytic activity of lysophosphatidylcholine is known to be inhibited by bovine serum albumin. When dilauroylglycerophosphocholine and didecanoylglycerophosphocholine were preincubated with bovine serum albumin their lytic activities were also inhibited; Table I shows the effects of preincubating dilauroylglycerophosphocholine with bovine serum albumin at 10 or 40°C for 30 min before adding it to erythrocytes. These phosphatidylcholine may combine with bovine serum albumin as lysophosphatidylcholine does.

The lysis of human red cells by dilauroylglycerophosphocholine at either 40 or 10°C showed a "lag time" of 30 min, as reported previously. Bovine serum albumin was found to shorten this lag time. Addition of bovine serum albumin to human erythrocytes that had been preincubated with dilauroylglycerophosphocholine for 15 min at 40°C, resulted in rapid and complete hemolysis (Fig. 2). The rate of hemolysis due to addition of phosphatidylcholine plus bovine serum albumin depended on the amounts of both bovine serum albumin (Fig. 3A) and phosphatidylcholine (Fig. 3B). Fig. 3B shows that the presence of bovine serum albumin decreased the concentration of phosphatidylcholine required for complete lysis from 16 nmol/ml to 10 nmol/ml. The presence of bovine serum albumin also reduced the concentration of didecanoylglycerophosphocholine required for lysis of human erythrocytes. Since fatty acid-free albumin had a similar effect of bovine serum albumin, the effect was not be due to contaminating fatty acids.

Effect of pre-incubation temperature on shortening by bovine serum albumin of the "lag phase" in lysis of human erythrocytes with dilauroylglycerophosphocholine. Bovine serum albumin shortened the lag phase in hemolysis when erythrocytes were preincubated with dilauroylglycerophosphocholine for more than 5 min at 40°C (Fig. 4). At 25°C, about 60 min preincubation was required for complete lysis with bovine serum albumin. However, when the erythrocytes had been preincubated with dilauroylglycerophosphocholine at

TABLE I

INHIBITION BY BOVINE SERUM ALBUMIN OF LYSIS OF HUMAN ERYTHROCYTES BY DILAUROYLGLYCEROPHOSPHOCHOLINE

Human erythrocytes (10^7 cells/ml) were incubated with 21 nmol/ml of dilauroylglycerophosphocholine, 4 mg of bovine serum albumin or mixtures of 21 nmol/ml of dilauroylglycerophosphocholine and 4 mg/ml of bovine serum albumin for 90 min at 10 or 40°C. Hemolysis was measured as described in text.

	Hemolysis percent at incubation temperature	
	10°C	40°C
None	6.5	13.5
Dilauroylglycerophosphocholine	94.0	98.0
Dilauroylglycerophosphocholine + bovine serum albumin	1.7	3.0
Bovine serum albumin	5.0	6.5

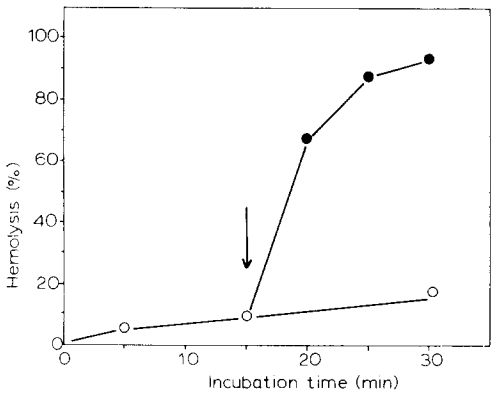

Fig. 2. Effect of bovine serum albumin on the "lag phase" in lysis of human erythrocytes by dilauroylglycerophosphocholine. Erythrocytes (10^7 cells/ml) were incubated with 21.1 nmol/ml of dilauroylglycerophosphocholine at 40°C for 15 min. Then samples were incubated further with (●) or without (○) 4 mg/ml of bovine serum albumin.

10°C for 30 min no lysis was observed on addition of bovine serum albumin and shift of the temperature to 25°C.

Osmotical fragility of human erythrocytes treated with dilauroylglycerophosphocholine. The osmotical fragility of erythrocytes that had been incubated with dilauroylglycerophosphocholine at 25°C for 30 min was less than that of untreated human erythrocytes (Fig. 5).

Effect of shift-down in temperature on lysis of human red blood cells pretreated with dilauroylglycerophosphocholine. The lag time in lysis of human erythrocytes treated with dilauroylglycerophosphocholine was also shortened by shifting the temperature from 40 to 10 or 0°C but not from 40 to 25°C during incubation (Fig. 6). The rate of hemolysis was also increasing by shifting the temperature from 30 to 0°C during incubation, but hemolysis was not com-

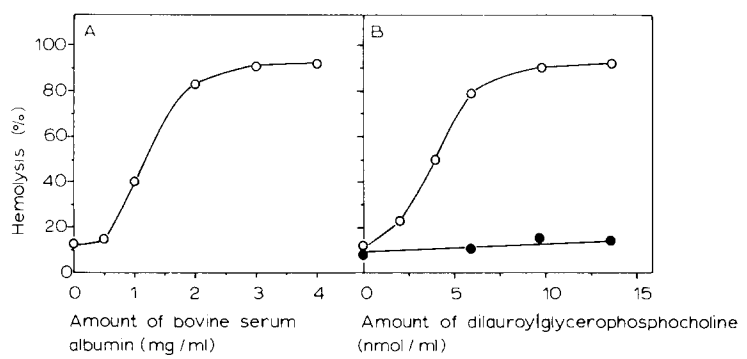

Fig. 3. (A) Effect of the amount of bovine serum albumin on lysis of human erythrocytes by dilauroylglycerophosphocholine. Human erythrocyes (10^7 cells/ml) were incubated with 21 nmol/ml of dilauroylglycerophosphocholine at 40°C for 15 min. Then various amounts of bovine serum albumin were added and hemolysis was measured after further incubation for 15 min at 25°C. (B) Effect of the amount of dilauroylglycerophosphocholine on bovine serum albumin-dependent lysis of human erythrocytes. Human erythrocytes (10^7 cells/ml) were incubated with various amounts of dilauroylglycerophosphocholine for 15 min at 40°C. Hemolysis was measured after further incubation at 25°C for 15 min with (○) or without (●) 4 mg/ml of bovine serum albumin.

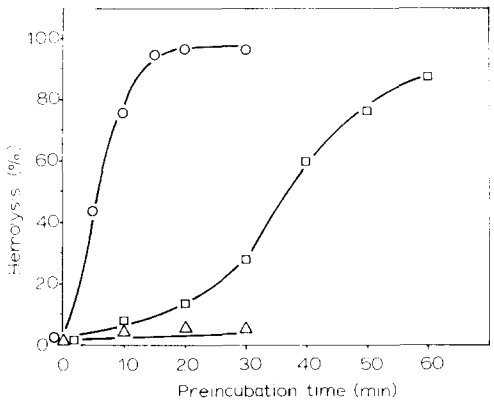

Fig. 4. Effect of preincubation temperature on bovine serum albumin-dependent lysis of human erythrocytes treated with dilauroylglycerophosphocholine. Human erythrocytes (10^7 cells/ml) were incubated with dilauroylglycerophosphocholine (21.1 nmol/ml) for various times at 40°C (○), 25°C (□), and 10°C (△). Hemolysis was measured after further incubation for 15 min at 25°C with 4 mg/ml of bovine serum albumin.

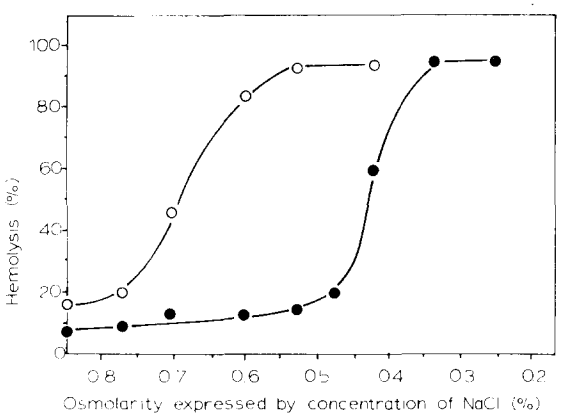

Fig. 5. Increase in osmotic fragility of human erythrocytes on treatment with dilauroylglycerophosphocholine. Erythrocytes were incubated with (○) and without (●) 21.2 nmol/ml of dilauroylglycerophosphocholine for 30 min at 25°C. Then various amounts of veronal-buffered saline were added, the mixtures were incubated further for 30 min at 25°C, and hemolysis was measured. Hemolysis is plotted against the final osmolarity of the reaction mixture.

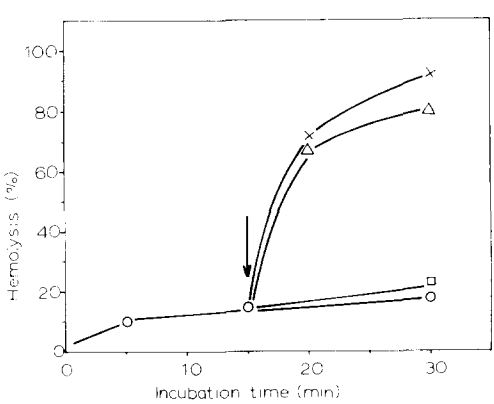

Fig. 6. Effect of shift-down in temperature on lysis of human erythrocytes pretreated with dilauroylglycerophosphocholine. Human erythrocytes (10^7 cells/ml) were incubated with 21 nmol/ml of dilauroylglycerophosphocholine at 40°C for 15 min. Then the reaction mixture were shifted from 40 to 0°C (X), 10°C (□) and 25°C (△) and incubated further for 15 min. The control was incubated at 40°C without shift-down of the temperature (○).

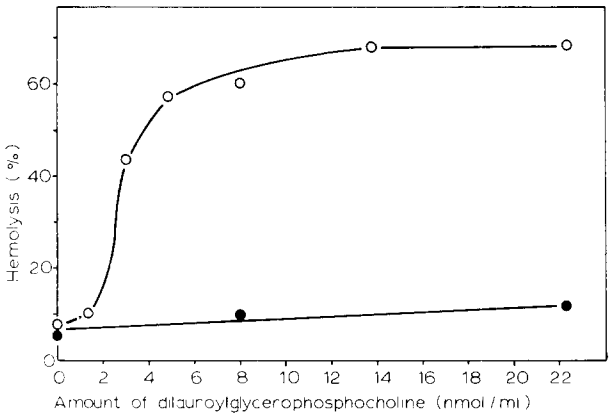

Fig. 7. Effect of shift down in temperature on the dependence of hemolysis on dilauroylglycerophosphocholine concentration. Human erythrocytes (10^7 cells/ml) were incubated with various amounts of dilauroylglycerophosphocholine for 10 min at 40° C. Then hemolysis was measured 5 min after shifting the temperature to 0° C (○) or further at 40° C (●).

plete within 5 min. Fig. 7 shows the effect of the dilauroylglycerophosphocholine concentration on lysis of erythrocytes incubated first at 40° C for 10 min and then at 0° C for 5 min: the amount of phosphatidylcholine required for lysis was less when the temperature was shifted down than when it was not. It should be noted that after the shift-down of temperature lysis was again not complete.

Discussion

Reman et al. [2] studied the lysis of beef erythrocytes by a series of short-chain phosphatidylcholines at 20° C; they showed that the minimum concentration of the most active phosphatidylcholine, diundecanoylglycerophosphocholine, was of the same order as that of stearoylglycerophosphocholine. However, with all the phosphatidylcholines tested, the "lag time", that is the time from the beginning of the incubation to the start of lysis, was much longer than with lysocompounds. Arnold and Weltzien [3] also examined the lysis of human erythrocytes by didecanoylglycerophosphocholine; they found that lysis by this phosphatidylcholine had a shorter lag time and was more rapid at 10 than at 37° C. We found that hemolysis of human erythrocytes by dilauroylglycerophosphocholine showed pronounced temperature-dependence, shift-down in temperature increasing lysis.

It is unknown why temperature affects the rate of hemolysis by phosphatidylcholines. The receptor for lysophosphatidylcholine or short-chain phosphatidylcholines on natural membranes have not been identified, though they could be lipid constituents of the membrane [2]. Liposomal membranes were found to be sensitive to lysophosphatidylcholine when they were in a state of phase separation [6,7]. Short-chain phosphatidylcholine may also interact mainly with parts of erythrocyte membranes showing phase separation. Shift-down in the incubation temperature was reported to result in damage by lysophosphatidylcholine of liposomal membranes prepared from egg phosphatidyl-

choline [6]. It has been suggested that shift-down of the temperature might cause temporary phase separation of lipid components of the bilayer, and this could result in increased hemolysis on shift-down of temperature.

In the present investigation on the temperature dependence of lysis of erythrocytes of various animals by dilauroylglycerophosphocholine, two types of erythrocytes were distinguished. The rate of lysis of the erythrocytes of cat, dog, cow, calf, pig and sheep increased linearly with increase in the incubation temperature. In contrast, the rate of lysis of human, rat, rabbit, monkey and chicken erythrocytes showed a biphasic relation with the incubation temperature. Previously de Gier et al. [8] showed that the red cells of various animals could be divided into two similar groups on the basis of the temperature dependence of penetration of glycerol into the cells. The erythrocytes from the first group described above showed a glycerol permeability which is strongly dependent on temperature. On the other hand glycerol permeation into the red cells of the second group was found to be much less dependent on temperature. Red cells can also be classified into two groups on the basis of their contents of Na^+ and K^+ [9]. These classification, however, do not necessarily correspond to the present one. Red cells of calf and pig, which are grouped to the cow type either by the glycerol permeability observation or by the present experiment, are involved in the cells of the human group which contains low Na^+ and high K^+.

It was shown that after nonhemolytic hydrolysis of membrane phospholipid by purified cobra venom phospholipase A_2, addition of bovine plasma albumin (Fraction V) results in hemolysis without any further hydrolysis of phospholipid [10,11]. The lysis by albumin does not seem to be entirely due to the ability of albumin to remove cleaved fatty acid from the membrane, but may result from its binding to and removal of lyso-derivatives of the membrane produced by the enzyme. It has also been reported that 1 mol of albumin binds 1 mol of lysophosphatidylcholine [12,13]. In this study we found that the "lag times" in hemolysis of erythrocytes by didecanoyl- or dilauroylglycerophosphocholine were shortened by addition of albumin to the medium.

Hemolysis may occur as follows: first phosphatidylcholine is adsorbed to the surface of the membrane; then there is a "deeper interaction" of the molecules with the membrane, resulting in disorganization of the structural array and change in permeability. During the "lag time" in the hemolysis by phosphatidylcholine, some step in these events may proceed without causing lysis. In the case of human erythrocytes, the rate of some early step may increase on increasing the incubation temperature, since the state of erythrocyte which was sensitive to albumin or to a shift-down of temperature, was reached more rapidly at a higher temperature. Addition of albumin or shift-down of the temperature may stimulate the step inducing a change of organization. It was calculated that when erythrocytes has been treated with 21 nmol of dilauroylglycerophosphocholine or 6.4 nmol of didecanoylglycerophosphocholine about 30—45 nmol or 7.5 nmol of bovine serum albumin (mol. wt. 67 000), respectively, were required for complete lysis. The binding of dilauroyl- or didecanoylglycerophosphocholine to albumin seems to be stoichiometric, 1 : 1 or 1 : 2, like the binding of lysophosphatidylcholine to albumin. Quantitative experiments are required to confirm that similar values were estimated from experiments on the interaction of short-chain phosphatidylcholine liposomes [14] and of lipo-

somes containing lysophosphatidylcholine [15] with albumin.

It is interesting that the amount of dilauroylglycerophosphocholine required for 50% lysis of erythrocytes in the presence of bovine serum albumin is the same as that for 50% lysis of erythrocytes at 10°C. This amount of dilauroylglycerophosphocholine ($4 \cdot 10^{-16}$ mol/cell), which presumably reacts with erythrocytes in some way, is more than the amount of phospholipids in erythrocytes (about $2 \cdot 10^{-16}$ mol/cell) [16]. Using benzylated lysophosphatidylcholine (*rac*-1-alkyl-2-benzylglycero-3-phosphocholine) derivatives, Weltzien [17] showed that at the beginning of hemolysis the molar amounts of three lysophosphatidylcholine derivatives, including stearoylglycerophosphocholine, bound to the red cells were similar ($1.5 \cdot 10^{-16}$ mol/cell). This amount is similar to the amount required for 50% hemolysis, and is of the same order as the minimum amount of dilauroylglycerophosphocholine required for 50% lysis, in spite of the fact that lysophosphatidylcholine and dilauroylglycerophosphocholine exist in different aggregated forms, as spherical micelles and liposomes, respectively. Recently, Weltzien et al. [18] also reported the aggregation state of the slow reacting lysophosphatidylcholine analog, 1-octadecyl-2-benzylglycero-3-phosphocholine, in aqueous solution. Aggregates of this were most similar to those of phosphatidylcholine liposomes than to usual lysophosphatidylcholine spherical micelles. They concluded that this liposomal structure is responsible for the slow adsorption of this lysophosphatidylcholine analog to erythrocytes and they proposed that the rate of adsorption may be determined by the rate of escape of single lysophospholipid molecules from the liposomes. These valuable experiments, together with the present study on dilauroylglycerophosphocholine which also has liposomal structures, will undoubtedly stimulate furter investigations.

Acknowledgements

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