Diminished Osmotic Fragility of Human Erythrocytes Following the Membrane Insertion of Oxygenated Sterol Compounds

By Rolf A. Streuli, Jeffrey R. Kanofsky, Robert B. Gunn, and Stanley Yachnin

Oxygenated sterol compounds (OSC), when incubated for 1 hr with human erythrocytes in lipoprotein-depleted medium at concentrations of 0.625-5 × 10⁻⁹M, are inserted into the cell membrane and remain there despite subsequent washing of the cells. The insertion results in expansion of the surface area of the red cell ghost membrane, an increase in critical hemolytic volume, and as a consequence, in diminished osmotic fragility of the erythrocytes. This effect is seen with echnocyte-forming as well as with non-echinocyte-forming OSC. Erythrocytes treated with OSC do not differ from control cells with respect to their mean cell volume (MCV) in isotonic solution, water content, ion fluxes, and filterability through polycarbonate filters. The shift of the osmotic fragility curve toward lower NaCl concentrations is proportional to the amount of OSC inserted into the red cell membrane.

In addition to their capacity to inhibit sterol synthesis in mammalian cells, oxygenated sterol compounds (OSC)* have wide-ranging effects on certain membrane-associated functions and on the membrane morphology of human peripheral blood cells. Exposure of red blood cells to certain OSC induces the formation of echnocytes. Also, OSC are capable of protecting red cells from the development of increased osmotic fragility that is observed after their incubation for 24 hr in a cholesterol-depleted medium. Furthermore, after such an incubation, 20α-hydroxycholesterol, which does not induce echnocytes, produces a population of osmotically resistant red cells. We postulated that the effects of OSC on red blood cells were a consequence of their physical insertion into the plasma membrane lipid bilayer. We have subsequently demonstrated that both echnocyte-forming and non-echinocyte-forming OSC enter red blood cell membranes rapidly when cells are exposed to OSC in vitro.

In order to study the effects of OSC on human red cell osmotic fragility under conditions where loss of cholesterol from the membrane is minimized by avoiding long incubations in vitro in lipoprotein-depleted medium (LPDM), we designed experiments in which such exposures in the presence or absence of OSC were limited to 1 hr. Our results show that the osmotic fragility of normal red cells is not altered by such incubation in the absence of OSC, but that the insertion of OSC into red cell membranes under these conditions renders the cells resistant to osmotic lysis. In addition, the increased osmotic fragility of red cells from patients with hereditary spherocytosis can be corrected by the insertion of OSC into their membranes. Our results show that the protective effects of OSC on red cell osmotic lysis are due to expansion of the membrane surface area of the cells and that the critical hemolytic volume of OSC-treated red cells as well as the volume of resealed ghosts

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317

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*The trivial names used are: cholesterol (5-cholesten-3\beta-ol), 4\beta-hydroxycholesterol (5-cholesten-3\beta,4\beta-diol), 5\alpha-hydroxy-6-ketocholesterol (cholestan-3\beta,5\alpha-diol-6-one), 6\beta-hydroxysterol (5\alpha-cholestan-3\beta,6\beta-diol), 6-ketocholesterol (5\alpha-cholestan-3\beta-ol-6-one), 7\alpha-hydroxycholesterol (5-cholesten-3\beta,7\alpha-diol), 7\beta-hydroxycholesterol (5-cholesten-3\beta,7\beta-diol), 7-ketocholesterol (5-cholesten-3\beta-ol-7-one), 20α-hydroxycholesterol (5-cholesten-3\beta,20α-diol), 22-ketocholesterol (5-cholesten-3-ol-22-one), 25-hydroxycholesterol (5-cholesten-3,25-diol), hydrocorotizone (4-pregnen-11\beta,17\alpha,21-triol-3,20-dione), epipregnanolone (5β-pregnan-3\alpha-ol-20-one), pregnanalone (5β-pregnan-3\beta-ol-20-one), pregnenolone (5β-pregnan-3\beta-ol-20-one), allopregnanolone (5α-pregnan-3\beta-ol-20-one), pregnanediol (5β-pregnan-3\alpha,20α-diol), pregnanediol (5β-pregnan-3\beta-ol-20-one), allopregnanolone (5α-pregnan-3\beta-ol-20-one), pregnanediol (5β-pregnan-3\alpha,20α-diol), pregnanediol (5β-pregnan-3\beta-ol-20-one).
prepared from OSC-containing red cells is increased compared to those in a control red cell population.

**MATERIALS AND METHODS**

Cholesterol, 6-ketocholesterol, 22-ketocholesterol, and 22-hydroxycholesterol were purchased from the Sigma Chemical Co. (St. Louis, Mo.). All other steroids were obtained from Steraloids, Inc. (Wilton, N.H.). 4-14C-Pregnenolone (55.7 mCi/m mole) was purchased from the New England Nuclear Corp. (Boston, Mass.). 4-14C-cholesterol (>50 mCi/m mole) from the Amersham Corp. (Arlington Heights, Ill.). 32Na (carrier-free) and 31Cl (12.7 mCi/g Cl) from ICN (Irvine, Calif.). Nigericin was a gift from Eli Lilly and Co. (Indianapolis, Ind.).

**Normal Subjects and Patients**

Most of our experiments were performed on red blood cells obtained from healthy control subjects. We also studied the erythrocytes from three nonplasenectomized patients with hereditary spherocytosis, who gave informed consent to phlebotomy. Their diagnosis was established on the basis of family history, splenomegaly, red cell morphology, the presence of reticulocytosis, and abnormal osmotic fragility.

**Incubation of Red Blood Cells**

Freshly drawn heparinized blood was washed 3 times in phosphate-buffered isotonic saline (0.15 M NaCl, 0.01 M PO4, pH 7.4) containing 0.25% crystalline bovine serum albumin (PBS-BSA, Miles Laboratories, Inc., Elkhart, Ind.) to remove plasma and buffy coat. The cells then were suspended in PBS-BSA at a hematocrit of 50% and were incubated with OSC for 1 hr (or longer if desired) as previously described.1

After incubation, the red cell suspension was transferred to sterile conical 50-ml plastic tubes (Falcon 2070) and centrifuged at 4°C for removal of the supernatant. The red cells were then washed twice with PBS-BSA at 4°C. After washing, the cells were suspended in PBS-BSA at a hematocrit of 40%. We then measured the mean corpuscular volume (MCV) of the cells with a Coulter Counter Model ZBI that was connected with an MCV computer Model MHR (Coulter Electronics, Hialeah, Fla.). Measurements of MCV were done in triplicate. These postincubation red blood cells were used for measurement of their osmotic fragility.

**Osmotic Fragility and Critical Hemolytic Volume**

For the measurement of osmotic fragility, we followed the method recommended by Beutler.1 The osmolarities of the NaCl solutions were verified with an Advanced Osmometer, Model 3R (Advanced Instruments, Inc., Needham Heights, Mass.). In certain experiments, ethanol (1% v/v) and various steroids (final concentration 5 x 10−3 M) were added directly to the glass tubes containing the salt solutions of graded concentrations, followed by addition of unincubated normal erythrocytes. The suspensions were allowed to stand at room temperature for 60 min and were then centrifuged, and the osmotic fragility curve was determined. The resulting curves were compared with osmotic fragility curves of red cells exposed to the various OSC and steroids in our lipoprotein-depleted serum (LPDS) incubation medium, followed by our usual washing procedure. All osmotic fragility curve determinations were performed in duplicate.

Osmotic fragility is expressed as the mean corpuscular fragility (MCF), i.e., the NaCl concentration at which 50% of the erythrocytes hemolyze. Changes in osmotic fragility induced by OSC and other compounds are expressed as the difference between the MCF of the sample incubated with the compound and the MCF of the control sample (ΔMCF).

The critical hemolytic volume of control and OSC-treated erythrocytes was determined by the microhematocrit method of Seeman et al.3

**Cholesterol Measurements**

Control and OSC-exposed red cells were subjected to chloroform:methanol extraction, saponification, neutral lipid isolation, and thin-layer chromatography (see below). The total cholesterol content of the red cells was determined on the neutral lipid extract by a modification of the method of Zlatkis et al.4 Cholesterol recovery by our lipid extraction procedure was monitored by addition of trace amounts (85,000 cpm) of 14C-cholesterol to the red cell sample prior to extraction, and ranged between 92.7% and 99.8%.

**Quantification of the Insertion of Steroids Into Red Cell Membranes**

Oxgenated sterol compounds. The quantification of the amount of a given OSC inserted into red cells was done by previously described methods, utilizing thin-layer chromatography.3 However, it was not possible to quantify 22-ketocholesterol with the method described because the Rf of this compound differs only slightly from that of cholesterol (Rf = 0.96). Therefore, we exposed the ethanol-solubilized neutral lipid extracts of cells incubated with 22-ketocholesterol to an equal volume of a solution of NaBH4 in methanol (10 mg/ml). This mixture was allowed to stand at room temperature for 2 hr and was then evaporated to dryness under N2. The residue was again dissolved in 0.25 ml absolute ethanol. The same procedure was performed with 0.25 ml of an ethanolic standard solution of 2.5 x 10−3 M 22-ketocholesterol in which was dissolved the neutral lipid extract of control erythrocytes not previously exposed to 22-ketocholesterol. The exposure to NaBH4 resulted in a partial (50%−75%) conversion of 22-ketocholesterol to a reduced derivative (presumably 22-hydroxycholesterol) characterized by its slower movement on the chromatography plate relative to that of 22-ketocholesterol (Rf = 0.88), and by its gray color after spraying with H2SO4 in contrast with the pink color displayed by 22-ketocholesterol. This method allowed an exact quantification of the amount of 22-ketocholesterol present in the OSC-exposed red cells by comparison with the standard samples.

In certain experiments, in order to assess the effect of repeated red cell washing on the amount of red-cell-inserted OSC, we performed OSC extractions and quantifications on red cell buttons that had been centrifuged and from which the supernatant OSC-containing medium had been aspirated as completely as possible. The amount of OSC remaining in the supernatant medium was measured by similar techniques. Other equal aliquots of the same OSC-exposed red blood cells were then subjected to 1, 2, 3, 4, or 5 cold PBS washes, and appropriate red cell extracts for OSC quantification were prepared from each.

**Pregnenolone.** Red cells were incubated with 5 x 10−3 M pregnenolone together with 3 μCi 4-14C-pregnenolone in LPDM in the usual manner. Following a 1-hr incubation, 0.5 ml of the red cell suspension was added in duplicate to 10 ml Aquasol (New England Nuclear Corporation, Boston, Mass.) together with 0.5 ml PBS. A duplicate 0.475 ml portion of the supernatant medium, following sedimentation of the red cells by centrifugation, was prepared for scintillation counting in a similar way, except that a 0.5 ml control red cell suspension in LPDM was also added to the counting fluid to avoid distortion in counting efficiency due to quenching. Equal amounts of red cells from the pregnenolone incubation mixture that had been washed in 100 volumes of cold PBS 1, 2, 3, 4, or 5 times
Measurement of the Volume of Red Cell Ghosts

For the measurement of the volume of red cell ghosts, we followed the method of Seeman et al. A suspension of incubated and washed erythrocytes (1 part) in 0.3% phosphate-buffered NaCl solution (50,000 parts) was prepared and allowed to stand at room temperature for 2 hr. In this suspension, the red cell ghosts attain a maximal volume and are resealed for 2 hr. The mean cell volume of the erythrocyte ghosts was then measured by means of the Coulter Counter and the MCV computer after the isotonic solution in the system had been replaced by a 0.3% NaCl solution. The Coulter probe had an aperture of 100 μ diameter, the aperture current setting was 0.125, the amplification was 8, and the threshold setting was 10 U. The MCV computer was calibrated by utilizing the volume of normal erythrocyte ghosts (147.0 cu μ) derived from the measurements by Seeman. All measurements were done in triplicate. Since Seeman has shown that under these conditions red cell ghosts maintain a spherical shape, a fact we verified by examination of both control and OSC-containing ghosts by phase microscopy, we then calculated the surface area (S) of the ghosts from their volume (V) by using the formula: 

$$ S = \frac{3(4\pi/3)^{1/3}}{V} $$

Red Cell Filterability

The effect of OSC on erythrocyte filterability was measured with 3μm Nucleopore polycarbonate filters (Nucleopore Corporation, Pleasanton, Calif.), as described by Gregersen et al. The cells were incubated for 1 hr under our usual conditions. After incubation, the suspensions were diluted to a hematocrit of 2% with PBS-BSA, and the filtration time for successive 2-ml aliquots of each sample was measured.

$^{22}$Na Efflux, $^{38}$Cl Efflux, and Ion and Water Content of Red Cells

Red blood cells were loaded with $^{22}$Na according to a modification of the method of Sarkadi et al. Fresh whole blood was centrifuged for 5 min at 17,000 g in the cold. The plasma and buffy coat were aspirated; the cells were washed 3 times in 160 mM choline chloride and resuspended to a hematocrit of 50%. The suspension was warmed to 37°C, and then 1 μCi of $^{22}$NaCl was added for each 6 ml. Loading with $^{22}$Na was performed by addition of 0.1 ml of 10$^{-5}$ M Nigericin in ethanol and mixing for 60 sec at 37°C. The suspension was divided into three 30-ml aliquots of ice-cold 160 mM choline chloride plus 0.5% albumin and 15 mM Tris-HCl, pH 7.4, at 0°C and centrifuged. The cells were washed 3 times in this solution and stored in the cold. More than 90% of the $^{22}$Na was retained by the cells. The cells were then incubated with 1% ethanol or with OSC under our usual conditions. After the incubation, 0.25 ml of packed red blood cells were washed 3 times in 110 mM MgCl₂ in the cold, then hemolyzed and analyzed for initial Na+, $^{22}$Na, and hemoglobin.

The $^{22}$Na efflux of control and OSC-incubated red blood cells was measured at 37°C, pH 7.4, in solutions containing 15 mM Tris-HCl, 5 mM D-glucose, and 150 mM NaCl or 150 mM choline chloride, with and without 10$^{-7}$ M ouabain or 5 mM KCl. Three-milliliter samples were placed on ice at various times after addition of 0.25 ml of packed cells to 25 ml of solution. The tubes were centrifuged immediately, and 1 ml of supernatant was counted in a Packard Instrument Autogamma scintillation counter along with samples containing 1 ml of whole suspension. The flux was calculated from the rate of tracer efflux and the initial specific activity of the cells.

The chloride flux was measured at 0°C after incubation of the red blood cells with either ethanol or OSC. The efflux of $^{38}$Cl, cell water, and chloride content was measured according to the methods of Gunn and Fröhlich.

RESULTS

The osmotic fragility of erythrocytes incubated for 1 hr at 37°C in LPDM containing 1% (v/v) ethanol does not differ from the osmotic fragility of unincubated red blood cells. Cholesterol and 25-hydroxycholesterol at concentrations of 2.5 × 10$^{-5}$ M and 5 × 10$^{-5}$ M in LPDM have no effect on osmotic fragility after incubation with normal erythrocytes for 1 hr at 37°C. The MCF of normal red blood cells was 0.403% ± 0.01% NaCl (mean ± SD, n = 14, range 0.391%–0.415%).

The following OSC diminish the osmotic fragility of erythrocytes when the cells are exposed to the compounds for 1 hr at a concentration of 2.5 × 10$^{-5}$ M or 5 × 10$^{-5}$ M in LPDM: 4β-hydroxycholesterol, 5α-hydroxy-6-ketocholesterol, 6β-hydroxycholesterol, 6-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 7-ketocholesterol, 20α-hydroxycholesterol, and 22-ketocholesterol (Table 1). This reduction in MCF depends on the concentration of the OSC present in the incubation mixture (Fig. 1) and is proportional to the amount of OSC inserted into the red cell (Table 2). The potency of the various OSC with respect to their capacity for protecting erythrocytes against osmotic lysis can be expressed as a quotient, with Δ MCF as the numerator and the

### Table 1. Effect of Various OSC on the Osmotic Fragility of Normal Red Blood Cells

<table>
<thead>
<tr>
<th>OSC Compound</th>
<th>Concentration in LPDM</th>
<th>ΔMCF (% NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4β-Hydroxycholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.035</td>
</tr>
<tr>
<td>5α-Hydroxy-6-Ketocholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.028 ± 0.006† (2)</td>
</tr>
<tr>
<td>6β-Hydroxycholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.021</td>
</tr>
<tr>
<td>6-Ketocholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.031</td>
</tr>
<tr>
<td>7α-Hydroxycholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.041 ± 0.009 (3)</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.061 ± 0.01 (2)</td>
</tr>
<tr>
<td>7β-Hydroxycholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.034 ± 0.003 (5)</td>
</tr>
<tr>
<td>20α-Hydroxycholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.045</td>
</tr>
<tr>
<td>22-Ketocholesterol</td>
<td>2.5 × 10$^{-5}$ M</td>
<td>0.021 ± 0.008 (2)</td>
</tr>
<tr>
<td></td>
<td>5 × 10$^{-5}$ M</td>
<td>0.034 ± 0.009 (4)</td>
</tr>
<tr>
<td></td>
<td>5 × 10$^{-5}$ M</td>
<td>0.062 ± 0.006 (4)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses are the number of experiments performed.
†Mean ± SD.
amount of OSC inserted into the red blood cells in micrograms as the denominator. The quotients found for 7β-hydroxycholesterol, 22-ketocholesterol, and 20α-hydroxycholesterol were higher than the quotient found for 7α-hydroxycholesterol; the latter, therefore, can be considered a less potent inhibitor of red cell osmotic lysis than the first three compounds (Table 2).

If erythrocytes are incubated for 1 hr in LPDM containing 22-ketocholesterol at a concentration of $5 \times 10^{-3} M$, the MCF is lowered by 0.065% NaCl. However, if the red blood cells are incubated in a medium that contains 20% normal AB serum instead of LPDS, 22-ketocholesterol at the same concentration lowers the MCF only by 0.023% NaCl (Fig. 2). Similar results were obtained with 7β-hydroxycholesterol; the addition of free cholesterol ($5 \times 10^{-5} M$) to the LPDM did not change the effect of this OSC (results not shown).

The following compounds ($5 \times 10^{-5} M$) failed to

**Table 2. Relationship between the Amount of Various OSC Inserted into Red Blood Cells and Their Effect on Osmotic Fragility**

<table>
<thead>
<tr>
<th>Compound</th>
<th>OSC Concentration in LPDM</th>
<th>µg OSC Inserted/0.5 ml Packed Red Cells (Mean ± SD)</th>
<th>∆MCF (%NaCl)</th>
<th>∆MCF µg OSC Inserted × 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>7β-Hydroxycholesterol*†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1.25 × 10^{-5} M</td>
<td>19.9 ± 1.9</td>
<td>0.018</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^{-5} M</td>
<td>51.9 ± 2.7</td>
<td>0.026</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-5} M</td>
<td>40.0 ± 9.9</td>
<td>0.037</td>
<td>0.9</td>
</tr>
<tr>
<td>22-Ketocholesterol</td>
<td>1.25 × 10^{-5} M</td>
<td>27.7 ± 0.4</td>
<td>0.021</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^{-5} M</td>
<td>46.2 ± 0.9</td>
<td>0.040</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-5} M</td>
<td>84.1 ± 2.1</td>
<td>0.070</td>
<td>0.8</td>
</tr>
<tr>
<td>20α-Hydroxycholesterol</td>
<td>2.5 × 10^{-5} M</td>
<td>32.4 ± 2.8</td>
<td>0.027</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-5} M</td>
<td>62.2 ± 7.5</td>
<td>0.047</td>
<td>0.8</td>
</tr>
<tr>
<td>7α-Hydroxycholesterol</td>
<td>2.5 × 10^{-5} M</td>
<td>68.6 ± 10.4</td>
<td>0.037</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-5} M</td>
<td>134.7 ± 17.9</td>
<td>0.054</td>
<td>0.4</td>
</tr>
</tbody>
</table>

For each OSC, all values shown were obtained in the course of a single experiment.

*Red cells exposed to $5 \times 10^{-5} M$ 7β-hydroxycholesterol displayed slight hemolysis during the washing procedure. Therefore this compound was studied only at the concentrations shown.

†The total cholesterol contents of the 0.5 ml red cell samples were (mean ± SD): 7β-hydroxycholesterol, experiment 1: 698 ± 50 µg; 7β-hydroxycholesterol, experiment 2, 20α-hydroxycholesterol, and 7α-hydroxycholesterol: 705 ± 61.4 µg; 22-ketocholesterol: 723 ± 49 µg.
Fig. 3. The anhemolytic effect of epipregnanolone is abolished by washing. When the osmotic fragility of unincubated red blood cells was measured by adding the cells to saline solutions to which was added epipregnanolone ($5 \times 10^{-5}$M), a marked reduction of osmotic fragility was observed (☆☆, not washed). However, if red blood cells were incubated with epipregnanolone ($5 \times 10^{-5}$M) in LPDM for 1 hr and were subsequently washed 2 times, their osmotic fragility (☆----☆, washed) was not different from that of control cells (not shown).

protect normal erythrocytes from osmotic lysis when the cells were incubated under our usual conditions and tested after washing twice in 80 volumes of PBS-BSA: hydrocortisone, 5-pregnen-3β, 20α-diol, epipregnanolone, pregnanolone, pregnenolone, allo-pregnanolone, pregnanediol, and pregnanedione. However, when we measured the osmotic fragility of unincubated red blood cells by adding the cells to saline solutions to which was added epipregnanolone, 5-pregnen-3β, 20α-diol, pregnanolone, or pregnenolone at a concentration of $5 \times 10^{-5}$M, a marked reduction of osmotic fragility was observed ($\Delta$MCF = 0.045%, 0.028%, 0.028%, and 0.021% NaCl, respectively) (Fig. 3).

Following incubation of red blood cells with $5 \times 10^{-5}$M pregnenolone in LPDM, the amount of this compound present in the erythrocytes was gradually reduced by a sequence of washing steps. This behavior contrasts with that of OSC; these compounds were not removed from the red blood cells despite repeated washing of the cells in PBS (Fig. 4).

The MCV of erythrocytes incubated with OSC did not differ from the MCV of control erythrocytes when measured in isotonic solution. However, the volume and calculated surface area of sealed ghosts of erythrocytes incubated for 1 hr with OSC in LPDM were larger than the volume and surface area of ghosts of control erythrocytes. This increase is proportional to the concentration of OSC in the medium to which the red cells are exposed (Table 3). Incubation of erythrocytes with 2.5 and $5 \times 10^{-5}$M 22-ketocholesterol resulted in a more pronounced increase of their ghost surface area than incubation with 20α-hydroxycholesterol at similar concentrations.

In order to confirm that the volume and surface area changes induced by OSC in red cell ghosts were not an artifact produced by lack of proper ghost releasing in OSC-treated cells, we also determined the changes induced by OSC in the critical hemolytic volume of OSC-treated red cells when compared to controls and found them to be comparable to the results obtained in Table 3. For example, by this technique, red cells exposed to $5 \times 10^{-5}$M 22-ketocholesterol underwent a 4.5% increase in critical hemolytic volume when compared to their control. Similar confirmatory results were obtained with 20α-hydroxycholesterol.

Erythrocytes incubated with OSC and control cells did not differ in their capacity to pass through polycarbonate filters. We tested red cells exposed to echino-
cytocyte-forming OSC (5α-hydroxy-6-ketocholestanol and 6β-hydroxycholestanol) as well as non-echinocyte-forming OSC (20α-hydroxycholesterol and 25-hydroxycholesterol). With the increasing cumulative volume of filtered cells, the flow rate through the filters gradually decreased due to plugging of the filter pores by unfilterable cells, as described by Gregersen et al. There was no difference in the initial flow rate \( (V_0) \), nor in the rate of flow decrement \( (\lambda) \) seen with successive 2-ml aliquots of the red cell suspension, between control cells and OSC-treated cells (Table 4).

Control cells and cells incubated with OSC (5α-hydroxy-6-ketocholestanol and 7β-hydroxycholesterol, \( 2.5 \times 10^{-5} M \)) did not differ in their water content. There was also no difference in the ouabain-sensitive, ouabain-insensitive, and external Na-dependent Na effluxes from high-sodium cells (intracellular Na \( \sim 40 \) mmole/kg cell water), and no difference was observed in the Cl efflux between control and OSC-exposed red blood cells.

In earlier studies we demonstrated that the degree of echinocyte formation induced by certain OSC reached a peak at 5–150 min exposure. Thereafter, it declined to a nadir at 2–8 hr with no change in the amount of OSC inserted into the red cell membrane, suggesting that OSC alter their equilibrium distribution between the two halves of the red cell membrane lipid bilayer. If such OSC redistribution indeed occurs, the experiments shown in Table 5 demonstrate that it does not alter the degree of membrane expansion caused by OSC, since there is no difference in the Δ MCF caused by OSC when it is measured at 1-hr and 3-hr exposure of red cells to OSC, despite the significant reduction in echinocyte formation noted at the later time.

We measured the effect of OSC on the osmotic fragility of erythrocytes from three patients with hereditary spherocytosis. Incubation of their red blood cells with 7α-hydroxycholesterol, 20α-hydroxycholesterol, and 22-ketocholesterol in LPDM at a concentration of \( 2.5 \times 10^{-5} M \) or \( 5 \times 10^{-5} M \) resulted in correction of the osmotic fragility curve (Fig. 5). The OSC not only corrected the abnormally high MCF, but also largely eliminated the "tail" of osmotically sensitive cells visible in the osmotic fragility curve of our patients with hereditary spherocytosis at NaCl concentrations of 0.5%–0.65%. The relationship

### Table 3. Insertion of OSC Into Red Blood Cell Membranes Increases Red Cell Ghost Volume and Surface Area

<table>
<thead>
<tr>
<th>Compound</th>
<th>OSC Concentration in LPDM</th>
<th>Red Cell Ghost Volume (μl; Mean ± SD)</th>
<th>Percent Increase of Ghost Volume</th>
<th>Red Cell Ghost Surface Area (μm²; Mean ± SD)</th>
<th>Percent Increase of Ghost Surface Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>—</td>
<td>147.0 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20α-Hydroxycholesterol</td>
<td>( 2.5 \times 10^{-5} M )</td>
<td>150.3 ± 0.8*</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22-Ketocholesterol</td>
<td>( 5 \times 10^{-5} M )</td>
<td>151.2 ± 0.6†</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( * p < 0.02 \) by comparison with control cells.

\( † p < 0.01 \) by comparison with control cells.

\( ‡ p < 0.001 \) by comparison with control cells.

### Table 4. Studies on the Filterability of Red Blood Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>OSC Concentration in LPDM</th>
<th>Percent Echinocytes</th>
<th>Extrapolated Initial Flow Rate ( (V_0) ), ml/sec; Mean ± SD*</th>
<th>Rate of Flow Decrement ( (\lambda) ), ml/sec; Mean ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>—</td>
<td>0</td>
<td>0.32 ± 0.02</td>
<td>0.024 ± 0.006</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>( 2.5 \times 10^{-5} M )</td>
<td>0</td>
<td>0.33 ± 0.01</td>
<td>0.024 ± 0.004</td>
</tr>
<tr>
<td>5α-Hydroxy-6-ketocholestanol</td>
<td>( 2.5 \times 10^{-5} M )</td>
<td>96</td>
<td>0.31 ± 0.03</td>
<td>0.027 ± 0.002</td>
</tr>
<tr>
<td>6β-Hydroxycholesterol</td>
<td>( 2.5 \times 10^{-5} M )</td>
<td>99.5</td>
<td>0.32 ± 0.02</td>
<td>0.031 ± 0.001</td>
</tr>
<tr>
<td>25-Hydroxycholesterol</td>
<td>( 2.5 \times 10^{-5} M )</td>
<td>0.5</td>
<td>0.33 ± 0.01</td>
<td>0.030 ± 0.001</td>
</tr>
</tbody>
</table>

Successive filtration of 2-ml aliquots of a red blood cell suspension (hematocrit 2%) through a 3-μm polycarbonate filter at constant filtration pressure \( (10 \text{ cm} \text{ H}_2\text{O}) \) results in a decrement of the flow rate of the red cells due to progressive plugging of the filter by the cells. The data can be fitted by an exponential function: \( V \propto V_0 e^{-\lambda t} \), where \( V_0 \) is the extrapolated initial flow rate (ml/sec), \( \lambda \) is the rate of flow decrement (ml/sec), and \( V \) is the cumulative filtered volume (ml). Echinocyte formation was monitored by methods previously described.

*There is no statistical difference between control cells and cholesterol- or OSC-treated cells.
between \( \Delta \) MCF and the amount of OSC inserted in the red blood cells of a patient with hereditary spherocytosis is shown in Table 6.

**DISCUSSION**

Our experiments show that oxygenated derivatives of cholesterol inserted into the erythrocyte membrane\(^3\) are capable of expanding the cell membrane without affecting the volume or the water content of the cell in an isotonic environment. An increase of the cell surface area-to-volume ratio allows the erythrocyte to accumulate a larger volume of water in a hypotonic environment before reaching the critical hemolytic volume, i.e., the maximal volume attainable before osmotic lysis.\(^4\) This results in reduced susceptibility of the erythrocyte to osmotic lysis.\(^5\)

A wide range of drugs and amphipathic substances have been shown to be capable of protecting the red blood cell against osmotic lysis by expanding its membrane surface area.\(^6,7\) The expansion of the red blood cell membrane by all of these substances cannot be explained simply by an increase of the membrane substance due to the bulk volume of the inserted molecules. Anesthetics, at doses producing general anesthesia, expand red blood cell membranes approximately 10 times more than would be expected from the bulk volume of the anesthetic molecules present in the membrane.\(^8\) In previous experiments, we have shown that red cells incubated in LPDM for 1 hr at 37°C lose 9.7% (\( \sim \)70 \( \mu \)g/0.5 ml packed red cells) of their membrane cholesterol.\(^3\) Approximately the same amount was replaced by OSC in those of our experiments in which we exposed the erythrocytes to LPDM containing 22-ketocholesterol or 20\( \alpha \)-hydroxycholesterol at 5 \( \times \) 10\(^{-3}\)M, or 7\( \alpha \)-hydroxycholesterol at 2.5 \( \times \) 10\(^{-3}\)M. Since the replacement of the cholesterol loss in red blood cells by a lesser or approximately equal amount of OSC results in enhanced osmotic resistance of the cells, and since the physical dimensions of the OSC molecules differ only slightly from those of cholesterol, the effect of OSC on red cell membranes must be explained by mechanisms other than the simple bulk volume effect of OSC molecules inserted in the membrane.

Cooper et al. have shown that cholesterol-loading of erythrocyte membranes also results in protection of the red cells against osmotic lysis.\(^9\) In their studies, in order for cholesterol-enriched red cells to achieve a \( \Delta \) MCF of 0.07% NaCl, their cholesterol content had to be increased to 140% of normal, which represents an increase of approximately 280 \( \mu \)g cholesterol/0.5 ml

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**Table 5. The Effect of Increasing Time of Exposure of Red Cells to OSC on Echinocyte Formation and Osmotic Fragility**

<table>
<thead>
<tr>
<th>OSC Compound</th>
<th>Time of Incubation (hr)</th>
<th>Echinocyte Formation (%)</th>
<th>( \Delta ) MCF (% NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7( \beta )-Hydroxycholesterol</td>
<td>1</td>
<td>85.8 ± 7.0</td>
<td>-0.042</td>
</tr>
<tr>
<td>7( \beta )-Hydroxycholesterol</td>
<td>3</td>
<td>62.8 ± 10.4</td>
<td>-0.044</td>
</tr>
<tr>
<td>5( \alpha )-Hydroxy-6-ketocholestanol</td>
<td>1</td>
<td>89.5 ± 4.5</td>
<td>-0.023</td>
</tr>
<tr>
<td>5( \alpha )-Hydroxy-6-ketocholestanol</td>
<td>3</td>
<td>24.8 ± 3.8</td>
<td>-0.028</td>
</tr>
</tbody>
</table>

Control red cells did not alter their osmotic fragility over the course of the 3-hr incubation.

*By comparison with the 1-hr echinocyte percentage.

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**Table 6. Amount of OSC Inserted Into the Red Blood Cells of a Patient With Hereditary Spherocytosis:**

<table>
<thead>
<tr>
<th>Compound</th>
<th>OSC Concentration in LPDM</th>
<th>( \mu )g OSC Inserted/0.5 ml Packed Red Cells (Mean ± SD)</th>
<th>( \Delta )MCF (% NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7( \alpha )-Hydroxycholesterol</td>
<td>2.5 ( \times ) 10(^-3) M</td>
<td>74.1 ± 15.0</td>
<td>0.040</td>
</tr>
<tr>
<td>20( \alpha )-Hydroxycholesterol</td>
<td>2.5 ( \times ) 10(^-3) M</td>
<td>37.8 ± 6.0</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>5 ( \times ) 10(^-3) M</td>
<td>70.5 ± 5.1</td>
<td>0.055</td>
</tr>
</tbody>
</table>

The cholesterol content of the red blood cell samples was 483 ± 43.5 \( \mu \)g (mean ± SD).

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**Fig. 5.** Correction of the osmotic fragility curve of red cells from a patient with hereditary spherocytosis by incubation of the abnormal erythrocytes with 20\( \alpha \)-hydroxycholesterol and 22-ketocholesterol (both at 5 \( \times \) 10\(^{-3}\)M) in LPDM for 1 hr.
packed red cells. The same degree of osmotic protection can be afforded 0.5 ml packed red cells by inserting 84 μg 22-ketocholesterol into their membranes. Thus, on a molar basis, 22-ketocholesterol is approximately 3.4-fold more effective than cholesterol in protecting red cells against osmotic lysis.

Anesthetics have been shown to cause disordering or fluidization of the red cell membrane.\textsuperscript{26} \textsuperscript{28} We have postulated such a mechanism of action for OSC following their insertion into red cell membranes.\textsuperscript{3} The OSC oxygen substitutions may reduce, through steric hindrance and, more importantly, through polar–apolar repulsions, the closeness of packing of the phospholipid hydrocarbon side chains in the lipid bilayer of the red cell membrane, and may render the membrane more fluid while expanding its surface area.

In our experiments, the degree of expansion of red cell ghost membranes by inserted OSC ranged from 1.5\% to 3.4\%, depending on the identity and concentration of the OSC used. This expansion can be related to the degree of protection against osmotic lysis provided by the OSC as well as to the amount of a given OSC inserted in the red cell membrane. The range of our values for red cell ghost membrane expansion relative to the resultant protection against osmotic lysis agrees with that found by Seeman et al.\textsuperscript{4} for the antihemolytic effect of anesthetics and other amphipaths. We have shown that OSC-containing erythrocytes do not differ from control cells in their water content, in their tracer and net sodium efflux from Na-loaded cells, and in their chloride exchange flux. The filterability of control cells did not differ from that of cells treated with OSC, whether or not the OSC were capable of producing echinocytes. Other echinoctyogenic agents have been shown not to affect the filterability of red blood cells.\textsuperscript{29}

The osmotic protective effect of progesterones has usually been demonstrated only with the compounds present in the hypotonic solutions used for measurement of the osmotic fragility of the erythrocytes.\textsuperscript{22} If, however, the cells are incubated with progesterones and then washed, their osmotic fragility does not differ from that of control cells. The difference in behavior of progesterones and OSC with respect to their capacity to protect red cells against osmotic lysis following washing is explained by our finding that progesterones do not stay in the membrane, but are removed by the washing process, whereas OSC are firmly inserted into the membrane and are not washed out. Conrad and Singer\textsuperscript{30} have recently shown that the binding of other amphipathic compounds (chlorpromazine, 2,4-dinitrophenol, 1-decanol) to cell membranes is weak; these compounds are also known to protect erythrocytes against osmotic lysis if they are added directly to the hypotonic salt solutions. The structural property that allows OSC molecules to be firmly anchored in the cell membrane may be their closer structural similarity to the cholesterol molecule, particularly with respect to the cholesterol side chain. The most precise evidence for the important role played by the sterol side chain in anchoring OSC molecules in the lipid bilayer is the finding that 5-pregnen-3-\beta,20α-diol, an analogue of 20α-hydroxycholesterol lacking only the last 6 carbons of the sterol side chain, does not stay in the red cell membrane and therefore has no effect on osmotic fragility if the cells are washed two times after their exposure to this compound. 20α-Hydroxycholesterol, by contrast, is a potent inhibitor of red cell osmotic lysis following its insertion and cannot be removed from the red cells even after repeated washings in PBS.

The reduced effect of OSC on osmotic fragility observed when red blood cells are exposed to OSC in a lipoprotein-containing medium may be due to the capacity of serum lipoproteins to bind 60\%–80\% of the OSC, thereby making it unavailable for entry into the erythrocyte membrane.\textsuperscript{31} However, the protection offered by lipoproteins against OSC membrane insertion is only relative and can be overcome by increasing OSC concentrations. If OSC concentrations are increased 2–3-fold, insertion of OSC into red cells membranes from lipoprotein-containing medium can equal the amount inserted from lipoprotein-depleted medium at lower concentrations, and the expected changes in red cell shape (echinocyte formation) and membrane expansion (protection against osmotic lysis) ensue secondarily to an equivalent extent.\textsuperscript{31}

Not only do OSC protect normal red blood cells against osmotic lysis, but they also normalize the MCF of erythrocytes from patients with hereditary spheroctosis. 7α-Hydroxycholesterol, 20α-hydroxycholesterol, and 22-ketocholesterol are capable of correcting the increased osmotic fragility of the red cells observed in this disease. The amounts of OSC inserted into the membrane of erythrocytes from patients with hereditary spheroctosis are not appreciably different from those inserted into normal red cells under the same conditions. In addition, the potency of OSC in producing decreases in osmotic fragility is the same in red cells of both normal individuals and those with hereditary spheroctosis.

These in vitro experiments suggest that it may be possible to develop a pharmacologic approach to the management of hereditary spheroctosis, since, as Cooper and Jandl have shown, expansion of the hereditary spheroctosis red cell membrane by the insertion of free cholesterol results in improved survival of such
DIMINISHED RED CELL OSMOTIC FRAGILITY

325

cholesterol-enriched cells in vivo. OSC may not be the ideal agents to contemplate in regard to clinical use, since they may have deleterious effects in vivo, particularly with regard to possible atherosclerotic changes. However, the model we have developed with these compounds may prove useful in searching for other, safer, membrane expanding drugs.

REFERENCES


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