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INTERACTION BETWEEN STEROIDS AND MEMBRANES. UPTAKE OF STEROIDS AND STEROID SULPHATES BY RESEALED ERYTHROCYTE GHOSTS

E. MULDER, G. J. M. LAMERS-STAHLHOFEN and H. J. VAN DER MOLEN
Department of Biochemistry, Division of Chemical Endocrinology, Medical Faculty at Rotterdam, Rotterdam, The Netherlands

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SUMMARY

Reconstituted cells were formed by haemolysis of human erythrocytes in hypotonic media followed by resealing of the membranes in isotonic solution. These so-called "resealed erythrocyte ghosts" could be filled with different protein solutions present in the haemolysing medium. Reconstituted cells containing bovine serum albumin or diluted plasma with a large amount of corticosteroid binding globulin (CBG) were prepared by this method.

Uptake of steroids by reconstituted cells was measured using equilibrium dialysis and incubation studies and was compared with the uptake by cells not containing proteins. After washing of the cells containing CBG, the steroid specifically bound by this protein inside the cell could be determined.

Free steroids (testosterone, dihydrotestosterone, androstenedione, dehydroepiandrosterone, oestradiol, progesterone, cortisol) penetrated quickly into the reconstituted cells, compared with the rather slow penetration of conjugated steroids (dehydroepiandrosterone sulphate and oestrone 3-sulphate).

A difference between the amount of cortisol and progesterone bound to CBG in resealed ghosts was only observed if plasma proteins were also present outside the reconstituted cells. Under these conditions the rate of uptake by binding sites inside the ghost was much slower for cortisol than for progesterone. This was not due to differences in membrane permeability but mainly to different distribution coefficients of the steroids between medium and ghosts.

INTRODUCTION

LITTLE is known about a possible regulatory function of the cell membrane for the transport of steroids into cells.

A function of the cell membrane has been implicated to explain the uptake of DHA and DHA-sulphate by liver cells [1], the secretion of testosterone by interstitial cells of the testis [2, 3] and the uptake of progesterone [4], and oestradiol [5] by uterine cells. However, the rapid distribution of steroids over the cell in studies on the uptake of steroids by target cells does not suggest a selective mechanism at the membrane level (see e.g. Ref. 6).

In a previous study [7] on conversion rates of steroids by an enzyme localized inside the cell we concluded that the erythrocyte membrane had different permeabilities for free and for conjugated steroids. DHA and DHA-sulphate were metabolized by 17β-hydroxysteroid dehydrogenase in erythrocyte haemolysates with comparable reaction rate, but the conjugated steroid was converted more slowly than the free steroid by intact erthrocytes.

In the present paper the uptake of different steroids in a model cell system is described. Reconstituted cells ("resealed ghosts" [8, 9]) containing different proteins were formed from membranes of erythrocytes. The advantage of the use of this system is the possibility to vary the protein content of the artificial cell.

The binding of steroids inside these cells varies with the differences in protein content of the cells and can therefore be estimated without homogenisation of the cell, thus avoiding a possible redistribution of steroid as a result of the homogenisation procedures.

EXPERIMENTAL

Materials

The following [^{3}H]-labelled steroids were obtained from New England Nuclear Corp., Frankfurt, Germany or the Radiochemical Centre, Amersham, Bucks., U.K.: [1, 2-$^3H_2$]-labelled 4-androstene-3, 17-dione (50 Ci/mmol), testosterone (37 Ci/mmol), 5α-dihydrotestosterone (49·0 Ci/mmol), progesterone (35 Ci/mmol), cortisol (50 Ci/mmol); [7α-^{3}H]-labelled dehydroepiandrosterone (16 Ci/mmol) and dehydroepiandrosterone sulphate (10 Ci/mmol); [6, 7-$^3H_2$]-labelled oestradiol (56 Ci/mmol) and oestrone 3-sulphate (40 Ci/mmol). When necessary, these steroids were purified by paper chromatography or t.l.c. to a radiochemical purity of over 98%[11]. [U-^{14}C]-sucrose (10 mCi/mmol) was obtained from The Radiochemical Centre.

Unlabelled steroids were obtained from Steraloids, Pawling, N.Y., U.S.A.; and bovine albumin (defatted) from Fluka, Buchs, Switzerland. In the experiments with plasma a dilution of third trimester human pregnancy plasma was used. Steroids were removed from this plasma by shaking with 50 mg/ml charcoal for 30 min at room temperature; followed by centrifugation (20 min, 30,000 g_{av} at 4°C) and filtration.

Preparation of resealed ghosts

Resealed erythrocyte ghosts were prepared as described by Passow[8]. Erythrocytes were isolated from 20 ml of fresh heparinized blood. The plasma layer and the leucocyte-containing layer were separated from the erythrocytes by centrifugation and carefully removed. After resuspension of the erythrocytes in isotonic Tris-buffered NaCl (5 mM Tris and 166 mM NaCl, pH 7·4) the cells were centrifuged again. This washing procedure was repeated twice. The packed erythrocyte suspension was cooled to 0°C, and 3 ml was added to 30 ml of the haemolysing fluid (a 4 mM $MgSO_4$ solution) at 0°C. Various substances were also added, depending on the type of experiment, e.g. bovine albumin (final concentration 4%), human pregnancy plasma (final concentration 3%) or ^{14}C-sucrose. Five min after the haemolysis of the erythrocytes, the haemolysate was made isotonic by addition of 1·75 ml of 3·32 M KCl. The haemolysate was kept at 0°C for another 5 min. Thereafter the suspension was incubated at 37°C for 60 min. Subsequently the ghosts were washed twice with isotonic Tris-buffered NaCl and centrifuged for 10 min at 30,000 g_{av} at 4°C. Approximately 1·5 ml of ghosts were obtained after the final centrifugation. This preparation was used in the experiments on steroid uptake.

Determination of [^{14}C]-*sucrose leakage of ghosts*

In order to estimate a possible leakage from the ghosts, [^{14}C]-sucrose (1 μCi/mg sucrose) was sealed into the ghosts by adding 1 μCi of this label to 30 ml of the haemolysing fluid.

For determination of the total amount of [^{14}C]-sucrose in the ghost suspension, part of this suspension was mixed with a trichloraceticacid solution (final con-

centration 4%). After centrifugation (10 min, 30,000 g_{av}), the supernatant was removed, the residue was again mixed with trichloraceticacid solution (final concentration 10%), centrifuged and a second supernatant was collected. From the combined supernatants containing all the [^{14}C]-sucrose present in the ghosts a sample was taken for counting radioactivity (see measurement of radioactivity).

After incubation with the steroids the ghost suspension was centrifuged (10 min, 30,000 g_{av}) and the amount of [^{14}C]-sucrose in an aliquot of the supernatant was determined.

The loss of [^{14}C]-sucrose by the ghosts was calculated from the ratio of the amount of [^{14}C]-sucrose in the supernatant after incubation and the total amount of [^{14}C]-sucrose in the suspension.

Dialysis of ghosts containing albumin

Ghost suspensions (1 ml) were dialysed in Visking dialysis tubing (type 8/32) against 15 ml of Krebs–Ringer buffer containing radioactive steroids, for 40 h at 4°C. After dialysis the concentration of radioactivity in the media inside and outside the dialysis sac was calculated. In some experiments the ghosts were disrupted by sonication (2 times, 5 sec, 21 K cycles/sec at 4°C).

Incubation experiments

Radioactive and non-radioactive steroids were dissolved in methanol, evaporated to dryness, solubilized by shaking with Krebs–Ringer buffer and transferred to a storage flask. Steroid concentration in this flask was calculated from the amount of radioactive steroid present.

For incubation the concentrated ghost suspension (0·3 ml) was mixed with Krebs–Ringer buffer containing 0·1% bovine albumin (0·7 ml) and an amount of radioactive steroid from the storage flask. After the incubation the ghost suspension (1 ml) was washed 3 times with 5 ml Krebs–Ringer buffer containing 0·1% albumin.

For the study of the uptake of cortisol and progesterone by ghosts as a function of the incubation time, the ghost suspension was washed the first time with 75 ml of Krebs–Ringer buffer containing 0·1% albumin and then twice with 5 ml of this buffer. From the residual ghosts the radioactive steroid was extracted.

When ghosts were incubated in medium containing both plasma and steroid, the binding of the steroid to specific plasma proteins was ensured by a pre-incubation period of 90 min at 0°C.

Separation of bound and unbound cortisol present in plasma samples

To 0·2 ml of diluted plasma incubated with radioactive cortisol 90 mg of ammonium sulphate was added and after 10 min shaking, the solution was centrifuged. The radioactivity was counted in aliquots of the supernatant and original plasma and the percentage steroid bound to plasma determined. For a 10% pregnancy plasma solution the percentage free cortisol ranged from 10% for 1×10^{-9} M to 92% for 3×10^{-7} M cortisol in the solution.

Extraction of steroid and measurement of radioactive samples

Non-conjugated steroids were extracted from the ghost suspension by extracting four times with ethyl acetate. Conjugated steroids were extracted with methanol, followed by two extractions with 80% ethanol [12]. Recovery of radioac-

tive steroids through these extraction procedures was more than 95%. Extracts were evaporated to dryness, the residues were dissolved in 0·2 ml methanol and mixed with 15 ml of a dioxane solution containing 100 g naphthalene, 7 g 2,5-diphenyloxazole (PPO) and 0·3 g 1,4-bis-(5-phenyloxazoly-2-)-benzene (POPOP) per 1 dioxane. Radioactivity in the samples was counted in a Nuclear Chicago Mark I or a Packard model 3375 liquid-scintillation spectrometer until a standard error of 1% was reached. Quench corrections were calculated from external standard ratios. Radioactivity in plasma samples and supernatant of ammonium sulphate precipitates was determined in a solution containing 0·1 ml of the sample and 15 ml of Instagel*.

RESULTS

Estimation of the leakage of the ghosts during the experiments

[^{14}C]-sucrose was added to the buffer used for haemolysis of the erythrocytes and sealed into the ghosts during the preparation procedure of the ghosts. The uptake of steroids into the ghosts was estimated either after incubation studies with incubation times ranging from a few min to several h or with equilibrium dialysis experiments which lasted 48 h. The loss of [^{14}C]-sucrose from the ghosts during the incubation of 4°C was less than 10%. After the incubations the ghosts suspensions were washed; therefore proteins leaked out of the ghosts were washed away and could not influence the figures obtained for the amount of steroid bound inside ghosts. After 48 h of dialysis the loss of [^{14}C]-sucrose ranged from 5 to 10%.

Uptake of steroids by resealed ghosts containing albumin

The uptake of several steroids by resealed ghosts was measured by equilibrium dialysis. A diluted suspension of ghosts inside a dialysis bag was dialysed against a buffer solution with steroid. In the control experiments the ghosts were ultrasonically disrupted before dialysis. The results of these experiments are given in Table 1 (a and b). In another series of experiments (c and d in Table 1) a 4% solution of albumin was added to the medium used for haemolysis of the ghosts. Therefore the ghosts prepared in this way contained a diluted solution of albumin in addition to some residual haemoglobulin. For the non-conjugated steroids almost no difference was observed in the uptake between intact and sonicated ghosts. The amount of steroid taken up by ghosts with albumin was higher in all instances. The amount of steroid bound by the ghosts varied also largely for the different steroids. The uptake increased (for the intact ghosts as well as for the sonicated ghosts) in the sequence cortisol, androstenedione, testosterone, dihydrotestosterone, DHA, progesterone, oestradiol. A difference was observed between intact and disrupted ghosts for the uptake of conjugated steroids. After 48 h of dialysis the amount of conjugated steroid bound by intact ghosts with albumin was only one third of the amount bound by the disrupted fragments and cell contents of the sonicated ghosts. These differences indicate that the uptake of conjugated steroids is a very slow process in contrast to the uptake of the non-conjugated steroids.

This difference could also be demonstrated by incubation of ghosts containing

*Obtained from Packard Instrument.

Table 1. Effect of intracellular contents of ghost and of sonication of ghosts on steroid uptake

Intracellular content of erythrocyte	Treatment	Steroid conc. in medium pmol/ml	Amount (pmol) of steroid uptake by ghost preparation: Testosterone pmol	Androstenedione pmol	DHT pmol	Cortisol pmol	Progesterone pmol	DHA pmol	DHA sulphate pmol	Oestradiol pmol	Oestradiol sulphate pmol
(a) buffer	—	3	3·5	3·5	4·0	3·1	9·7	7·1	5·0	9·4	5·5
	—	75	89	83	96	79	240	181	143	235	119
	—	1500	2430	2150	2700	1380	4860	2530	2420	7720	2390
(b) buffer	S*	3	3·8	4·0	4·9	3·2	11·5	8·4	7·3	—	5·2
	S	75	98	92	123	80	295	210	177	243	134
	S	1500	2690	2150	2580	1460	6080	3930	3300	—	2510
(c) 4% albumin in buffer	—	3	9·2	7·0	13·8	3·5	14·8	21·1	6·3	20·1	13·3
	—	75	243	159	334	89	345	521	224	459	379
	—	1500	6460	3770	8170	1620	7160	10020	3890	16410	7550
(d) 4% albumin in buffer	S	3	9·7	6·7	13·4	3·4	16·1	21·2	20·7	20·2	35·0
	S	75	234	160	370	89	367	477	659	452	1095
	S	1500	6700	3380	7940	1680	7500	9270	11200	15730	20780
Ratio of the amount of steroid in dialysis sacs with intact and with sonicated ghosts (c/d)†			0·9–1·1	0·9–1·1	0·9–1·1	0·9–1·1	0·9–1·0	1·0–1·1	0·30–0·35	1·0–1·1	0·3–0·4

The uptake of steroid by the ghost preparations was estimated using dialysis as described in the method section. Uptake of steroid (pmol) was estimated using dialysis sacs containing 0·5 ml ghosts and 0·5 ml buffer.

Dialysis was carried out against Steroid Solution in Krebs–Ringer bicarbonate buffer.

*S = ghosts were disrupted by sonication.

$$\dagger \text{ratio} = \frac{\text{pmol in dialysis sac with intact ghosts containing albumin}}{\text{pmol in dialysis sac with sonicated ghosts containing albumin}}.$$

The range of the values obtained for the 3 steroid concentrations is given.

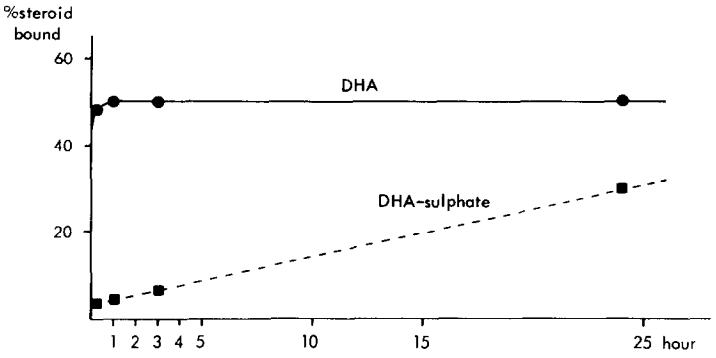

Fig. 1. The rate of uptake of DHA and DHA-sulphate by resealed ghosts containing 4% albumin. Ghosts were incubated in $1{\cdot}5 \times 10^{-6}$ M steroid solution. The amount of steroid bound by the ghosts was expressed as percent of the total amount of steroid present per ml suspension and was plotted on the vertical axis.

4% albumin for different periods of time in a buffer containing DHA or DHA-sulphate (Fig. 1).

Uptake of cortisol and progesterone by resealed ghosts containing diluted plasma

The uptake of steroids by the ghosts containing albumin inside the ghosts as described in the preceding section may reflect both adsorption and non-specific interaction with protein. More specific binding of a steroid to intracellular proteins generally occurs in target cells for steroid hormones [13]. These binding proteins are available only in limited amounts and bind with high affinity. In target tissues the non-specific bound steroid can be distinguished from specific bound steroid by washing of the tissue. The non-specific bound steroid is removed during the washing procedure and the specific bound steroid is retained because this steroid dissociates only slowly from the receptor protein.

The contents of a resealed ghost prepared with diluted plasma containing corticosteroid binding globulin (CBG) has some resemblance to the cytoplasma of a target cell, in as much as a membrane separates a specific steroid binding protein from the medium outside the cell. The Scatchard-type plot [14] obtained after incubation of resealed ghosts containing diluted plasma with different amounts of steroids is given in Fig. 2A. A similar effect as found after washing of target tissues was obtained after washing of the ghosts. The non-specific bound steroid is almost completely removed and a plot characteristic for binding with high affinity at sites with a low capacity was obtained (Fig. 2B). The ratio bound–unbound shows a sharp decrease with increasing amount of steroid.

With ghosts resealed without plasma, but incubated in diluted plasma solutions with steroids and subsequently washed, only a horizontal line in a Scatchard type plot, characteristic for non-specific binding at sites with a large capacity and low bound–unbound value, was obtained. This demonstrated that the specific binding of steroid to ghosts resealed in a diluted plasma solution is not due to contamination with plasma on the outside of the ghosts. Horizontal lines were also obtained with heat denaturated plasma sealed inside the ghosts (see dotted lines in Fig. 2B).

The number of specific binding sites for cortisol did not differ significantly from those available for progesterone (Fig. 2). However, non-specific binding was much higher for progesterone than for cortisol as reflected by the much higher

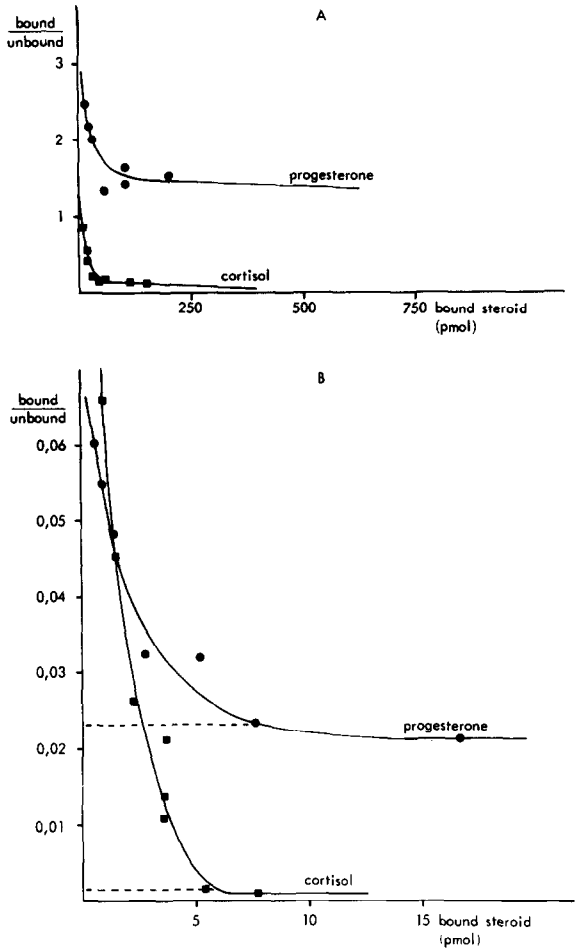

Fig. 2. Effect of washing of ghosts on the retention of progesterone and cortisol by resealed ghosts containing 3% plasma. A. Ghosts were incubated for 30 min at 4°C in Krebs–Ringer bicarbonate buffer containing different amounts of steroid. Ghosts were separated from the medium by centrifugation and the amount of steroid bound by the ghosts was determined. B. The ghosts were incubated for 30 min at 4°C in steroid solutions in Krebs–Ringer buffer as indicated in Fig. 2A. After the incubation the ghosts were washed three times with 0·1% albumin solution in Krebs–Ringer bicarbonate buffer as described in the method section. Thereafter the amount of steroid bound was determined. The dotted lines were obtained with ghosts containing heat denaturated plasma proteins (heated 15 min at 60°C). "Bound–unbound" on the vertical axis reflects the ratio of the amount of steroid bound by the ghosts to the amount of free steroid in the incubation medium. On the horizontal axis the pmol steroid bound by 0·3 ml of ghosts suspended in 1 ml buffer is plotted.

value of the bound–unbound ratio for progesterone at the right side of the graph. This is in agreement with the results obtained with ghosts containing albumin, as described in the preceding section. In most experiments the incubation of the ghosts with steroids was performed at 4°C. At 37°C the uptake of cortisol and progesterone by ghosts containing diluted plasma was similar to the uptake at 4°C. Only the amount of both steroids bound inside the cell was somewhat lower.

Results on the influence of the incubation time on the uptake of steroid by the

ghosts containing plasma are presented in Fig. 3. After the incubation with steroid the ghosts were washed quickly in a large volume of buffer in order to prevent a further uptake of steroid. The concentration of steroid in the incubation medium was high enough to ensure that a large part of the steroid is bound specifically to the plasma inside the ghost (Curves 1 and 2 in Fig. 3A). With heat denaturated plasma sealed into the ghosts a much lower value for the amount of steroid bound was obtained after washing (Curve 3 and 4 in Fig. 3A). Incubation of ghosts containing plasma for different periods of time in a Krebs–Ringer buffer (Fig. 3A) showed no measurable effect of the incubation time on the specific binding to the CBG in the ghosts. There was no difference in specific binding for incubation times ranging from a few minutes to several hours. When the ghosts with plasma inside the membrane were incubated in a medium containing plasma together with a small amount of steroid, an effect of incubation time on uptake of cortisol was observed, but no effect on progesterone uptake was measurable (Fig. 3B). During this last type of incubation experiment a large part of the steroid was bound to plasma proteins outside the cell (Table 2). Of the free cortisol a small

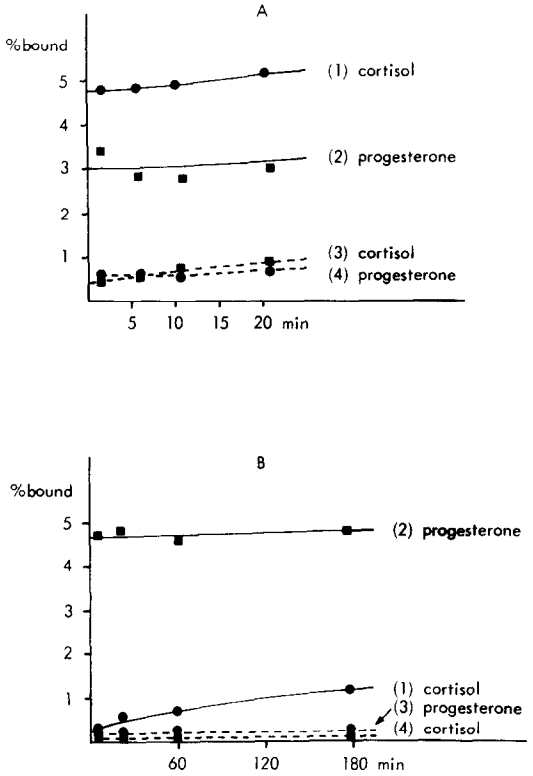

Fig. 3. Effect of incubation time on the specific binding of progesterone and cortisol to steroid binding globulin inside resealed ghosts. Curves 1 and 2 reflect results obtained with ghosts which contained plasma proteins, including CBG, from diluted pregnancy plasma. Curves 3 and 4 were obtained with ghosts containing heat denaturated plasma protein (heated for 15 min at 60°C). A. Incubations of ghosts in Krebs–Ringer buffer with 2×10^{-8} M radioactive steroid. B. Incubations of ghosts in Krebs–Ringer buffer containing 10% plasma and 1×10^{-9} M radioactive steroid. The percentage of the steroid bound to the ghosts was measured after washing the ghosts in a large volume of isotonic saline as described in the method section.

Table 2. Effect of incubation time on uptake of cortisol by CBG containing ghosts. Resealed ghosts containing plasma CBG were incubated in medium with 10% plasma and 1×10^{-9} M ^{3}H-cortisol for various periods of time

Incubation time min	Steroid bound to plasma protein outside ghosts (1) %	Free steroid in medium (1) %	Steroid bound to ghosts before washing (2) %	Steroid bound to CBG in ghosts after washing (3) %
0	87–89	11–13	—	—
5	72–73	15–16	11–12	0·25–0·35
20	66–69	14–17	14–19	0·50–0·55
60	67–75	11–13	15–21	0·60–0·65
180	64–67	15–18	18–19	1·0–1·1

(1) The amount of free steroid in the medium and steroid bound to plasma proteins was determined by an ammonium sulphate precipitation procedure as described in the method section.

(2) The amount of steroid bound to ghosts before washing was calculated from the total amount of steroid added and the amount of steroid recovered from the medium after incubation.

(3) These figures are also plotted in Fig. 3B (curve 1). Ghosts were washed after incubation as described in the method section.

part was immediately bound in a non-specific way to the ghost. The binding of cortisol inside the cell to CBG increases slowly during the incubation period.

DISCUSSION

The uptake of steroids by cells in a tissue or by isolated cells cannot be studied by simply measuring the steroid bound to the cell fragments obtained after homogenisation because a redistribution of steroids occurs during the homogenisation procedure [7]. In the reconstituted cell system used for the experiments described in this paper, the content of the model cell can be varied. The binding of steroids by proteins inside these resealed ghosts depends on the cell contents and differences in the amount and quality of these proteins inside the cell result in differences in the quantity of total steroid bound by the cell preparation. The total amount of steroid bound by the resealed ghost preparation therefore reflects binding to proteins inside these ghosts. This is clearly demonstrated for example by curves 1 and 3 in Fig. 3A where a difference in heat treatment of the protein sealed into the ghosts resulted in large differences of the total amount of steroid bound by the ghosts.

It has been reported that resealed ghost membranes retain a number of functional characteristics of the original cell membrane, such as active transport of cations and impermeability for proteins and sugars [8, 9]. On the other hand the ghost preparations are heterogeneous in volume distribution and a difference with the original erythrocyte membrane has been shown by electron microscopic studies [10]. The changes which occur in the membrane during ghost formation cannot be completely reversed by a resealing procedure as demonstrated by the use of certain phospholipases [15]. The resealed ghost is, however, a model system which is more closely related to the original cell membrane than for example liposomes (artificial cell system formed from phospholipids) [16].

In a previous communication we have observed differences in conversion rates of free and conjugated steroids by 17β-hydroxysteroid dehydrogenase inside erythrocytes [7] and we concluded that conjugated steroid (DHA-sulphate and oestradiol-sulphate) penetrated more slowly into intact erythrocytes than free steroids.

The penetration rate of DHA-sulphate and oestradiol-sulphate into the resealed ghosts containing a diluted albumin solution was also slow compared to the rate of penetration of non-conjugated steroids. For the free steroids the distribution between ghosts and medium is in agreement with the results found for the distribution of steroids between erythrocytes and buffer [17] and between liposomes and buffer [16]. In general a lower percentage of the more polar steroid was bound than the less polar steroids. In the course of a study on the uptake of progesterone and cortisol by uterine tissue Milgrom and Baulieu [4] observed a higher binding of progesterone by a CBG-like protein inside the uterine cells. These authors considered the higher rate of metabolism of cortisol in the circulation and a permeability barrier for cortisol as an explanation for this difference.

We have, however, not observed a barrier effect of the membrane for the uptake of cortisol in the experiments with ghosts containing CBG. Only in the more complicated system in which plasma proteins were present both inside and outside the ghosts was the rate of uptake of cortisol much slower than the uptake of progesterone.

In some recent publications on uptake of steroids by cells a carrier function for a plasma protein was suggested. For liver cells CBG (transcortin) is suggested as a possible steroid carrier protein [18, 19] and in uterine tissue a protein might be involved in oestradiol uptake [5]. This hypothesis is supported by indirect evidence, because direct measurements of the uptake of steroids were not possible due to the redistribution effects during homogenisation. In the present studies uptake of steroids through an inert membrane structure (the red cell membrane) is relatively fast and addition of plasma proteins to the outside medium did not increase the rate of steroid uptake. Although this model membrane system will only have some general membrane characteristics in common with the target cell membranes, our results could reflect that a carrier system need not necessarily be present to ensure a fast uptake of steroid hormones.

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