Biochimica et Biophysica Acta, 394 (1975) 120–128

BBA 76974

CHOLESTEROL SULFATE

II. STUDIES ON ITS METABOLISM AND POSSIBLE FUNCTION IN CANINE BLOOD

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(Received October 28th, 1974)

SUMMARY

Previous in vitro studies to evaluate the possible role of cholesterol sulfate in the stabilization of the human erythrocyte membrane have been extended to the dog in vivo. Thus, following the injection of labelled cholesterol sulfate, a large fraction of the administered sterol conjugate is taken up by the membrane of the canine erythrocyte. Peak membrane levels were obtained within 30–60 min. Measurement of radioactivity associated with the plasma and red cell fractions in serial samples allowed the calculation of the half-life of cholesterol sulfate in each fraction. From the data obtained from the plasma of four dogs, the half-life was calculated to 5.8 ± 0.9 h. The half-life of cholesterol sulfate associated with the erythrocyte membrane was calculated to be 6.7 ± 1.2 h.

In addition, following the intravenous administration of 0.2–0.7 mg of cholesterol sulfate/kg of body weight and withdrawal of serial blood samples, a significant diminution in the degree of hemolysis was observed when the red cells were exposed to hypotonic saline solutions. Maximal stabilization effects were observed at approx. 6–7 h after the administration of the sterol conjugate. Hemolytic properties returned to normal at approx. 24 h following the injection.

INTRODUCTION

In previous publications we have reported on the occurrence of cholesterol sulfate in human plasma and erythrocytes [1, 2]. The concentration of cholesterol sulfate associated with the red cell was found to be at least 2-fold higher than the corresponding plasma level. Following the incubation of red cells with [^{3}H]cholesterol sulfate in physiologic saline for 1 h, a 50 % uptake of the label was observed. Subsequently, it was found that the sterol sulfate was associated almost entirely with the erythrocyte membrane and was firmly bound, since several washing procedures failed to dislodge the label.

In in vitro experiments with human erythrocytes, it was found that a marked diminution in hemolysis occurred when the cells were exposed to hypotonic saline solutions containing physiological concentrations (10^{-5} M) of cholesterol sulfate. Scanning electron microscope studies of human erythrocytes in hypotonic media, containing 10^{-5} M concentrations of cholesterol sulfate, illustrate the retention of the normal biconcave structure of the cell while in the absence of the sterol sulfate the usual spherical and crenated shapes are observed [3].

In studies to determine the specific structural requirements of the sterol molecule, it was shown that the hydrophobic side chain, as well as the sulfate moiety, was essential in order to obtain this effect of stabilization. The foregoing data would suggest, albeit indirectly, that this sterol conjugate may play a role in the maintenance of structure of the erythrocyte membrane.

In order to evaluate further this possibility, a series of studies was performed with the dog. Thus, following intravenous administration, the effect of cholesterol sulfate on the in vitro stability of the red cell in hypotonic solutions was determined. In addition, the uptake of the tritiated sterol conjugate by the red cell in vivo, as a function of time, was studied.

Finally, following the assay of radioactivity in serial samples of plasma, as well as of the red cell membrane fraction, the half-life of cholesterol sulfate associated with these fractions was calculated. The results of these experiments are the subject of the present report.

MATERIALS AND METHODS

All solvents were redistilled prior to use. [1,2-$^3H_2$]Cholesterol (40–60 Ci/mmol) was purchased from New England Nuclear Corp. and purified as described previously [4]. [1,2-$^3H_2$]Cholesterol sulfate, ammonium salt, was prepared from purified [1,2-$^3H_2$]cholesterol as reported in a previous communication [5]. [4-^{14}C]Cholesterol sulfate, ammonium salt, was prepared from [4-^{14}C]cholesterol (New England Nuclear Corp., 50 Ci/mol) in a similar manner. Prior to intravenous injection, a mixture of radioactive and non-radioactive cholesterol sulfate was dissolved in 0.6 ml of ethanol and then diluted with 4 ml of homologous plasma. This mixture was incubated for 2 h at 37 °C with agitation to ensure a homogeneous solution. Following intravenous injection of the sterol sulfate, serial blood samples (10 ml) were withdrawn. Each blood specimen was defibrinated using glass beads and centrifuged at 1 500 rev./min.

Each sample of plasma and erythrocytes was processed in the following manner.

Plasma. A tracer amount of ^{14}C-labelled cholesterol sulfate was added to each specimen in order to correct for procedural losses. Plasma proteins were precipitated with cold methanol (1 vol. plasma/4 vols methanol). The precipitate was removed by centrifugation. The aqueous methanol was then extracted twice with hexane in order to remove the free cholesterol liberated during the course of the experiment. The aqueous methanol fraction was evaporated to dryness and redissolved in 3 ml of 1.5 M NH_4OH. This aqueous fraction was extracted twice with equal volumes of *n*-butanol. This final partition served to eliminate a large fraction of the residue prior to thin-layer chromatography. Random samples of the butanol extracts were

chromatographed on Chromar-500 (Mallinkrodt) to verify that the radioactivity in these extracts was indeed associated with the sterol conjugate. The system isooctane/ethyl acetate/*t*-butanol/methanol/1 M NH_4OH (upper phase only) (2 : 4 : 2 : 2 : 4, by vol.) was used. In each instance the radioactivity was found to migrate with authentic cholesterol sulfate.

Preparation of erythrocyte ghosts. The red cell fraction was washed three times with cold physiological saline buffered with 0.01 M phosphate, pH 7.4. Erythrocyte ghosts were prepared according to the method of Dodge et al. [6]. The protein content of the membrane fraction was measured according to the technique of Lowry et al. [7].

Four mongrel dogs, weighing approx. 10 kg each, were injected intravenously with a solution of cholesterol sulfate prepared as described above. The quantity of the sterol sulfate injected varied between 0.22 and 0.76 mg of cholesterol sulfate per kg of weight. Using an average value of 79 ml/kg for the whole blood volume of the dog and a hematocrit of 45, the quantity of cholesterol sulfate injected into the plasma represents an increase of circulating cholesterol sulfate of approx. 50–200 μg/100 ml. In a limited series of plasma cholesterol sulfate measurements in the dog, values ranging between 50 and 100 μg/100 ml have been observed (Roberts, K.D., unpublished). Following the injection of the above quantities of the sterol sulfate, peak circulating levels would be below the levels found in human plasma [2].

To serve as a control experiment, a tracer quantity of [^{3}H]cholesterol sulfate was injected under the same conditions used for the experimental animals to eliminate any possible effect of the injection procedure itself (dog 5).

Washed red cells were obtained as described above at specified time intervals following the injection of cholesterol sulfate. Each sample of red cells was divided into three fractions.

(1) The first fraction was added to distilled water and served as a control where 100 % hemolysis occurred.

(2) The second fraction was added to hypotonic saline solutions of two concentrations, 136 and 142 mosM. At these concentrations, approx. 50 % hemolysis is observed. The mixtures were incubated for 1 h at room temperature. At the end of the incubation period, the suspensions were centrifuged at 2000 rev./min for 5 min. The supernatant fractions were analyzed spectrophotometrically at 540 nm to determine the degree of hemoglobin release into the medium.

(3) Erythrocyte ghosts were prepared as described above. Aliquots of the individual membrane samples were analyzed for their content of radioactivity.

Serial plasma samples were also assayed for their radioactive content. In this manner the uptake of the labelled cholesterol sulfate by the erythrocyte, as well as its disappearance from plasma, were determined as a function of time. In addition, the half-life of the sterol conjugate associated with the erythrocyte as well as with the plasma was calculated according to previously published procedures [8–10].

RESULTS

In vitro stabilization of the erythrocyte in hypotonic saline solutions following the intravenous administration of cholesterol sulfate

Following the intravenous injection of cholesterol sulfate in amounts ranging from 0.2 to 0.7 mg/kg, serial blood samples were withdrawn to evaluate the stabilization effects of increased levels of the circulating sterol sulfate when the red cells are subsequently exposed to hypotonic salt solutions. Fig. 1 illustrates the percent diminution of hemolysis plotted as a function of time following the administration of cholesterol sulfate to four dogs. In each instance a highly significant diminution in the degree of hemolysis is observed. This diminution of hemolysis varied between 20 and 45 % of the control values. In two dogs that were studied for a longer period of time it was observed that maximum stabilization occurs between 6 and 7 h. Following this, a slow return to the control levels of hemolysis is observed over a period of 24 h.

Uptake of [^3H]cholesterol sulfate, following intravenous injection, by the erythrocyte membrane

In four dogs, a maximal uptake was observed between 30 and 60 min which was followed by a slow release of the label from the red cell ghost. This slow decline of radioactivity is undoubtedly due to an exchange of the membrane-bound sterol conjugate with plasma cholesterol sulfate which is undergoing constant dilution due to endogenous production. That this disappearance of the sterol sulfate from the erythrocyte is due to such an exchange rather than to metabolism is indicated by the absence of sterol sulfohydrolase in the erythrocyte (Roberts, K. D., unpublished). The latter portion of the curve was used to calculate the half-life of cholesterol sulfate that is associated with the red cell ghost.

Calculation of the half-life of circulating cholesterol sulfate associated with the plasma and erythrocyte ghost

Fig. 2 represents a typical plot, on a semi-logarithmic scale, of the disappear-

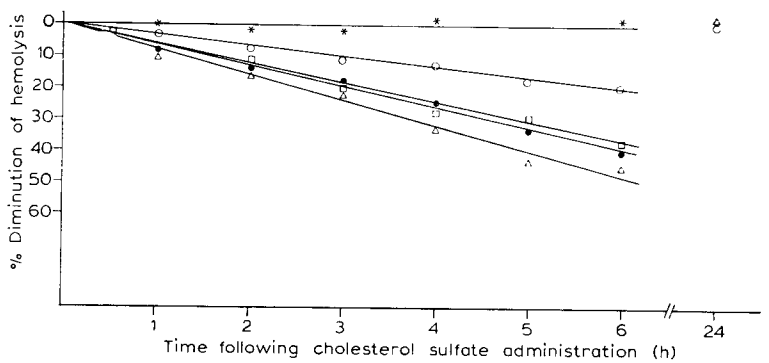

Fig. 1. Stabilization effect of intravenous-administered cholesterol sulfate on erythrocytes in hypotonic media. The following amounts (mg/kg) of cholesterol sulfate were injected; ○—○, dog 1, 0.22 mg; ●—●, dog 2, 0.54 mg; △—△, dog 3, 0.63 mg; □—□, dog 4, 0.74 mg. Dog 5 (*—*) served as the control animal, i.e. the same injection procedure minus cholesterol sulfate.

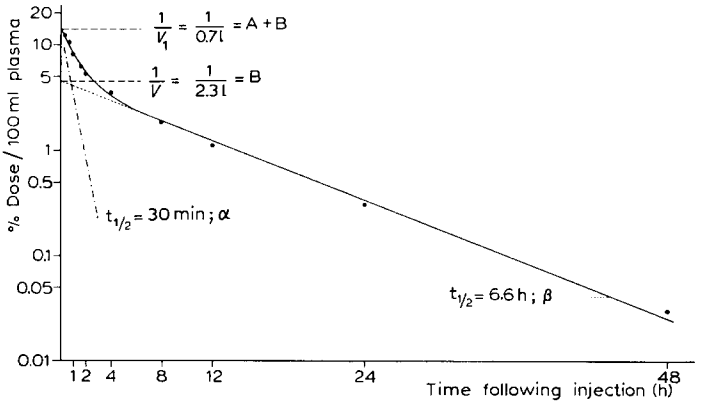

Fig. 2. Disappearance of cholesterol sulfate tracer from plasma. Following the intravenous injection of [^{3}H]cholesterol sulfate ($284 \cdot 10^6$ dpm), serial blood samples were withdrawn at the specified time intervals. The plasma levels of radioactivity associated with the sterol sulfate fraction are plotted on a semi-logarithmic scale as the percent dose administered per 100 ml of plasma vs time (h) following the injection.

ance of the sterol conjugate from the plasma and is characteristic of a double exponential curve or a two-compartmental model, for the time period under study. The plasma levels of radioactivity are plotted as the percent dose injected per 100 ml of plasma vs the time following the injection of the ^{3}H-labelled sterol conjugate. The curve appeared to be linear between 4 and 48 h and these points were used to calculate a regression line by least square analysis. The plasma half-life of cholesterol sulfate calculated from this portion of the curve was 6.6 h. Extrapolation of this curve allowed the calculation of an apparent distribution volume (V) of 2.3 l according to a single compartment model. In four dogs, a mean plasma half-life value was calculated to be 5.8 ± 0.9 h. When the latter part of the curve is extrapolated (intercepting the ordinate at B, Fig. 2) and the resulting calculated plasma concentration is subtracted from the measured values for the first 2 h, a second log-linear curve with a half-life of 30 min is resolved [10]. The curves of disappearance were similar in the four dogs that were studied. At approx. 4 h following injection of the label, the curve flattens out and follows a straight line up to 48 h. In terms of a two-compartment model, there is a rapid distribution into a space of approx. 0.7 l (V_1). This is followed by a relatively slow movement into a total volume ($V_1 + V_2$) of approx. 1.6 l which is the more meaningful volume of distribution than the apparent volume (V) of 2.3 l. The volume of 1.6 l, as calculated from A, B, α and β (Fig. 2) according to Tait et al. [9], does not exceed the volume of the extra-cellular fluid (2.7 ± 0.7 l for dogs of 9.5 kg [11]).

Using the same method of calculation, the half-life of cholesterol sulfate associated with the red cell ghost was found to be 6.7 ± 1.2 h in three dogs. A typical disappearance curve is illustrated Fig. 3. Pertinent data used for the calculation of the disappearance curves (Figs 2 and 3) of labelled cholesterol sulfate from the plasma and erythrocyte membranes (dog 6) are presented in Table I.

Columns four and six of Table I represent the hexane-extractable radioactivity. This data has not been corrected for procedural losses. The amount of radioactivity

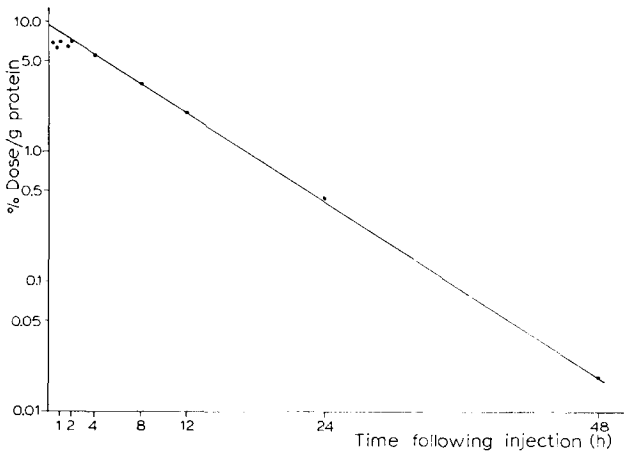

Fig. 3. Disappearance of cholesterol sulfate tracer from erythrocyte ghosts. Following the intravenous injection of [^{3}H]cholesterol sulfate ($284 \cdot 10^6$ dpm), serial blood samples were withdrawn as indicated and the erythrocyte membrane fraction was prepared as described in the text. The radioactivity associated with this fraction is plotted on a semi-logarithmic scale, as the percent of dose administered per g of membrane protein vs time (h) following the injection.

TABLE I

DATA OBTAINED FROM POST-INJECTION PLASMA AND ERYTHROCYTE GHOST SAMPLES FROM DOG 6

Sample	Time after injection of [^{3}H]cholesterol sulfate (h)	Dose/100 ml of plasma (%)	Dose in hexane extract of 100 ml plasma (%)	Dose/g membrane protein (%)	Dose in hexane extract of ghosts/g protein (%)	Circulating cholesterol sulfate associated with erythrocytes (%)
1	0.33	12.1	0.05	6.93	0.10	17.4
2	0.67	10.7	0.07	6.35	0.08	17.9
3	1.0	8.1	0.14	7.01	0.17	24.2
4	1.67	6.1	0.26	6.47	0.35	28.0
5	2.0	5.2	0.46	7.09	0.48	33.2
6	4.0	3.5	0.55	5.54	1.42	36.5
7	8.0	1.9	0.62	3.36	3.34	39.9
8	12.0	1.1	0.72	2.04	3.29	40.5
9	24.0	0.3	0.49	0.45	2.99	35.4
10	48.0	0.03	0.31	0.02	0.78	—

in this fraction illustrates the degree of cleavage of the sterol sulfate as a function of time. This radioactivity is associated with the free sterol fraction as well as the fatty acid-esterified sterol fraction.

That fraction of the circulating sterol sulfate that is associated with the erythrocytes is presented in the last column of Table I. It has been shown previously [2], as well as from the present data, that essentially all of this radioactivity is associated with the erythrocyte ghost fraction.

DISCUSSION

Previous studies to evaluate the possible biological function of cholesterol sulfate have revealed that this sterol conjugate is a naturally occurring entity in plasma and the red cell membrane [1, 2]. The effect of cholesterol sulfate on the in vitro stabilization of the red cell has also been reported [1–3]. That the human red cell avidly binds this sterol sulfate has been demonstrated in vitro [1, 2]. Ina recent publication, Hochberg et al. [12] have reported that, following the intravenous administration of [^{3}H]cholesterol sulfate to the rat, a significant portion of the label was found to be associated with the erythrocytes. In patients with metachromatic leukodystrophy, Moser et al. [13] reported that parenterally administered $Na_2{}^{35}SO_4$ became associated with red cell lipids. The major portion of this radioactivity was tentatively identified as cholesterol sulfate after chromatographic purification.

The above findings have provided the incentive to continue the investigation of the possible role of cholesterol sulfate in the red cell membrane. The amphipathic nature of the sterol sulfate would suggest that cholesterol sulfate possesses an ideal structure for interaction between the hydrophilic and lipophilic components of membrane structure. In the present report, an attempt has been made to verify certain in vitro observations using in vivo techniques with the dog as the experimental animal.

Thus, we have previously reported [2] that the in vitro incubation of human erythrocytes in physiological saline with [^{3}H]cholesterol sulfate results in a 50 % uptake of the label after 1 h. In the in vivo experiments in the dog reported here, a rapid and significant uptake of the label by the red cells is also observed even in the presence of possible competing proteins or lipoproteins of the plasma. As observed in vitro with human red cells [2], in the present experiments virtually all of the radioactivity found to be associated with the dog erythrocyte remained firmly bound to the erythrocyte ghost fraction.

The maximum uptake of the sterol conjugate by the erythrocytes was found to occur between 30 and 60 min, while the maximum stabilization effect of the sterol conjugate on the red cells occurred at approx. 6–7 h. It would appear, therefore, that once the sterol sulfate is in contact with the membrane, a period of time is required for its integration with the membrane structure before the maximal stabilization effects are observed. In two dogs that were studied for longer periods of time, a slow return to the normal hemolytic properties was observed. Normal pre-injection values were attained at approx. 24 h following the administration of the sterol sulfate.

The diminution of hemolysis in vitro as a function of the injected dose of cholesterol sulfate was studied in four dogs. In three of the four dogs the effect of the conjugate on the stabilization of the red cells in hypotonic salt solutions was found to increase with the amount of cholesterol sulfate administered (Fig. 1). Since the circulating levels of cholesterol sulfate in each dog were not analyzed, no correlation betweeen these levels with the response to the dose of the sterol conjugate could be made.

In comparison to the human, circulating levels of cholesterol sulfate in the dog are considerably lower. This finding, along with the report that canine red cells are predisposed to osmotic hemolysis [14], indicating that these cells are relatively leaky, would also support the contention that cholesterol sulfate is implicated in erythrocyte membrane stability.

Several investigators have reported the uptake of steroids by the erythrocyte [15–21]. In addition, steroids such as pregnanolone and testosterone are reported to protect human erythrocytes from osmotic hemolysis [22]. However, in the case of cholesterol sulfate we believe that this is the first instance of a rapid uptake, a firm binding of the entire fraction to the erythrocyte ghost, as well as a significant diminution of hemolysis using physiological levels of a naturally occurring steroidal compound.

The half-life of circulating cholesterol sulfate in the dog was calculated to be of the order of 6–7 h from the second linear portion of the curve. This value is approx. one half of the corresponding value in human subjects where a half-life of 14–15 h was reported [8]. In the latter study, the erythrocyte fraction, following the intravenous administration of labelled cholesterol sulfate, was not examined.

It has been shown previously that, under certain conditions [5], adrenal tissue may transform cholesterol sulfate directly into Δ^3-3β-hydroxysteroid sulfates [5]. In the normal individual, however, this transformation of circulating cholesterol sulfate does not occur to any significant extent [8]. In their recent report, Hochberg et al. [12] have suggested that the latter experiment may be equivocal since radioactive cholesterol sulfate is rapidly and tightly absorbed onto the red cell membrane and, therefore, it is possible that the labelled precursor was not available to the adrenal for subsequent transformation during the experimental period. The present experiments have shown, at least in the dog, that while this uptake is indeed rapid, the sterol sulfate re-enters the plasma after a relatively brief period of time. In fact, the half-life of the sterol conjugate that is associated with the red cell is quite similar to the half-life found in the plasma. Whether a different situation exists in man can be established only by experimentation.

In vivo studies to determine the uptake and half-life of the sterol sulfate associated with the human erythrocyte are currently in progress in this laboratory. In addition, attempts will be made to correlate possible alterations in the binding capacity of red cells for cholesterol sulfate, as well as levels of the sterol conjugate, with the degree of red cell fragility in various pathological conditions.

ACKNOWLEDGEMENTS

The authors are indebted to Mrs G. Huneault for skilfull technical assistance and to Miss M. Généreux for secretarial assistance. Thanks are also due to Dr Erlio Gurpide, New York, for helpful suggestions. This work was supported by Grant MT-3646 of the Medical Research Council of Canada. K.D.R. is an associate of the Medical Research Council of Canada.

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