The Effect of Nonionic Surfactant Structure on Hemolysis

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The hemolytic properties of polyoxyethylene-type nonionic surfactants were investigated in the concentration range of 0.1–5.0 wt/vol%. The effects of hydrophilic-lipophilic balance, molecular weight, chemical structure and solubilizing ability of the nonionic surfactants in hemolysis are discussed. Bulky surfactants with three or four alkyl chains in their molecules, which show low solubilizing ability of lipids, were the least likely to induce hemolysis. Morphological observations of erythrocytes by video-enhanced microscopy showed that linear surfactants induce the formation of spherocytes and cause hemolysis, whereas bulky surfactants induce only morphological changes from discocytes to spheroechinocytes.

KEY WORDS: Bulky structure, hemolysis, morphological change, nonionic surfactant.

For intravenous medicines it is important to develop safer solubilizers without hemolytic activity. It is also necessary to clarify the relationship between the hemolytic properties of nonionic surfactants and their structures for future synthesis of new types of surfactants. Many surfactants induce hemolysis near the critical micelle concentration (CMC) (1–4). Hemolysis studies have usually been carried out in the CMC range (10^{-3}–10^{-5}M) (5–7). However, it is more important to understand the hemolytic properties of nonionic surfactants in the larger concentration range used for their practical applications. Nonionic surfactants are used in intravenous medicines as solubilizers at a concentration of around 7% (8). We studied the hemolytic activity of polyoxyethylene-type (POE-type) nonionic surfactants in the concentration range of 0.1–5.0 wt/vol% and focussed on the relationship between the structure of surfactants and their hemolytic ability.

The hemolytic properties of surfactants depend on factors such as the chemical structure, solubilizing ability and hydrophilic-lipophilic balance (HLB). Isomaa *et al.* (9) reported that the hemolytic potency of ionic and amphoteric surfactants decreases with increasing alkyl chain-length. Fukuda (10) showed that POE(n) alkyl ethers comprising 9–10 oxyethylene units have maximum hemolytic activity, and Kondo *et al.* (5) reported that hemolytic activities of POE(n) dodecyl ethers decrease with increasing length of the POE chain. Segal *et al.* (6) showed that POE chainlength does not affect hemolysis in terms of molarity. Miyajima *et al.* (3) showed that POE(30) cholesteryl ether has the maximum hemolytic activity in the POE(n) cholesteryl ether series, and Zaslavsky *et al.* (11) reported that hemolysis does not correlate with the HLB of surfactants. It seems from these results that surfactants with long alkyl and POE chains show low hemolytic activity, but this has not yet been clarified. We studied the hemolytic activities of nonionic surfactants containing several alkyl chains in the molecule and considered the effects of structure on hemolysis.

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MATERIALS AND METHODS

Surfactants. POE(n) oleyl ether, POE(n) monostearate, POE(20) sorbitan monooleate, POE(n) hydrogenated castor oil, POE(n) sorbitol tetraoleate and POE(20) polyoxypropylene (POP) (6) 2-decyltetradecyl ether were purchased from Nikko Chemicals Co. (Tokyo, Japan). POE(20) 2-decyltetradecyl ether was obtained from Nihon Emulsions Co. (Tokyo, Japan). The formulas of the surfactants are shown in Table 1. Egg yolk lecithin (PL-100LE, 95% purity) was purchased from QP Inc. (Tokyo, Japan). Fluorescence spectroscopy-grade 5(6)carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY). These materials were of commercial grade and were used without further purification.

Erythrocytes. Blood was drawn from Japanese white rabbits (either sex, 2.0–3.0 kg). Erythrocytes were separated by centrifugation (2,000 rpm; 2,400 × *g*) for 5 min and resuspended after washing in an isotonic buffer solution (0.15 M NaCl and 0.01 M sodium phosphate at pH 7.0). After repeating this procedure three times, the suspension was adjusted to 2 vol/vol%. The concentration was measured at 1.14×10^8 cells/mL with a Coulter counter.

Hemolysis. Two mL of the erythrocyte suspension and 2 mL surfactant buffer solution were mixed in a test tube. After gentle mixing, the tubes were stored in a temperature-controlled water bath at 37°C for 15, 30 or 120 min and then centrifuged at 3,500 × *g* for 15 min. The extent of hemolysis (as a percentage) was obtained from the absorbance (540 nm) of the supernatant. A completely hemolyzed control sample was prepared by dilution of the erythrocyte suspension with a 1.0 wt% aqueous solution of Triton X-100. A stable erythrocyte sample was prepared by the addition of the isotonic buffer solution to the erythrocyte suspension.

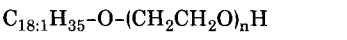
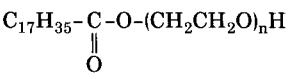
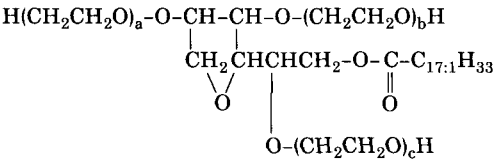
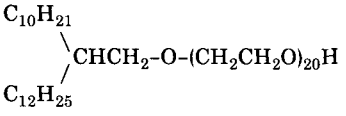
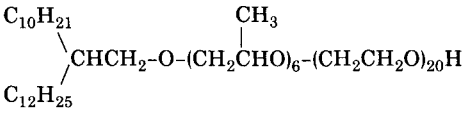
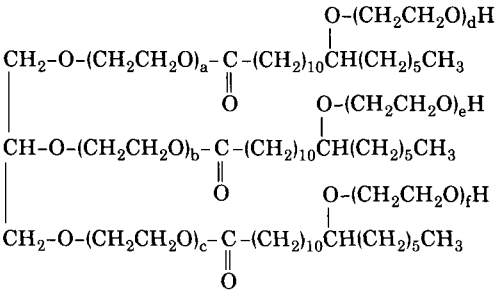
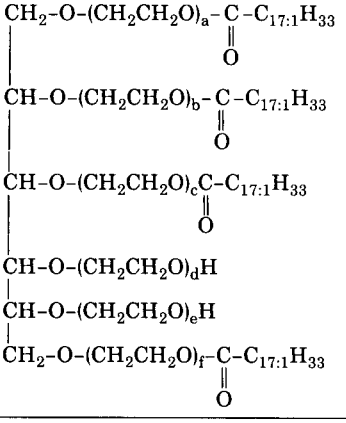
Preparation of liposomes. Liposomes containing CF were prepared by a similar method to that of Miyajima *et al.* (4). Egg yolk lecithin (0.075 g) and cholesterol (0.025 g) were dissolved in 5 mL chloroform, and this solution was dried to a thin film with an evaporator and stored in vacuum overnight. The lipid mixture was dispersed in 200 mM CF Tris HCl buffer solution (20 mM, pH 7.4) by vortexing and sonicating with a probe-type sonicator (Branson Sonic Power Co., Danbury, CT) at 30 W for 15 min under cooling in an ice bath. Unencapsulated CF was removed from the liposome solution in a Sepharose 4B column at 5°C. The liposome solution (2.8 mL) was mixed with 0.2 mL of 0.1 wt% aqueous surfactant solution at 23°C and stored for 1 h. The percentage of leaked CF was determined by the change in fluorescence intensity at 520 nm with an exitation wavelength of 470 nm. Complete leakage of CF is equivalent to hemolysis.

Phase diagrams. The solubility regions were determined by visual inspection of samples in glass vials kept at 37°C. The samples were prepared by titration of water into a mixture of the other components.

Observation of morphology. Erythrocyte suspensions were diluted with 0.15 M NaCl and 0.01 M phosphate buffer at pH 7.0. This solution was then placed on a capillary microslide (Vitro Dynamics Inc., Rockaway, NJ) and was

TABLE 1

Chemical Formulas of Nonionic Surfactants[a]

POE(n) oleyl ether	
POE(n) monostearate	
POE(20) sorbitan monooleate	
POE(20) 2-decyltetradecyl ether	
POE(20)POP(6) 2-decyltetradecyl ether	
POE(n) hydrogenated castor oil	
POE(n) sorbitol tetraoleate	

[a]POE, polyoxyethylene; POP, polyoxypropylene.

brought into contact with 2 wt% aqueous surfactant solution containing 0.15 M NaCl and 0.01 M phosphate on the microslide. The hemolytic process was followed with a video-enhanced microscope (VEM) (Nikon, HPD Microscope; Hamamatsu System Inc., Argus 100, Hamamatsu, Japan) and was recorded on floppy disk.

RESULTS AND DISCUSSION

The surfactants tested in this study were all POE-type nonionic surfactants. The hemolytic activities of these surfactants are shown as the extent of hemolysis expressed as a percentage after mixing erythrocytes and surfactants at 37°C. Table 2 summarizes the percentage hemolysis of erythrocytes. Hemolysis decreased as the POE chain-length increased in the POE(n) oleyl ether and POE(n) monostearate series, and hemolysis was not observed in the POE(n) hydrogenated castor oil or the POE(n) sorbitol tetraoleate series. The percentage hemolysis in the POE(20) 2-decyltetradecyl ether solution was lower than that in the POE(20) oleyl ether solution. Branching of the lipophilic part is effective in decreasing the hemolytic activity of surfactants. The introduction of a POP chain in POE(20) 2-decyltetradecyl ether induces a further reduction in hemolytic activity. POE(20) POP(6) 2-decyltetradecyl ether has methyl branches in the POP chain. The bulky structure of the binding site between the hydrophilic group (POE chain) and the lipophilic group (alkyl chain) may be more effective in reducing hemolytic activity than the branched alkyl chain. POE(n) hydrogenated castor oil and POE(n) sorbitol tetraoleate have three and four binding sites with alkyl chains in their molecules, respectively, so there is no hemolysis with these surfactant systems because of their bulky structure.

Time dependence of hemolysis was observed in the POE(20) sorbitan monooleate, POE(20) 2-decyltetradecyl ether, POE(20) POP(6) 2-decyltetradecyl ether, POE(50) oleyl ether and POE(55) monostearate systems. POE(20) sorbitan monooleate, POE(20) 2-decyltetradecyl ether and POE(20) POP(6) 2-decyltetradecyl ether do not have linear structures. Although POE(50) oleyl ether and POE(55) monostearate have linear structures, their hemolytic ability may be low because of their high hydrophilicity and, therefore, these surfactants belong to the category of slow-reaction hemolytic surfactants described by Weltzien (12).

In hemolysis, concentration dependence was not observed in any surfactant systems except in the POE(55) monostearate, POE(20) 2-decyltetradecyl ether and POE(20) POP(6) 2-decyltetradecyl ether systems. All these surfactants belong to the surfactant group showing time-dependent hemolysis. Surfactants with moderate hemolytic activity show time- and concentration-dependent hemolysis; however, surfactants of low hemolytic activity do not show such time and concentration dependence in this concentration range.

The influence of molecular weight of surfactants on hemolysis is shown in Figure 1, which compares percentage hemolysis and the molecular weight of surfactants. The percentage hemolysis has a tendency to decrease with an increase in molecular weight of nonionic surfactants. Only the results of the 5 wt/vol% surfactant aqueous solutions are shown in Figure 1 because the 0.1 wt/vol% and 1.0 wt/vol% solutions showed the same tendency. The solubilizing power of surfactants with long oxyethylene chains decreases because of their low ability to aggregate (13,14). The hemolytic ability of surfactants with high molecular weight was low in the POE-type nonionic

TABLE 2

Percentage of Hemolysis by Some Types of Nonionic Surfactants[a]

Surfactant concentration (wt/vol%):			0.1			1.0			5.0		
Incubation time (min):			15	30	120	15	30	120	15	30	120
Surfactant	MW	HLB-n[b]	Hemolysis in %[c,d]								
POE(10) oleyl ether	708	14	100	100	100	100	100	100	100	100	100
POE(20) oleyl ether	1148	17	100	100	100	100	100	100	100	100	100
POE(50) oleyl ether	2468	18	52	74	78	49	57	65	32	32	53
POE(25) monostearate	1429	15	—	80	82	—	83	87	—	85	85
POE(40) monostearate	2089	17	—	72	76	—	78	81	—	66	77
POE(55) monostearate	2749	18	—	0	18	—	1	44	—	26	72
POE(20) sorbitan monooleate	1326	15	0	−1	27	0	−1	65	0	48	65
POE(20) 2-decyltetradecyl ether	1233		2	65	100	100	100	100	100	100	100
POE(20) POP(6) 2-decyltetradecyl ether	1582	11	−1	−1	0	−2	−2	7	−2	−2	35
POE(20) hydrogenated castor oil	1874	10	−1	−1	−2	−1	−1	0	0	2	2
POE(40) hydrogenated castor oil	2754	12	−2	−2	−2	−2	−2	−2	−2	−2	−2
POE(60) hydrogenated castor oil	3634	14	−1	−1	−1	−2	−2	−1	−2	−2	−3
POE(40) sorbitol tetraoleate	3072	12	0	0	−1	−1	−1	−2	0	0	−1
POE(60) sorbitol tetraoleate	3952	14	−2	−2	−2	−1	−1	−1	−4	−4	−3

[a]Abbreviations as in Table 1. MW, molecular weight; HLB, hydrophilic-lipophilic balance.
[b]HLB numbers were calculated by the emulsification method (Ref. 15).
[c]Erythrocyte concentration: 1 vol/vol%.
[d]0% and 100% hemolysis were obtained by the addition of isotonic buffer solution and 1.0 wt% Triton X-100 aqueous solution, respectively.

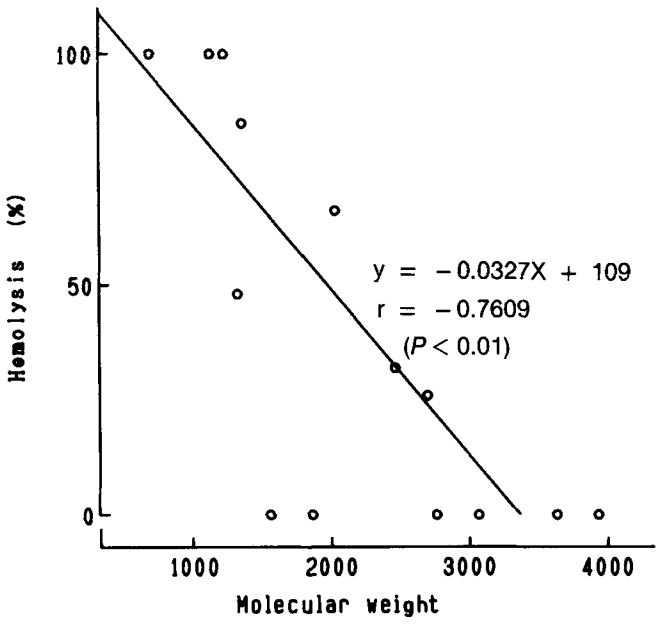


FIG. 1. Hemolysis and molecular weight of nonionic surfactants. Surfactant concentration: 5 wt/vol%/system, incubation time, 30 min.

surfactant system because hydrophilicity increases with an increase in the POE chain. Therefore, the relationship between molecular weight and hemolysis by surfactants of the same HLB number should be considered to understand the effects of molecular weight on hemolysis. The HLB number of POE(10) oleyl ether, POE(60) hydrogenated castor oil and POE(60) sorbitol tetraoleate is 14 (15) (Table 2). The hemolytic abilities of POE(60) hydrogenated castor oil and POE(60) sorbitol tetraoleate, having higher molecular weights, are lower than that of POE(10) oleyl ether. Molecular weight is also important in reducing hemolysis, as well as in reducing HLB.

The molecular weight of POE(50) oleyl ether, POE(55) monostearate and POE(40) hydrogenated castor oil are within the 2,500–3,000 range (Table 2), and the hemolytic abilities of the former two are higher than that of the latter. The former surfactants have a single alkyl chain in the molecule and the latter has three alkyl chains. It is effective to introduce a bulky structure at the binding site to decrease hemolysis.

Hemolysis starts with solubilization of erythrocyte lipids and proteins by surfactants (6,16,17). This is why many investigations concerned with hemolysis and surfactants were carried out with surfactant solutions near the concentration range of CMC (1–4). However, POE(n) hydrogenated castor oil and POE(n) sorbitol tetraoleate do not induce hemolysis at 5 wt/vol%. The CMC of POE(60) hydrogenated castor oil was shown to be below 0.01 wt/vol% by the solubilization of *p*-dimethylaminoazobenzene; therefore, the mechanism of hemolysis of surfactants cannot be explained only by solubilization. Miyajima *et aL.* (3) pointed out that the hemolytic activity of POE(n) cholesteryl ethers was not the same as the order of their CMC.

Lecithin liposomes encapsulating CF were prepared as an erythrocyte model, and the leakage of CF from liposomes caused by the addition of surfactants was measured. The percentage hemolysis and the leakage of CF are shown in Table 3. There is a good relationship between these two results. Percentage hemolysis and leakage of CF decrease with an increase in polyoxyethylene chainlength in the linear alkyl nonionic surfactant series. POE(n) hydrogenated castor oil shows the lowest percentage hemolysis and leakage. From these results, hemolysis is related to the interaction of lipids in erythrocyte membranes and nonionic surfactants. If a nonionic surfactant is a good solubilizer of lipids (lecithin), a mixed solution of lecithin and surfactant gives an isotropic micellar solution. The solubilization of lecithin by nonionic surfactants was investigated from ternary phase diagrams of lecithin/nonionic surfactant/aqueous solution systems (Fig. 2). Aqueous ethanol solution was used as a solvent instead of pure water to avoid complicating the phase diagrams. The mixture of lecithin and nonionic surfactants was dissolved in ethanol, and water was then added to the solution. The isotropic solution region is shown as the shaded area in Figure 2. POE(10) oleyl ether aqueous solution shows a wide region of isotropic surfactant solution [Fig. 2(a)]. More hydrophilic POE(50) oleyl ether does not show such a wide range of isotropic solution [Fig. 2(b)]. These two phase diagrams indicate that POE(50) oleyl ether cannot solubilize as much lecithin in the micelles as POE(10) oleyl ether can. POE(50) oleyl ether may be too hydrophilic to produce mixed micelles with lecithin, and POE(60) hydrogenated castor oil also cannot solubilize much lecithin in the micelles [Fig. 2(c)]. POE(60) hydrogenated castor oil is a good solubilizer for lipophilic medicines (*e.g.,* vitamin K), but is not good for lecithin.

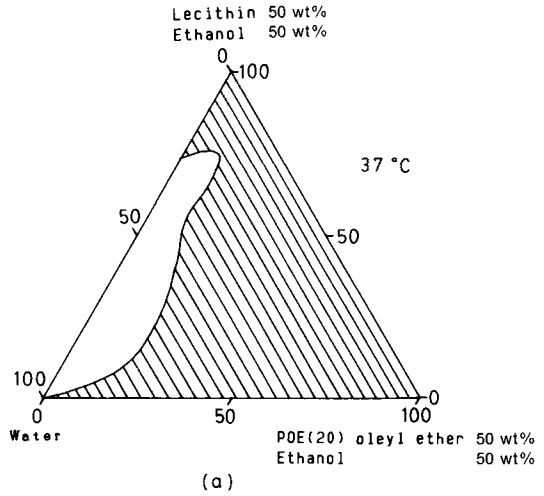
Morphological changes of erythrocytes by nonionic surfactants are shown in Figure 3. The erythrocyte structure rapidly changed into a spherocyte within 30 s of contact when POE(10) oleyl ether was added to the erythrocyte solution. The size gradually increased, and the cells burst within 1 min. Thus, hemolysis caused by POE(10) oleyl ether appears to be due to osmotic lysis (18). After about the same contact time, erythrocytes remained as discocytes in the POE(60) hydrogenated castor oil solution. The

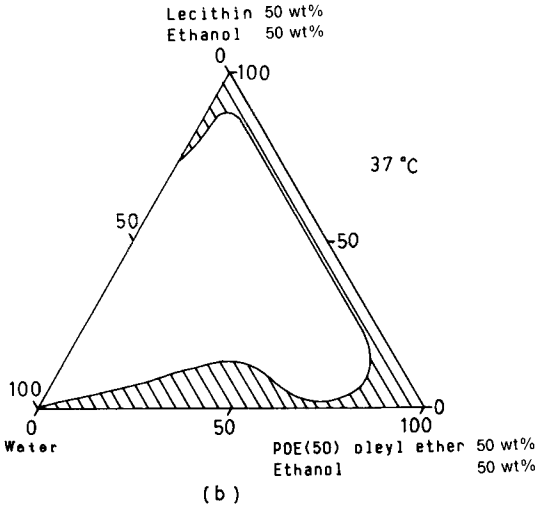
TABLE 3

Correlation Between Hemolysis and Carboxyfluorescein (CF) Release from Liposomes Caused by the Addition of Nonionic Surfactants

Surfactant	Hemolysis (%)[a]	CF release (%)[b]
POE(10) oleyl ether	100	98
POE(20) oleyl ether	100	96
POE(50) oleyl ether	22	17
POE(25) monostearate	85	54
POE(40) monostearate	66	26
POE(55) monostearate	26	13
POE(20) POP(6) 2-decyltetradecyl ether	−2	24
POE(20) hydrogenated castor oil	2	8
POE(40) hydrogenated castor oil	−2	9
POE(60) hydrogenated castor oil	−2	7
POE(60) sorbitol tetraoleate	−4	37

[a]Surfactant concentration: 5 wt/vol%, incubation, 30 min. Abbreviations as in Table 1.
[b]Surfactant concentration: 0.0067 wt/vol%, incubation, 1 h.





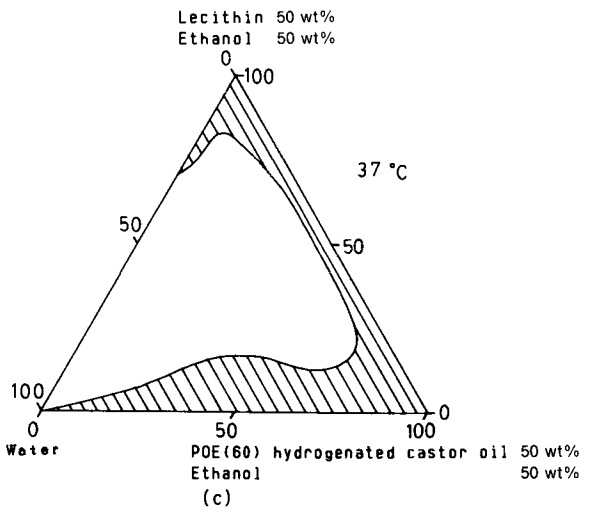


FIG. 2. Ternary phase diagrams of 50 wt% lecithin ethanol solution/50 wt% nonionic surfactant ethanol solution/water system. Shaded area, isotropic solution region. (a) POE(10) oleyl ether system; (b) POE(50) oleyl ether system; and (c) POE(60) hydrogenated castor oil system. POE, polyoxyethylene.

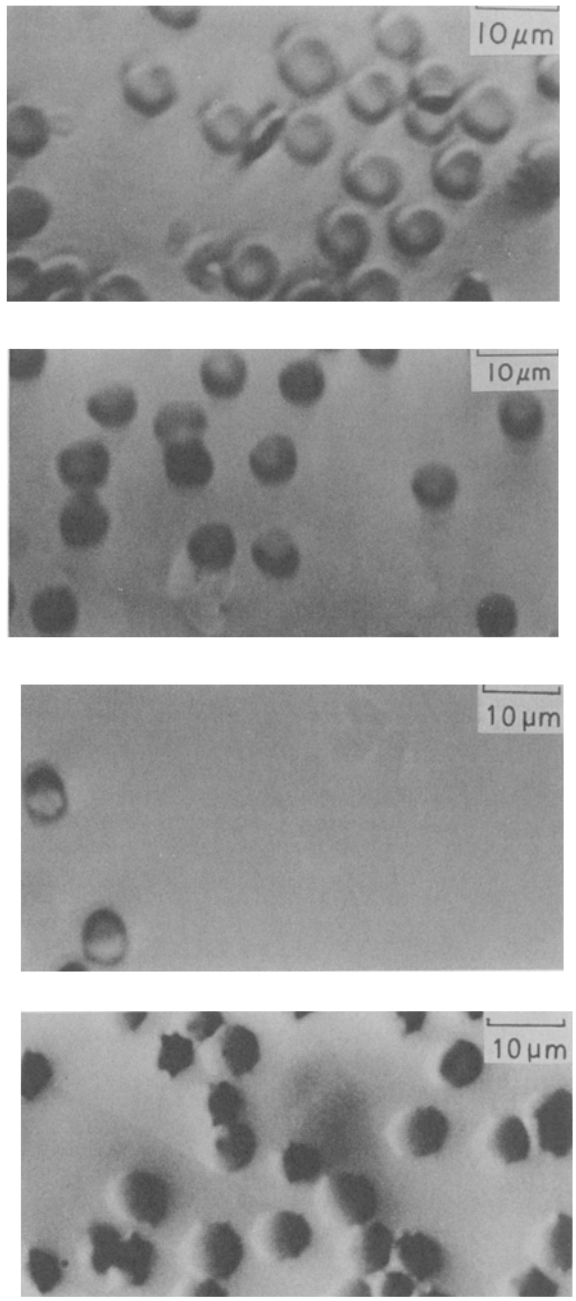


FIG. 3. Morphological changes of erythrocytes caused by the addition of nonionic surfactants. (a) before contact with surfactant; (b) 15 s after contact with POE(10) oleyl ether; (c) 30 s after contact with POE(10) oleyl ether; and (d) 30 min after contact with POE(60) hydrogenated castor oil. POE, polyoxyethylene.

first biconcave-shaped erythrocytes gradually changed into spheroechinocytes after 30 min of contact with POE(60) hydrogenated castor oil. However, the subsequent spheroechinocyte-spherocyte change did not occur in this system. The discocyte-echinocyte change indicated the adsorption of POE(60) hydrogenated castor oil onto the erythrocyte surface. POE(60) hydrogenated castor oil may not be able to penetrate the erythrocyte membrane because of its bulky structure and low solubilizing ability with lecithin. POE(60) hydrogenated castor oil does

not induce hemolysis but affects erythrocyte morphology. POE(10) oleyl ether, having linear alkyl chains, may easily penetrate into the lipophilic layer of the erythrocyte membrane and lead to uptake of water. Curvature of the erythrocyte membrane will change with the penetration of single alkyl chain nonionic surfactants because of the tendency for micellar aggregation. However, surfactants with polyalkyl chains in their molecules will not change the lamellar structure of the erythrocyte membrane, even though they penetrate the membrane (19). We conclude from these results that nonionic surfactants with several alkyl chains and high molecular weights are useful as solubilizers for intravenous drugs.

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