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Membrane Cholesterol and Cell Fusion of Hen and Guinea-Pig Erythrocytes

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1. The cholesterol content of hen erythrocytes was modified by treating the cells with phospholipid liposomes. 2. Depletion of cellular cholesterol, by using liposomes of dipalmitoylglycerophosphocholine or phosphatidylcholine from hen erythrocytes, had no effect on the susceptibility of the cells to fusion induced by oleoylglycerol, but markedly decreased fusion induced by Sendai virus. 3. By contrast, enrichment of cellular cholesterol by using liposomes of dipalmitoylglycerophosphocholine and cholesterol increased cell fusion induced by oleoylglycerol, poly(ethylene glycol) and Sendai virus. 4. Virus-induced cell fusion of guinea-pig erythrocytes, which were enriched in cholesterol by feeding a cholesterol-rich diet to the animals, was also enhanced. 5. Hen erythrocytes that were treated with liposomes prepared from egg phosphatidylcholine contained increased quantities of phospholipid phosphorus and fused readily on incubation with retinol, independently of their cholesterol content. 6. It is suggested that cholesterol may enhance cell fusion by acting to facilitate a phase separation of protein-free areas of lipid bilayer, which subsequently provide the sites for cell fusion.

Cholesterol is an important constituent of plasma membranes and it is known to modify the fluidity of membrane phospholipids (Chapman, 1973). Relatively little is known, however, of the effects that changes in the content of membrane cholesterol have on the process of cell fusion, although it has been suggested that the fluidity of membranes may be an important factor in the ability of membranes to fuse (Ahkong *et al.*, 1973; Papahadjopoulos *et al.*, 1973).

From studies on the virus-induced fusion of cells with different molar ratios of cholesterol/phospholipid in their plasma membranes, it was concluded that this facet of lipid composition does not determine the susceptibility of plasma membranes to fuse (Poste *et al.*, 1972). Dispersions of dipalmitoylglycerophosphocholine and of cholesterol have, however, been shown to inhibit the fusion of myoblasts (van der Bosch *et al.*, 1973). Further, the fusion of cultured baby-hamster kidney cells and of fibroblasts, which is promoted by liposomes composed of phosphatidylserine, is inhibited by the presence of cholesterol in the liposomes (Papahadjopoulos *et al.*, 1973). A similar inhibitory effect of cholesterol was observed on fusion occurring between negatively charged liposomes of phosphatidylserine (Papahadjopoulos *et al.*, 1974). Cholesterol has also been shown to inhibit the fusion of spherical phospholipid membranes of phosphatidylcholine and phosphatidylserine (Breisblatt & Ohki, 1976).

Cholesterol in liposomes thus has inhibitory effects, although there is apparently no evidence that a high concentration of cholesterol in a biological membrane inhibits membrane fusion. Attention has previously been drawn to this paradox (Lucy, 1974) in relation to the membranes of secretory organelles, e.g. those of zymogen granules, which fuse readily with the plasma membrane in exocytosis even though both membranes contain high proportions of cholesterol. Techniques are now available for decreasing or increasing the cholesterol content of erythrocyte membranes *in vitro* (Bruckdorfer *et al.*, 1968, 1969; Grunze & Deuticke, 1974; Cooper *et al.*, 1975). The cholesterol content of guinea-pig erythrocytes can also be increased *in vivo* by dietary means (Kroes & Ostwald, 1971). In the work reported here, we have therefore used these procedures to enable us to study the effects of the cholesterol content of erythrocytes on their ability to fuse after treatment with Sendai virus or with various chemical fusogens. The results obtained indicate that an increase in membrane cholesterol enhances fusion by Sendai virus and by chemical fusogens, whereas a depletion

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of the cholesterol content decreases virus-induced cell fusion and has little or no effect on chemically induced cell fusion.

Materials and Methods

Cholesterol, oleoylglycerol, dipalmitoylglycerophosphocholine (Table 1), 1-palmitoylglycerophosphocholine, dextran (mol.wt. 82000), fatty acid-free bovine serum albumin and *o*-phthalaldehyde were all from Sigma (London) Chemical Co. (London S.W.6, U.K.). Purified egg phosphatidylcholine (Table 1) was supplied by Lipid Products (Redhill, Surrey, U.K.); commercial grade phosphatidylcholine (Table 1), which contained approx. 7mol% of lysophosphatidylcholine and a trace of phosphatidylethanolamine, was from Merck (Darmstadt, Germany). Crystalline retinol was from Roche Products (Welwyn Garden City, Herts., U.K.). Neuraminidase (*Vibrio cholerae*) was from Behringwerke (Hoechst Pharmaceuticals, Hounslow, Middlesex, U.K.). Poly(ethylene glycol) of mol.wt. 6000 was from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Phospholipids (Table 1) from washed hen erythrocytes were extracted by the method of Rose & Oklander (1965). Phosphatidylcholine (Table 1) was prepared from the mixed lipids by chromatography on silicic acid [100–200 grade; Mallinckrodt (St. Louis, MO 63160, U.S.A.), 100mm×10mm glass columns]. A phosphatidylcholine fraction, which contained some sphingomyelin, was eluted in 50% (v/v) chloroform/methanol.

Lipid analyses

Lipids were extracted from 0.1ml of packed erythrocytes as described above, and from liposome preparations by the method of Folch *et al.* (1957). Cholesterol was determined in the extracts by using *o*-phthalaldehyde (Rudel & Morris, 1973), and lipid phosphorus was determined by the direct method of Raheja *et al.* (1973).

Fatty acid profiles of phospholipids were determined by g.l.c., by using a Pye 104 chromatograph with a dual flame-ionization detector fitted to a DP 88 Minigrator (Pye–Unicam, Cambridge, U.K.). The 1.5m glass columns contained 10% poly(ethylene glycol) adipate on Diatomite CAW (Pye–Unicam) and were operated at 190°C in argon at gas-flow rate of 50ml/min. Methyl esters of the phospholipid fatty acids were prepared by the procedure of Christie (1973). Fatty acid analyses obtained from triplicate runs on two methylated samples of each phospholipid showed a maximum variation of ±4%.

The purity of the phospholipids was checked by t.l.c. on silica gel H (Merck) run in a solvent of chloroform/methanol/acetic acid/water (25:15:4:2, by vol.), and developed with a H_2SO_4 spray (50%, v/v) at 180°C for 30min.

Preparation of liposomes

Solutions of lipids in chloroform were evaporated under N_2. The lipids were dried under vacuum and then, after addition of 0.9% NaCl (10ml) or 10ml of a solution containing 140mM-NaCl and 45mM-sucrose buffered at pH7.4 with 12.5mM-sodium phosphate buffer, the lipids were sonicated for 5–10min, or longer as specified in the text, in a 150W ultrasonic disintegrator (MSE, Crawley, Sussex RH10 2QQ, U.K.) fitted with a 1.9cm titanium probe and operated at the medium setting with maximum tuning. Solutions used for the preparation of liposomes were saturated with N_2 and contained streptomycin sulphate (200mg/litre) and penicillin G (80mg/litre). Sonication vessels were cooled by chilled water. Excess of undispersed lipid and any debris present from the probe were removed by centrifugation at 21000*g* for 30min.

Guinea pigs

Male albino Dunkin-Hartley guinea pigs (500g) (Redfern Animal Breeders, Brenchley, Kent, U.K.) were fed for 4–5 weeks on BOCM Coney Brand pellets (Lillico, Betchworth, Surrey, U.K.) mixed with

Table 1. *Fatty acid composition of phospholipids used to prepare liposomes*
The phospholipids are obtained and analysed as described in the Materials and Methods section.

Phospholipid	Composition (mol% of total fatty acid)				
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Dipalmitoylglycerophosphocholine	100	0	0	0	0
Purified egg phosphatidylcholine (Lipid Products)	33	14	31	18	4
Egg phosphatidylcholine (Merck)	31	17	26	19	6
Phospholipids from hen erythrocytes	19	16	23	31	8
Phosphatidylcholine from hen erythrocytes	24	11	22	36	6

100g of corn oil/kg of diet and 10g of cholesterol/kg of diet. A control group of guinea pigs was fed on an identical diet, but without the cholesterol.

Erythrocytes

Blood (approx. 2ml) was removed from the brachial vein of an adult hen and taken into a syringe containing citrate anticoagulant solution (1ml) (De Gowin *et al.*, 1949). Alternatively, blood was collected from the jugular vein on slaughter. Guinea pigs were anaesthetized with CO_2; blood was removed by cardiac puncture and collected in the citrate anticoagulant solution. Blood thus obtained was centrifuged at 3000*g* for 5min, and the erythrocytes were then washed by resuspension in the anticoagulant solution (10ml). Subsequently, the cells were washed twice either in the 12.5mM-phosphate buffer, pH7.4, containing NaCl and sucrose (see above) (Grunze & Deuticke, 1974), or in modified Eagle's basal salt solution, pH7.4 (Ahkong *et al.*, 1973), that was further modified in the experiments described in the present paper to contain 45mM-sucrose. In some cases, the modified Eagle's basal salt solution contained fatty acid-free bovine serum albumin (20g/litre), as indicated in the text. The washed cells were pelleted by centrifugation at 3000*g* for 5min.

Preincubation of erythrocytes with liposomes

Packed erythrocytes (0.3ml) were mixed in stoppered tubes (capacity 12ml) with liposomes suspended in the 12.5mM-phosphate buffer (pH7.4, containing NaCl and sucrose; see above) (2.7ml). In some experiments, the liposomes which had been prepared in 0.9% NaCl solution were diluted appropriately to make the suspension of liposomes iso-osmotic with respect to the modified Eagle's basal salt solution containing bovine serum albumin (20g/litre) where indicated in the text. This dispersion of liposomes (6ml) was then added to the packed erythrocytes (0.3ml). Incubation tubes were rotated at 60rev./min by a slow-speed motor in a water bath at 37°C for up to 20h. After incubation, the cells were washed with 4×10ml of the modified Eagle's basal salt solution, pH6.8, and then centrifuged into a pellet at 3000*g* for 5min.

Incubation of erythrocytes with chemical fusogens

Retinol. Packed erythrocytes (0.1ml) were mixed with 0.9ml of the modified Eagle's basal salt solution, pH6.8 (see above), which additionally contained dextran (80g/litre) as described by Ahkong *et al.* (1973). Retinol was stored and handled as described by Dingle & Lucy (1962); stock and working solutions were prepared as described previously (Ahkong *et al.*, 1973). Retinol (100μg) was injected, with a Hamilton syringe (Micromeasure N.V., The Hague, The Netherlands), as a solution in ethanol (25μl) into the modified Eagle's basal salt solution, pH6.8, containing dextran (6ml). The dispersion of retinol (0.4ml) prepared in this way was added to the suspension of erythrocytes (0.2ml). The cells were then incubated at 37°C, and samples taken at intervals for observation by optical microscopy with phase-contrast or Nomarski differential-interference-contrast optics (Standard WL Research Microscope; Carl Zeiss, Degenhardt and Co. London W1P 8AP, U.K.), at a magnification of ×400.

Oleoylglycerol. Dispersons of oleoylglycerol were prepared by sonication of the lipid (1mg) in the modified Eagle's basal salt solution (6ml) used above with retinol, by the procedure described by Ahkong *et al.* (1973). Erythrocytes were treated with suspensions of oleoylglycerol as described above for experiments with retinol.

Poly(ethylene glycol). Packed erythrocytes (0.1ml) were diluted to 1ml by addition of the modified Eagle's basal salt solution, pH6.8 (see above), not containing dextran. This cell suspension (0.2ml) was centrifuged at 3000*g* for 5min. The packed erythrocytes were then re-suspended in 0.4ml of poly(ethylene glycol) (400g/litre) at 37°C and incubated for 15min at 37°C. Subsequently, the erythrocytes were diluted further by the addition of modified Eagle's basal salt solution not containing dextran (2ml), and the cells then centrifuged at 3000*g* for 5min. The packed erythrocytes were resuspended in the same salt solution (0.4ml) and incubated at 37°C. Samples were taken at intervals for observation by optical microscopy.

Evaluation of chemically induced cell fusion

The time of incubation before the onset of extensive cell fusion was measured as previously described (Ahkong *et al.*, 1973). Thus the 'time to fusion', t_f, was determined, i.e. the number of minutes of incubation that elapsed before fused cells were observed in high incidence, with the optical microscope, in samples taken at frequent intervals from a preparation of treated cells. The time to fusion can normally be determined to within $\pm 1\frac{1}{2}$min.

Cells (0.6ml of cell suspension) were fixed for microscopy by the addition of an ice-cold solution of glutaraldehyde (5%, v/v) in 0.083M-sodium cacodylate/HCl buffer, pH7.4. The cells were left for 1h, and then washed twice in water, suspended in Harris's Haematoxylin (BDH Chemicals, Poole, Dorset BH12 4NN, U.K.) solution (0.2ml) and washed twice in tap water after 20min. Finally, the cells were suspended in 3.2mM-NH_3 (1ml) and

counted, by using the optical microscope, at a magnification of ×400. The percentage polykaryocytosis was defined as the number of nuclei present in polykaryocytes expressed as a percentage of the total number of nuclei observed: 500–1000 nuclei were counted in each determination.

Cell fusion induced by Sendai virus

Sendai virus was grown and assayed in HA units (Salk, 1944) and indices of cell fusion were evaluated as described by Hart *et al.* (1976). For the determination of virus-induced haemolysis, the samples used in estimating cell fusion were centrifuged for 10min at 1500*g*. Water (3ml) was then added to the supernatant (0.5ml) and the A_{541} was measured by using a Unicam SP.500 spectrophotometer. The A_{541} values obtained were compared with those given by cells that were totally haemolysed by suspension in water.

Counting of cells

After incubation of hen erythrocytes with liposomes, the cells were centrifuged at 3000*g* for 5min. Packed cells (0.05ml) were then resuspended in a solution containing 0.1% NaN_3 (2ml) (Isoton; Coulter Electronics, Harpenden, Herts., U.K.). The number of cells was counted with a Coulter counter model F_n (Coulter Electronics) fitted with a 100μm aperture. Cell counts (in triplicate) were corrected for coincidence before calculating mean values.

Results

Hen erythrocytes treated with liposomes prepared from dipalmitoylglycerophosphocholine

The procedure devised by Cooper *et al.* (1975) for enriching or depleting the cholesterol pool of human erythrocyte membranes by treating the cells with dipalmitoylglycerophosphocholine liposomes in the presence of albumin, was used. Hen erythrocytes were treated in this way with liposomes having a cholesterol:phospholipid molar ratio of 0.5:1.0. There was little change, by comparison with the untreated cells, in the fusion of cholesterol-depleted erythrocytes on subsequent incubation with oleoylglycerol (Table 2*a*). By contrast, cells that were treated with cholesterol-rich liposomes (choles-

Table 2. *Properties of hen erythrocytes treated with liposomes prepared from dipalmitoylglycerophosphocholine*
Liposomes were prepared from 10mg of dipalmitoylglycerophosphocholine, with 0, 5 or 20mg of cholesterol, in 0.9% NaCl solution (10ml) by sonication for 20min for cholesterol-poor liposomes (cholesterol:phospholipid molar ratio, 0.5:1.0) or 40min for the cholesterol-rich liposomes (2.5:1.0) (*a*), or 7min (*b* and *c*). Hen erythrocytes, washed in modified Eagle's basal salt solution containing fatty acid-free bovine serum albumin (20g/litre) and sucrose (45mM) were preincubated with the liposomes in this solution, and then treated with oleoylglycerol as described in the Materials and Methods section. The values given in the Tables are either the individual values from two separate experiments, or are mean values (±S.D.) obtained from three separate experiments. Assays for cholesterol and phospholipid phosphorus were done in duplicate, and percentage polykaryocytosis and time to fusion (t_f) were measured in triplicate, within each experiment.

Molar ratio of cholesterol:phospholipid in liposomes	10^7 × Cholesterol content (nmol/cell)	10^7 × Phospholipid content (nmol/cell)	t_f (min)	Percentage polykaryocytosis in 15min
(*a*)				
No liposomes	2.98	8.28	12.7	1.00
	2.80	7.64	10.2	1.03
0.5:1.0 ($n=3$)	2.31±0.01	9.16±0.45	11.5±1.8	1.00±0.00
2.5:1.0 ($n=3$)	7.04±0.50	8.76±0.06	8.5±1.0	11.38±2.17
(*b*)				
1:1 without lysophosphatidylcholine	—	—	—	5.0
				11.5
1:1+1 mol% of lysophosphatidylcholine	—	—	—	5.5
				7.5
1:1+10mol% of lysophosphatidylcholine	—	—	—	7.5
				5.0
(*c*)				
No liposomes ($n=3$)	4.44±0.30	10.33±0.55	—	1.67±0.58
0:1 ($n=3$)	3.14±0.29	9.17±0.35	—	1.20±0.00
1:1 ($n=3$)	5.04±0.17	9.53±0.31	—	2.17±1.15
2:1 ($n=3$)	8.35±0.56	10.37±0.58	—	48.17±6.83

terol:phospholipid molar ratio 2.5:1.0), and which had an increased content of cholesterol, fused more quickly and more extensively than control cells (Table 2*a*). Since up to 30% of the cholesterol of hen erythrocytes is present in the nuclear membrane (Zentgraf *et al.*, 1971), which is probably less affected in these experiments than the plasma membrane, the changes in the cholesterol content of the plasma membrane are likely to be greater than those for total cellular cholesterol shown in Table 2.

The highly cholesterol-rich liposomes used, in which some of the cholesterol may have been present in a separate phase (Freeman & Finean, 1975), were found to contain 10mol% of lysophosphatidylcholine, presumably because of the protracted sonication (40min) found necessary for their preparation. However, experiments shown in Table 2(*b*), in which up to 10mol% of 1-palmitoylglycerophosphocholine was incorporated into the liposomes used, showed no increase in the extent of polykaryocytosis occurring on subsequent incubation of the erythrocytes with oleoylglycerol for 15min.

Table 2(*c*) refers to experiments in which liposomes were prepared by sonicating the lipids for only 7min, when no lysophosphatidylcholine was detected. A marked increase in polykaryocytosis induced by oleoylglycerol again occurred with cholesterol-rich cells that had been preincubated in the presence of cholesterol-rich liposomes. By contrast, cells that were treated with cholesterol-free liposomes in the presence of albumin, and which had lost cholesterol, did not fuse more extensively than the control cells.

In a further experiment in which liposomes were prepared by sonicating lipids for 7min, hen erythrocytes were preincubated with the liposomes and then treated with a variety of different agents (Table 3). As in Tables 2(*a*) and 2(*c*), cholesterol-enriched erythrocytes showed more extensive fusion than control cells on subsequent incubation with oleoylglycerol (stearoylglycerol was not fusogenic; cf. Ahkong *et al.*, 1973). Similarly, these cells were fused more extensively on treatment with poly(ethylene glycol), and on incubation with Sendai virus (Table 3). Table 3 shows that depletion of cholesterol did not affect the behaviour of the erythrocytes towards oleoylglycerol (cf. Table 2), but the depleted cells were fused much less extensively by Sendai virus than were the control cells. Virus-induced haemolysis of the cells was nevertheless approximately constant for all groups of cells (Table 3), except for cholesterol-depleted cells, where haemolysis was slightly less, possibly indicating a decreased binding of virus. It was not possible to determine the effect of depletion of cellular cholesterol on the response of hen erythrocytes to poly(ethylene glycol), because the depleted osmotically sensitive cells were lysed during aggregation by this substance.

The absence of any effect of cholesterol depletion on the response of hen erythrocytes to fusion with oleoylglycerol in the above experiments might be attributed to a compensatory exchange of dipalmitoylglycerophosphocholine from the liposomes with polyunsaturated phospholipids in the plasma membranes of the cells. Small quantities of polyunsaturated lipid were in fact detected in the supernatant solution after preincubation of hen erythrocytes with liposomes prepared from dipalmitoylglycerophosphocholine. In addition, the presence of serum albumin during the preincubation with liposomes might perhaps have stabilized the plasma membranes of the cells during the subsequent fusion process.

To exclude these possibilities, liposomes were prepared from phosphatidylcholine obtained from hen erythrocytes. These liposomes were used to pre-treat hen erythrocytes in the presence and in the absence of serum albumin. [Egg phosphatidylcholine has previously been shown (Bruckdorfer *et al.*, 1968) to be capable of depleting human erythrocytes of cholesterol in the absence of serum albumin.] Incubations of hen erythrocytes with two different

Table 3. *Fusion of pretreated hen erythrocytes induced by glycerol esters, poly(ethylene glycol) and Sendai virus*

Liposomes were prepared from dipalmitoylglycerophosphocholine and cholesterol as described for Tables 2(*b*) and 2(*c*). Hen erythrocytes were prepared, preincubated with liposomes, and then treated with oleoylglycerol (or stearoylglycerol) as in Table 2. Cells were treated with poly(ethylene glycol), or Sendai virus, and handled as described in the Materials and Methods section. The values given in the Table are mean values (±S.D.) obtained from three separate experiments. Assays for cholesterol, phospholipid phosphorus, fusion index and haemolysis were done in duplicate, and for percentage polykaryocytosis in triplicate, within each experiment.

Molar ratio of cholesterol: phospholipid in liposomes	$10^7\times$ Cholesterol content (nmol/cell)	$10^7\times$ Phospholipid content (nmol/cell)	Percentage polykaryocytosis			Fusion index with Sendai virus	A_{541}
			In 15min with oleoyl-glycerol	In 24h with stearoyl-glycerol	In 6min with poly(ethylene-glycol)		
No liposomes	4.36±0.43	7.87±0.96	1.27±2.19	0	6.97±2.17	0.093±0.031	0.41±0.02
0:1	2.82±0.28	7.79±1.11	1.57±1.36	0	Lysis	0.003±0.002	0.32±0.01
1:1	4.91±0.34	8.09±0.66	2.9 ±1.76	0	4.30±2.08	0.075±0.002	0.38±0.03
2.3:1	6.69±0.45	8.34±0.03	16.03±6.00	0	20.57±5.73	0.500±0.011	0.39±0.01

Table 4. *Properties of hen erythrocytes treated with liposomes prepared from hen erythrocyte phosphatidylcholine*
Liposomes were prepared from 10 or 20mg of hen erythrocyte phosphatidylcholine in 0.9% NaCl solution (10ml) by sonication for 7min. Hen erythrocytes, washed in modified Eagle's basal salt solution, were preincubated with the liposomes in this medium (with or without 20g of fatty-acid free bovine serum albumin/litre), and then treated with oleoylglycerol as described in the Materials and Methods section. The values given are from two separate experiments. Assays for cholesterol, phospholipid phosphorus and time to fusion (t_f) were done in duplicate, and percentage polykaryocytosis in triplicate, within each experiment.

Liposome incubation	$10^7 \times$ Cholesterol content (nmol/cell)	$10^7 \times$ Phospholipid content (nmol/cell)	t_f (min)	Percentage polykaryocytosis in 15min with oleoylglycerol
No liposomes	3.50	8.55	6–7	7.0
	3.20	7.55		8.0
Phosphatidylcholine (10mg)+albumin	2.40	8.06	6–7	10.0
	2.10	7.61		6.0
Phosphatidylcholine (20mg)+albumin	1.82	8.36	6–7	4.0
	1.58	8.10		9.0
Phosphatidylcholine (10mg)	2.80	8.36	6–7	10.0
	2.80	8.10		6.0
Phosphatidylcholine (20mg)	2.80	8.25	6–7	7.0
	2.80	8.55		10.0

Table 5. *Properties of hen erythrocytes treated with liposomes prepared from egg phosphatidylcholine*
Hen erythrocytes were preincubated with liposomes for 0, 7 or 20h at 37°C in 12.5mM-phosphate buffer, pH7.4 (containing NaCl and sucrose). The liposomes were prepared by sonication of 20mg of phosphatidylcholine (Merck), with or without 10mg of cholesterol, in the above medium as described in the Materials and Methods section. The values for cholesterol, phospholipid phosphorus and t_f, are mean values (±S.D.) obtained from the number (n) of separate experiments shown. Assays for cholesterol and phospholipid phosphorus were done in duplicate, and time to fusion (t_f) in triplicate, within each experiment. Untreated erythrocytes contained 4.3×10^{-7}nmol of cholesterol/cell, 5.2×10^{-7}nmol of phospholipid phosphorus/cell, and exhibited a t_f of 30min.

Preincubation period with liposomes (h)	Molar ratio of cholesterol: phospholipid in liposomes	Cholesterol in preincubated cells (% of that in untreated cells)	Phospholipid P in preincubated cells (% of that in untreated cells)	Time to fusion, t_f, on incubation with retinol (% of that in untreated cells)
0 ($n=4$)	0:1	102± 7	112±15	99±10
7 ($n=4$)	0:1	92± 8	106±17	75±17
20 ($n=9$)	0:1	86± 8	116± 6	42±16
20 ($n=4$)	1:1	120±12	111± 5	60± 9

concentrations (0.17 and 0.34g/litre) of phosphatidylcholine from hen erythrocytes, in the absence of serum albumin, each resulted in a 16% depletion of the total cholesterol of the cells. In the presence of albumin, almost 50% of the cellular cholesterol was removed. The loss of cholesterol had, however, no effect on the subsequent behaviour of the modified cells on treatment with oleoylglycerol (Table 4), indicating once again that cholesterol depletion does not influence the extent of cell fusion induced by oleoylglycerol.

Hen erythrocytes treated with liposomes prepared from egg phosphatidylcholine

Grunze & Deuticke (1974) have reported that treatment of bovine erythrocytes with purified preparations of egg phosphatidylcholine readily causes cell lysis. We have confirmed this finding with hen erythrocytes. Grunze & Deuticke (1974) also observed, however, that 55% of the membrane cholesterol was removed from the mammalian cells before lysis occurred when the cells were treated with a less-pure preparation of phosphatidylcholine in the presence of sucrose. We applied this procedure to hen erythrocytes and found that approx. 14% of the total cellular cholesterol was removed on treatment of the cells with cholesterol-free liposomes in the presence of sucrose for 20h at 37°C (Table 5). When cholesterol was present in the liposomes, the total cellular cholesterol was increased.

Erythrocytes that had been preincubated for 7 or 20h with cholesterol-free liposomes exhibited de-

creases in the time required for the induction of cell fusion on subsequent incubation with retinol, a lipid-soluble fusogen (Ahkong *et al.*, 1973) that behaves like oleoylglycerol (Maggio & Lucy, 1975). This was unlikely to be due to the decreased cholesterol content of the erythrocytes, however, as cells which were preincubated with liposomes that contained cholesterol were also fused relatively rapidly (Table 5). These experiments indicate the need for caution in interpreting the behaviour of cells that have been depleted of cholesterol by treatment with liposomes. In this instance, Table 5 shows that all of the preparations of erythrocytes that were treated with liposomes, including the zero-time controls, contained increased quantities of phospholipid phosphorus despite repeated washing of the cells after treatment with liposomes.

Guinea-pig erythrocytes

Since it is relatively easy to modify the cholesterol content of guinea-pig erythrocytes by feeding cholesterol to the animals (Kroes & Ostwald, 1971), this procedure was used as an alternative to the liposome technique to obtain erythrocytes containing different quantities of cholesterol.

In experiments with the erythrocytes of guinea pigs that had been fed on a standard laboratory diet, no cell fusion could be satisfactorily demonstrated in the presence of oleoylglycerol or of retinol because the cells aggregated extensively into very large clumps of indeterminate structure. Aggregation was decreased slightly by increasing the concentration of dextran present from 80 to 120g/litre. However, at the higher concentration the medium was so viscous that intercellular contacts were also decreased, and this effect militated against the occurrence of cell fusion.

Some guinea-pig erythrocytes were pretreated with neuraminidase as described by Ahkong *et al.* (1975), and the released sialic acid was detected in the medium by the method of Warren (1959). With such neuraminidase-treated cells, fusion was observed on using oleoylglycerol and retinol as fusogens. Nevertheless, the problem of cellular aggregation remained, and this prevented any useful comparison being made of the behaviour of normal and cholesterol-loaded guinea-pig erythrocytes.

In other experiments guinea-pig erythrocytes were washed in modified Eagle's basal salt solution (without albumin or sucrose) and then treated with Sendai virus, as described in the Materials and Methods section. [Mean values (±S.D.) of the molar ratio of cholesterol:phospholipid in lipids extracted from erythrocytes of three animals fed on the cholesterol-rich diet, and from erythrocytes of three animals fed on the standard diet, were 1.4 ± 0.0 and 0.90 ± 0.05 respectively.] Erythrocytes from each animal were treated with a range of concentrations of Sendai virus (100–400 HA units), and the fusion index and the percentage haemolysis were determined. Data for the cholesterol-rich cells were normalized with respect to the corresponding values found for control cells; the normalized value of the fusion index thus obtained was 4.28 ± 3.32 (S.D.; ten determinations), showing that the cholesterol-rich erythrocytes were fused more extensively than the control cells. No difference in virus-induced haemolysis was found between the two groups of cells; the normalized value for percentage haemolysis in cholesterol-rich cells was 1.01 ± 0.08 (S.D.; ten determinations).

Discussion

In phospholipid systems that are above their transition temperature, the steroid nucleus of cholesterol effectively prevents flexing of the lipid hydrocarbon chains, thereby making them less mobile (Chapman, 1973). Spin-label derivatives of stearic acid (Kroes *et al.*, 1972) and fluorescent probes (Vanderkooi *et al.*, 1974) indicate a decreased fluidity in the membranes of cholesterol-enriched cells. Conversely, a decrease in membrane cholesterol increases the fluidity of biological membranes (Radda & Vanderkooi, 1972; Tanaka & Ohnishi, 1976).

Attention has been drawn by Papahadjopoulos *et al.* (1976*a*) to the possibility that some of the reported observations on membrane fluidity and membrane fusion in model experiments with phospholipid vesicles may be capable of more than one interpretation and may not actually involve membrane fusion. There nevertheless remain a number of studies which clearly involve membrane fusion, and which indicate that an increase in membrane fluidity favours cell fusion (Ahkong *et al.*, 1973; Papahadjopoulos *et al.*, 1973; Kosower *et al.*, 1975; Breisblatt & Ohki, 1976; Papahadjopoulos *et al.*, 1976*b*). It might be expected therefore that the presence of cholesterol would inhibit the fusion of membranes and, as indicated in the introduction of the present paper, this has in fact been reported for membrane fusion involving liposomal systems and spherical lipid bilayers.

By contrast, lysosomal membranes and zymogen-granules membranes are rich in cholesterol and yet they participate readily in membrane fusion reactions occurring *in vivo*. Interestingly, a decrease in the membrane sterol content of L cells decreases endocytosis (Heiniger *et al.*, 1976), and fusion of the cholesterol-rich lipoprotein LP-X, from patients with hepatic cholestasis, with erythrocyte membranes has been observed (Verkleij *et al.*, 1976). Further, the experiments reported in the present paper show not only that a deficiency in cellular

cholesterol decreases the fusion of erythrocytes by Sendai virus, but also that an increase in cellular cholesterol enhances fusion occurring in the presence of oleoylglycerol, poly(ethylene glycol) and Sendai virus.

It seems reasonable to assume in discussing the present experiments that an increase in cellular cholesterol in fact represents an increase in plasma-membrane cholesterol. How then is the apparently contradictory behaviour of cholesterol in membranes to be explained? It is relevant that dimethyl sulphoxide, which induces cell fusion (Ahkong *et al.*, 1975) and also enhances the rate of fusion of acidic phospholipid vesicles (Papahadjopoulos *et al.*, 1976*a*), has been found to produce an increase in the transition temperature of acidic dimyristoylglycerophosphoglycerol membranes (Lyman *et al.*, 1976). It has therefore been suggested that the fusogenic properties of dimethyl sulphoxide are related to its ability to induce a phase transition in the lipids of membranes, and that its actions may resemble those of Ca^{2+} in promoting vesicle fusion (Papahadjopoulos *et al.*, 1976*a*,*b*). Significantly, the intramembranous particles of unfixed T and B mouse lymphocytes have been found to aggregate on incubation at 0°C in dimethyl sulphoxide (McIntyre *et al.*, 1974).

Since the emergence of protein-free areas of lipid bilayer and an increase in membrane fluidity are both important in membrane fusion, it has been proposed that fusion actually occurs between the regions of fluid lipid bilayer [cf. Fig. 2 in Ahkong *et al.* (1975)]. We suggest that the enhancement of cell fusion by cholesterol that is reported in the present paper may be due to the sterol acting to facilitate an initial phase separation of protein-free areas of lipid bilayer; location of cholesterol in the outer half of the lipid bilayer of erythrocytes (cf. Fisher, 1976) may be important in this respect. It is relevant that the adenosine triphosphatase of rabbit sarcoplasmic reticulum is excluded from solid lipid regions of phospholipid bilayer membranes containing more than 10mol% of cholesterol (Kleeman & McConnell, 1976); cholesterol loading of bovine erythrocytes also decreases the surface density of intramembranous particles, as shown by freeze-fracture techniques (Deuticke & Ruska, 1976). Further, it is noteworthy in relation to our suggestion that Brûlet & McConnell (1976), in an immunochemical study with model membranes, consider the possibility that cholesterol has at least two effects on complement fixation in liposome membranes, one being due to the effects of cholesterol on membrane fluidity and one related to enhancement of hapten exposure at the membrane surface.

From studies on phospholipid monolayers it has been proposed that poly(ethylene glycol) decreases membrane surface potential, thus permitting cell fusion to occur more readily (Maggio *et al.*, 1976). Cholesterol-rich regions of membranes will presumably have a lower surface potential, and this may also facilitate membrane fusion.

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