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CHANGES OF NONELECTROLYTE PERMEABILITY IN CHOLESTEROL-LOADED ERYTHROCYTES

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SUMMARY

Membrane cholesterol in porcine and bovine erythrocytes was elevated up to 165 % of its normal value by incubation of the cells in cholesterol/phosphatidylcholine dispersions with or without serum. This alteration of membrane lipid composition brought about only a minor (10–40 %) decrease of the permeability to glycerol, erythritol and to organic acids penetrating by non-ionic diffusion, although additional cholesterol had actually been incorporated into the lipid bilayer, as indicated by determinations of cell surface area from the critical hemolytic volume, in combination with quantitative evaluation of freeze-etch electron micrographs.

On the basis of this finding and of the previously demonstrated (Grunze, M. and Deuticke, B. (1974) Biochim. Biophys. Acta 356, 125–130) considerable increase of permeability in cholesterol-depleted cells, it is proposed that in the erythrocyte membrane a pronounced "specific" reduction of permeability by cholesterol occurs only up to a molar ratio cholesterol/polar lipid of 0.6. At higher ratios cholesterol affects permeability only slightly, owing to an "unspecific" rigidifying effect on the membrane lipid phase.

INTRODUCTION

The influence of cholesterol on the fluidity of the hydrophobic core of artificial phospholipid bilayer membranes [1–5] and on their permeability to water [6] and nonelectrolytes [7–9], as well as ions [10–12], has been unequivocally established in numerous studies. Recent results obtained on erythrocytes [13] and the microorganism *Acholeplasma laidlawii* [14–16] have furthermore provided clear evidence that in biological membranes, too, cholesterol reduces the transfer rates of solutes penetrating via the membrane lipids. Such results agree well with the cholesterol-induced decrease of fluidity, to be anticipated in plasma membranes in view of their rather high degree of unsaturation [8, 14, 17].

Abbreviation: phenopyrazone, 1,4-diphenyl-3,5-dioxopyrazolidin.

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Nearly all these studies on artificial and natural membranes have been concerned with the effects of cholesterol up to molar ratios cholesterol/phospholipid of 1.0. This is not only the highest ratio found in plasma membranes under physiological circumstances [18, 19] but it is also assumed to be the limiting ratio for an interaction between cholesterol and phospholipid [20, 21].

On the other hand, investigations of erythrocyte disorders in hepatic and biliary diseases [22–24], as well as results obtained with artificial cholesterol/phospholipid systems [25–29], have demonstrated that cholesterol is able to coexist with phospholipids in membrane systems at ratios exceeding 1.0. Little is known about the physical state of cholesterol and its influence on membrane permeability under these conditions. In cholesterol-loaded erythrocytes from guinea pigs fed a high-cholesterol diet and in normal erythrocytes loaded with cholesterol in the plasma of such animals, Kroes and Ostwald [30] observed a reduction of nonelectrolyte permeability, as determined by osmotic swelling techniques and tracer methods. High-cholesterol diet, however, not only increases erythrocyte cholesterol levels, but also induces changes of the concentration and the pattern of membrane phospholipids, of their fatty acid composition [31] and of the plasma lipoproteins [32]. Since these changes might also contribute to the effects observed we investigated the effect on erythrocyte permeability of cholesterol incorporated into the membrane under more controlled conditions.

METHODS

Cholesterol loading procedure

Porcine and bovine erythrocytes were loaded with cholesterol by incubation either in saline suspensions of sonicated cholesterol/phosphatidylcholine liposomes or in mixtures of such dispersions with heated serum as described by Cooper et al. [27].

In a typical experiment a round bottom flask was coated with 200 mg egg lecithin (Merck No. 5331, used without further purification) or dipalmitoyl phosphatidylcholine (Sigma No. 6138) and 50–400 mg of cholesterol (Merck No. 2471) under N_2.

Pure dipalmitoyl phosphatidylcholine was used, since purified egg lecithin proved to cause considerable lysis of the erythrocytes. The lipids were then suspended in 30 ml of a medium containing (mM): NaCl 140, Na_2HPO_4/NaH_2PO_4 12.5; sucrose 45 (medium A), and sonicated in an ice-cooled glass beaker using the standard tip of a Branson sonifier B 12 at 100 W output. After 5 sonication periods of 3 min the liposome suspension was centrifuged (15 min, 0 °C, Sorvall RC 2B 50 000 $\times g$) to remove undispersed cholesterol. These liposome stock suspensions were then diluted to 100 ml with either 70 ml of medium A or 20 ml medium A and 50 ml serum, previously heated to 56 °C for 30 min in order to inactivate phosphatidylcholine-cholesterol acyltransferase.

Whereas no phospholipid was sedimented by the centrifugation, the percentage of cholesterol removed increased with rising cholesterol content but could not be kept constant even for the same initial amount of cholesterol. Since, moreover, the initial saline dispersions were diluted with sera of varying phospholipid and cholesterol content, the ratio cholesterol/phospholipid in the final incubation medium was not

exactly known, which seemed tolerable since the mechanism of cholesterol incorporation was not the subject of the present studies.

The preparation and sonication of the lipid dispersion did not affect the composition of both phosphatidylcholines used, as determined by thin-layer chromatography [33], nor could any peroxide formation be detected by the spectrophotometric technique of Klein [34].

Erythrocytes, washed 3 times in 154 mM NaCl, were incubated in the lipid suspensions at 37 °C and pH 7.35 under gentle rotation. Penicillin (8 mg/100 ml) and streptomycin (20 mg/100 ml) were added to prevent bacterial contamination. Cells incubated in the corresponding media without phosphatidylcholine and cholesterol served as controls.

Analytical procedures

At the end of the loading period the suspension medium was removed by centrifugation and the cells washed 3 times in medium A. An aliquot was lysed with an equal volume of water and the lysate extracted with 11 volumes of isopropanol and 7 volumes of chloroform [35]. Cholesterol and total lipid phosphorus (after removal of water-soluble phosphates [36]) were assayed by routine procedures [37, 38] and their concentrations related to the hemoglobin content of the cells, determined spectrophotometrically as cyanhemiglobin at 546 nm in the lysate.

Osmotic fragilities of the erythrocytes were determined by mixing 50 μl of a 50 % cell suspension in medium A with 5 ml of NaCl solutions of different tonicities. After 30 min, the remaining cells were spun down. The hemoglobin content of the supernatant was determined as described above.

Permeability measurements

Erythrocyte permeabilities to nonelectrolytes (erythritol, glycerol) and to organic acids penetrating by non-ionic diffusion (acetic acid, propionic acid) were determined by measuring the efflux of the ^{14}C-labeled compounds from preloaded cells under equilibrium conditions as previously described [39].

The incubation media contained (mM): (a) glycerol (erythritol) 30, Na_2HPO_4/NaH_2PO_4 5, NaCl 140, glucose 8, sucrose 40, pH 7.35; (b) acetate (propionate) 20, Na_2HPO_4/NaH_2PO_4 40, NaCl 90, pH 8.0. The rate coefficients of tracer efflux served as a measure of permeabilities. In case of acetic acid and propionic acid these values were corrected for a constant pH in view of the marked pH dependency of the rate coefficients [40].

Freeze-fracture electron microscopy

For freeze-etching packed samples of normal, cholesterol-depleted [13] and cholesterol-loaded ox erythrocytes were rapidly frozen, without treatment by cryoprotecting agents, in Freon 22 cooled by liquid nitrogen, fractured in a Balzers high vacuum freeze-etch unit BA 500 R at −100 °C, etched for 90 s and shadowed with platinum/carbon at an angle of 45 °C using an electron beam gun (EVM 052 with EK 552, Balzers AG) in connection with a quartz crystal film thickness monitor to obtain approx. 20 Å platinum. For the following carbon shadowing at an angle of 90° a carbon arc evaporator was used. The replicas were treated with 40 % chromic acid, washed with distilled water and examined at 80 kV with a Siemens Elmiskop I

electron microscope. Particle counts were performed on high contrast positives at a final magnification of 186 000×. Only those parts of the fracture face perpendicular to the axis of the microscope, by visual aspect, over an area of at least 0.1 μm^2 were used for counting. In order to minimize the statistical error, all countings were carried out by the same person with the aid of an electronic counting device coupled to the tip of the ink pen used for marking the counted particles.

RESULTS

(*a*) *Permeability changes of cholesterol-loaded erythrocytes*

Mammalian erythrocytes incubated for prolonged periods of time in media composed of serum and cholesterol/egg lecithin suspension gradually accumulate cholesterol [27]. Under our experimental conditions membrane cholesterol of bovine erythrocytes could be elevated approximately 65 % above its original value (Table I). Somewhat lower cholesterol values were obtained by incubating the cells with serum and cholesterol/dipalmitoyl phosphatidylcholine or with cholesterol/egg lecithin in saline media without addition of serum. The total concentration (μmol phosphorus/μmol hemoglobin) of membrane phospholipids as well as their distribution pattern proved to be independent of the extent of cholesterol loading as verified by thin-layer chromatography (Table II). The increase of membrane cholesterol can thus be regarded as being due to a selective uptake of cholesterol. Fusion with or adsorption of whole liposomes would elevate membrane cholesterol and phospholipids (per unit of hemoglobin).

In the light of the well documented interaction between cholesterol and phospholipids [1–13], such changes of membrane cholesterol should affect the transfer of

TABLE I

ACCUMULATION OF MEMBRANE CHOLESTEROL IN BOVINE ERYTHROCYTES DURING INCUBATION IN EGG LECITHIN/CHOLESTEROL SUSPENSIONS

	H of incubation	Cholesterol***	Accumulation (%)	Lipid phosphorus***	Cholesterol/ phospholipid
A*	0	0.85	–	1.09	0.78
	3	1.06	+ 25	1.15	0.93
	10	1.24	+ 46	1.13	1.11
	24	1.42	+ 67	1.16	1.23
	27	1.41	+ 65	1.15	1.22
B**	0	0.89	–	1.11	0.81
	4	0.99	+ 11	1.12	0.89
	6	1.13	+ 27	1.14	0.99
	10	1.16	+ 31	1.13	1.03
	24	1.22	+ 37	1.12	1.09

* Lipids (cholesterol 4 mg/ml, egg lecithin 2 mg/ml) (These numbers refer to the concentrations initially suspended and sonicated. The true concentrations and ratios are lower, particularly for cholesterol.) dispersed in mixtures (1:1) of heated serum with medium A. Composition of medium A see Methods. Undispersed cholesterol removed by centrifugation (see Methods).

** Lipids dispersed in medium A.

*** μmol/μmol hemoglobin.

TABLE II

LIPID COMPOSITION OF BOVINE ERYTHROCYTES AFTER 22 h. INCUBATION IN DIPALMITOYL PHOSPHATIDYLCHOLINE/CHOLESTEROL SUSPENSIONS

Cells incubated in mixtures (1:1) of heated serum and medium A plus the indicated concentrations of lipids.

	Chol*	Lipid-P*	Cholesterol/ lipid-P	% of total sum			
				PC	Sph	PS	PE
Fresh cells	0.88	1.10	0.80	1.6	45.7	22.2	30.5
Controls	0.88	1.15	0.77	0.2	47.1	21.7	31.0
DPC (2 mg/ml)							
+Cholesterol (1 mg/ml)	0.81	1.06	0.77	1.6	47.1	21.7	29.4
+Cholesterol (1.5 mg/ml)	1.09	1.09	1.00	2.7	44.7	21.9	30.5
+Cholesterol (3 mg/ml)	1.22	1.09	1.12	1.7	44.6	22.8	30.8

Abbreviations: DPC, dipalmitoyl phosphatidylcholine; chol, cholesterol; Lipid-P, Lipid-phosphorus; PC, Phosphatidylcholine; Sph, Sphingomyeline; PS, Phosphatidylserine; PE, Phosphatidylethanolamine.

* μmol/μmol hemoglobin.

compounds penetrating via the lipid phase of the membrane.

Since in bovine and porcine erythrocytes small hydrophilic polyols as well as lipophilic organic acids can be assumed, on the basis of a number of criteria [42], to penetrate by simple diffusion ("nonionic diffusion" in case of the organic acid) via the erythrocyte membrane lipid, such compounds were chosen as test permeants. The possible contribution of ionic transfer of the organic acids, as an anion, was sup-

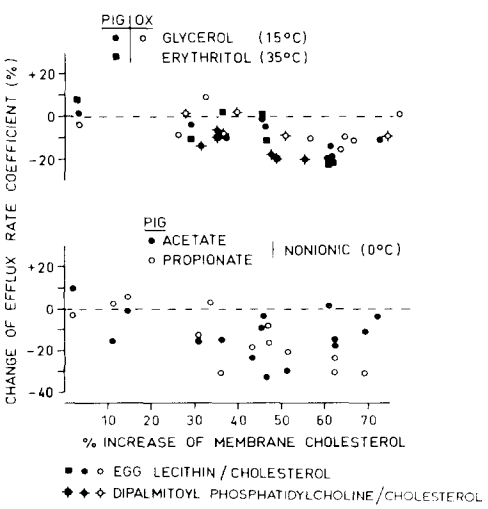

Fig. 1. Influence of cholesterol loading on the transfer of non-electrolytes in mammalian erythrocytes. Cells were loaded for 22 h as described in Table I. After washing, fluxes were determined as described in Methods. The ionic components of the fluxes of acetate and propionate were blocked by phenopyrazone (8 mM).

pressed by addition to the media of phenopyrazone (1,4-diphenyl-3,5-dioxopyrazolidin), a potent inhibitor of anion transfer [40].

As is evident from Fig. 1, cholesterol loading induced a slight but definite diminution of the transfer of all test solutes, although the changes of transfer obtained at the same increase of membrane cholesterol varied to some extent in different experiments. The effect was independent of the nature of the phospholipid in the loading medium. Permeabilities of cells containing approximately 1.6 times the normal amount of cholesterol were reduced by 10–20 % and 10–35 %, respectively, for the two groups of compounds. These changes are smaller than those reported by Kroes and Ostwald [30] for erythritol tracer fluxes in cholesterol-loaded guinea pig erythrocytes. The fluxes of the control erythrocytes, preincubated for the same time periods without added lipid remained unaffected as compared to fresh cells, within the range of reproducibility of our measurements (±5 %).

In order to substantiate this lack of pronounced changes of permeability in cholesterol-loaded erythrocytes, two possible artifacts had to be excluded. Firstly, cholesterol might not have been incorporated into the bilayer phase of the erythrocyte membrane, but only remained attached to the membrane in spite of thorough washings of the cells. Secondly, cholesterol, although incorporated into the membrane, might not have been inserted in vitro into the same position and with the same consequences for permeability as in vivo. The first problem was studied by measuring the surface area of cholesterol-loaded erythrocytes, the second one by measuring permeabilities in erythrocytes first depleted of cholesterol and then reloaded to their normal cholesterol content.

(*b*) *Increase of surface area of cholesterol-loaded erythrocytes*

Incorporation of cholesterol into the lipid bilayer of the erythrocyte membrane should go along with predictable changes of the cell surface area. The surface area of the erythrocyte can be determined by measurements of its "critical hemolytic volume" [22, 43], assuming that osmotic lysis of the cell occurs without prelytic increase of surface area when the cell has reached the volume of a sphere in the course of osmotic swelling. This indirect procedure is so far the only one available for determination of the erythrocyte surface area.

The critical hemolytic volume, V_h, was computed according to Castle and Daland [43] from the relative extracellular tonicity at 50 % hemolysis, obtained from osmotic fragility curves for control and cholesterol-loaded cells. The calculations were carried out for ox erythrocytes, using a value of 0.43 for the apparent osmotically inactive fraction of the cell volume [44]. Relative increases of the surface area A were calculated from changes of V_h by the equation $(V_{h_m}/V_{h_c})^{\frac{2}{3}} = A_m/A_c$, where indices m and c refer to modified and control cells. Relative increases of surface area were converted into absolute values using the normal area of the ox erythrocyte of 95 μm^2 [45]. The constancy of the mean cellular volume, at normal tonicity, of cholesterol-loaded cells, which is pertinent to the above calculations, was ascertained by measurements of cellular water content.

As is evident from Fig. 2, the tonicity producing 50 % hemolysis diminished considerably when the ratio cholesterol/phospholipid was increased. The increases of cell surface area calculated from a number of such fragility curves could be linearly related to the elevation of the molar ratio cholesterol/phospholipid (Fig. 3). The

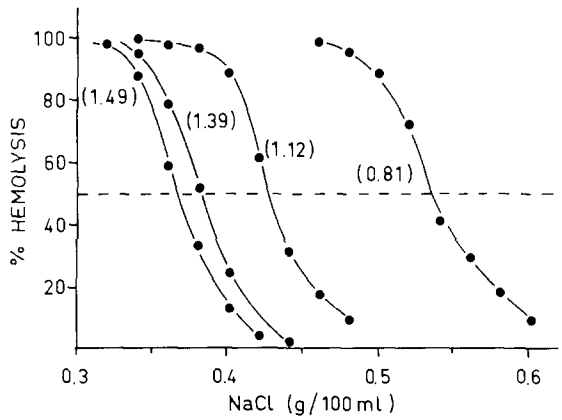

Fig. 2. Osmotic fragility curves of normal and cholesterol-loaded bovine erythrocytes. Cells loaded for 22 h with egg lecithin (2 mg/ml) and cholesterol (1, 2, 4 mg/ml) dispersed in serum/medium A (1:1). Molar ratios cholesterol/phospholipid are given in brackets. The phospholipid content of the cells remained constant.

linearity indicates that at least a constant proportion of the additional cholesterol contributes to the cell surface area.

This result, obtained with bovine erythrocytes incubated in normal serum, cholesterol and egg lecithin is consistent with data obtained for human high-cholesterol erythrocytes produced in the same way [27], as well as for cholesterol-rich erythrocytes obtained by incubation in the serum of patients suffering from biliary obstruction [22, 23]. The altered lipoprotein composition and the elevated bile acid levels of these sera [24] are thus not essential for the insertion of cholesterol into the lipid bilayer phase of the erythrocyte membrane.

The calculation of the surface area of cholesterol loaded erythrocytes from

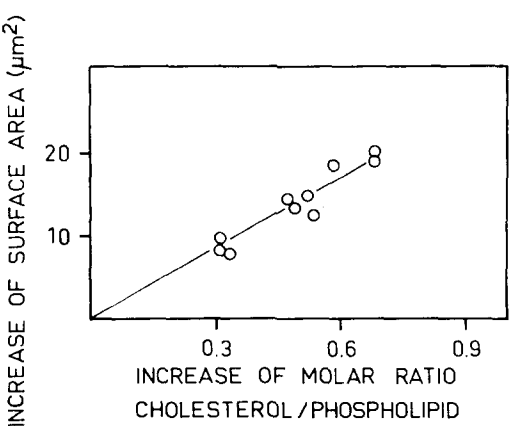

Fig. 3. Relationship between the increase of cell surface area and the increase of the cholesterol/phospholipid ratio in cholesterol-loaded cells. Cells loaded as described in Fig. 2. Increases of surface area determined as described in the text.

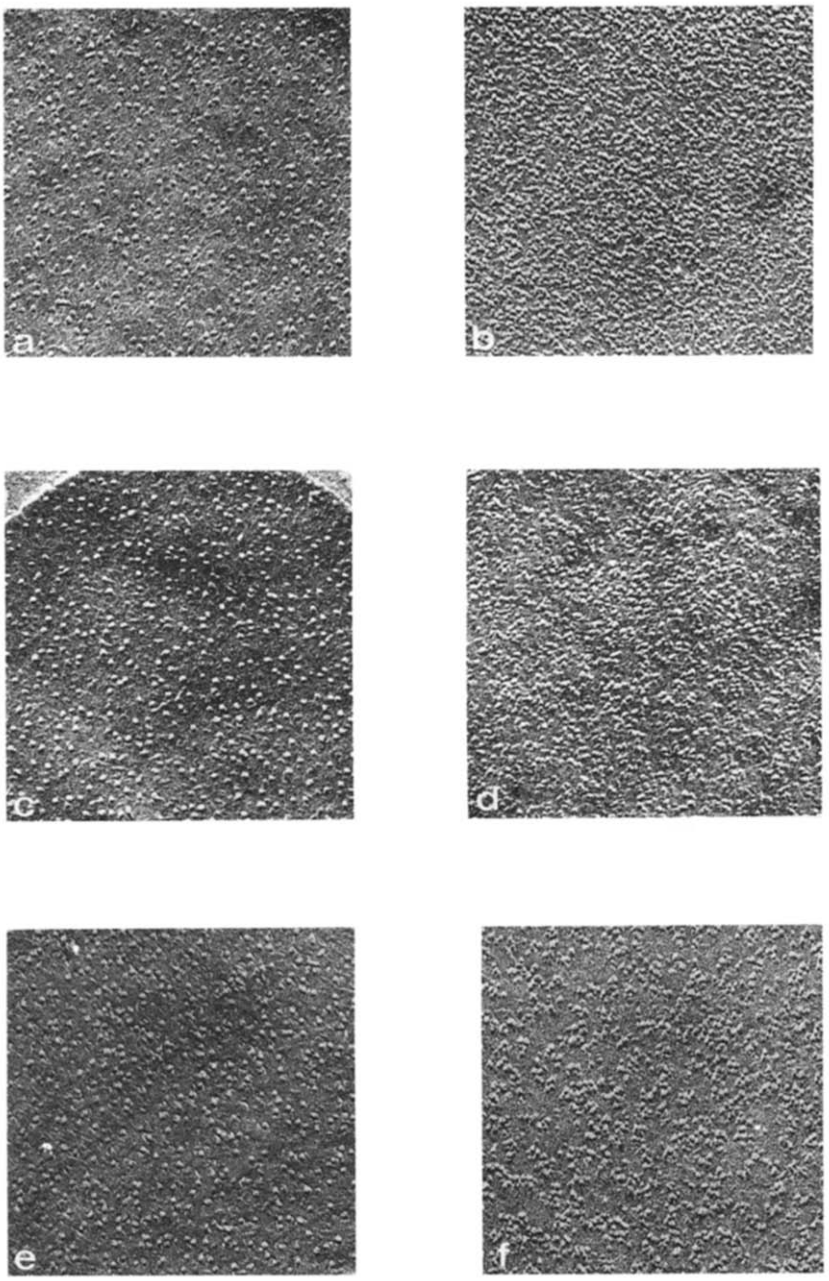

Fig. 4. Membrane fracture faces of bovine erythrocytes with different concentrations of cholesterol. a, b, Cholesterol-depleted cells (−42 %); c, d, Controls; e, f, Cholesterol-loaded cells (+ 58 %); a, c, e, outer fracture face; b, d, f, inner fracture face. For further details see text. Final magnification: × 150 000.

osmotic fragility requires that membrane resistance to biaxional (areal) strain remains very high under these conditions, so that no prelytic increase of surface area can occur. This prerequisite, although most probably fulfilled, cannot be tested independently at present. Therefore, we used freeze-etch electron microscopy to obtain further evidence for the increase of surface area. The aspect of the intramembrane fracture faces did not exhibit any major changes after variation of the molar ratio cholesterol/phospholipid between 0.4 and 1.36 (Fig. 4). In particular, no inhomogeneities of the matrix, which is assumed to represent the hydrophobic domain of the membrane interior, could be detected. The particle distribution was regular in all the outer fracture faces (face B) and in the inner fracture faces (face A) of cells having a normal or reduced cholesterol content. Elevation of membrane cholesterol went along with slight but consistent particle aggregation, corresponding to grade 1 of the classification of Elgsaeter and Branton [46]. This finding provides evidence for a minor perturbation due to extra cholesterol.

Particle counts, performed on the outer fracture faces, varied with the cholesterol content of the membrane in a manner expected to result from changes of the area of the hydrophobic matrix (Table III). Assuming that the number of particles per cell is not affected by the elevation of membrane cholesterol, the decrease of particle density in the cholesterol-loaded cell would correspond to a 7 % increase of surface area. This value agrees qualitatively with the increase of surface area calculated from osmotic fragility and supports the contention that the decrease in osmotic fragility observed in cholesterol-loaded cells is due to an increase of surface area and not to a lowering of the strain resistance of the membrane.

In order to determine more quantitatively the percentage of the additional cholesterol which is actually incorporated into a bilayer, the increases in surface area calculated from V_h were plotted against the increases in surface areas as computed from the number of cholesterol molecules taken up by a single erythrocyte and the areas per molecule of cholesterol and phospholipids. The calculations were based on the following assumptions concerning concentrations and space requirements of membrane lipids arranged in a bilayer.

(1). *Concentrations.* Phospholipids: $2 \cdot 10^8$ molecules per cell (constant, as calculated from our measurement of 1.1 μmol lipid phosphorus/μmol hemoglobin);

TABLE III

INFLUENCE OF CHANGES IN VITRO OF THE MEMBRANE CHOLESTEROL CONCENTRATION ON THE DENSITY OF MEMBRANE-INTERCALATED PARTICLES IN BOVINE ERYTHROCYTES (OUTER FRACTURE FACE)

Cells were depleted of or loaded with cholesterol by 24 h incubation in serum/medium A plus egg lecithin 2 mg/ml, or egg lecithin 2 mg/ml plus cholesterol 3 mg/ml.

Cholesterol (μmol/μmol hemoglobin)	Lipid phosphorus	Cholesterol/ phospholipid	Particles per μm^2 (mean ± S.E.M.)	
0.52	1.06	0.49	1084 ± 8 (n = 94)	P < 0.005*
0.90	1.05	0.86	1010 ± 20 (n = 42)	
1.42	1.04	1.36	937 ± 10 (n = 165)	P < 0.005*

* P values relate to the respective differences from the control.

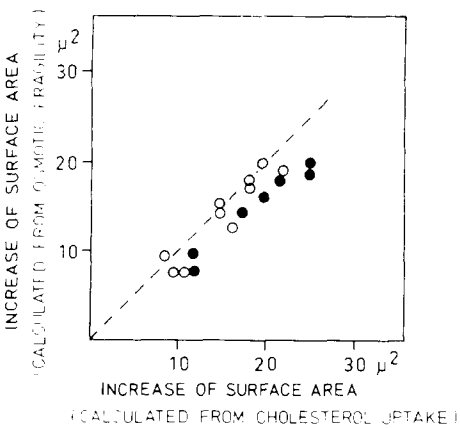

Fig. 5. Correlation between increases of erythrocyte surface area determined in different ways. For details see text. ○, Values calculated under the assumption that the incorporated cholesterol interacts with phospholipids up to a ratio of 1.0; ●, Values calculated under the assumption that incorporated cholesterol does not interact with phospholipids.

hemoglobin: 5 mmol/l cells; number of cells/l cells: $1.72 \cdot 10^{13}$, as computed from the mean cellular volume (58 μm^3 [45]); cholesterol: variable, as determined in the individual experiments.

(2) *Areas per molecule.* Phospholipids: 64 $Å^2$ [4, 9, 47]; cholesterol: 37 $Å^2$ [4, 47]; cholesterol+phospholipid: 85 $Å^2$ [4, 47], all values relating to a mean surface pressure of approximately 30 dyne/cm.

According to Fig. 5, the agreement between changes of surface area calculated by the two completely different approaches is surprisingly close. The best fit to the ideal 45° relationship is obtained when interaction of cholesterol with phospholipid, leading to a common molecular area of 85 $Å^2$, is allowed up to a ratio of 1.0. This particular detail however, has to be regarded with some reservation in view of the

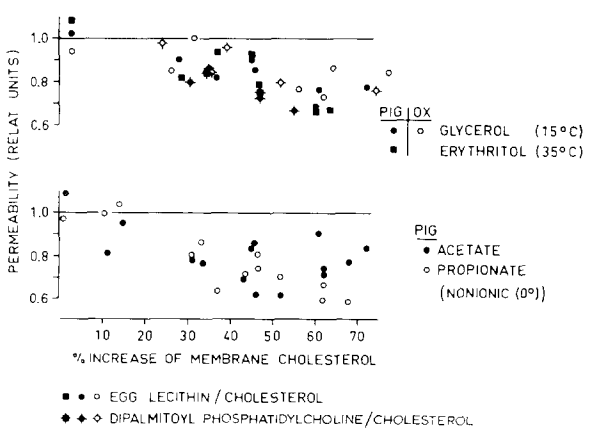

Fig. 6. Changes of nonelectrolyte permeabilities in cholesterol loaded erythrocytes. Data corrected for the cholesterol induced increase of surface area.

limited reliability of the underlying numerical assumptions. Nevertheless, our data provide strong evidence that at least 90 % of the cholesterol incorporated into the erythrocyte under our conditions are arranged as a bilayer.

This statement implies that the rate coefficients given in Fig. 1 for different extents of cholesterol loading have to be corrected for the changes in surface area, the rate coefficient k of tracer efflux being related to the permeability coefficient via the ratio cell volume/cell surface area. The correction factor required can be obtained from the data in Fig. 3.

Corrected data for relative permeabilities are given in Fig. 6. The decrease of permeability with increasing cholesterol content is more clearly evident from these data, although it is not comparable in its extent to the opposite permeability changes brought about by cholesterol depletion [13].

(*c*) *Permeability changes in cholesterol-depleted and -reloaded erythrocytes*

The low response of permeability to cholesterol loading might also be due to the fact that, in contrast to "native" cholesterol, cholesterol incorporated in vitro does not interact with phospholipids. In order to test this assumption, ox and pig erythrocytes were first depleted of cholesterol as previously described [13] and then reloaded by the procedure used for the loading of fresh cells. The time course of loading observed under these conditions is compared in Fig. 7 to that observed with normal erythrocytes. Obviously, the depleted cells accumulate cholesterol faster than the normal cells, indicating that the difference between membrane and medium cholesterol influences the rate of cholesterol uptake. The data compiled in Table IV demonstrate that increases in permeability due to cholesterol depletion are fully reversible in reloaded cells, i.e. cholesterol incorporated in vitro into depleted cell membranes is able to interact with the phospholipid in the same way as native cholesterol. On the basis of these findings, the small changes of permeability of erythrocytes loaded in vitro with cholesterol can be regarded as the true response to cholesterol accumulation.

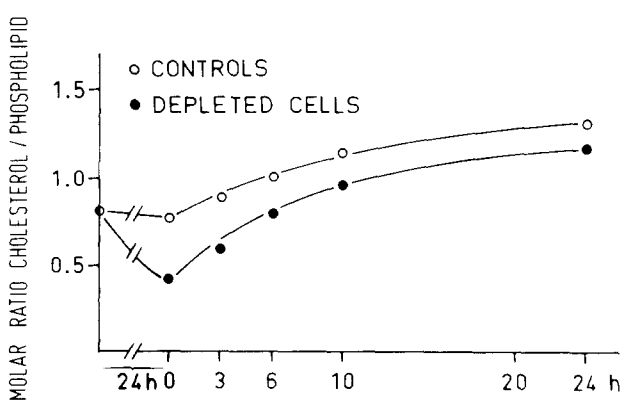

Fig. 7. Uptake of cholesterol into normal and cholesterol-depleted bovine erythrocytes. Cells depleted by 24 h incubation with egg lecithin (2 mg/ml) dispersed in serum/medium A (1:1). Controls incubated without lecithin. Cholesterol loading as described in Methods.

TABLE IV

REVERSIBILITY OF THE INCREASE OF PERMEABILITY IN CHOLESTEROL DEPLETED BOVINE ERYTHROCYTES AFTER RELOADING WITH CHOLESTEROL

	Cholesterol content (relative units)	Permeability (relative units)	
		Glycerol	Acetate (nonionic)
Normal cells	1.0	1.0	1.0
Depleted cells	0.57	1.35	1.31
Reloaded cells	1.03	0.96	1.04

DISCUSSION

The "specific" interaction of cholesterol with phospholipids in the liquid-crystalline state, as indicated by a decrease of space requirement ("condensing reaction", cf. e.g. refs. 4 and 9), by a diminution of the acyl chain mobility in the vicinity of the glycerol back bone [3, 5] and by a suppression of the thermotropic phase transition [1, 4, 21] has been studied in many laboratories (for recent reviews see refs. 1, 47–49). Nevertheless, the nature of this cholesterol/phospholipid interaction is still a matter of debate [3, 4, 47–51].

It has been claimed [20, 21] that in aqueous cholesterol/phospholipid systems above a critical molar ratio of 1 : 1 cholesterol should crystallize and be expelled from the lipid bilayer. The results presented here provide further evidence for the alternative concept [22–24, 27–29] that in artificial and natural membranes cholesterol is capable to coexist with phospholipid at ratios exceeding unity. In addition, our results demonstrate that cholesterol can be introduced into the erythrocyte membrane even in the absence of serum (Table I). Thus, lipoproteins are not essentially required for the insertion of additional cholesterol.

The question arises, by what mechanism cholesterol molecules in excess of the phospholipid molecules present affect permeability and how they are arranged in the lipid bilayer.

For a discussion of this problem it seems useful to consider the influence of cholesterol on the nonelectrolyte permeability of erythrocytes and liposome systems over the whole range of cholesterol/lipid ratios investigated as yet. Fig. 8 provides the information available in a slightly schematized way.

In this diagram, relative permeabilities of cholesterol-depleted erythrocytes as obtained previously [13], and of the cholesterol-loaded cells studied in the present report (Fig. 6) are plotted against the ratio cholesterol/polar lipid. This ratio, which also takes into account the glycolipids present in the erythrocyte membrane*, seems best suited for a comparison between cholesterol-induced permeability changes of the

* The mean total glycolipid content in the erythrocyte membrane was taken to amount to 10 mol % of the phospholipid content, assuming a mean molecular weight of 1120 for a typical erythrocyte glycosphingolipid and a mean ratio (on a weight basis) of 1:6.6 between glyco- and phospholipids [17].

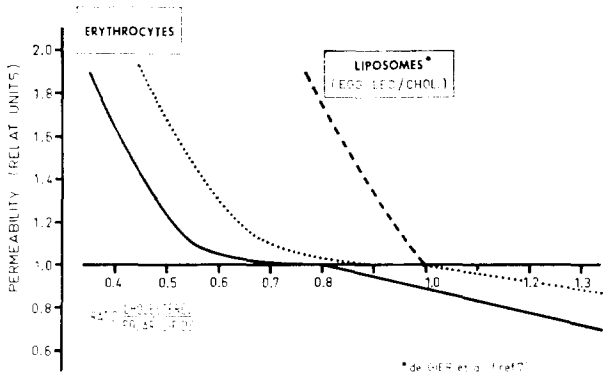

Fig. 8. Schematized diagram representing the relationship between erythrocyte permeability towards compounds penetrating via the lipid phase of the membrane and the cholesterol content of this phase. Data are corrected for cholesterol induced changes of surface area, using our data and data from ref. 22. Data for egg lecithin/cholesterol liposomes are given for reasons of comparison. The curve for cholesterol depleted cells is taken from ref. 13. Relationships calculated under the assumptions that all polar lipids (continuous line) or 80 % of the polar lipids (dotted line) are "free" for an interaction with cholesterol.

erythrocyte membrane and of artificial phospholipid bilayers, since the physicochemical properties of glycolipid systems have recently been shown also to be influenced by cholesterol [52, 53]. According to the diagram, an increase of cholesterol in the range of low ratios cholesterol/polar lipid greatly reduces the nonelectrolyte permeability of the erythrocyte membrane.

The maximum effect is attained at a ratio of approximately 0.6, i.e. at 37 mol% cholesterol. In contrast, the permeability of phosphatidylcholine liposomes is considerably reduced by cholesterol up to a ratio of 1.0 (cf. the hatched line in Fig. 8 and refs. 7 and 9).

The reasons for this discrepancy between erythrocytes and egg lecithin liposomes are not clear as yet. Among others, two interpretations have to be considered. Firstly, a certain amount of the erythrocyte polar lipids may be expected to interact with membrane proteins, analogous to the situation in other membrane systems [54–56], and therefore may not be "free" for an interaction with cholesterol. As a consequence, the curve in Fig. 8 has to be shifted to the right in order to relate erythrocyte permeability to the ratio of cholesterol/free polar lipid. Assuming that, as in other membranes [54–56], approximately 20 % of the polar lipids are withdrawn from the interaction with cholesterol in the erythrocyte membrane by this mechanism, one arrives at the dotted curve in Fig. 8 for the relationship between permeability and the ratio cholesterol/free polar lipids. This curve still differs from that obtained for egg lecithin liposomes since lowering of the cholesterol/phospholipid ratio from 1 to 0.8 does not produce an increase of permeability. In order to explain this difference one would either have to assume that 40 % of the erythrocyte polar lipids interact with protein, which seems an unreasonably high value, or consider an additional phenomenon: a certain fraction of the polar lipids in the erythrocyte membrane, although free for an interaction, may not be affected "specifically" by cholesterol in the sense defined above, i.e. a marked decrease of their acyl chain flexibility and of their mean

area per molecule [1, 9, 17, 57–59]. Such an inability might for instance be due to the presence in the erythrocyte membrane, in contrast to egg lecithin, of polyunsaturated phospholipid species [17, 57], which are not influenced by cholesterol [58, 59], and of saturated phospholipids [17, 57], which are fluidized by cholesterol below the temperature of their phase transition [60].

As a consequence, cholesterol should induce a notable decrease of permeability of the erythrocyte membrane lipid domain only up to a limiting ratio of cholesterol/polar lipid lower than in egg lecithin liposomes, in which apparently all molecular species interact specifically with cholesterol. Physicochemical studies of the interaction of cholesterol with extracted total erythrocyte membrane polar lipids at molar ratios between 0.8 and 1.0 may provide further insight into this problem. On the other hand, in such isolated systems the proposed asymmetry of the membrane lipid [65] which may also contribute to the different response of erythrocyte membrane and liposomes, would no longer be maintained.

The small permeability effects induced by cholesterol above the limiting ratio cholesterol/polar lipid, at which the permeability increment $\Delta P/\Delta$ ratio changes rather abruptly from a high to a low value, may operationally be defined as "unspecific". This unspecific influence would be exerted by approximately 20 % of the cholesterol normally present in the erythrocyte membrane and by all the cholesterol additionally incorporated in the present study. It seems possible that this unspecific effect is due to a rigidifying action of cholesterol resulting from the simple insertion of a large number of rigid sterol nuclei into the liquid-crystalline membrane lipid phase. Such an effect, although less pronounced than the "specific" effect of cholesterol should still influence permeability. Experimental support for this interpretation can be derived from spin resonance [61], fluorescence polarisation and viscosity [63] measurements in cholesterol-rich liposomes and erythrocyte membranes. Furthermore, an increased mechanical rigidity of cholesterol loaded erythrocytes has been demonstrated by measurements of erythrocyte filtrability [27]. Interestingly, the rigidification seems to affect not only the transfer of compounds penetrating via the lipid phase of the membrane but also mediated cation movements [64].

The arrangement of cholesterol in excess of polar lipids remains to be elucidated. The concept of an unspecific rigidifying action at high ratios cholesterol/polar lipid requires that additional cholesterol is distributed to a first approximation statistically over the whole membrane area. The alternative arrangement, namely areas of pure cholesterol, would be difficult to reconcile with membrane integrity.

In conclusion, the influence of cholesterol on the permeability of the lipid phase of the erythrocyte membrane has revealed some features different from those known or anticipated for artificial lipid membranes, illustrating once more, that the study of such artificial systems can provide valuable qualitative information on the permeability properties of a biomembrane, but cannot replace their detailed investigation.

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