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THE EFFECT OF PARTIAL REPLACEMENTS OF MEMBRANE CHOLESTEROL BY OTHER STEROIDS ON THE OSMOTIC FRAGILITY AND GLYCEROL PERMEABILITY OF ERYTHROCYTES

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SUMMARY

1. (A) Sonicated dispersions of lecithins were incubated with aliquots of washed human erythrocytes, leading to the removal of part of the cholesterol complement, eventually followed by lysis. (B) Sonicated dispersions of lecithin and cholesterol with one of a range of other steroids were prepared and incubated with aliquots of washed human erythrocytes. By a simple exchange process, the steroid composition of the erythrocytes were modified.

2. (A) The osmotic fragility of erythrocytes was considerably increased after removal of part of the cholesterol complement with pure lecithin dispersions. (B) The osmotic fragilities of the modified erythrocytes were measured, and it was found that relatively small differences were detectable in view of the substantial replacement of part of the cholesterol with one of the other steroids.

3. Significant differences in the glycerol permeabilities of the modified human and pig erythrocytes were demonstrated. In the presence of 3-ketosteroids, the rates of penetration of glycerol were significantly higher than those containing 3β-hydroxy steroids. Cholesterol-depleted erythrocytes also exhibited considerably increased glycerol permeability.

INTRODUCTION

The structural and functional importance of cholesterol in natural and artificial membranes has been studied in several laboratories. The interaction of cholesterol with phospholipids in monolayers and liposomes has been demonstrated[1-3], although direct confirmation of these effects in natural systems has not yet been achieved. The exchange of cholesterol between erythrocytes and plasma, which reaches completion within a few h, is a well-known phenomenon[4]. A more detailed study of this exchange process indicated the importance of hydrophobic bonding for the binding of cholesterol in the erythrocyte membrane[5]. It has been shown that membrane preparations other than those of the erythrocyte exhibit cholesterol exchange with plasma or pure lipoproteins[6].

The demonstration of the ability of aqueous dispersions of egg lecithin to remove cholesterol from erythrocyte membranes and the exchange of cholesterol

between lecithin–cholesterol dispersions and erythrocyte ghosts[7] opened up new possibilities in the study of steroid function in natural membranes. It was shown that large proportions of the membrane cholesterol could be replaced by other lecithin-solubilized steroids using the exchange process[8].

Although cholesterol is the principal steroid in animal cellular membranes, other steroids have been reported in appreciable concentrations in various tissues[9–13]. In the following report, using the exchange process, steroids other than cholesterol were introduced into intact human erythrocytes in order to study the effect of this action on the structural integrity and permeability properties of the membrane.

MATERIALS AND METHODS

Steroids

The steroids were obtained from the following sources: cholest-5-en-3β-ol (cholesterol), cholest-5,7-dien-3β-ol (7-dehydrocholesterol), cholest-5,7,22-trien-24-methyl-3β-ol (ergosterol), cholest-4-en-3-one and 5α cholestan-3β-ol (cholestanol) (Fluka AG, Buchs, Switzerland); cholest-5-en-3-one, 5α cholestan-3-one and cholest-5,22-dien-24 ethyl-3β-ol (stigmasterol) (Koch-Light Laboratories, Colnbrook, Bucks, England); 5β cholestan-3β-ol (coprostanol) (K. and K. Laboratories, Hollywood, Calif., U.S.A.); cholest-7-en-3β-ol (lathosterol) (Ikapharm, Ramat-Gan, Israel); cholest-4,6-dien-3-one (British Drug Houses, Poole, England). A sample of cholest-B nor-5-en-3β-ol (B norcholesterol) was kindly provided by Dr. J. Joska (Institute of Organic Chemistry and Biochemistry, Prague, Czechoslovakia). The steroids were examined on silica gel plates with a light petroleum (b.p. 40–60°)–ether–formic acid (50:50:2, by vol.) solvent system and also checked by gas–liquid chromatography (see below). A high degree of purity was indicated in most of the samples. Those requiring purification were recrystallized several times from ethanol.

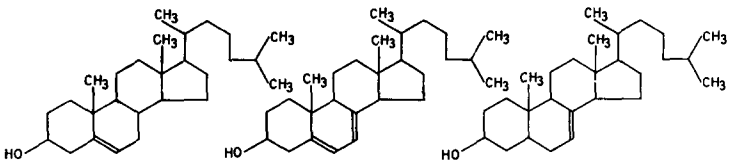
The separation of the steroids from the major phospholipids was achieved by elution with chloroform from the total lipid extracts on silicic acid columns as described by NELSON[14]. The steroid contents of the eluents were estimated quantitatively on a Carlo Erba Fractovap Model C gas chromatograph. The apparatus was fitted with a glass, direct-injection system and a 200-cm glass column (internal diameter, 4 mm) which was packed with Gaschrom 3% QF1 (Applied Science Laboratories) and maintained at 233° with a gas flow of 120 ml N_2 per min. 7-Dehydrocholesterol was estimated from its ultraviolet absorption spectrum in ethanol.

Phospholipids

Lecithin was obtained from egg yolks *via* the $CdCl_2$ adduct and examined for purity on silica gel plates using a chloroform–methanol–water (65:35:4, by vol.) solvent system. The phospholipid content of erythrocytes and dispersions was estimated from aliquots of the total lipid extract by the method of KING[15].

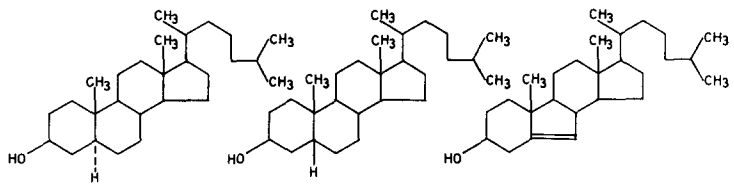
Lipid dispersions

Chloroform solutions of mixtures of lecithin (20 mg), cholesterol (6 mg) and one other steroid (10 mg) were each transferred to a round-bottomed tube and the solvent removed under vacuum. The lipid mixtures were covered with 10 ml of 30 mM NaCl and subjected to ultrasonic radiation (Branson Sonic Power Instrument



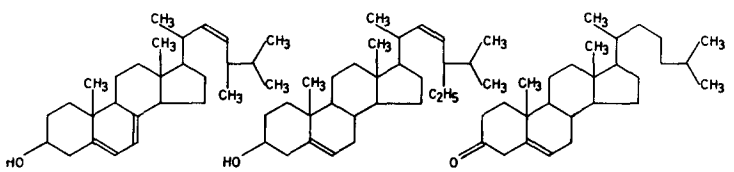
1 2 3

Cholest-5-en-3β-ol (cholesterol) | Cholest-5,7-dien-3β-ol (7-dehydrocholesterol) | Cholest-7-en-3β-ol (lathosterol)



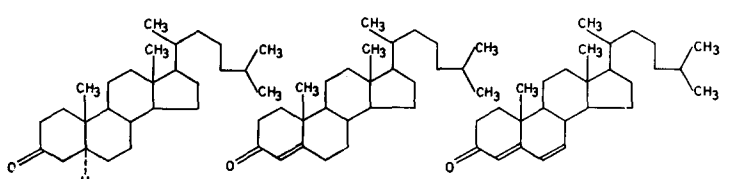
4 5 6

5α Cholestan-3β-ol (cholestanol) | 5β Cholestan-3β-ol (coprostanol) | Cholest-B nor-5-en-3β-ol (B norcholesterol)



7 8 9

Cholest-5,7,22-trien-24 methyl-3β-ol (ergosterol) | Cholest-5,22-dien-24 ethyl-3β-ol (stigmasterol) | Cholest-5-en-3-one



10 11 12

5α Cholestan-3-one | Cholest-4-en-3-one | Cholest-4,6-dien-3-one

Model S-125, position 4). During the sonications, the tubes were cooled with an ice–water mixture. The dispersions were then centrifuged at 48000 $\times$ *g* for 1 h and the supernatants removed for use. On average, 70% of the original phospholipid and steroid was dispersed. Of the original 10 ml, 7 ml were used. During the subsequent incubations with erythrocytes no obvious precipitation could be observed.

Erythrocytes

Fresh samples of human blood were obtained and stored in heparinized saline at 4° and used within 18 h. Prior to use, the erythrocytes were freed of plasma with 3–4 washings in cold isotonic saline, the buffy interfacial layer having been removed after each centrifugation. Centrifuge-packed erythrocytes were diluted (1 : 1, v/v) with isotonic saline before use. Pig erythrocytes were prepared in a similar manner.

Incubation conditions

Incubations of erythrocytes with lipid dispersions were carried out in 16 ml of isotonic medium containing saline and Tris–HCl buffer (pH 7.2) with gentle shaking at 37° (in one experiment the medium also contained 10% dimethylsulphoxide). Samples of the mixture were removed for determination of osmotic fragility or glycerol permeability, and the remaining erythrocytes were washed 3 times with isotonic saline before the lipids were extracted and analysed.

Osmotic fragility determinations

The osmotic fragilities were determined using a Fragiligraph Model D-2 (Elron Electronic Industries Ltd., Israel). The technique was developed and described by DANON[16]. A 40-μl aliquot of the erythrocyte–lipid dispersion mixture introduced into the special dialysis cuvette of the fragiligraph and dialysed against 11 ml of water (or 10% dimethylsulphoxide in water in cases where this was used in the incubation mixtures). The times for 50% haemolysis were measured from 2–4 readings of the haemolysis curves and for normal erythrocytes were on the order of 2–3 min. The relative fragilities of the erythrocyte samples are expressed simply by dividing the 50% lysis time for erythrocytes incubated with lecithin–cholesterol dispersions by the 50% lysis times of the erythrocytes with modified steroid contents and multiplying by 100.

Glycerol permeability

The swelling of erythrocytes in the presence of glycerol was recorded using the same optical methods as employed by DE GIER *et al.*[17] in their experiments with liposomes, also assuming that the erythrocyte acted as a perfect osmometer. The initial rate of decrease in absorbance value $d(1/A)/dt$ was calculated and taken to represent the initial rate of increase in erythrocyte volume. The erythrocyte swellings at 37° were observed at 824 mμ using a Vitatron UFD photometer. From each incubation mixture, 50 μl was rapidly stirred into 5 ml of 154 mM glycerol–55 mM NaCl–22 mM Tris–HCl buffer (pH 7.2) and the change in light transmission in the cuvette was traced by a recorder.

RESULTS

The dispersion of steroids with lecithins

In previous experiments it has been shown that many steroids were solubilized by lecithin to a lesser extent than cholesterol[8]. When erythrocyte ghosts were incubated with lecithin sols containing less than the full complement of cholesterol, there was a reduction in the ghost cholesterol[7]. In the following experiments, in order to maintain the steroid level of the erythrocytes, cholesterol was employed in the

preparation of all lecithin dispersions, which therefore contained mixtures of cholesterol and another steroid. This ensured a high steroid content in the dispersions. The molar ratios of lecithin and cholesterol to the other steroids in each dispersion are shown in Table I. The absolute amounts solubilized varied somewhat, but the ratios obtained with identical sonication mixtures were constant.

TABLE I

THE SOLUBILIZATION OF A NUMBER OF STEROIDS IN THE PRESENCE OF CHOLESTEROL BY SONICATION WITH EGG LECITHIN

Mixtures of lecithin (20 mg), cholesterol (6 mg) and one other steroid (10 mg) were sonicated in 10 ml 30 mM saline for 7 min and centrifuged at 48000 × *g* for 1 h. The phospholipid and steroid contents (gas–liquid chromatography) were analysed in the lipid extracts. Each ratio was determined from 2–3 independently prepared dispersions which had the same presonication lipid content. The molar ratios of lecithin to total steroid were calculated from the weights obtained in the analysis, the molar equivalents of lecithin in each dispersion being expressed as 100.

Dispersed steroid (in addition to cholesterol)	*Molar ratio lecithin : total steroid*	*Cholesterol / test steroid*
None*	100 : 104	—
7-Dehydrocholesterol	100 : 106	5.00
Lathosterol	100 : 129	0.57
Cholestanol	100 : 109	0.53
Coprostanol	100 : 120	0.83
B norcholesterol	100 : 124	0.195
Ergosterol	100 : 60	6.70
Stigmasterol	100 : 49	3.70
Cholest-5-en-3-one	100 : 131	5.55
5α Cholestan-3-one	100 : 60	1.43
Cholest-4-en-3-one	100 : 88	1.39
Cholest-4,6-dien-3-one	100 : 96	0.87

* In this sonication, 12 mg of cholesterol was used to obtain maximum saturation of the lecithin.

As has often been demonstrated, the molar ratio of lecithin to cholesterol was close to unity. Where steroid mixtures were employed, the molar ratios of lecithin to steroid also approximated to unity except in the case of the plant steroids ergosterol and stigmasterol (see Table I). Cholestanol, coprostanol and lathosterol were solubilized in approximately the same ratios to cholesterol as those in the presonication mixtures. B norcholesterol appeared to be preferentially solubilized compared to cholesterol. All the 3-ketosteroids except cholest-5-en-3-one were solubilized well but to a lesser extent than cholesterol. However, 7-dehydrocholesterol, ergosterol and stigmasterol were rather poorly solubilized. In the case of cholest-5-en-3-one, it can be seen from the low ratio of lecithin to total steroid and from the limited availability of dispersable steroid, that somewhat less than the usual amount of lecithin was solubilized. It has been suggested that varying degrees of solubilization may not only be the direct consequence of small structural differences, but may also be influenced by the crystal lattice energies of the steroids (P. A. EDWARDS AND C. GREEN, unpublished observations). This may account for the poor solubilization of 7-dehydrocholesterol from the solid state in contrast to its ready acceptance by the erythrocyte membrane[8].

Steroid composition of erythrocytes after incubation

It has previously been established that steroids other than cholesterol are capable of exchange between lipid dispersions and erythrocyte ghosts[8]. Cholest-4,6-dien-3-one exchanged much more rapidly than cholesterol in this system. The exchange properties of several of the other steroids employed in this study were hitherto unknown, and therefore preliminary experiments were necessary to determine whether it was possible to incorporate significant quantities of these 'foreign' steroids into the erythrocytes.

Aliquots of human erythrocytes were incubated 6.5 h with the lipid dispersions under the conditions described in MATERIALS AND METHODS. The ratios of the total steroid contents of the dispersions and the erythrocytes were approx. 2:1, the initial erythrocyte cholesterol content being the same (2.96 mg) in all the incubations. The final steroid contents of the erythrocytes were determined on gas–liquid chromatography, the results of which are shown in Table II. Routine determinations of the total phospholipid contents of the erythrocytes were performed, and it was found that these values varied by no more than $\pm 5\%$ from the original.

TABLE II

INCUBATIONS WITH SONICATED LIPID DISPERSIONS AND HUMAN ERYTHROCYTES

Erythrocyte samples were incubated with lipid dispersions containing steroids 2–3 times the weight of erythrocyte cholesterol content (2.96 mg cholesterol) in 16 ml of isotonic saline–Tris–HCl buffer (pH 7.2) at 37° for 6.5 h. The lipids of the washed cells were extracted and analysed. Coprostanol was not available for this experiment. Figures are averages of results obtained from two identical incubations, using the same source of aqueous dispersions of lipid and erythrocytes, under identical conditions. The variations in the duplicates did not vary by more than $\pm 3\%$ from the average results expressed below. The phospholipid contents of the incubated erythrocytes did not vary by more than $\pm 5\%$.

Test steroid (in addition to cholesterol)	*Steroid analysis of erythrocytes after incubation*		*Replacement of cholesterol (%)*
	Cholesterol (mg)	*Test steroid (mg)*	
7-Dehydrocholesterol	2.57	0.50	16
Lathosterol	2.23	1.01	31
Cholestanol	2.78	Trace	—
B norcholesterol	2.25	0.97	30
Ergosterol	3.14	0	—
Stigmasterol	2.76	Trace	—
Cholest-5-en-3-one	3.06	Trace	—
5α Cholestan-3-one	2.62	0.39	15
Cholest-4-en-3-one	2.02	0.77	27
Cholest-4,6-dien-3-one	2.56	0.72	22

The results show that several steroids can be incorporated into the erythrocytes in considerable quantities, providing a basis for further experimentation. The small degree of incorporation of most of the other steroids was a result of their poor solubilization by lecithin. However, this cannot be the reason for the low incorporation of cholestanol into the erythrocyte, since it was highly solubilized. It has previously been shown that ergosterol is not well incorporated into the erythrocyte[8].

Although the percentage of incorporated steroid varies from experiment to experiment, incorporations in subsequent experiments were of a similar order, though

even higher values were obtained. During the course of the incubations, the total steroid contents of the erythrocytes were reasonably constant, with a maximum deviation of $\pm 8\%$ from the original content.

Osmotic fragility

It has already been demonstrated that dimethylsulphoxide can accelerate the rate of exchange of cholesterol[5] and cholest-4,6-dien-3-one[8]. Dimethylsulphoxide also enables cholesterol-depleted erythrocyte ghosts to reattain their normal steroid complement in the presence of lecithin–steroid dispersions[7,8]. These properties were an

TABLE III

THE DETERMINATION OF THE OSMOTIC FRAGILITIES OF HUMAN ERYTHROCYTES WITH MODIFIED STEROID COMPOSITIONS

Expt. a. Erythrocyte samples were incubated with lipid dispersions containing steroids 3–4 times the weight of the erythrocyte cholesterol content (1.05 mg) in isotonic saline–Tris–HCl buffer (pH 7.2) at 37° for 6.5 h. *Expt. b.* Conditions as in Expt. a except that the erythrocytes contained 1.10 mg cholesterol and were incubated in the presence of 10% dimethylsulphoxide. The osmotic fragilities of all the erythrocyte samples were determined with the fragiligraph and their steroid contents analysed.

	*Fragility units**	*Test steroids*	*Replacement of erythrocyte cholesterol* (%)
Expt. a			
Fragility	186	None (lecithin alone)	(33% cholesterol removed)
↑ Increasing	127	Cholest-5-en-3-one	2.3
	126	5α Cholestan-3-one	20.5
	109	Stigmasterol	2.0
	104	Cholest-4-en-3-one	30.0
	104	Cholest-4,6-dien-3-one	25.0
	104	Cholestanol	3.0
↓ Decreasing	100	Cholesterol	0
	96	Ergosterol	4.0
	85.5	Lathosterol	26.1
	85	B norcholesterol	20.5
	80	7-Dehydrocholesterol	7.3
Expt. b			
Lysed during incubation		None (lecithin alone)	(48% cholesterol removed)
Fragility	138	Cholest-5-en-3-one	3.0
↑ Increasing	121	Cholest-4-en-3-one	31.1
	112.5	Stigmasterol	4.0
	109.5	5α Cholestan-3-one	32.3
	109.5	Cholestanol	3.0
	100	Cholesterol	0
	100	Ergosterol	2.0
↓ Decreasing	91.5	Cholest-4,6-dien-3-one	32.1
	91	Lathosterol	41.0
	89	7-Dehydrocholesterol	18.3
	80	B norcholesterol	50.0

* Fragility units were calculated as follows:

$$\frac{\text{50\% lysis time of erythrocyte incubated with lecithin–cholesterol}}{\text{50\% lysis time of erythrocytes incubated with test dispersions}} \times 100.$$

asset in these experiments, since the ideal situation was that in which the total steroid weight in each of the erythrocyte samples would be identical. However, there existed the possibility that dimethylsulphoxide may have influenced osmotic fragility measurements and so these were repeated in the absence and the presence of dimethylsulphoxide in separate experiments. The results in Table III, Expts. a and b, show a basically similar pattern in the absence or presence of dimethylsulphoxide.

In most cases, the erythrocytes remained intact despite quite considerable changes in their steroid composition. However, as has been previously demonstrated[18], the removal of cholesterol from the erythrocytes produced a larger increase in the fragility, eventually leading to haemolysis. Both 3-keto- and 3β-hydroxysteroids were able to compensate for the loss of cholesterol from the erythrocytes without, in most cases, gross changes in the osmotic fragility.

Glycerol permeability

Human erythrocytes were incubated with a series of lipid dispersions containing steroids which, from experience of the previous experiments, were known to replace over 5% of the original cholesterol. As in the previous experiments, a standard lecithin–cholesterol and a pure lecithin dispersion were also employed. The initial ratios of dispersion to erythrocyte steroid were in the range 4 : 1–5 : 1, the erythrocytes having an initial cholesterol content of 1.22 mg. After a 6-h incubation period under the usual conditions (without dimethylsulphoxide), the rates of swelling of the erythrocytes in glycerol were determined. The results in Table IV indicate considerable differences in the glycerol permeability of erythrocytes with diverse steroid compositions. A 5-fold increase in the swelling rate of erythrocytes was observed following the simple removal of cholesterol with a pure lecithin dispersion. This indicates a

TABLE IV

THE GLYCEROL PERMEABILITY OF HUMAN ERYTHROCYTES WITH MODIFIED STEROID COMPOSITION

Samples of human erythrocytes were incubated with lipid dispersions containing steroids 4–5 times the weight of erythrocyte cholesterol contents (1.22 mg) in 16 ml isotonic saline–Tris–HCl buffer (pH 7.2) for 6 h at 37°. The swelling rates of these erythrocytes in 154 mM glycerol–55 mM saline–22 mM Tris–HCl buffer (pH 7.2) were determined spectrophotometrically and their steroid contents analysed.

Test steroid	*Erythrocyte lipid analyses (mg)*			*Replacement of cholesterol* (%)	*Glycerol permeability* $d(1/A)/dt$
	Total phospholipid	*Cholesterol*	*Test steroid*		
No dispersion	2.81	1.22	—	—	1.200
Lecithin only	2.70	0.75	—	39% cholesterol removed	7.950
Cholesterol	2.83	1.27	—	0	1.580
7-Dehydrocholesterol	2.63	1.04	0.083	7.4	0.514
Lathosterol	2.90	0.87	0.39	31	1.570
Coprostanol	2.72	0.90	0.30	25	0.525
B norcholesterol	2.73	0.78	0.50	39	1.940
5α Cholestan-3-one	2.59	0.94	0.24	20	3.160
Cholest-4-en-3-one	2.69	0.89	0.28	24	4.340
Cholest-4,6-dien-3-one	2.78	0.78	0.42	38	2.830

significant increase in the permeability of glycerol. The presence of 3-ketosteroids also caused an increase in the glycerol permeability. Coprostanol and 7-dehydrocholesterol caused some decrease in permeability, but the presence of lathosterol and B norcholesterol caused little deviation from the normal values.

With respect to their glycerol permeability characteristics, erythrocytes of several species have been divided into two distinct groups[19]. The first group, those of rat, man, rabbit and guinea pig, exhibit rapid haemolysis in 0.303 M glycerol, the process being almost independent of temperature. Pig, dog, cat, sheep and ox erythrocytes have a relatively slow rate of haemolysis, but there is a significant temperature dependence, so that at 37° the difference between the two groups is less marked. Therefore, a comparison was made on the effects of steroid content modification on the glycerol permeabilities of human and pig erythrocytes. The experiment was identical to the previous one except that in addition to the swelling measurements after 6 h, determinations were made at zero time for each individual incubation mixture. Aliquots from the same lipid dispersions were used with both human and pig erythrocytes which were incubated concurrently.

TABLE V

A COMPARISON OF THE GLYCEROL PERMEABILITIES OF HUMAN AND PIG ERYTHROCYTES WITH MODIFIED STEROID COMPOSITIONS

Equal volumes of lecithin–steroid (3–4 mg) dispersion were incubated with samples of human (0.80 mg cholesterol) and pig (0.91 mg cholesterol) erythrocytes for 6 h at 37°. The osmotic swelling of these erythrocytes in 154 mM glycerol–55 mM saline–22 mM Tris–HCl buffer (pH 7.2) was determined spectrophotometrically and their steroid contents analysed.

Test steroid	*Replacement of erythrocyte cholesterol (%)*	*Glycerol permeability d(1/A)/dt (rate of decrease in absorbance)*	
		Zero time	*6 h*
(a) Human			
No dispersion	0	0.91	1.17
Lecithin alone	22% cholesterol removed	1.02	3.14
Cholesterol	0	1.38	1.12
7-Dehydrocholesterol	7.1	1.27	0.86
Lathosterol	19.8	1.01	1.24
Coprostanol	26.1	1.21	1.16
B norcholesterol	46.3	1.24	1.56
5α Cholestan-3-one	32.7	1.59	2.85
Cholest-4-en-3-one	29.6	0.942	2.55
Cholest-4,6-dien-3-one	35.7	0.850	2.50
(b) Pig			
No dispersion	0	0.152	0.138
Lecithin alone	—*	0.134	—
Cholesterol	0	0.124	0.142
7-Dehydrocholesterol	8.0	0.120	0.097
Lathosterol	17.1	0.093	0.156
Coprostanol	17.5	0.163	0.136
B norcholesterol	39.8	0.132	0.183
5α Cholestan-3-one	29.6	0.134	0.306
Cholest-4-en-3-one	41.0	0.130	0.435
Cholest-4,6-dien-3-one	35.2	0.143	0.295

* Erythrocytes lysed during incubation.

In general, the results in Table V agree with those found in the first experiment. Lysis occurred before 6 h elapsed in the case of pig erythrocyte-pure lecithin incubation, and therefore no permeability determination could be made. However, the results for human and pig erythrocytes compared favourably; although the steroid compositions were not identical, they were not too dissimilar. The permeability-increasing effect of the 3-ketosteroids was apparent also in the pig erythrocytes. The effect of 7-dehydrocholesterol and coprostanol in decreasing the permeability rate was less marked than in the previous experiment. The effect of cholesterol removal from human erythrocytes, though less extensive than in the first experiment, produced an appreciable increase in the rate of glycerol penetration.

DISCUSSION

The above results demonstrate that the steroid composition of the red blood cell membrane can be changed drastically by incubations with sonicated lipid dispersions. In agreement with earlier observations[8], incubation of the red blood cells with a pure lecithin sol causes a depletion of the steroid content of the cell. The incubations with lecithin sols saturated with cholesterol and other steroids demonstrate that the erythrocyte membrane is able to accept a wider range of steroids, the structures of which vary in several ways from the native cholesterol. The extent to which these steroids exchange with the cholesterol is highly variable, and this is not only determined by the degree of their solubilization in the lecithin dispersions. Many of the 3β-hydroxysteroids were well solubilized and incorporated in large amounts, replacing an equivalent weight of cholesterol. The plant sterols ergosterol and stigmasterol were poorly solubilized in the lecithin dispersions, and consequently there was little incorporation in the red cells. However, cholestanol was rather poorly incorporated despite the high solubilization. This observation is rather difficult to explain, particularly when the *cis*-form coprostanol replaced 25% of the cholesterol.

The removal of cholesterol from erythrocytes by lecithin dispersions as shown in this paper or by incubation with fresh plasma[18] does increase the osmotic fragility significantly. To explain this phenomenon the following alternative or perhaps superimposed effects as a direct result of the cholesterol removal can be envisaged:

(a) The osmotic resistance of the erythrocyte is often thought to be related to the shape of the cell. From direct microscopic observations of red blood cells from various mammalian species and of those from pathological sources, CASTLE AND DALAND[20] suggested that the percentage difference between the volume of the erythrocytes in isotonic plasma and that of a sphere of equal surface determines the osmotic resistance. Therefore the increased osmotic fragility of the cholesterol-depleted cells may be the result of a reduced membrane surface area, as a consequence of which the spherical form is reached in much higher salt concentrations. In this respect it is relevant to mention that in patients with diseases of the liver and biliary tract, the erythrocytes often demonstrate 'macroplania', and the increased surface area of these cells appeared to be correlated with an increased cholesterol content and a decreased osmotic fragility[21,22].

(b) The increased osmotic fragility of the depleted cells may be the consequence of an increased volume in the isotonic medium (a higher ratio of thickness to diameter) as a result of an increased ion content of the cells due to an enhanced permeability.

An increased permeability for glycerol molecules is obvious from the highly increased swelling rates of the sterol-poor erythrocytes in isotonic solutions of this non-electrolyte.

(c) The differences in osmotic fragility may be due to changes in the resistance of the membrane to breaking. One could consider that the molecular cohesion of the membrane constituents is reduced by the removal of cholesterol.

Although it is likely that changes in surface area (a) and/or volume (b) are of primary importance with respect to increase in fragility, it can be assumed that the main reason for the increased glycerol permeability is to be found in the mutual association of the membrane molecules. Experiments with artificial lipid membranes have demonstrated that the presence of cholesterol in these lipid films is able to retard the passage of various components. In direct correlation with the erythrocyte experiments, liposomes prepared from lecithins with cholesterol demonstrated a much slower swelling rate in isotonic glycerol than without this sterol[3]. In addition it was shown that the leak of glucose[2] and Cl^- (ref. 23) from these model systems was reduced quite considerably in the presence of cholesterol. Using the 'black film' system it has been shown that the presence of this steroid retards the passage of water through the lecithin bilayers[24].

When part of the cholesterol was replaced with other steroids the total steroid–phospholipid content remained constant within rather close limits. Therefore we presume that the observed variations in osmotic fragility are primarily due to the modified steroid composition and are only secondary to reduction in the lipid composition. From the relatively small differences in fragility it can be tentatively concluded that the presence of 3-ketosteroids causes a slight increase, whereas increased unsaturation or the absence of one carbon atom from the B ring, may cause a slight decrease in the osmotic fragility.

As the variations in osmotic fragility are small, the surface areas of the erythrocytes with various steroids can be considered to be nearly identical, and therefore the differences in swelling rate of red cells in isotonic glycerol solutions indicate real differences in the permeability coefficients for this compound. In the case of human and pig erythrocytes, it has been shown that the replacement of cholesterol with 3-ketosteroids is accompanied by a marked increase in the glycerol permeability. On the other hand the 3β-hydroxysteroids in the membrane appear to be as potent and in some cases slightly more so than cholesterol in limiting the rate of influx of glycerol into the erythrocytes. The preliminary conclusion that can be drawn from these experiments is that the OH-group of the molecule is very important in the associations of the steroid with the other membrane constituents. Such interactions may serve to strengthen the binding of cholesterol into the membrane, although this is primarily believed to be effected by hydrophobic bonding. However, there remains the possibility that the OH-group may play a more direct role in the regulation of membrane permeability. This could only be established by further experimentation.

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