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CHOLESTEROL SULFATE
I. OCCURRENCE AND POSSIBLE BIOLOGICAL FUNCTION AS AN AMPHIPATHIC LIPID IN THE MEMBRANE OF THE HUMAN ERYTHROCYTE

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SUMMARY

Cholesterol sulfate is a normal constituent of human erythrocytes at a concentration which is approximately 2-fold higher than plasma cholesterol sulfate. In these cells, the major fraction of the cholesterol sulfate is firmly bound to the membrane.

Cholesterol sulfate as well as certain analogs can protect the red blood cell against hypotonic hemolysis. This effect is produced in vitro at physiological concentrations of the sterol sulfate and both the sulfate moiety as well as the side chain of the molecule are necessary for biological activity.

INTRODUCTION

Theoretical analysis of membrane structure made it obvious that the introduction of cholesterol sulfate in the design of membrane models would explain several aspects of the stability and function of these organelles. Like many other lipids of biological membranes, this sterol sulfate is an amphipathic molecule. The sulfate moiety has a pK_a of approximately 3.3 (Bleau, G., unpublished), therefore cholesterol sulfate is ionized in its physiologic microenvironment. The non-polar portion of the molecule has dual properties; the sterol nucleus confers rigidity while some flexibility is maintained as a result of the side chain. It is proposed that because of its unique structure and properties, cholesterol sulfate is ideally suited for the formation of interactions between membrane components.

The discovery of cholesterol sulfate in a wide variety of tissues from many

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species [1] including purified myelin (Roberts, K. D., unpublished), raises the hypothesis that cholesterol sulfate may be an important constituent of many types of membranes. The subject of this report is one of the approaches used to test this hypothesis where cholesterol sulfate levels were measured in human plasma and erythrocyte membranes and studies were made concerning the effect of this compound and several analogs on the stability of erythrocytes in hypotonic media.

MATERIALS AND METHODS

The methods used for the preparation and purification of the steroids as well as the radioactive counting techniques have been described previously [2, 3]. The enzymatic-radioisotope displacement assay for the measurement of cholesterol sulfate [3] consists, in brief, of using human placental steroid sulfatase from the microsomal fraction to obtain standard displacement curves by plotting the number of cpm of [^{3}H]cholesterol liberated (product of the reaction with [^{3}H]cholesterol sulfate as substrate) versus the amount (in nanograms) of exogenous cholesterol sulfate added to the incubation medium. A typical standard curve is presented in Fig. 1. Unknown concentrations were estimated from the curve and corrected for procedural losses.

Procedure for the preparation of erythrocyte ghosts

All experiments were performed on freshly drawn blood from normal men. Blood was collected in tubes containing EDTA and centrifuged to separate the erythrocytes. Ghosts were prepared as described by Dodge et al. [4].

Studies of the effects of cholesterol sulfate and analogs on erythrocyte hemolysis

Blood was freed of fibrin by gentle agitation with glass beads for 15 min. The erythrocytes were sedimented by centrifugation at $900 \times g$ for 10 min, washed three times and resuspended in isotonic saline, 0.01 M phosphate buffer (pH 7.4). An aliquot of 50 μl of the erythrocyte suspension (approx. $5 \cdot 10^9$ cells per ml) was added to 5 ml of the test solutions. The test solutions consisted of known concentrations of cholesterol sulfate or analogs in saline of different osmolalities. Compounds of low solubility in aqueous media were first dissolved in 50 μl of ethanol, this solvent was added at the same concentration to control solutions. After 1 h of incubation at room temperature the erythrocytes were centrifuged at $1500 \times g$ for 10 min and the percentage hemolysis was determined by measuring the absorbance (at 540 nm) of hemoglobin in the supernatant. The optical density reading obtained for erythrocytes in distilled water was used as a reference point for complete hemolysis.

Determination of the uptake of [^{3}H]cholesterol sulfate by erythrocytes

Erythrocytes ($4.7 \cdot 10^8$) were washed twice with 5 ml of isotonic saline. They were resuspended in 5 ml of isotonic saline containing non-radioactive cholesterol sulfate at 10^{-5} M and $1.35 \cdot 10^5$ dpm of [^{3}H]cholesterol sulfate (cholesterol sulfate and [^{3}H]cholesterol sulfate were first dissolved in 50 μl of ethanol). After 1 h of incubation at room temperature followed by centrifugation at $1500 \times g$ for 10 min, a 4 ml aliquot of the supernatant was removed and the erythrocytes were washed

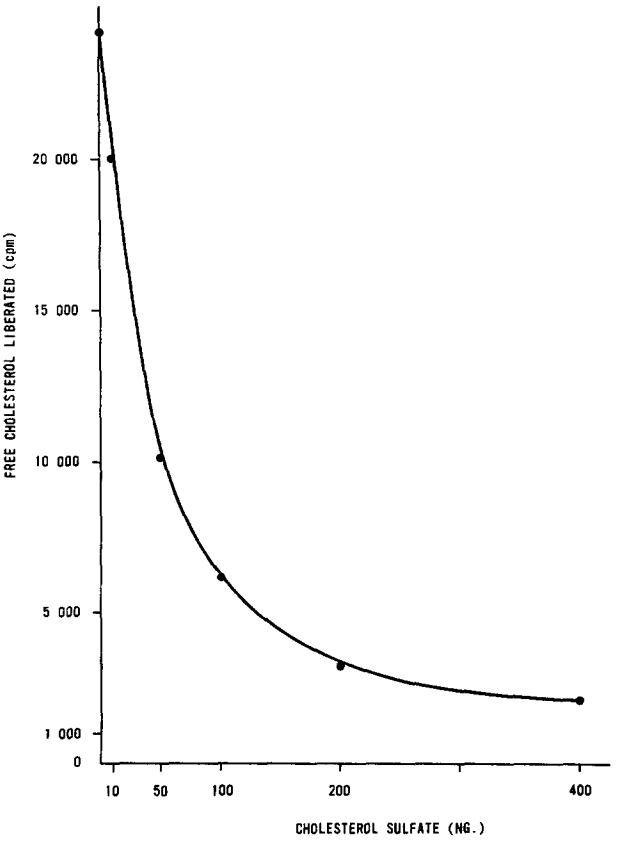

Fig. 1. Enzymatic-radioisotope displacement curve for the measurement of cholesterol sulfate. The technique is based upon the displacement of tritiated cholesterol sulfate from human placental sulfatase by the addition of unlabelled cholesterol sulfate. The details and reproducibility of the assay system have been reported previously [3].

three times with the same volume of isotonic saline. Distilled water was added to cause hemolysis. After centrifugation at 20 000 $\times g$ for 30 min at 0 °C, the first supernatant (hemolysate) was removed and the ghosts were washed twice and finally resuspended in 5 ml of distilled water. Radioactivity was determined after extracting [^{3}H]cholesterol sulfate from all of the fractions with ether: ethanol (3 : 1, v/v) as described by Edwards et al. [5].

RESULTS

Concentration of cholesterol sulfate in plasma and erythrocyte ghosts

Levels of cholesterol sulfate in plasma and erythrocyte ghosts are listed in Table I. For each subject (age: 26–41 years) duplicate values were obtained from three samples of plasma and triplicate values from a sample of erythrocyte ghosts. For the purpose of comparison, levels of plasma cholesterol are also presented. These results demonstrate that the concentration of cholesterol sulfate in the ery-

TABLE I

CONCENTRATION OF CHOLESTEROL SULFATE IN MALE PLASMA AND ERYTHROCYTE MEMBRANES

Subject	Plasma cholesterol sulfate (μg/100 ml plasma)			Mean	Membrane cholesterol sulfate (μg/100 ml red cells)			Mean	Plasma cholesterol (mg%)
A	371	417	347	376	885	987	920	930	237
	343	421	356						
B	338	472	405	387	652	699	673	675	207
	349	346	410						
C	302	361	302	307	454	440	486	460	140
	266	325	286						
D	326	430	338	373	785	801	752	779	210
	385	423	388						

throcytes is approx. 2-fold higher than the plasma level of this conjugate. Calculated on a dry weight basis using the value of $13 \cdot 10^{-13}$ g/erythrocyte ghost [4], cholesterol sulfate accounts for approx. 1 mg per g dry wt of erythrocyte ghosts.

Distribution of [^3H]cholesterol sulfate following incubation with erythrocytes

The data in Table II indicate that 46% of the [^{3}H]cholesterol sulfate incubated was found to be associated with the erythrocytes. The radioactivity in the first and second wash of the erythrocytes merely represents residual [^{3}H]cholesterol sulfate in the medium (only 4 out of the 5 ml of medium were removed after centrifugation). More than 98% of the cholesterol sulfate retained by the erythrocyte is firmly bound to the membrane fraction, as the hemolysate and membrane washings were virtually devoid of [^{3}H]cholesterol sulfate.

Stabilization of erythrocytes by cholesterol sulfate and analogs

As shown in Fig. 2, cholesterol sulfate reduces the hemolysis in hypotonic solutions. This in vitro effect was consistently observed with erythrocytes obtained

TABLE II

DISTRIBUTION OF [^{3}H]CHOLESTEROL SULFATE FOLLOWING INCUBATION FOR 1 h WITH HUMAN RED BLOOD CELLS IN PHYSIOLOGIC SALINE SOLUTION

Fractions	Percentage of [^{3}H]cholesterol sulfate incubated
Supernatant	44.5
1st wash of erythrocytes	7.0
2nd wash of erythocytes	2.1
Hemolysate	0.5
Membrane wash	0.4
Erythrocyte membranes	46.0

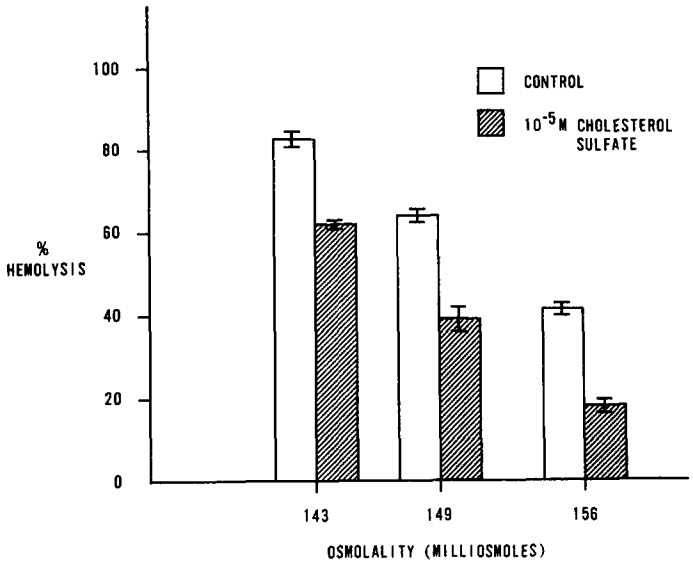

Fig. 2. Effect of cholesterol sulfate (10^{-5} M) on the hemolysis of human erythrocytes in hypotonic saline solution. Percent hemolysis represents the percentage of the total hemoglobin released into the medium. The figure illustrates the data obtained in one subject. Three determinations were done at each salt concentration. The bars represent the mean $\pm$ the standard deviation. This experiment was repeated several times and provided the same conclusion with each subject i.e. increased stabilization by the sterol sulfate is observed as one increases the osmolality. The typical data illustrated above indicate a 25 % diminution of hemolysis at 143 mosM, 39 % at 149 mosM and a diminution of 56 % at 156 mosM.

from both men and women. The optimal level of cholesterol sulfate required at the cell concentration used was 10^{-5} M; higher concentration (up to 10^{-4} M) did not increase the protection significantly and did not cause hemolysis of the red blood cells. Due to the low solubility of cholesterol sulfate in saline, concentrations higher than 10^{-4} M could not be tested. Cholesterol sulfate offers protection against hemolysis over a wide range of osmolality in the osmotic fragility curve but the maximum effect, of those concentrations examined, was observed at 156 mosM i.e. a greater stabilization effect occurs as one approaches isotonicity.

In order to establish the molecular requirements and the specificity of the cholesterol sulfate induced protection, a series of natural and synthetic compounds which are structurally related to cholesterol sulfate was tested at equimolar concentrations (Table III). Dehydroisoandrosterone sulfate which differs from cholesterol sulfate by a ketone group at carbon 17 in place of a C_8 side chain is completely devoid of activity. A negative result was also obtained with cholenic acid 3-sulfate. This would indicate that the Δ^5-3β-sulfoxy steroid structure is not the only portion of the cholesterol sulfate molecule that is involved in the interaction with membrane components. In addition, those compounds possessing the side chain but lacking the sulfate moiety were also inactive (cholesterol, cholesterol chloride and bromide). The observation that cholesterol phosphate is devoid of activity provides further evidence of the importance of the sulfate moiety.

TABLE III

EFFECT OF CHOLESTEROL SULFATE AND ANALOGS ON HEMOLYSIS

The compounds were tested at a concentration of 10^{-5} M in 156 mosM saline. Steroid and sterol sulfates were synthesized as the ammonium salt.

Compounds	Relative potency (% of cholesterol sulfate activity)
Cholesterol sulfate	100
Dehydroisoandrosterone sulfate	2
Cholenic acid 3-sulfate	10
Cholesterol	0
Cholesterol chloride	0
Cholesterol bromide	6
Cholesterol phosphate	0
Progesterone	16
Na_2SO_4	0
NH_4Cl	0
7-Ketocholesterol sulfate	65
22-Ketocholesterol sulfate	65
Sitosterol sulfate	82
Ergosterol sulfate	129

A comparison of cholesterol sulfate with compounds possessing a more closely related structure provided further insight into the structural requirements for such stabilization. Thus, 7-ketocholesterol sulfate, 22-ketocholesterol sulfate and sitosterol sulfate also provide a certain degree of protection from hypotonic hemolysis but are all less potent than cholesterol sulfate. The most potent compound evaluated to date is ergosterol sulfate which almost completely prevents hemolysis.

In a recent study by Raz and Livne [6] it was reported that C_{18} fatty alcohols also stabilize the human erythrocyte in hypotonic media. In studies to determine the relationship between the degree of unsaturation of the hydrocarbon chain and the degree of stabilization it was found that alcohols with an increasing number of double bonds were more potent as stabilizers of the red cell. In this respect it is interesting to note that in our experiments with ergosterol sulfate, which possesses a double bond in the hydrophobic side-chain, was more active than cholesterol sulfate in stabilizing the erythrocyte. Raz and Livne [6] also reported that the protective effect of the lipids tested was reversible in that it could be removed following a single washing procedure. In contrast, in our experiments with cholesterol sulfate it was found that once the sterol conjugate is associated with the red cell membrane it cannot be removed following several washings.

DISCUSSION

The biological function of cholesterol sulfate as well as other sterol sulfates is unknown. Cholesterol sulfate is a ubiquitous entity in mammals [1] and its isolation from lower species such as the sea star, *Asterias rubens*, has been reported by Björkman et al. [7].

If cholesterol sulfate is indeed present in this organism, this finding would be in contradiction to the report of Gupta and Scheuer [8]. The latter investigators have reported that the sea star does not synthesize Δ^5 sterols but rather a mixture of Δ^7 mono- and di-unsaturated sterols. It is possible that the sterol conjugate isolated by Björkman et al. [7] is Δ^7 cholestenol sulfate since Δ^7 cholestenol is one of the major sterols synthesized by *A. Rubens* [9]. In further support of this contention is the fact that Δ^7 cholestenol exhibits a mass spectrum similar to but not identical to cholesterol [10]. Furthermore, the infrared spectrum of Δ^7 cholestenol sulfate is similar to but not superimposable on that of cholesterol sulfate (Bleau, G., unpublished). In a recent report of Voogt [11], gas chromatographic data demonstrated that, following saponification, Δ^7-cholestenol was by far the major sterol present in *A. Rubens*. Only a trace amount of cholesterol was reported.

In a previous study [3], the identity of the sterol conjugate isolated from the erythrocyte has been established on the basis of its migration with authentic cholesterol sulfate on thin-layer chromatography and by a positive methylene blue reaction of Roy [12]. The identity of the sterol moiety has been confirmed by solvolysis, thin layer chromatography of the free sterol, acetylation with [^{14}C]acetic anhydride and by crystallization of the doubly-labelled acetate with authentic cholesterol acetate [3].

From the data presented here and from current knowledge of the structure of biological membranes, it is proposed that cholesterol sulfate and possibly other sterol sulfates be considered as important constituents of membranes.

In the experiment where the uptake of [^{3}H]cholesterol sulfate was investigated, the assumption was made (probably false) that the molecule of cholesterol sulfate lies flat on the membrane surface and with the data summarized in Table IV, it was calculated that less than 0.5% of the surface area of the membrane is covered by cholesterol sulfate. However, we have shown that under these conditions the sterol sulfate offers maximal stabilization of the erythrocyte. One interpretation of this finding is that the interaction involving cholesterol sulfate is critical for the structural organization of the entire membrane.

The binding of cholesterol sulfate to erythrocytes differs from the binding of free steroids or of other steroid sulfates. Holzbauer [13] and Brinkmann et al. [14, 15]

TABLE IV

DATA FOR THE CALCULATION OF THE ERYTHOCYTE SURFACE AREA COVERED BY CHOLESTEROL SULFATE

Cholesterol sulfate concentration	10^{-5} M
Percent uptake of cholesterol sulfate	46
Erythrocyte concentration	$4.7 \cdot 10^9$ cells/ml
Surface area of the cholesterol sulfate molecule*	120 Å^2
Surface area of an erythrocyte	150 μm^2
Avogadro's number	$6.022 \cdot 10^{23}$ molecules/g mole
Calculated number of cholesterol sulfate molecules per erythrocyte	$6.0 \cdot 10^5$
Calculated maximal percent of cell surface covered by cholesterol sulfate molecules	0.5

* Value estimated from the projection of a Dreiding model of the cholesterol sulfate molecule lying flat.

have reported that non-conjugated steroids taken up by the erythrocyte in vitro can be easily removed by washing with physiological saline. This would indicate that these compounds are loosely associated with the cell. These investigators have also observed that a large percentage of the individual steroids was bound to proteins of the membrane-free hemolysate. In contrast, in the present study it was found that the major fraction of the cholesterol sulfate is firmly bound to the erythrocyte membrane.

Specific interactions are also involved in the binding of cholesterol sulfate to membranes. Thus, Benes et al. [16] have reported that intact erythrocytes take up only 3% of [^{3}H]dehydroisoandrosterone sulfate from the incubation medium, of which less than 1% is associated with the membrane fraction. This demonstrates that the mere sulfo-conjugation of the free steroid molecule is not the sole criteria determining the extent of uptake by the erythrocyte membrane. The lack of effect of dehydroisoandrosterone sulfate on the stabilization of erythrocytes agrees with this interpretation.

Many surface-active compounds are known to protect or stabilize membranes at certain concentrations and to cause hemolysis at higher concentrations. This has been clearly established by Seeman [17] and by Seeman et al. [18]. These compounds include tranquilizers, antihistamines, fatty acids, alcohols, anesthetics and steroids. In general, both synthetic compounds and naturally occurring substances are only effective at concentrations considerably above physiologic levels. In the case of cholesterol sulfate, a substance normally present in the erythrocyte membrane, the stabilization effect was observed at physiologic concentrations. At the present time the possibility cannot be excluded that these surface-active compounds alter membrane properties by an effect on membrane-bound cholesterol sulfate. Seeman et al. [18] have proposed several possible mechanisms to explain the effect of such drugs. One of these mechanisms involves the displacement of a "membrane-associated component" which normally retains the membrane in a condensed state. Such a component may possibly be cholesterol sulfate.

In preliminary experiments in our laboratory it was found that, following the intravenous administration of physiological concentrations of cholesterol sulfate to the dog, the red cells exhibited a significantly increased stability when exposed to hypotonic salt solutions in vitro. These studies will be the subject of a forthcoming communication.

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