Biochimica et Biophysica Acta, 600 (1980) 126—139

BBA 78834

INTERACTION OF DILAUROYLGLYCEROPHOSPHOCHOLINE WITH ERYTHROCYTES

PRE-HEMOLYTIC EVENTS AND HEMOLYSIS

YUTAKA TANAKA, KEIZO INOUE and SHOSHICHI NOJIMA

Department of Health Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo (Japan)

(Received November 6th, 1979)

Key words: Dilauroylglycerophosphocholine; Hemolysis; K^+ leakage; Erythrocyte

Summary

The process of interaction of dilauroylglycerophosphocholine with erythrocytes that eventually results in hemolysis was examined.

1. The rate of uptake of dilauroylglycerophosphocholine by human erythrocytes was rather slow, but increased with increasing temperature.

2. The first observable change of human erythrocytes induced by dilauroylglycerophosphocholine was a morphological change from discocytes to spherechinocytes. This change preceded K^+ leakage.

3. Adsorption of dilauroylglycerophosphocholine to human erythrocytes caused K^+ leakage. The rate of K^+ leakage was also temperature-dependent. The temperature-dependence was due to the temperature-dependence of lipid uptake, because in order to cause K^+ leakage a given amount of dilauroylglycerophosphocholine must be bound to the erythrocytes, irrespective of the temperature.

4. The temperature-dependence of hemolysis of human erythrocytes was different from that of pre-hemolytic events (morphological change, adsorption of lipids and K^+ leakage). Hemolysis was rapid below 10 and above 37°C, but slow at about 25°C. The hemolysis observed below 10°C seemed to be a 'colloid osmotic lysis', since it occurred immediately after K^+ leakage, but the hemolysis above 37°C may not be a colloid osmotic lysis. Above 37°C, additional binding of dilauroylglycerophosphocholine to the erythrocyte membrane may cause hemolysis by a different mechanism from that working below 10°C.

5. Above 25°C, most human erythrocytes are resistant to colloid osmotic

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholine)-ethanesulfonic acid.

lysis induced by dilauroylglycerophosphocholine. It can be concluded that human erythrocytes are composed of two types of population, one resistant, and one sensitive to colloid osmotic lysis. The mechanism of the resistance was sensitive to temperature, pH and various sulfhydryl agents.

6. Pig erythrocytes were hemolysed immediately after K^+ leakage even above 25° C, indicating that they were sensitive to a colloid osmotic mechanism over the whole temperature range tested. Pig erythrocytes may lack the 'mechanism' giving resistance to colloid osmotic lysis.

The process of hemolysis of erythrocytes by dilauroylglycerophosphocholine is shown schematically.

Introduction

Interactions of liposomes with various animal cells have recently attracted widespread interest because these interactions may provide models of various physiological processes, such as fusion, adhesion, cytosis and exchange of membrane components [1]. Weltzien and coworkers studied the mechanism of lysis of erythrocytes by benzylated lysophosphatidylcholine derivatives, which are known to form liposomes and cause characteristic slow lysis [2,3].

Previously, we reported [4,5] two characteristics of lysis of human erythrocytes by dilauroylglycerophosphocholine. (1) The lag time of hemolysis was rather longer than that of hemolysis by lysolecithin, hemolysis taking 30 min under our experimental conditions. (2) The rate of hemolysis showed biphasic temperature-dependence: hemolysis was rapid at 5—10° C and above 37° C, but slow at about 25° C.

These two characteristics seem useful because the long lag time enables us to study the interaction of the liposomes with erythrocytes before hemolysis more easily, and the biphasic temperature-dependence should provide clues to the nature of the erythrocyte membrane.

In this work, we examined the process of interaction of dilauroylglycerophosphocholine with erythrocytes that leads eventually to hemolysis of the cells.

Materials and Methods

Buffer. Tris-buffered saline (150 mM NaCl and 5 mM Tris-HCl, pH 7.4) was used throughout, except when otherwise noted. In experiments on the pH-dependence of hemolysis, 150 mM NaCl buffered with 5 mM Mes or with 5 mM Hepes was used. When K^+ leakage from erythrocytes and hemolysis were measured simultaneously, 150 mM choline chloride buffered with 5 mM Tris-HCl (pH 7.4) was used. The kinetics of hemolysis were not appreciably affected by the monovalent cation species.

Erythrocytes. The human erythrocytes used were from freshly drawn, heparinized blood of healthy donors. The blood was centrifuged at $300 \times g$ for 5 min and the plasma and buffy coat were discarded. The precipitated cells were then washed three times with the medium used in the experiment and used with 48 h.

Pig erythrocytes were obtained in a similar way.

Lipid. Dilauroylglycerophosphocholine was prepared by using the method of Robles and van den Berg [6]. Vesicles of dilauroylglycerophosphocholine were prepared by suspending the dried sample of lipid in the medium used in the experiments and sonicating the suspension in a sonicator equipped with a microprobe (Branson Sonifier Model W185, operating at 18 W) for 10 min at room temperature. [*N*-C^3H_3]Dilauroylglycerophosphocholine (5.1 Ci/mol) was prepared by introducing the ^{3}H-labeled methyl group into the choline moiety with [^{3}H]methyl iodide (Radiochemical Centre, Amersham) by using the method of Stoffer et al. [7].

Uptake of [^{3}H]dilauroylglycerophosphocholine by erythrocytes. A series of plastic tubes (Eppendorf 3810) containing 500 μl of aqueous [^{3}H]dilauroylglycerophosphocholine suspension were placed in a water bath at an appropriate temperature. Then 100-μl of erythrocyte suspension ($6 \cdot 10^7$ cells/ml) were added, and after incubation for appropriate times, the mixtures were centrifuged ($10\,000 \times g$, 20 min) and the radioactivities in the top 300 μl of the supernatant and in the sediment plus the lower 300 μl of the supernatant were measured. For this, both samples were transferred to scintillation vials and oxidized with H_2O_2 and the resulting colorless solutions were mixed with 10-ml of scintillation cocktail, and their radioactivities were determined in a liquid scintillation counter (Packard 3320). The number of moles of dilauroylglycerophosphocholine adsorbed to the erythrocytes was calculated from the total radioactivity in the sediment fraction minus the radioactivity in the buffer, measured as that in the top 300 μl of buffer [2].

Scanning electron microscopic observations. Erythrocyte suspensions were fixed by adding 4 vols. of 0.9% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4. The mixture was allowed to stand for 1 h at room temperature and then centrifuged and the precipitated cells were washed three times with distilled water and resuspended in water. A small amount of this suspension was placed on an aluminum brick and dried at room temperature. The specimen was coated with gold in a vacuum chamber and examined in a scanning electron microscope (Shimadzu, type ASM) with an accelerating voltage of 15 kV.

Measurement of hemolysis. In some experiments, hemolysis was measured using cells labeled with radioactive chromate by the method of Inoue et al. [8]. An appropriate amount of aqueous dilauroylglycerophosphocholine suspension (500 μl) was preincubated for 10 min at the required temperature and the reaction was started by adding 100-μl of ^{51}Cr-labeled erythrocyte suspension ($6 \cdot 10^7$ cells/ml). The mixture was occasionally gently shaken during the reaction. After incubation, the mixture was centrifuged at $300 \times g$ for 5 min, then 300-μl of the supernatnat were carefully removed for counting in an auto-well gamma counter (Aloka, JDC 751). The percentage hemolysis was calculated as follows:

$$\frac{\text{counts in 300 } \mu\text{l of the supernatant} \times 2}{\text{counts in } 6 \cdot 10^6 \text{ erythrocytes}} \times 100$$

Hemolysis was also assayed by measuring turbidity change. An aliquot (0.5 ml) of washed erythrocyte suspension ($6 \cdot 10^7$ cells/ml) was transferred to a

cuvette containing 2.5 ml of isotonic medium identical to the washing medium. The cuvette was placed in a double-beam spectrophotometer (Shimadzu, UV 140) and equilibrated at the desired temperature. The erythrocyte suspension was agitated continuously with a small magnetic stirrer and the change in turbidity at 675 nm with time was recorded. Then dilauroylglycerophosphocholine suspension (5—20 μl) was added and the mixture was incubated for various periods. The percentage hemolysis was calculated from the change in turbidity. A control experiment showed that there was no difference between the percentage hemolysis calculated from the change of turbidity and that calculated using ^{51}Cr-labeled erythrocytes.

Measurement of K^+ leakage from the erythrocytes. The release of K^+ from erythrocytes was monitored with a miniature K^+ electrode and reference electrode (Microelectrodes Inc., NH, U.S.A., MI-440 and MI-409) which were immersed in the cuvette. The K^+ electrode and reference electrode were connected to a pH meter (Hitachi-Horiba, F-7ss) and a recorder. The amount of K^+ in the erythrocytes was determined by lysing them with Triton X-100. The electrode was calibrated at each temperature by measurements of standard KCl solutions. The leakage of K^+ was expressed as the percentage of K^+ released. Hemolysis was measured simultaneously by measuring turbidity change.

Results

Uptake of dilauroylglycerophosphocholine liposomes by human erythrocytes. Uptake of [^{3}H]dilauroylglycerophosphocholine by human erythrocytes was examined by incubating erythrocytes with excess dilauroylglycerophosphocholine at various temperatures for various periods. After incubation, the radioactivity bound to erythrocytes was calculated as described in Materials and Methods. The liposomes seemed to be taken up by erythrocytes irreversibly, since the radioactivity in the erythrocytes did not change when the cells were washed. The uptake was temperature-dependent; it proceeded faster at higher incubation temperatures (Fig. 1A). At 10°C, only a small amount of dilauroylglycerophosphocholine was bound to erythrocytes even after incubation for 90 min. Fig. 1B shows the dose-dependence of the uptake of dilauroylglycerophosphocholine by erythrocytes. These results also again indicate the temperature-dependence because uptake at 37°C was more efficient than that at 10°C.

Morphological change of human erythrocytes induced by dilauroylglycerophosphocholine. Fig. 2 shows the time course of the dilauroylglycerophosphocholine-induced hemolysis at 10°C. Erythrocytes gave a higher noise level in the turbidity curve before they were treated with dilauroylglycerophosphocholine. When they were treated with dilauroylglycerophosphocholine, noise in turbidity gradually decreased and disappeared within 4 min. During incubation for a further 15 min there was no appreciable change of turbidity. Then, an abrupt decrease of turbidity was observed, indicating that hemolysis occurred suddenly after a lag of about 20 min. The process of hemolysis determined by measuring turbidity was very similar to that determined by measuring chromate release [4].

Under a scanning electron microscope, untreated erythrocytes had a normal biconcave shape, as shown in Fig. 3B. The biconcave shape of erythrocytes

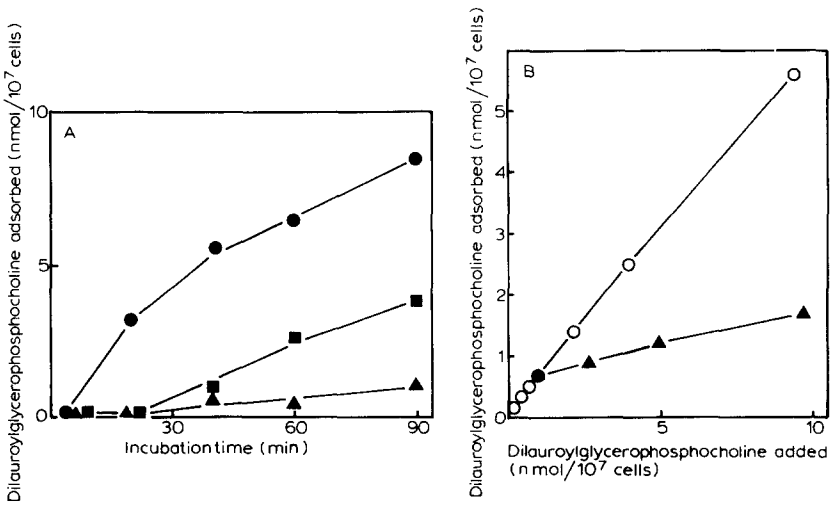

Fig. 1. Uptake of [^{3}H]dilauroylglycerophosphocholine by human erythrocytes at various temperatures. (A) Time course of uptake of [^{3}H]dilauroylglycerophosphocholine by erythrocytes at 10 (▲), 25 (■) and 40°C (●). Human erythrocytes (10^7 cells/ml) were incubated with 20 μM of dilauroylglycerophosphocholine at various temperatures. Uptake of dilauroylglycerophosphocholine by erythrocytes was determined as described in Materials and Methods. (B) Uptake of [^{3}H]dilauroylglycerophosphocholine by erythrocytes as a function of the amount of dilauroylglycerophosphocholine added. Erythrocytes (10^7 cells/ml) were incubated with various amounts of dilauroylglycerophosphocholine for 90 min at 10 (▲) and 37°C (○).

might be responsible for the high noise level in the turbidity curve, since the cells tend to become aligned along the turbulent flow created by the stirrer. Most erythrocytes appeared as spheroechinocytes after 5-min treatment with dilauroylglycerophosphocholine (Fig. 3A). The time-dependent change in shape from discocytes to spheroechinocytes, which was observed under a scanning

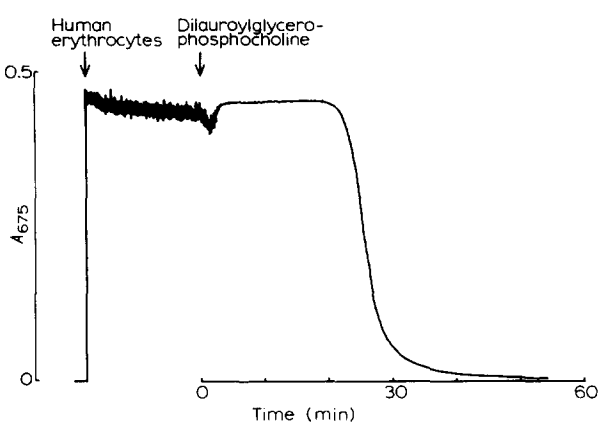

Fig. 2. Turbidity change of human erythrocytes induced by dilauroylglycerophosphocholine at 10°C. An aliquot (0.5 ml) of washed erythrocyte suspension ($6 \cdot 10^7$ cells/ml) was transferred to a cuvette containing 2.5 ml of 5 mM of Tris-buffered saline equilibrated at 10°C in a spectrophotometer. Then 15-μl of dilauroylglycerophosphocholine suspension (5 mM) were added and the change in turbidity at 675 nm with time was recorded as described in Materials and Methods.

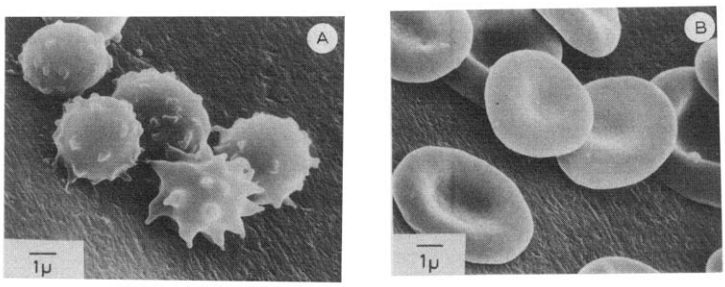

Fig. 3. Scanning electron micrographs of human erythrocytes. (A) Erythrocytes were incubated with 25 μM dilauroylglycerophosphocholine in 5 mM Tris-buffered saline (pH 7.4) for 10 min at 10°C. Samples were then fixed, dried and coated with gold as described in Materials and Methods. (B) Erythrocytes were incubated in Tris-buffered saline without dilauroylglycerophosphocholine for 10 min at 10°C, then treated as described above.

electron microscope, corresponds well to the decrease in noise observed on turbidity assay, indicating that the decrease in noise might be due to shape change.

The temperature-dependence of the change in shape induced by dilauroylglycerophosphocholine was next examined. The time required for morphological change (change in shape) was plotted against the incubation temperature. As described above, incubation of erythrocytes with dilauroylglycerophosphocholine at 10°C for 4 min was required for complete disappearance of the noise in turbidity. With increasing incubation temperature, the rate of transformation seemed to increase (Fig. 4); change in shape was complete within 1 min when erythrocytes were incubated with dilauroylglycerophosphocholine above 25°C.

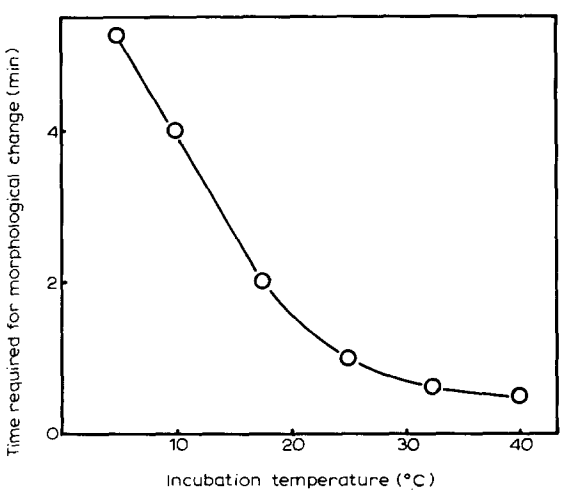

Fig. 4. Temperature-dependence of change in shape of human erythrocytes induced by dilauroylglycerophosphocholine. Erythrocytes (10^7 cells/ml) were incubated with 25 μM dilauroylglycerophosphocholine at various temperatures and the turbidity changes were recorded as described for Fig. 2. The time required for complete disappearance of the noise in turbidity (time required for the morphological change) is plotted against the incubation temperature.

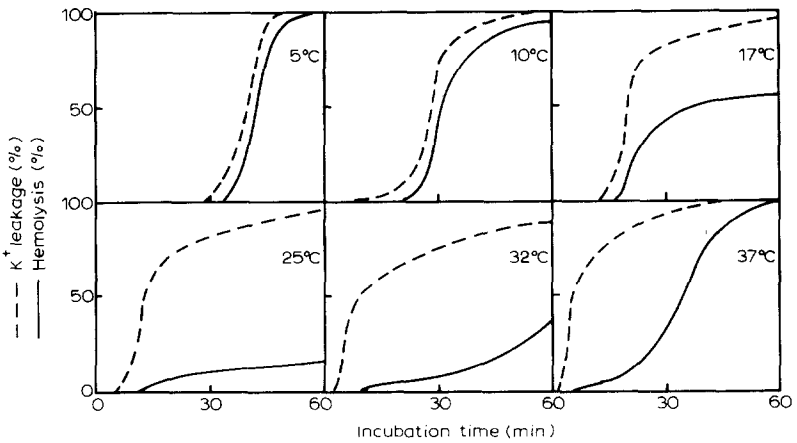

Fig. 5. Time course of K^+ leakage and hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine at various temperatures. Erythrocytes (10^7 cells/ml) were incubated with 25 μM dilauroylglycerophosphocholine. The percentage of K^+ leakage from erythrocytes (- - - - -) and the percentage of hemolysis (———) were monitored simultaneously as described in Materials and Methods.

No appreciable amount of dilauroylglycerophosphocholine was adsorbed on the erythrocytes when the change occurred (Fig. 1A).

Temperature-dependence of K^+ leakage and hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine. The kinetics of K^+ leakage from human erythrocytes and of hemolysis induced by dilauroylglycerophosphocholine were measured simultaneously as described in Materials and Methods (Fig. 5).

When erythrocytes were incubated with 20 μM of dilauroylglycerophosphocholine at 5°C, K^+ leakage started after 35 min. Almost immediately after K^+ leakage, the turbidity of the erythrocyte suspension began to decrease, indicating that hemolysis occurred almost immediately. Incubation at 10°C shortened both the lag phase of K^+ leakage and that of hemolysis; about 27 and 30 min were required for 50% of the K^+ leakage and hemolysis, respectively. At 25°C, K^+ leakage occurred rather faster than at 10°C; 13 min was required for 50% of the K^+ leakage. At this temperature, however, very little hemolysis (less than 20%) was observed even after 60-min incubation. Electron microscopic examination showed that erythrocytes changed in morphology from normal discocytes to spherocytes via spheroechinocytes. Spherocytes, which were extensively observed after incubation at 25°C for 30 min, might be formed by some osmotic mechanism due to enhanced flux of ions. When erythrocytes were incubated with dilauroylglycerophosphocholine at 37°C, apparent hemolysis was again observed. This observation is consistent with results reported in previous papers [4,5]. In the reaction at 37°C, unlike that at 5 or 10°C, the interval between K^+ leakage and hemolysis was rather long; i.e., 35 min were required for 50% hemolysis, while only 5 min were required for 50% K^+ leakage. At 17°C, K^+ leakage and hemolysis showed kinetics intermediate to those at 10 and 25°C.

Dose-response of K^+ leakage and hemolysis of human erythrocytes by

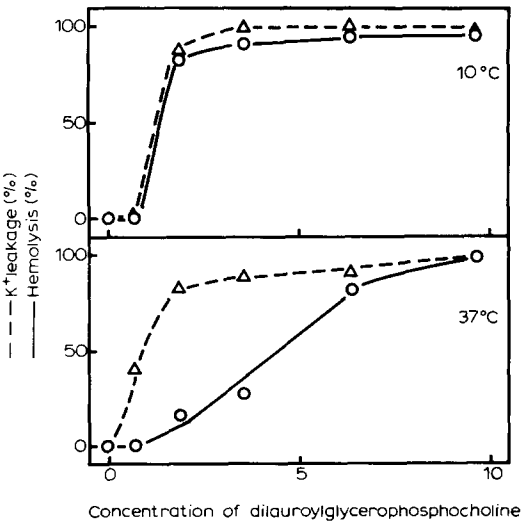

Fig. 6. Dose-response of K^+ leakage from human erythrocytes and hemolysis induced by dilauroylglycerophosphocholine at 10 and 37°C. Erythrocytes (10^7 cells/ml) were incubated with various amounts of dilauroylglycerophosphocholine for 90 min. The percentages of K^+ leakage and hemolysis were measured as described in the legend for Fig. 5.

dilauroylglycerophosphocholine. The amounts of dilauroylglycerophosphocholine required for K^+ leakage and hemolysis were affected by temperature (Fig. 6).

Human erythrocytes were incubated with various concentrations of dilauroylglycerophosphocholine at 10 or 37°C for 90 min and then K^+ leakage and hemolysis were measured. At 10°C, 1.3 μM dilauroylglycerophosphocholine was required for 50% K^+ leakage and 1.4 μM for 50% hemolysis. In contrast, at 37°C, 0.9 μM dilauroylglcyerophosphocholine was required for 50% K^+ leakage and 5.0 μM for 50% hemolysis. Thus, at 10°C, the amount of dilauroylglycerophosphocholine required for hemolysis was very similar to that required for K^+ leakage, whereas at 37°C, a larger amount was required for hemolysis than for K^+ leakage.

Temperature-dependence of hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine. As reported previously [4], hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine increased with decrease in the incubation temperature from 25 to 10°C. When human erythrocytes were incubated with excess dilauroylglycerophosphocholine, they all lysed at 10°C, but only a few lysed at 25°C. In this work we examined the hemolysis at various temperatures between 10 and 25°C in more detail. As shown in Fig. 7A, biphasic kinetics of hemolysis were observed at all temperatures examined. For example, at 20°C, 45% of the chromate was released rapidly within 40 min, and then release continued very slowly for another 50 min.

The dose-dependence of hemolysis at temperatures between 10°C and 25°C was next examined (Fig. 7B). With excess dilauroylglycerophosphocholine,

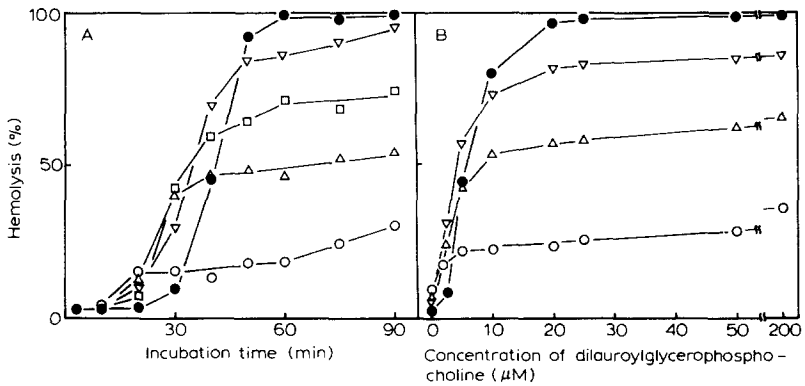

Fig. 7. (A) Time course of hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine at various temperatures. Erythrocytes (10^7 cells/ml) were incubated with 20 μM of dilauroylglycerophosphocholine at 10 (●), 15 (▽), 17.5 (□), 20 (△) and 25°C (○). The percentage of hemolysis was determined at various times using ^{51}Cr-labeled erythrocytes as described in Materials and Methods. (B) Dose-response of hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine at various temperatures. Erythrocytes (10^7 cells/ml) were incubated with various amounts of dilauroylglycerophosphocholine for 60 min at 10 (●), 15 (▽), 20 (△) and 25°C (○).

55% of the chromate was released at 20°C, whereas only 25% was released at 25°C. Counting of cell numbers after hemolysis indicates that about 50 and 75% of the erythrocytes remained unhemolysed at 20 and 25°C, respectively. Thus, partial hemolysis observed here is due to a certain number of cells failing to lyse, rather than to partial release of hemoglobin from all the cells.

Next, erythrocytes were incubated with excess dilauroylglycerophosphocholine at 25°C, and then the temperature was decreased to various lower values. Fig. 8 shows that the change of turbidity was greatly enhanced only during a short period, just after decreasing the temperature.

The possibility that hemolysates, once formed, prevent further hemolysis was excluded by the finding that addition of a certain amount of hemolysate did not have any effect in preventing hemolysis.

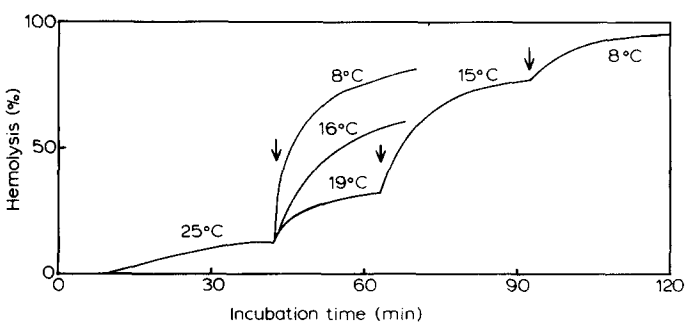

Fig. 8. Time course of hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine at various temperatures. Arrows indicate the time of shift-down to the indicated temperature. Erythrocytes (10^7 cells/ml) were incubated with 20 μM of dilauroylglycerophosphocholine in a cuvette. Hemolysis was monitored by measuring turbidity change as described in Materials and Methods.

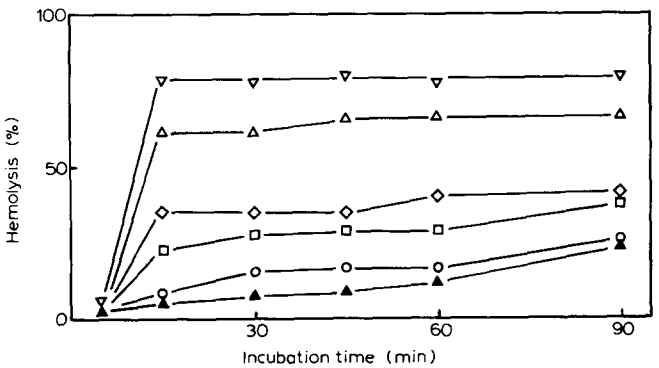

Fig. 9. Time course of hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine at various pH values. Human erythrocytes (10^7 cells/ml) were incubated with 20 μM dilauroylglycerophosphocholine at 25°C and pH 5.5 (▽), 6.0 (△), 6.5 (◇), 7.0 (□), 7.5 (○) and 8.0 (▲). Solutions of 150 mM NaCl buffered with 5 mM Mes were used as media for experiments at pH 5.5, 6.0 and 6.5 and solutions of 150 mM NaCl buffered with 5 mM Hepes were used for experiments at pH 7.0, 7.5 and 8.0. Percentage hemolysis was determined at various times using ^{51}Cr-labeled erythrocytes.

Effect of pH on hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine. As described above, hemolysis by dilauroylglycerophosphocholine in a medium of pH 7.4 at 25°C showed biphasic kinetics. In the first phase only 25% of the erythrocytes was hemolysed. The effect of pH on hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine at 25°C is shown in Fig. 9. Decrease in the pH from 7.5 to 5.5 increased hemolysis in the first phase and at pH 5.5, 80% hemolysis was observed within 15 min even at 25°C. Without dilauroylglycerophosphocholine, no hemolysis was observed under these conditions. It is also noteworthy that the first phase of hemolysis was completed within the same period, irrespective of the pH. Under these experimental conditions, no change of pH was observed during or after hemolysis.

Effects of Cu^{2+}, Hg^{2+} and sulfhydryl agents on hemolysis of human erythrocytes induced by dilauroylglycerophosphocholine. The kinetic study on hemolysis of erythrocytes induced by dilauroylglycerophosphocholine at 25°C in the presence of $CuSO_4$ was performed. With increase in the concentration of $CuSO_4$, hemolysis in the first phase increased; with 10 μM $CuSO_4$, 70% hemolysis was observed within 15 min (data not shown). $CuCl_2$ had the same effect as $CuSO_4$.

Sulfhydryl agents such as $HgCl_2$, *p*-chloromercuriphenylsulfonic acid and *N*-ethylmaleimide had similar effects. $HgCl_2$ was more potent than $CuSO_4$, since 2 μM $HgCl_2$ had a similar effect to 10 μM $CuSO_4$, but *p*-chloromercuriphenylsulfonic acid and *N*-ethylmaleimide had rather weaker effects. In the absence of dilauroylglycerophosphocholine these agents, including $CuSO_4$, did not cause any hemolysis, even at the highest concentrations tested.

Temperature-dependence of K^+ leakage and hemolysis of pig erythrocytes induced by dilauroylglycerophosphocholine. In the previous work [5] we examined the sensitivities of erythrocytes of various animals to dilauroylglycerophosphocholine at temperatures between 0 and 40°C, and classified the

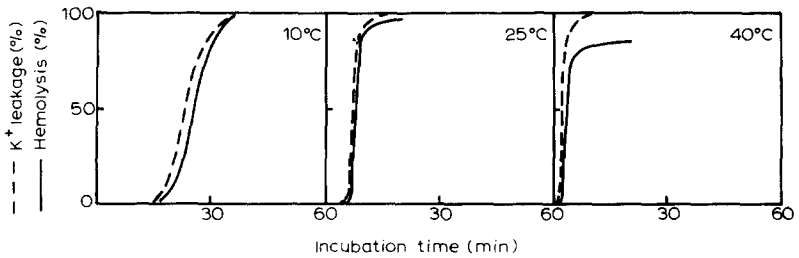

Fig. 10. Time course of K^+ leakage and hemolysis of pig erythrocytes induced by dilauroylglycerophoschocholine at various temperatures. Erythrocytes (10^7 cells/ml) were incubated with 13.5 μM dilauroylglycerophosphocholine. The percentages of K^+ leakage from erythrocytes (- - - - -) and hemolysis (———) were monitored simultaneously as described in the legend for Fig. 5.

erythrocytes into two types on the basis of the results obtained. The rate of hemolysis of monkey, rabbit, rat and human erythrocytes (human-type) was rapid at 10 and 40°C but slow at 25°C. In contrast, the rate of hemolysis of cow, calf, sheep, pig, dog and cat erythrocytes (cow-type) increased with increasing incubation temperature. Next, we examined the temperature-dependence of dilauroylglycerophosphocholine-induced K^+ leakage of cow-type erythrocytes. Pig erythrocytes were used for these studies because their intracellular K^+ concentration is high. When pig erythrocytes were incubated with excess dilauroylglycerophosphocholine at 10°C, K^+ leakage started after 15 min and was followed immediately by hemolysis. With increase in the incubation temperature, both K^+ leakage and hemolysis occurred faster and always simultaneously (Fig. 10). These results are in contrast to those obtained with human erythrocytes (Fig. 5).

Discussion

The process of interaction of erythrocytes with dilauroylglycerophosphocholine, which leads eventually to hemolysis, was studied and is summarized schematically in Fig. 11.

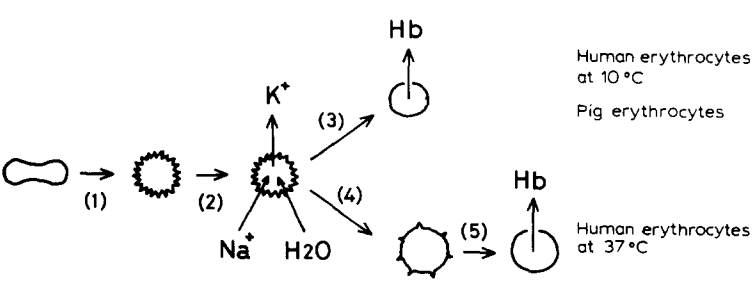

Fig. 11. Process of hemolysis of erythrocytes induced by dilauroylglycerophosphocholine. From the present experiments, steps 1, 2 and 3 proceed in both human erythrocytes at 10°C and pig erythrocytes at all temperatures, while steps 1, 2, 4 and 5 proceed in human erythrocytes at 37°C. (1) Morphological transformation. (2) K^+ leakage. (3) Hemolysis by colloid osmotic mechanism. (4) Resistance to hemolysis (swelling?). (5) Further uptake of dilauroylglycerophosphocholine, resulting in hemolysis, by a mechanism which may be different from that of colloid osmotic lysis in step 3.

When human erythrocytes were treated with dilauroylglycerophosphocholine, morphological change from discocytes to spheroechinocytes was observed first. The rate of this morphological change was temperature-dependent; with increase in the incubation temperature, the rate of change increased. The rate of uptake of dilauroylglycerophosphocholine by erythrocytes also increased with increasing incubation temperature. Therefore, it may be concluded that the interaction of erythrocytes with an amount of dilauroylglycerophosphocholine so small that it could not be detected under our experimental conditions is probably the cause of the morphological change. The discocyte-echinocyte transformation of human erythrocytes is produced both by intrinsic factors, such as ATP and Ca^{2+}, and by extrinsic factors, such as lysolecithin and methochlorpromazine [9—11]. Weltzien proposed in his review [12] that lysophosphatidylcholine may induce a change in shape as a result of increased ion permeability in the pre-hemolytic phase. The change in shape observed in the present experiment, however, is probably not simply a result of increased ion permeability, since it occurred without appreciable K^+ leakage from the erythrocytes.

Upon further incubation, a certain amount of dilauroylglycerophosphocholine was adsorbed to erythrocytes, causing K^+ leakage. The time required for K^+ leakage was also dependent on the incubation temperature. The amount of bound dilauroylglycerophosphocholine required for 50% K^+ leakage was calculated to be $7.3 \cdot 10^{-17}$ mol/cell at 10°C and $6.9 \cdot 10^{-17}$ mol/cell at 37°C, indicating that the interaction of about $7 \cdot 10^{-17}$ mol/cell of dilauroylglycerophosphocholine had the same effect on the erythrocytes irrespective of the incubation temperature.

The temperature-dependence of the kinetics of hemolysis of human erythrocytes was different from that of pre-hemolytic events (morphological change and K^+ leakage). Hemolysis was rapid at 5—10 and above 40°C, but slow at about 25°C. Hemolysis below 10°C seems to be a typical colloid osmotic lysis [13,14], since it occurred immediately after K^+ leakage. Permeation of Na^+ and K^+ towards equilibrium, together with the excess osmotic pressure due to the presence of intracellular hemoglobin, probably lead to swelling and eventual hemolysis of the erythrocytes. On the other hand, hemolysis above 37°C may not be a colloid osmotic lysis, since it was observed only after incubation for a further 30 min. It is probably due to a different mechanism from that operating below 10°C and may involve additional adsorption of dilauroylglycerophosphocholine to the erythrocytes. This possibility is supported by the fact that hemolysis was suppressed by washing the erythrocytes immediately after K^+ leakage was complete. The amount of bound dilauroylglycerophosphocholine required for 50% hemolysis at 37°C was $31 \cdot 10^{-17}$ mol/cell, which was much more than that required for K^+ leakage or for hemolysis at 10°C (about $7 \cdot 10^{-17}$ mol/cell). At 25°C, the hemolysis due to the mechanism operating at 37°C can be observed during a very long incubation time. This long delay of hemolysis may be due to the slow uptake of dilauroylglycerophosphocholine at this temperature. About 80% of the erythrocytes remained unhemolysed for 50 min after K^+ leakage was complete. Thus, 20% of the erythrocytes seemed to be sensitive to colloid osmotic lysis at 25°C. The percentage of erythrocytes that was sensitive to colloid osmotic lysis increased with decreasing tempera-

ture. A preliminary experiment showed that no appreciable percentage of the erythrocytes was sensitive to colloid osmotic lysis above 37°C. At present, it is unknown why most human erythrocytes are resistant to colloid osmotic lysis above 25°C. Some protein(s) in the membrane might be responsible for the resistance, since the pH of the medium and sulfhydryl agents had marked effects on the resistance of erythrocytes to colloid osmotic lysis. There could be an alternative explanation for the apparent resistance of erythrocytes to lysis at above 25°C. The lack of hemolysis after K^+ leakage may be attributed to the resealing of the damage caused by dilauroylglycerophosphocholine after the initial flux of ions. In fact, consistent with resealing, the K^+ release curves in the middle temperature range indicate that all of the K^+ is not released in the early phase. Hemolysis observed at the lower temperature may be related to a slow resealing process.

It is noteworthy that human erythrocytes treated with a benzylated lysophosphatidylcholine derivative showed similar kinetics of K^+ leakage and hemolysis [12]. Whereas at 37°C, intracellular K^+ can be released without any loss of hemoglobin, the difference between the release of the two markers is considerably smaller at low temperature. Weltzien postulated that temperature-dependence may be accounted for by change in the 'rigidity' of the membrane with temperature. It could be expected that above 25°C, the membrane would become more elastic and more resistant to colloid osmotic pressure. Some protein component(s) may be responsible for this increase in 'elasticity'.

Unlike human erythrocytes, pig erythrocytes were hemolysed immediately after K^+ leakage even above 25°C. Thus, it seems likely that pig erythrocytes lack this pH- and sulfhydryl agent-sensitive 'mechanism' of resistance to colloid osmotic lysis. Possibly, sulfhydryl agents modify some membrane protein(s) in human erythrocytes thus converting them to 'pig-type' erythrocytes. In a previous paper [5], we showed that the erythrocytes of various mammalian species could be divided into two groups on the basis of their sensitivity to dilauroylglycerophosphocholine. This classification may be based on whether or not this mechanism of resistance operates in the erythrocytes. Erythrocytes can also be classified into two groups on the basis of the temperature-dependence of penetration of glycerol into the cells [15]. It is noteworthy that the latter classification corresponds to that for sensitivity toward dilauroylglycerophosphocholine. Moreover, glycerol penetration showed sensitivities to Cu^{2+} [16] and pH variation [15] similar to the mechanism for resistance observed here. Conceivably, the same protein(s) is responsible for both glycerol permeation and the mechanism of resistance to colloid osmotic lysis.

The heterogeneity in sensitivity of human erythrocytes observed here may reflect their considerable heterogeneity in age. In fact, preliminary studies showed that the sensitivity of rat reticulocytes to dilauroylglycerophosphocholine was different from that of normal rat erythrocytes.

Acknowledgements

This work was supported in part by Grants-in-Aid (Nos. 237051 and 348372) for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

1 Pagano, R.E. and Weinstein, J.N. (1978) Annu. Rev. Biophys. Bioeng. 7, 435—468
2 Weltzien, H.U. (1973) Biochim. Biophys. Acta 311, 6—14
3 Weltzien, H.U., Arnold, B. and Kalkoff, H.G. (1976) Biochim. Biophys. Acta 455, 56—65
4 Kitagawa, T., Inoue, K. and Nojima, S. (1975) J. Biochem. (Tokyo) 78, 431—434
5 Kitagawa, T., Tanaka, Y., Inoue, K. and Nojima, S. (1977) Biochim. Biophys. Acta 467, 137—145
6 Robles, E.C. and van den Berg, D. (1969) Biochim. Biophys. Acta 187, 520—526
7 Stoffel, W., LeKim, D. and Tschung, T.S. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1058—1064
8 Inoue, K., Graf, L. and Rapport, M.M. (1972) J. Lipid Res. 13, 119—127
9 Weed, R.I. (1975) Clin. Hematol. 4, 3—28
10 Sato, T. and Fujii, T. (1974) Chem. Pharm. Bull. 22, 152—156
11 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4457—4461
12 Weltzien, H.U. (1979) Biochim. Biophys. Acta 559, 259—287
13 Wilbrandt, W. (1941) Pflüger's Arch. Ges. Physiol. 245, 22—52
14 Whittam, R. (1964) Transport and Diffusion in Red Blood Cells, pp. 176—191, Edward Arnold Ltd., London
15 De Gier, J., van Deenen, L.L.M. and van Senden, K.G. (1966) Experientia 22, 20—21
16 Stein, W.D. (1958) Nature 181, 1662—1663