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Induction of Acetylcholinesterase Release from Erythrocytes in the Presence of Liposomes[1]

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When human erythrocytes are incubated with liposomes, the release of acetylcholinesterase (AChE) occurs following an induction period [Cook *et al.* (1980) *Biochemistry* 19, 4601-4607]. However, the mechanism of the induction has not been elucidated. We examined the relationships among the lipid transfer from liposomes to erythrocytes, the morphological change of erythrocytes, the fluidity of the erythrocyte membrane and the start of AChE release. The AChE release into the liposomes and into shed-vesicle fractions started simultaneously after an induction period. The morphological index (MI) of erythrocytes was approximately 2.8 at the beginning of the release, regardless of the induction period. AChE was not released from the erythrocytes of index 2.8 even in the presence of liposomes if the MI remained at 2.8. Therefore, for the release, erythrocytes needed a further increase of the MI from 2.8. As the rate of lipid transfer increased, the induction period became shorter. No significant lipid release from erythrocytes was detected during the induction period. The initiation of the AChE release was not simply affected by the change in the membrane fluidity of erythrocytes upon interaction with liposomes. These results first demonstrate that AChE release into the shed-vesicle and liposome fractions is triggered by a further increase of the MI from 2.8, which is induced by lipid transfer from liposomes to erythrocytes.

Key words: erythrocyte, induction of AChE release, lipid transfer, liposome, morphological index.

The incubation of cells with liposomes results in the release of membrane proteins from the cells (*1-9*). Huestis and coworkers first carried out substantial studies on this subject, and found four major proteins including band 3 and acetylcholinesterase (AChE) in both the shed-vesicle fraction from erythrocytes, and the liposome fraction after incubation of human erythrocytes and dimyristoylphosphatidylcholine (DMPC) liposomes (*2*). Hence, the release of integral membrane proteins from erythrocytes in the presence of liposomes seems to come from the shed-vesicle release and the protein transfer to liposomes.

The induction period is always followed by protein release (*3, 9*). Ott *et al.* reported that after the incubation of erythrocytes and DMPC liposomes only the proportion of phosphatidylcholine in the lipid composition of the erythrocyte membrane increased and the composition resembled one of shed-vesicles (*3*). Their results suggest that the transfer of liposome phospholipids to erythrocytes is involved in the shed-vesicle release. However, this work addressed neither what is essential for the induction process, nor the relationship between the induction of shed-vesicle release and that of protein transfer to liposomes. A systematic study regarding this issue is needed.

In this study, in order to elucidate what triggers the protein release, we quantitatively investigated the time course of the release of a marker protein, AChE, the morphological change of erythrocytes, the amount of lipid transferred from liposomes, the membrane fluidity of erythrocytes, and the relationships among them. We employed an artificial lipid, 1,2-dimyristamido-1,2-deoxyphosphatidylcholine (D_{14}DPC), in addition to DMPC as a component of liposomes. D_{14}DPC has two acyl amide groups instead of the ester groups of DMPC, and was originally developed as an artificial boundary lipid (*10-12*). The release of membrane proteins from intact cells was accelerated by the inclusion of D_{14}DPC (*4-7*). The use of different types of phosphatidylcholines allows us to generalize the phenomena.

MATERIALS AND METHODS

Materials—α-Linolenic acid, dioleoylphosphatidylcholine (DOPC), and DMPC were purchased from Sigma

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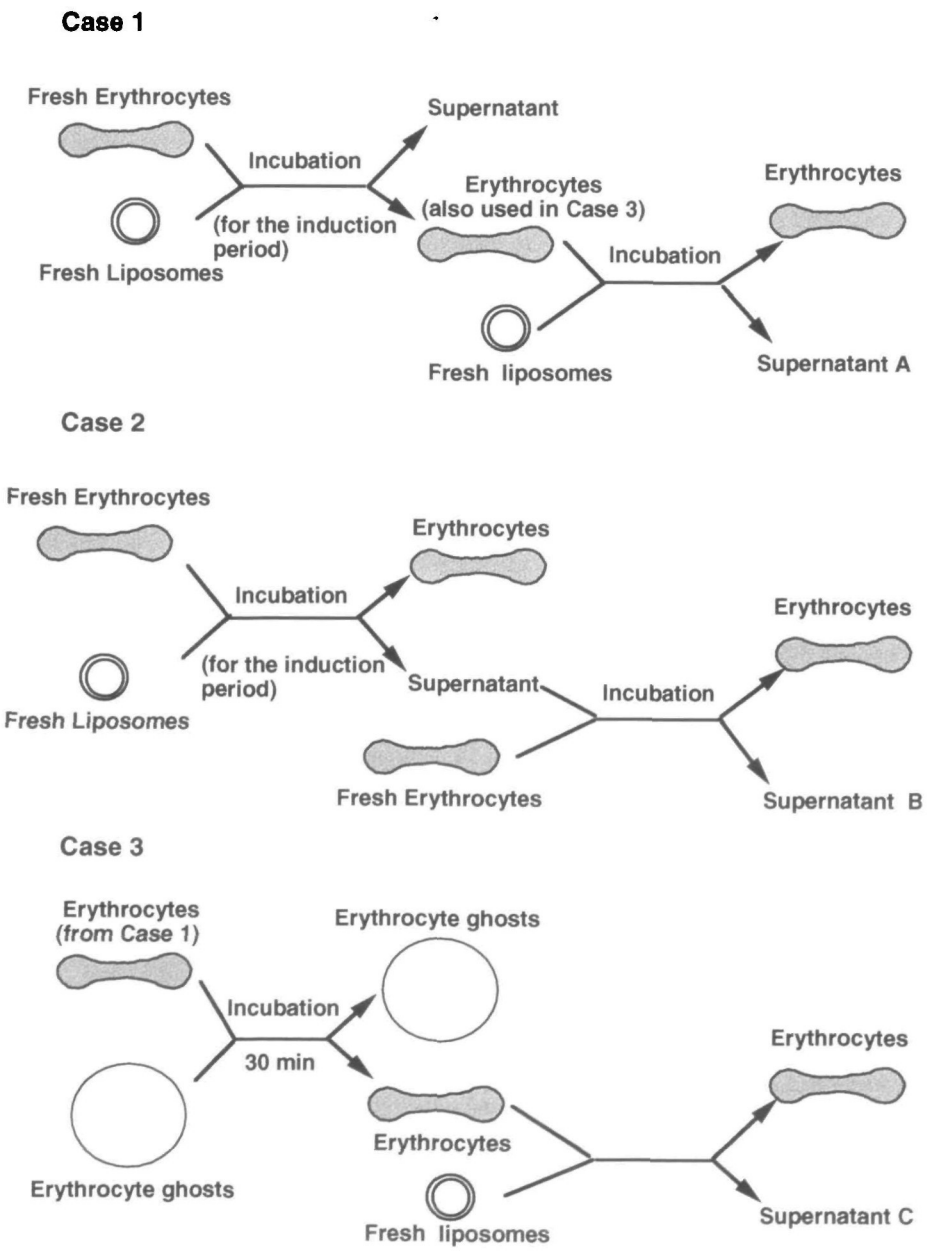
Abbreviations: AChE, acetylcholinesterase; D_{14}DPC, 1,2-dimyristamido-1,2-deoxyphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; HCT, hematocrit; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MI, morphological index.

Chemical (St. Louis, USA). D_{14}DPC was from Dojindo Laboratories (Kumamoto). 1,2-Dimyristoylphosphatidyl [*N*-methyl-^{3}H]choline ([^{3}H]DMPC, 75 Ci/mmol), 1,2-dimyristamido-1,2-deoxyphosphatidyl[*N*-methyl-^{3}H]choline ([^{3}H]D_{14}DPC, 80–85 Ci/mmol), and cholesteryl [1-^{14}C]oleate (53 mCi/mmol) were obtained from Amersham (Buckinghamshire, UK). 5-Doxylstearoyl acid was from Aldrich (Milwaukee, USA). All other reagents were commercially available and used without further purification. Throughout all the runs, 10 mM HEPES containing 150 mM NaCl (pH 7.4) was used as the buffer unless stated otherwise.

Preparation of Liposomes—Liposomes were made from DMPC, DOPC, or a mixture of 40 mol% DMPC and 60 mol% D_{14}DPC [referred as to DMPC(40)/D_{14}DPC(60) below]. The maximum content of D_{14}DPC was limited to 60 mol% because of the stability of the liposomes. The 100 nm liposomes were prepared by the extrusion method as previously reported (*4–7*).

Preparation of an Erythrocyte Suspension—Human erythrocytes were separated from plasma by centrifugation (1,000 × *g*) at 4°C for 10 min, and then washed three times with the HEPES buffer. The erythrocytes obtained were suspended in the buffer at 26.3% hematocrit (volume ratio of erythrocytes in the suspension, referred as to HCT below). Throughout all the runs, HCT was 26.3% unless stated otherwise. All the experiments were carried out within 72 h after the collection of blood. Erythrocyte ghosts were prepared according to the Dodge method (*13*). Per erythrocyte, 5.35×10^{10} α-linolenic acid was included.

AChE Release from Erythrocytes in the Presence of Liposomes—Human erythrocytes were heated at 37.0°C for 10 min, and then incubated with an equal volume of a liposome suspension at 37.0°C in a water bath. After the



Scheme 1. **Three different experiments involving incubation of erythrocyte with liposomes.** Case 1: Erythrocytes incubated with DMPC(40)/D_{14}DPC(60) liposomes in advance were incubated again with fresh liposomes. Case 2: Separated liposomal supernatant was incubated again with fresh erythrocytes. Case 3: Erythrocytes incubated with the liposomes in Case 1 were first incubated with erythrocyte ghosts and then with fresh liposomes. See the text for details.

incubation, the sample suspension was centrifuged at 2,000×g, 4°C for 3 min to pellet the erythrocytes. The AChE activity in the supernatant was measured by the method of Ellman et al. (14).

Sucrose Density Gradient Centrifugation—A continuous sucrose concentration gradient from 10 to 40% (w/v), was prepared in a 12.8 ml tube (Hitachi Koki, ϕ14 mm×89 mm). A supernatant separated from erythrocytes (100 μl) was placed on top of the sucrose gradient, followed by centrifugation at 200,800×g, 4°C for 2 h (Hitachi CP56GII, with a P40ST swinging bucket rotor). After the centrifugation, the gradient was immediately fractionated (0.41 ml per fraction) to isolate the liposome fraction [10-20% sucrose (w/v)] and the shed-vesicle fraction [30-40% sucrose (w/v)].

Effect of Erythrocyte Exposure to Liposomes or Liposome Exposure to Erythrocytes on AChE Release—The time course of AChE release was examined using erythrocytes incubated with liposomes in advance, and using liposomes incubated with erythrocytes in advance (Cases 1-3, Scheme 1). In Case 1, an equal volume mixture of an erythrocyte suspension and a DMPC(40)/D_{14}DPC(60) liposome suspension (lipid concentration, 0.5 mM) was incubated at 37°C. The incubation was terminated just before the AChE release started (at 7 min). After separation of the erythrocytes from the liposomes, the erythrocyte pellet was washed and suspended in the buffer. The erythrocytes thus obtained were further incubated with fresh liposomes (0.5 mM) at 37°C. In Case 2, DMPC(40)/D_{14}DPC(60) liposomes (2 mM) and an equal volume of fresh erythrocytes were incubated. At the end of the induction period (3 min), the liposomes were separated from the erythrocytes. Then, the liposomes (0.5 mM) were incubated at 37°C with fresh erythrocytes. In Case 3, 1 ml of the incubated erythrocyte suspension, as prepared as for Case 1, was incubated again with 50 ml of an erythrocyte ghost suspension at 37°C for 30 min (ghost:erythrocyte=155:1, by cell number). The erythrocytes were separated from the ghosts by centrifugation and the washed five times with the buffer. Then an equal volume mixture of the erythrocytes and the fresh liposomes (0.5 mM) was incubated at 37°C.

Morphological Change of Erythrocytes—Erythrocytes were fixed with 0.5 wt% glutaraldehyde in 150 mM aqueous NaCl for 10 min at room temperature and the observed under an Olympus IMT-2 light microscope. The erythrocyte morphology was assessed according to the method of Ferrell and Huestis (15, 16), and graded according to the scale proposed by Bessis (17). Erythrocytes of the normal shape (discocytes) are graded as zero. Crenated ones (echinocytes) and cupped or invaginated cells (stomatocytes) are given scores from +1 to +5 and from −1 to −4 depending on the extent of the morphological change, respectively. The average of the grades of one hundred erythrocytes is defined as the morphological index (referred as to MI below). All the determinations were performed in duplicate.

Lipid Transfer from Liposomes to Erythrocytes—Radiolabeled liposomes were prepared in a similar manner to that adopted for non-labeled liposomes. At the beginning, 20 μCi of [^{3}H]DMPC or [^{3}H]D_{14}DPC and 5 μCi of a non-exchangeable marker, cholesteryl [1-^{14}C]oleate (18), were mixed with non-radiolabeled phospholipids. The radioactivities of ^{3}H and ^{14}C nuclei were counted separately with an Aloka LSC 1000 scintillation counter. All the measurements were performed in duplicate. The amount of phospholipid spontaneously transferred from liposomes to erythrocytes was estimated according to Jones and Thompson (18). The amount of cholesterol was determined by the method of Zlatkis et al. (19). The phospholipid composition was determined by thin layer chromatography with a flame ionization detector (Iatron, Tokyo).

Membrane Fluidity—An ethanol solution of a spin probe, 5-doxylstearic acid (2.5 mg/ml, 10 μl), was dried under a flow of nitrogen gas at the bottom of a tube. In the tube 2 ml of an erythrocyte suspension (HCT, 13.2%) was incubated at 37°C for 10 min to embed the probe in the erythrocyte membranes (20). After washing with the buffer, the erythrocytes were submitted to electron spin resonance (ESR) measurement (JEOL, JES-FE1XG).

RESULTS

Time Course of AChE Release—The time courses of the AChE release to the supernatant from erythrocytes in the presence of DMPC or DMPC(40)/D_{14}DPC(60) liposomes are shown in Fig. 1. In both cases, the AChE release started after an induction period and eventually reached a plateau. These results agree with those of Cook et al. (9), and suggest that the mechanism of the AChE release should be essentially the same regardless of the lipid composition. On the other hand, the induction period and the release rate depend on the lipid composition. The inclusion of D_{14}DPC in DMPC liposomes shortened the induction period from 80 min to 6 min, and enhanced both the release rate and extent (Fig. 1). Meanwhile, no AChE release occurred at all when DOPC liposomes (5 mM) were employed. Figure 1 also shows that AChE started to be released into the liposome and shed-vesicle fractions simultaneously. This suggests that the same factor triggered the AChE release into both fractions.

Effect of Erythrocyte Exposure to Liposomes or Liposome Exposure to Erythrocytes on AChE Release—Because AChE release was observed not only in the shed-vesicle fraction, but also in the liposome fraction, we should consider a certain change on both liposomes and erythrocytes, which occurs with the induction. To clarify this point, two different experiments were carried out, as shown in Scheme 1. When erythrocytes were treated with liposomes in advance (Case 1), the induction period became significantly shorter (Fig. 2). When liposomes were treated with erythrocytes in advance (Case 2), the induction period was the same as that in the case of incubation of fresh erythrocytes and fresh liposomes. No AChE was released if erythrocytes were incubated without liposomes in either case (control experiment). These results suggest that the induction of AChE release is mostly governed by a change on erythrocytes.

Morphological Change of Erythrocytes and Lipid Transfer from Liposomes—Among the changes in erythrocyte membranes during the induction period, we first focused on the morphological change because it was already known that the incubation of DMPC liposomes and erythrocytes causes a morphological change (15, 16). Figure 3 shows the time course of the MI change under the conditions in Fig. 1, and indicates that in both types of the liposomes, the MI

increased up to 5. Although there was a large difference in the induction period between the two systems, the AChE release started when the MI reached approximately the same value (2.8) for both types of liposomes.

The relationship between the AChE release and the MI of the erythrocytes was further investigated. The MIs at the beginning of the AChE release under various conditions are summarized in Table I. The MIs were almost identical (between 2.7 and 2.9), regardless of the induction period. Furthermore, the erythrocytes incubated with diluted DMPC(40)/D_{14}DPC(60) liposomes (0.05 mM) for 1000 min scarcely showed the MI change (MI = 1.2), and the AChE release did not take place at all. These observations strongly suggest it is a common characteristic that the MI is approximately 2.8 at the beginning of the AChE release.

In order to determine whether or not it is essential for the induction of the AChE release that the erythrocytes undergo the MI change to 2.8 once, or if the erythrocytes need a further change of the MI, the experiment described in Case 3 in Scheme 1 was carried out. It was already known that the MI of erythrocytes incubated with liposomes decreases after further incubation with erythrocyte ghosts (*21*). Also in Case 3, the MI of the intact erythrocytes recovered from 2.8 to 0.8. The erythrocytes of the MI 0.8 were further incubated with fresh liposomes (Fig. 2). As a result, the induction period was 6 min, which is close to that in the case of intact erythrocytes (8 min). This result indicates that it is not enough for the induction that erythrocytes undergo the MI change to 2.8 once. Even if DOPC liposomes (5 mM)

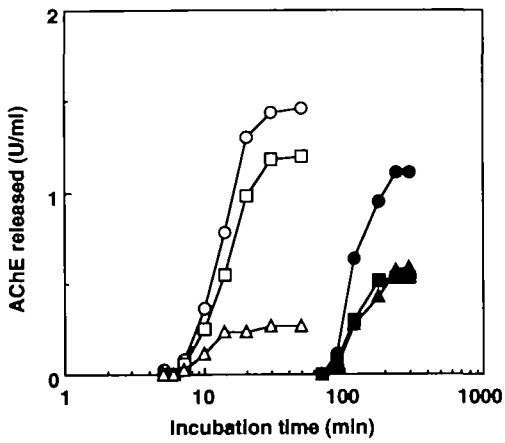


Fig. 1. **AChE release in the presence of DMPC liposomes (closed symbols) or DMPC(40)/D_{14}DPC(60) liposomes (open symbols).** Erythrocytes (hematocrit, 26.3%) were incubated with liposomes (lipid concentration, 1 mM) at 37°C. AChE activity in the supernatant (circles), liposome fraction (squares), and shed-vesicle fraction (triangles) was measured.

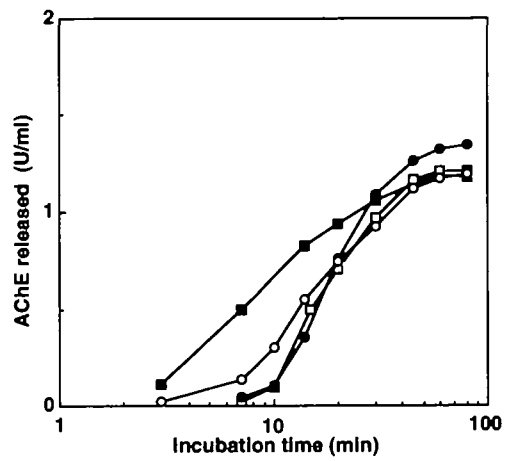


Fig. 2. **AChE release in Scheme 1.** AChE activity was measured in the supernatants obtained by incubation of fresh erythrocytes with fresh DMPC(40)/D_{14}DPC(60) liposomes (□), supernatant A in Case 1 (■), supernatant B in Case 2 (●), and supernatant C in Case 3 (○).

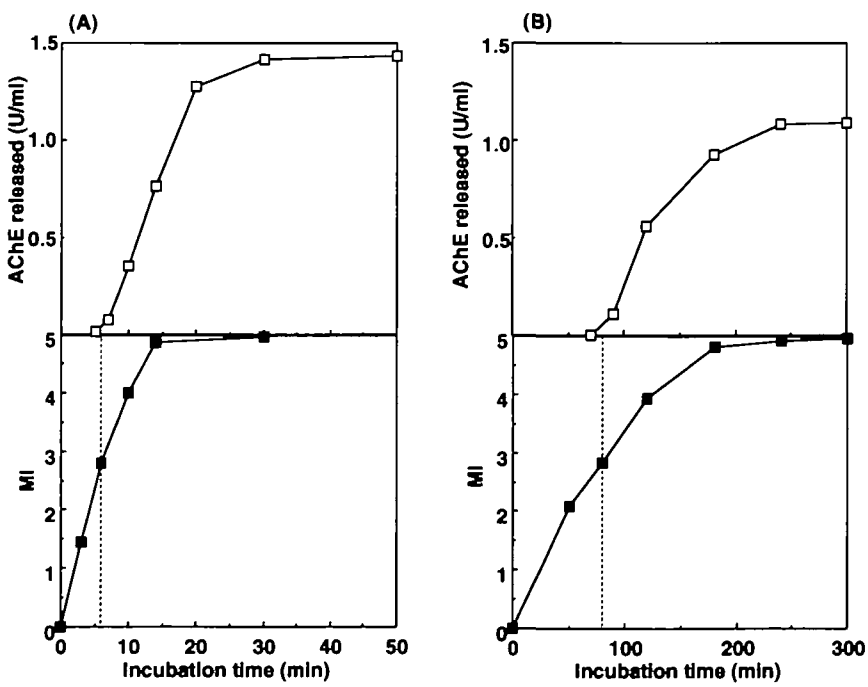


Fig. 3. **AChE release (□) and morphological index (MI) of erythrocytes (■) during incubation with liposomes.** An equal volume mixture of an erythrocyte suspension (HCT, 26.3%) and a DMPC(40)/D_{14}DPC(60) (A) or DMPC (B) liposome suspension (lipid concentration, 1 mM) was incubated at 37°C.

and the erythrocytes of the MI 2.8 were incubated for 90 min, no AChE release was observed, and the MI remained at 2.8. This strongly supports that a further change of the MI is important for the induction.

The morphological change of erythrocytes exposed to liposomes is already known to be correlated with lipid transfer from liposomes to erythrocytes (*15, 16*). Phosphatidylcholines incorporated into the erythrocyte membrane are mainly located in the outer leaflet of the lipid bilayer and generate echinocytes (crenated erythrocytes) (*15, 16*). Therefore, the observed morphological change is most likely caused by the transfer of liposomal lipid to the erythrocytes. In order to confirm this point in our experimental system, we determined the amount of phosphatidylcholine transferred from liposomes to erythrocytes. Table I shows that both a higher lipid concentration of liposomes and the inclusion of D_{14}DPC in DMPC liposomes resulted in a higher rate of lipid transfer and a shorter induction period. These results suggest that the higher rate of lipid transfer shortened the period for the MI to reach 2.8, namely the induction period.

In all of the present cases, determination of cholesterol (*19*) and TLC-FID analysis showed no significant cholesterol or phospholipid transfer (less than 0.7 and 0.6 μg, respectively) from erythrocytes to liposomes during the induction period. In addition, the incubation time seemed be too short to induce the spontaneous morphological change due to ATP depletion (*3*). In fact, the erythrocytes themselves (HCT, 13.2%) retained the discoid shape and no AChE was released even after 300 min at 37°C in the buffer. Under the present conditions the depletion of lipids or ATP in erythrocytes must be irrelevant as to both the morphological change and the initiation of the AChE release, which indicates that only the lipid transfer from liposomes is involved in this process.

Although a shorter induction period is certainly accompanied by a faster lipid transfer from the liposomes, a larger amount of lipid has to be transferred for the MI to reach 2.8 with a higher liposomal lipid concentration (Table I). This suggests the possibility that only a part of the lipids transferred from liposomes is utilized to change the erythrocyte morphology. To clarify this point, further investigation is needed.

Membrane Fluidity—Because it was reported that membrane fluidity dominates the induction of AChE release (*9*), we investigated the relationship between the erythrocyte membrane fluidity and the AChE release with 5-doxylstearic acid, which is a spin probe with fluidity in the relatively hydrophilic membrane region (*22*). During the induction period the order parameter (S') decreased from 0.635 to 0.622, indicating an increase in fluidity.

In order to determine if the enhancement of the membrane fluidity is essential for the induction of AChE release, α-linolenic acid was incorporated into erythrocytes before the incubation with liposomes (Fig. 4). Although α-linolenic acid increased the membrane fluidity (S' decreased to 0.614) the induction period was lengthened from 8 to 13 min. No AChE was released if the erythrocytes were incubated without liposomes for 90 min (control experiment). Therefore, the initiation of the AChE release cannot be simply attributed to an increase in the fluidity of erythrocyte membranes upon the interaction with liposomes.

In order to determine if the delay in the induction period observed here is also due to the morphological change of the erythrocytes, the MI was determined. In fact, the inclusion of α-linolenic acid decreased the MI to -0.6 before the incubation, and 2.2 at 8 min, which is the incubation time needed for the AChE release in the case of intact erythrocytes. Continued incubation increased the MI to 2.9 at 13

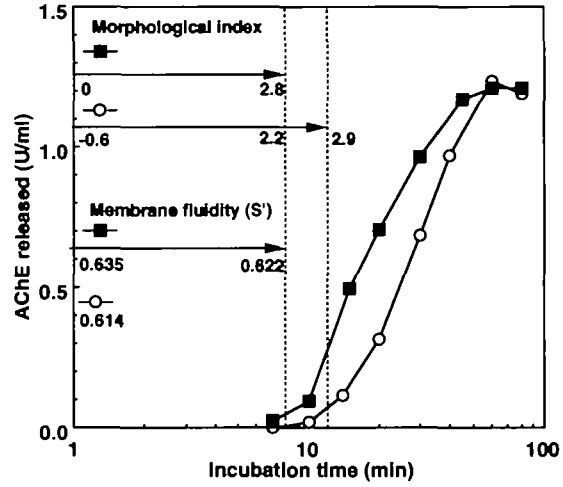


Fig. 4. **AChE release from erythrocytes containing α-linolenic acid in the presence of liposomes.** Erythrocytes (HCT, 26.3%) containing α-linolenic acid were incubated with an equal volume of a DMPC(40)/D_{14}DPC(60) liposome suspension (phospholipid concentration, 0.5 mM) at 37°C. The number of α-linolenic acid molecules per erythrocyte was zero (■) or 5.35×10^{10} (○). The MI of erythrocytes and the membrane fluidity are shown in the figure.

TABLE I. **Induction period, morphological index (MI) of erythrocytes, and amount and rate of phosphatidylcholine transfer to erythrocytes at the beginning of AChE release in the presence of different kinds of liposomes.** Equal volumes of erythrocytes (HCT, 26.3%) and liposomes were incubated at 37°C.

Liposomes	Liposomal lipid conc. (mM)	Induction period (min)	MI	Transferred phosphatidylcholine (μg)	Rate of lipid transfer (μg/min)
DMPC(40)/D_{14}DPC(60)	0.1	40	2.7±0.1	29.1±1.35	0.73
	0.2	17	2.8±0.0	36.6±2.03	2.15
	0.5	8	2.8±0.0	35.9±1.35	4.49
	1.0	6	2.8±0.0	68.4±2.03	11.40
DMPC	0.2	190	2.8±0.0	45.4±0.00	0.24
	0.5	100	2.8±0.0	78.5±8.12	0.79
	1.0	80	2.8±0.0	113.1±13.5	1.41
	5.0	33	2.9±0.0	264.0±15.6	8.00
	20.0	28	2.9±0.1	341.2±17.6	12.19

min, when the AChE release started. The MI remained at −0.6 if the erythrocytes were incubated without liposomes for 90 min (control experiment). These results strongly support that the morphological change of erythrocytes (with the MI at around 2.8) is critical for the initiation of AChE release.

DISCUSSION

Ott *et al.* reported that only the proportion of phosphatidylcholine in the lipid composition of erythrocyte membranes changed (increased) after the incubation of erythrocytes and DMPC liposomes, and the composition resembled that of shed-vesicles (*3*). This suggests that the transfer of phosphatidylcholine to erythrocyte membranes is involved in the induction of the shed-vesicle release. But it does not manifest what triggers the shed-vesicle release and which step is critical for the release. Of course, the relationship between the induction of shed-vesicle release and that of protein transfer to liposomes can not be determined from their results. The results presented here revealed that AChE release into the shed-vesicle and liposome fractions is triggered by elevation of the MI from 2.8, which is induced by lipid transfer from liposomes. This fact and the results of Ott *et al.* indicate that the induction of shed-vesicle release can be exclusively attributed to the geometric factor of the erythrocyte membrane, which is modified by the accumulation of exogenous phospholipids.

Because shed-vesicles encapsulate hemoglobin (*3*), the release of shed-vesicles should be accompanied by membrane fusion following close apposition between the inner monolayers of the erythrocyte membrane. It is well-known that a large amount of energy is necessary for membrane fusion because there is repulsion between membranes (*23*). Judging from that the induction of shed-vesicle release depends on the geometry of erythrocytes, the driving force of the fusion would also be generated by a geometry-related factor. Hence, as one of the candidates for the source that overcomes the large amount of energy, we propose "curvature energy," which is produced through curvature-related packing stress (*24*). It is known that high packing-stress vesicles need less polyethylene glycol for the induction of fusion (*24*). Therefore, it may be important that the "curvature energy" stored in the crenated membrane has to reach a certain threshold to induce the membrane fusion and the shed-vesicle release.

The simultaneous initiation of the AChE release into the liposome and shed-vesicle fractions (Fig. 1) suggests that the two different phenomena are triggered by the same event occurring in the erythrocyte membrane. We predict that a significant membrane defect, which could be produced by such a non-bilayer phase, appears during the fusion at the step of shed-vesicle release. The defect could weaken the interaction between proteins and lipids in the erythrocyte membrane, which would induce membrane protein transfer to liposomes.

Cook *et al.* previously concluded that an increase in the fluidity of liposome membranes results in a shorter induction period (*9*). However, our study revealed that DOPC liposomes, in which the membrane is in a fluid phase (*25*), could not induce the AChE release. This clearly contradicts their conclusion. Their proposal is based on the idea that one can control the membrane fluidity of either liposomes or erythrocytes by changing the temperature and/or the lipid composition (DMPC, DPPC and DSPC). However, such factors must not be confined to the membrane fluidity, but simultaneously affect the transfer of liposomal lipids to erythrocytes. The transfer rate increases with temperature (*26*), and a lipid bearing a shorter acyl chain is transferred faster than one bearing a longer one (*27*). Therefore, judging from the results of this study, the change in the induction period should not be attributed to membrane fluidity, but to the lipid transfer rate.

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