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METABOLISM OF FREE AND CONJUGATED STEROIDS BY INTACT AND HEMOLYSED MAMMALIAN ERYTHROCYTES

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SUMMARY

1. Experiments on the reduction rate of dehydroepiandrosterone and dehydroepiandrosterone sulfate and of estrone and estrone sulfate by human and rat erythrocytes indicate that both conjugated and non-conjugated steroids can be reduced by the 17β-hydroxysteroid dehydrogenase located in erythrocytes. Rat erythrocytes contain a much higher amount of this enzyme than human erythrocytes.

2. Whereas conversion rates of free and of conjugated steroids by hemolysed erythrocytes showed only small differences, the intact erythrocytes converted the conjugated steroids at a much slower rate than the free steroids. This effect was most pronounced for dehydroepiandrosterone sulfate.

3. The differences in rates of metabolism of dehydroepiandrosterone sulfate and of estrone sulfate by intact and lysed erythrocytes were not paralleled by similar differences in binding characteristics for steroids by intact and lysed cells.

4. The observed differences in reduction rate suggest a difference in permeability of the intact erythrocyte cell membrane for these steroid sulfates and the corresponding free steroids.

INTRODUCTION

It has been established that erythrocytes contain 17β-hydroxysteroid dehydrogenase[1-4]. This $NADP^+$-requiring enzyme has a broad specificity for steroids with a 17-oxo or 17β-hydroxyl group and acts on both estrone and dehydroepiandrosterone as well as on their sulfates[5]. Although the metabolism of steroids by erythrocytes might only be of limited importance compared to metabolism by other tissues[3], the erythrocyte is by virtue of its simple isolation procedure and internal structure a good model

Trivial and systematic names: Dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; dehydroepiandrosterone sulfate, 17-oxo-5-androsten-3β-yl sulfate; androstenediol, 5-androstene-3β, 17β-diol; androstenediol sulfate, 17β-hydroxy-5-androsten-3β-yl sulfate; estrone, 3-hydroxy-1,3,5(10)-estratrien-17-one; estrone sulfate, 17-oxo-1,3,5(10)-estratrien-3-yl sulfate; estradiol, 1,3,5(10)-estratriene-3,17β-diol; estradiol sulfate, 17β-hydroxy-1,3,5(10)-estratrien-3-yl sulfate.

system for some aspects of the study of uptake and metabolism of steroids by cells[6].

The present paper describes the relative rates of reduction of dehydroepiandrosterone and dehydroepiandrosterone sulfate, and of estrone and estrone sulfate by the 17β-hydroxysteroid dehydrogenase during incubation with intact and hemolysed erythrocytes.

Whereas the dehydrogenase is located inside the cell, and is not bound to the membrane, the reduction rates may be considered to be affected by the relative rates of permeation of steroids through the erythrocyte membrane.

MATERIALS AND METHODS

Labeled [4-^{14}C]- and [7α-^{3}H]dehydroepiandrosterone and [4-^{14}C]dehydroepiandrosterone sulfate; [6,7-^{3}H]estrone and [6,7-^{3}H]estrone sulfate were obtained from New England Nuclear Corporation and if necessary they were purified by paper or thin-layer chromatography to a radiochemical purity of over 98%. Unlabeled steroids, $NADP^+$, NADPH and glucose 6-phosphate were purchased from Steraloids and Boehringer.

Incubation experiments

Blood was either withdrawn from normal human subjects by venepuncture or obtained from rats by decapitation. Heparin was used as an anticoagulant. The blood was centrifuged and the upper layer and buffy coat (containing the leucocytes) were carefully removed and the red cells were washed twice with Krebs–Ringer bicarbonate buffer[7]. The concentrated erythrocyte suspension was diluted with the Ringer buffer (containing 5.5 mM glucose) as indicated in the tables and the mixture used as incubation medium. The radioactive substrates were added to the incubation medium in 0.02 ml propylene glycol. For experiments with hemolysed erythrocytes, the red cells were disrupted by sonication (2 times, 5 sec, 21 Kcycles/sec at 4°). In experiments with hemolysed erythrocytes, NADPH (final concentration 0.3 μmole/ml) and glucose 6-phosphate (final concentration 1 μmole/ml) were also added to the incubation medium.

Extraction and identification of steroids

At the end of the incubation the medium was divided in two parts. One part was used for determination of steroid binding to the red cells or to cell fragments as described below. The other part was diluted with water, and free steroids were extracted with ethyl acetate[6]. For removal of conjugated steroids the diluted incubation mixture was extracted once with 4 ml methanol (added very slowly with constant shaking) and twice with 4 ml 80% ethanol per ml of cell suspension. Recovery of the radioactive steroid through this type of extraction was better than 95%. The steroid sulfates were then solvolysed with ethyl acetate at pH 1 (see ref. 8). The ethyl acetate layer was concentrated and chromatographed on paper or thin-layer plates. Dehydroepiandrosterone was separated from 5-androstene-3β,17β-diol and estrone was separated from estradiol-17β in a Bush B1 system or on thin-layer plates in the solvent system benzene–ethyl acetate (2:1, v/v)[9]. After paper chromatography the areas containing radioactivity were localized by scanning with a Packard radiochromatogram scanner and eluted with methanol. The concentrated eluate was dissolved in a modi-

fied BRAY's solution[10]. Radioactivity was counted in a Packard liquid scintillation spectrometer, model 3375. Quench corrections were calculated from external standard ratios.

Binding studies

Part of the incubation mixture was used for determination of the amount of steroid bound to erythrocyte constituents. Binding to intact erythrocytes and to hemolysed erythrocytes was determined by equilibrium dialysis as described previously[6].

Cell-suspension or hemolysate (1 ml) were dialysed in Visking dialysis tubing (type 8/32) against 15 ml of Krebs–Ringer buffer for 40 h at 4°. Under these conditions the steroid dehydrogenase is inactive and the steroids are not reduced. Radioactive steroids in these media were determined as described above. Binding was expressed as steroid bound in percent of the total amount of steroid present in 1 ml of the original incubation mixture.

RESULTS

Conversion of free and conjugated steroids by intact and lysed human erythrocytes

Initial experiments with a partially purified 17β-hydroxysteroid dehydrogenase from human erythrocytes demonstrated that steroid sulfates with a free 17-oxo group could be reduced by this enzyme in the presence of NADPH. Under the conditions used the reduction rate of dehydroepiandrosterone sulfate was about 75% of the re-

TABLE I

REDUCTION OF DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULFATE, ESTRONE AND ESTRONE SULFATE BY INTACT AND HEMOLYSED HUMAN ERYTHROCYTES

Abbreviations: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone 3-sulfate; E, estrone; E-S, estrone 3-sulfate; %, % of incubated amount of steroid reduced. Incubation mixtures with intact erythrocytes contained equal volumes erythrocytes and Ringer solution (5.5 mM glucose). Hemolysed erythrocytes were prepared by sonication of mixtures of intact cells; glucose 6-phosphate and NADPH were added to these mixtures as described in MATERIALS AND METHODS. Purified enzyme preparation (17β-hydroxysteroid dehydrogenase) was obtained from human red blood cells as described elsewhere[5]. NADPH was added to the incubation medium containing this purified enzyme. Incubations were done for 5 h, at 37°. The figures represent average values from at least three experiments.

	Substrate concentration nmole/ml	*% steroid reduced after incubation*		*Ratio (1)**	*% steroid reduced after incubation*		*Ratio (2)***
		DHEA	*DHEA-S*		*E*	*E-S*	
Intact erythrocytes	1.75	33	1.1	30	16	7.5	2.1
	0.035	46	1.0	46	21	7.0	3.0
Hemolysed erythrocytes	1.75	18	10.9	1.6	6.1	10.4	0.59
	0.035	25	12.2	2.0	11.0	14.7	0.75
Purified enzyme from erythrocytes	1.75	38	33	1.2	22	40	0.55
Control incubation without erythrocyte preparation	1.75	0.3	0.8	—	1.0	0.7	—
	0.035	0.5	0.7	—	1.1	1.4	—

* Ratio (1) = $\frac{\%\ \text{DHEA reduced}}{\%\ \text{DHEA-S reduced}}$

** Ratio (2) = $\frac{\%\ \text{E reduced}}{\%\ \text{E-S reduced}}$

duction rate of dehydroepiandrosterone (Table I). Estrone sulfate was reduced at a somewhat higher rate than estrone.

Intact human erythrocytes were incubated in a medium containing glucose in order to provide a sufficient NADPH level in the cell and to favor the reduction of steroids by erythrocyte 17β-hydroxysteroid dehydrogenase[3]. Under these conditions the intact erythrocytes showed marked differences in the reduction rate of the conjugated and non-conjugated steroids (Table I). During a 5-h incubation 33–46% of the dehydroepiandrosterone was converted to androstenediol. In contrast the amount of dehydroepiandrosterone sulfate converted to androstenediol sulfate (1.0–1.1%) did not differ significantly from control incubations.

Conversion of dehydroepiandrosterone to androstenediol by erythrocytes lysed by sonication was about half that by intact cells. In contrast, the amount of dehydroepiandrosterone sulfate converted to androstenediol sulfate by the hemolysate was at least 10 times higher than the amount converted by intact cells (Table I). After lysis of the erythrocytes the ratio in Table I expressing the percent reduction of dehydroepiandrosterone *versus* the percent reduction of dehydroepiandrosterone sulfate was at least 10 times smaller. For estrone and estrone sulfate a comparable, but much less pronounced effect was observed and the difference between the reduction of estrone sulfate by intact and lysed cells was smaller.

Conversion of free and conjugated steroids by intact and lysed rat erythrocytes

The relatively low reduction of steroid conjugates by human erythrocytes required long incubation times (5 h) in order to obtain sufficient product for accurate estimations. Since rat erythrocytes contain a more active 17β-hydroxysteroid dehydrogenase than human erythrocytes the reduction of sulfate-conjugated steroids by in-

TABLE II

REDUCTION OF DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULFATE, ESTRONE AND ESTRONE SULFATE BY INTACT AND HEMOLYSED RAT ERYTHROCYTES

Abbreviations: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone 3-sulfate; E, estrone; E-S, estrone 3-sulfate; %, % of incubated amount of steroid reduced. Incubation mixtures with intact erythrocytes contained 1 vol. erythrocytes and 7 vol. Ringer solution (5.5 mM glucose). Hemolysed erythrocytes were prepared by sonication of mixtures of intact cells; glucose 6-phosphate and NADPH were added to these mixtures as described under MATERIALS AND METHODS. 1.75 nmoles steroid were added per ml incubation medium. Incubations were done for 15 min, at 37°. The figures represent average values from duplicate experiments

	Albumin concentration in medium	*% steroid reduced after incubation*		*Ratio (1)**	*% steroid reduced after incubation*		*Ratio (2)***
		DHEA	*DHEA-S*		*E*	*E-S*	
Intact erythrocytes	—	33.2	7.3	4.5	25	23	1.0
	6%	4.8	1.5	3.2	7.8	2.9	2.7
Hemolysed erythrocytes	—	35.4	71.5	0.49	25	39	0.64
	6%	5.2	5.7	0.91	7.5	4.5	1.6
Control incubation without erythrocyte preparation	—	1.8	0.9	—	1.7	1.8	—

* Ratio (1) = $\frac{\%\ \text{DHEA reduced}}{\%\ \text{DHEA-S reduced}}$

** Ratio (2) = $\frac{\%\ \text{E reduced}}{\%\ \text{E-S reduced}}$

tact and lysed erythrocytes of the rat was also investigated (Table II). The rate of conversion of dehydroepiandrosterone to androstenediol was almost similar for the diluted erythrocyte suspension and the hemolysate. On the other hand the amount of androstenediol sulfate formed from dehydroepiandrosterone sulfate by a hemolysate was much higher than the amount formed by the intact rat erythrocytes.

The reduction rate for estrone sulfate by erythrocytes increased less dramatically following hemolysis. Compared to dehydroepiandrosterone sulfate, conversion of estrone sulfate was much higher in intact cells. When albumin was added to the incubation mixtures, only a small amount of both free and conjugated 17-oxo steroids was reduced.

Binding of free and conjugated steroids to intact and lysed erythrocytes studied by equilibrium dialysis

The binding of free and conjugated steroids to intact erythrocytes and to hemolysates was also studied with the same concentration of steroids and erythrocyte suspension as shown in Table I and II. The amount of different steroids bound to erythrocytes is comparable at 4° and 37°, and remains constant after the first few minutes of incubation. Binding figures obtained in incubation experiments and by equilibrium dialysis are comparable[6]. Binding of steroids to the proteins of an erythrocyte hemolysate can not be easily studied by incubation. Part of the incubation medium was therefore put into a dialysis sac and the binding was studied by equilibrium dialysis (Table III).

TABLE III

BINDING OF FREE AND SULFATE-CONJUGATED DEHYDROEPIANDROSTERONE AND ESTRONE TO INTACT AND HEMOLYSED ERYTHROCYTES

The binding of the steroids was determined by equilibrium dialysis as described in MATERIALS AND METHODS.

$$\% \text{ bound} = \frac{\text{disint./min per ml inside dialysis sac} - \text{disint./min per ml outside dialysis sac}}{\text{disint./min per ml inside dialysis sac}} \times 100$$

The inside of the dialysis sac contained the human erythrocytes in a 1:1 suspension in Krebs–Ringer solution (compare Table I) and the rat erythrocytes in a 1:7 suspension with Krebs-Ringer (compare Table II). The initial concentration of the steroid in the dialysis sac was 1.75 nmoles/ml. The figures are the mean of at least three experiments, the individual measurements differing within the given range.

	% *steroid bound*	
	Intact cells	*Hemolysate*
Human Erythrocytes:		
Dehydroepiandrosterone	72 (70–73)	67 (66–68)
Dehydroepiandrosterone sulfate	54 (45–63)	92 (90–93)
Estrone	82 (81–83)	78 (77–79)
Estrone sulfate	73 (66–80)	84 (81–87)
Rat Erythrocytes:		
Dehydroepiandrosterone	38 (36–39)	39 (39–40)
Dehydroepiandrosterone sulfate	26 (22–29)	27 (22–30)
Estrone	67 (67–68)	66 (65–67)
Estrone sulfate	25 (20–31)	40 (39–42)

During dialysis at 4° only minor amounts (less than 5%) of steroid was reduced by steroid dehydrogenase. The percentage conjugated steroid bound differed significantly from the percentage free steroid bound, but the great difference in the amounts

of steroid sulfate reduced by 17β-hydroxysteroid dehydrogenase by intact and lysed human and rat erythrocytes was not reflected in similar differences in steroid binding capacity by these preparations.

DISCUSSION

The experiments described in this paper demonstrate a marked difference in reduction capacity between intact and hemolysed human erythrocytes for dehydroepiandrosterone sulfate and estrone sulfate. In contrast to this observation for steroid sulfates, steroid reduction by intact and lysed cells was similar for the non-sulfated substances.

Dehydroepiandrosterone sulfate was metabolized very slowly by the human erythrocytes, in the presence as well as in the absence of albumin. Albumin is known to bind both conjugated and free steroids in a non specific manner[11,12], and appears to inhibit reduction of 17-oxo steroids[13]. The presence of the intact membrane resulted in different effects for the conversion rate of free and conjugated steroids. In contrast the addition of a non-specific steroid binding protein such as albumin, has rather similar effects on the conversion rates of these two groups of steroids. Even if after thorough washing, the erythrocytes would have been contaminated with small amounts of plasma proteins it might be expected that the presence of such impurities would influence the conversion rates of steroids in the same way as the presence of albumin. Therefore the differences between the conversion of dehydroepiandrosterone and dehydroepiandrosterone sulfate by intact erythrocytes might still be considered to reflect the presence of the erythrocyte membrane rather than the presence of plasma impurities.

Rat erythrocyte preparations showed similar differences in reduction of conjugated and non-conjugated steroid as human erythrocytes. However, the reduction rate of steroid by rat erythrocytes was much higher in all instances. According to PORTIUS AND REPKE[1] the steroid dehydrogenase activity of rat erythrocytes is at least 100 times higher than that of human erythrocytes.

In a system where the enzyme is freely accessible to the substrate, the reaction rate should be related to the concentration of the unbound steroid. In several experiments it was observed (Table III), however, that the concentration of unbound conjugated steroid was lower in incubations with hemolysed erythrocytes and that such incubations resulted in high rates of steroid reduction. These data indicate that disruption of the membrane structure is more important for conversion of the steroid by the enzyme studied than substrate binding to the cell constituents in this system. Previous studies have indicated that membrane fractions prepared according to different methods contained almost no steroid dehydrogenase activity[5]. The enzyme was completely solubilized on hemolysis. Exogenous NADPH does not effect the reduction rate of steroids by intact erythrocytes with a high $NADP^+$/NADPH ratio, but has a considerable stimulating effect on the reduction of steroids by lysed cells. Therefore the enzyme is probably present in the inside of the erythrocytes. WINS AND SCHOFFENIELS[14] have suggested that most dehydrogenases may be more or less firmly bound to the cell membrane. Regardless of the exact location of this enzyme our data suggest that the intact erythrocyte membrane is a greater barrier for the conjugated steroid than for the non-conjugated steroids to reach the enzyme.

Some of the results obtained with erythrocyte membranes might perhaps be compared with recently published data by HEAP *et al.*[15] concerning uptake of steroids by liposomes (liquid crystals of phospholipids). The ratio of the conversion rates of dehydroepiandrosterone and dehydroepiandrosterone sulfate by the enzyme in intact erythrocytes (Table I) is qualitatively comparable to the ratio of the distribution coefficients for these steroids between saline solutions and liposomes. This could indicate that the enzymic activities are a reflection of these differences in distribution coefficient of free and conjugated steroids. These ratios can of course only be compared on a qualitative base while distribution coefficients of steroids for natural membranes and liposomes may differ considerably.

The rate of dehydrogenation of conjugated steroids was affected by the intact membrane structure of both human and rat erythrocytes in a qualitatively comparable way (Tables I and II), although these membranes differ considerably in lipid composition and magnitude of phospholipid renewal[16]. The much higher rate of steroid metabolism in rat erythrocytes is not a consequence of the differences in membrane structure, however, since erythrocytes of the rat contain more 17β-hydroxysteroid dehydrogenase[1] than human erythrocytes.

Little is known about a possible regulatory function of the plasma membranes for the transport of steroids into cells. In such widely different problems, as the uptake of dehydroepiandrosterone and dehydroepiandrosterone sulfate by liver cells[17] and the secretion of testosterone by testicular interstitial cells[18,19] a role of the cell membrane in steroid transport has been implicated. It may therefore be of importance to know to which extent the results of the experiments described in this paper, which show an effect of the intact erythrocyte membrane structure on steroid metabolism, are also valid under conditions *in vivo* for permeation of steroids, not only into erythrocytes but also through other plasma membranes.

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