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LOCALIZATION AND CHARACTERIZATION OF STEROID BINDING SITES OF HUMAN RED BLOOD CELLS*

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SUMMARY

It has been described previously that human erythrocytes contain steroid dehydrogenase activities and that erythrocytes can bind steroids. In the present studies the binding of steroids by human erythrocyte fractions (haemoglobin-containing membranes, haemoglobin-free membranes, the membrane-free haemolysate, a membrane protein preparation and a 17β-hydroxysteroid dehydrogenase preparation) was investigated by equilibrium dialysis.

1. The membrane protein preparation showed the highest combining affinity for testosterone. The binding sites of all the membrane preparations could not be saturated with testosterone. In the sequence of Δ^5-pregnenolone, 20α-dihydroprogesterone, progesterone, oestradiol, Δ^5-androstenediol and testosterone a decrease in combining affinity of haemoglobin-free membranes for the steroids was observed.

2. The binding of testosterone by the membrane-free haemolysate showed a lower combining affinity than the value obtained with the membrane preparations for this steroid.

3. Testosterone was bound in a specific way by a fraction from the membrane free haemolysate, containing 17β-hydroxysteroid dehydrogenase activity. An apparent association constant for binding of testosterone by this fraction was found to be in the order of magnitude of 10^8 M^{-1}. This enzyme fraction was further characterized by gel chromatography.

INTRODUCTION

Relatively little is known about the mechanism by which steroid hormones are taken up by tissues. Extracellular plasma proteins and intracellular receptors may be the most important regulators of this process[1]. The passage of steroid hormones

The following trivial names have been used throughout this paper: Δ^5-androstenediol, 5-androstene-3β,17β-diol; androstenedione, 4-androstene-3,17-dione; 20α-dihydroprogesterone, 20α-hydroxy-4-pregnen-3-one; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; oestradiol, 1,3,5(10)-estratriene-3,17β-diol; Δ^5-pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; testosterone, 17β-hydroxy-4-androsten-3-one.

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through the cell membrane has been considered mainly as a simple diffusion process[2,3], during which the steroids are in an unconjugated form and not bound to proteins. The role of the cell membrane in this process is not known. In order to obtain more information about the uptake of steroids by cells and especially about the function of the membrane in this uptake process, the human erythrocyte was chosen as a model system. The human erythrocyte is a relatively uncomplicated cell type, which can easily be isolated and which contains no nucleus or mitochondria.

From our previous studies concerning the uptake of steroids by intact erythrocytes *in vitro*[4], it appeared that the uptake was independent of time, temperature and steroid concentration. However, it was impossible to conclude where the bound steroid was localized in the intact erythrocyte. In the present investigation an attempt was made, therefore, to localize and characterize possible binding sites for steroids in the erythrocyte. For these studies cell membranes and membrane-free fractions of human erythrocytes were used.

MATERIALS AND METHODS

The following radioactive steroids were employed: Δ^5-[7α-^{3}H]androstenediol (spec. act. 15.9 Ci/mmole); [4-^{14}C]androstenedione (spec. act. 60.0 mCi/mmole); 20α-[1,2-$^3H_2$]dihydroprogesterone (spec. act. 32.7 Ci/mmole); 5α-[1,2-$^3H_2$]dihydrotestosterone (spec. act. 49.0 Ci/mmole); [6,7-$^3H_2$]oestradiol (spec. act. 40.0 Ci/mmole); Δ^5-[7α-^{3}H]pregnenolone (spec. act. 14.7 Ci/mmole); [1,2-$^3H_2$]progesterone (spec. act. 50.3 Ci/mmole); [1,2-$^3H_2$]testosterone (spec. act. 37.0 Ci/mmole); [4-^{14}C]-testosterone (spec. act. 58.8 mCi/mmole).

All radioactive and non-radioactive steroids were obtained from commercial sources and were purified by chromatography before use. [^{131}I]Albumin was obtained from Hoechst with a specific activity of 0.1 mC [^{131}I] per mg Albumin. Techniques for chromatography have been described elsewhere[5].

Preparation of erythrocyte fractions

Heparinized human blood was centrifuged at 1200 × *g* for 15 min. The plasma and leucocyte layer were discarded and the cells were washed 3 times with 2 vol. 0.9 % NaCl solution. The washed erythrocytes were haemolysed with 2.5 vol. 0.05 M phosphate buffer (pH 7.4). The total haemolysate was centrifuged at 30000 × *g* for 20 min. The membrane fraction was collected. Haemoglobin-free membranes were prepared by washing the membrane fraction several times with phosphate buffer, according to the method of Dodge *et al.*[6]. Membrane protein was isolated by a method described by Maddy[7]. The membrane-free haemolysate was fractionated by a modification of the $(NH_4)_2SO_4$ fractionating procedure used by Jacobsohn and Hochberg[8]. Equal amounts of supernatant and a saturated $(NH_4)_2SO_4$ solution (pH 7) were mixed and stirred overnight at 4 °C. The precipitate was collected after centrifuging at 30 000 × *g* for 20 min and dissolved in a solution with a final concentration of $(NH_4)_2SO_4$ of 20 % of saturation (140 g $(NH_4)_2SO_4$/l). This solution was again centrifuged and any residual precipitate discarded. The supernatant was mixed with $(NH_4)_2SO_4$ to a concentration of 50 % of saturation (350 g $(NH_4)_2SO_4$/l) and the $(NH_4)_2SO_4$ precipitation procedure was repeated.

Desalting of $(NH_4)_2SO_4$-containing solutions was carried out by chromatography on Sephadex G-25.

Extractions

For isolation of steroids 0.5-ml samples of the erythrocyte fractions were extracted 4 times with 2 ml ethyl acetate. Recoveries of added radioactive steroids were in the order of 98–100 %.

Radioactivity

Radioactivity in the samples was measured in a Nuclear Chicago Mark I liquid scintillation counter. Aqueous samples were counted after mixing 0.5 ml of the samples 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-phenyloxazolyl-2)-benzene (POPOP) per l dioxane. Ethyl acetate extracts were evaporated under N_2, the residues were dissolved in 0.1 ml methanol and counted after addition of 15 ml dioxane scintillator.

Protein estimations

Protein was estimated by the method of Lowry[9].

Equilibrium dialysis

Equilibrium dialysis was performed using 1 ml of a protein solution in a dialysis sac (Visking dialysis Tubing, Type 8/32) placed in a vial with 14.5 ml 0.05 M phosphate buffer (pH 7.4), containing an appropriate amount of radioactive (0.05 μCi) and non-radioactive steroid. The steroid concentrations used in the dialysis systems are given in the legends to Fig. 1–4. The steroid concentration in the dialysis sac [B] + [U] can be calculated from the value B/U and the corresponding B value. Equilibrium was obtained within 40 h. The percentage binding was calculated as:

$$\% \text{ Binding} = \frac{\text{(dpm/ml) inside} - \text{(dpm/ml) outside}}{\text{(dpm/ml) inside}} \cdot 100$$

Binding results were represented in so-called Scatchard curves[10,11], $B/U = f(B)$, based on the relationship: $B/U = K\ (n\text{P}—\text{B})$. B = bound steroid in moles/l; U = unbound steroid in moles/l; K = association constant; n = number of binding sites per mole of protein; P = protein in moles/l. When B = 0, then $B/U = K \cdot n \cdot P$ and when B/U = 0, then B = nP. If U approaches zero then B/U approaches its maximum value, which occurs when B approaches zero. When in a Scatchard curve B/U is independent of B then the formula $B/U = K\ (n\text{P}—\text{B})$ becomes $B/U = K \cdot n \cdot P$. In most cases the molecular weight of the binding protein is not known, therefore K cannot be calculated. An impression about the affinity of the steroid for the protein, however, can be obtained from the "combining affinity", which is defined as $C = B/U \cdot 1/n\text{P}$ l per g protein. nP is expressed in g protein/l[12]. All binding experiments were done at 4 °C unless otherwise stated.

A plasma protein preparation rich in the testosterone-binding plasma protein was prepared from Cohn fraction IV using the isolation procedure described by Mercier-Bodard[13].

Gel chromatography

This was performed on a 25 mm × 1000 mm column of Sephadex G-150 equilibrated at 4 °C with 0.05 M phosphate buffer (pH 7.0), containing 0.1 M KCl, 1 mM EDTA, 1 mM mercaptoethanol and 0.02 mM NADP. Elution of 4-ml protein samples (10–25 mg protein/ml) was performed at 4 °C using an upward flow of 15–20 ml/h. Fractions of 6 ml each were collected.

17β-Hydroxysteroid dehydrogenase activity

Activity in the column eluates was estimated by measuring the conversion of testosterone to androstenedione. Fractions (1 ml) from the column eluates were incubated with 0.4 ml 0.4 M Tris–HCl buffer (pH 8.5) containing 0.15 M NaCl, 7 mM $MgCl_2$, 0.3 mM NADP, 0.05 μCi [1,2-$^3H_2$]testosterone and 10 μg non-radioactive testosterone. The incubations were carried out for 3 h at 37 °C in a shaking water bath. At the end of the incubation period known amounts of [^{14}C]testosterone and [^{14}C]androstenedione were added to correct for losses during isolation and the steroids were extracted immediately with ethyl acetate. Androstenedione was separated from testosterone on silicagel thin-layer plates in the solvent system benzene–ethyl acetate (3:2, v/v). The areas containing radioactivity were localized after chromatography by scanning with a thin-layer plate scanner and were eluted with methanol. The concentrated eluate was dissolved in 15 ml dioxane scintillator and counted. Corrections for loss of steroid during isolation were calculated from $^{14}C/^3H$ ratios as described previously[5].

RESULTS

After haemolysis of intact erythrocytes the total haemolysate was separated into membranes and a membrane-free haemolysate.

Each fraction was diluted with buffer to the same protein concentration. Most of the protein in the membrane-free haemolysate consisted of haemoglobin. The membrane fraction contained much less but still a considerable amount of haemoglobin. The results of the binding studies of testosterone by the haemolysate fractions are shown in Fig. 1. The membrane fraction showed a higher combining affinity for testosterone in comparison with the total haemolysate and the membrane-free haemolysate. It was not possible to saturate the binding sites of any of these preparations with steroid within the concentration range used.

Binding by membrane preparations

The membrane fraction was further investigated in binding studies with haemoglobin-free membranes. The haemoglobin-free membranes[6] were resuspended in a volume of phosphate buffer, equal to the volume of the original amount of packed cells. This dilution was taken in order to compare binding data for this membrane suspension with binding data from erythrocytes suspended in an equal volume of isotonic buffer. The results of these binding studies are shown in Fig. 2. Δ^5-Pregnenolone showed the highest combining affinity. Within the steroid concentration range used no saturation of binding sites could be observed.

The haemoglobin-free membranes were further fractionated by a butanol-

extraction procedure according to the method of Maddy[7]. With this method it is possible to prepare a lipid-free protein fraction which contains almost all the membrane proteins. The results of binding studies with testosterone by this membrane

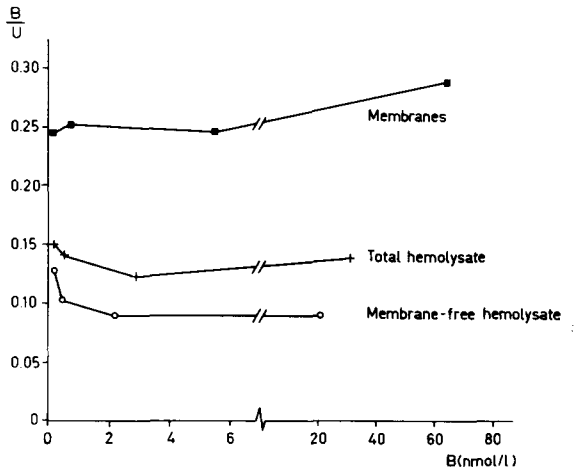

Fig. 1. Binding of testosterone by different erythrocyte fractions. Each protein fraction was dialysed at 4 °C against 0.058, 2.373, 23.73 and 237.3 nM testosterone, respectively. Protein concentration of each fraction: 20 mg/ml.

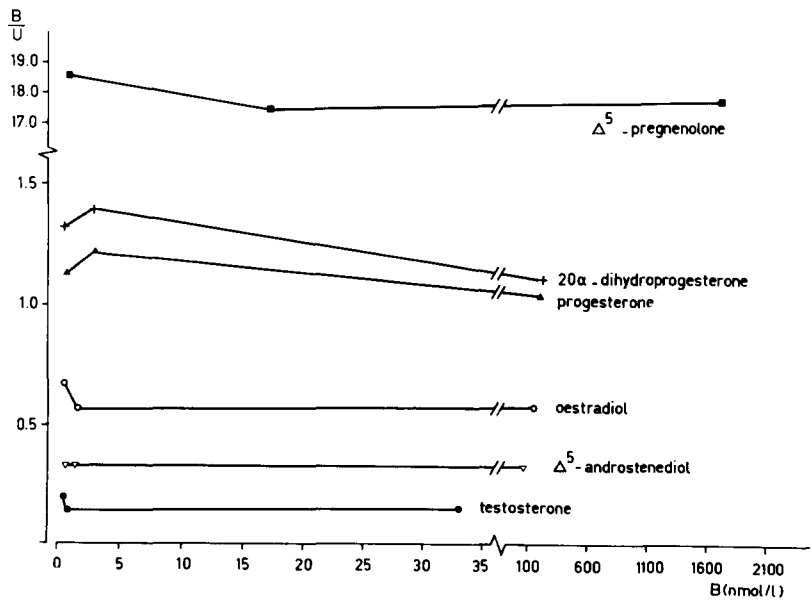

Fig. 2. Binding of different steroids by haemoglobin-free membranes. The membranes were dialysed at 4 °C against the following steroid concentrations: 0.095, 2.205 and 211.1 nM Δ^5-pregnenolone; 0.170, 2.293 and 212.5 nM progesterone; 0.690, 2.989 and 230.6 nM Δ^5-androstenediol; 0.042, 2.493 and 245.1 nM oestradiol; 0.063, 2.173 and 211.0 nM 20α-dihydroprogesterone; 0.289, 2.604 and 231.8 nM testosterone. Protein concentration: 2 mg/ml.

protein fraction and by the haemoglobin-free membranes are presented in Fig. 3. Both preparations were diluted to the same protein concentration. The binding sites of both preparations could not be saturated within the steroid concentration range used. The combining affinity of testosterone for the membrane protein was found to be higher than for the haemoglobin-free membranes. The binding studies presented in Fig. 3 were carried out at 4 °C. Because DeVenuto *et al.*[14] found only

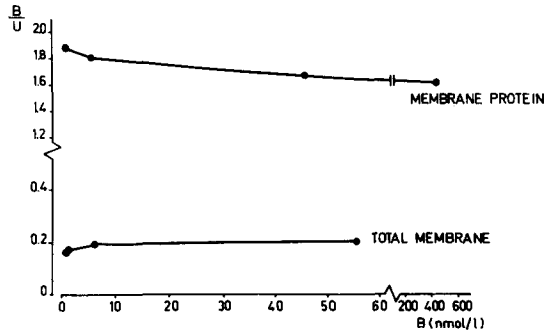

Fig. 3. Binding of testosterone by haemoglobin-free membranes (total membrane) and by a membrane protein fraction. Each fraction was dialysed at 4 °C against 0.058, 2.373, 23.73 and 237.3 nM testosterone, respectively at 4 °C. Protein concentration of each fraction: 2 mg/ml.

TABLE I

BINDING OF PROGESTERONE BY HAEMOGLOBIN-FREE MEMBRANES (TOTAL MEMBRANE) AND BY A MEMBRANE-PROTEIN FRACTION AT 4 °C AND AT 37 °C

Binding was estimated by equilibrium dialysis. Binding data are expressed as:

$$\frac{\text{bound steroid}}{\text{unbound steroid} \times \text{protein concn in mg/ml}}$$

Observed ranges of the combining affinity are given in parentheses.

	4 °C	*37 °C*
Total membrane	0.83 (0.81–0.85)	1.05 (0.93–1.18)
Membrane-protein	4.45 (3.93–5.21)	1.63 (1.53–1.65)

a small difference between the binding of progesterone by haemoglobin-free membranes and by a membrane protein fraction at 37 °C, we have investigated the binding of progesterone by the haemoglobin-free membranes and our membrane protein fraction both at 4 °C and at 37 °C. The results of these studies are presented in Table I. Although the combining affinity of progesterone for haemoglobin-free membranes was higher at 37 °C than at 4 °C, the combining affinity of progesterone for the membrane protein fraction showed the opposite result. At 37 °C the combining affinity of progesterone for the membrane proteins was smaller than at 4 °C.

Binding by membrane-free preparations

From previous studies it was concluded by Mulder *et al.*[15] that the 20–50 % saturated $(NH_4)_2SO_4$ precipitate of the membrane-free haemolysate contained a relatively high amount of 17β-hydroxysteroid dehydrogenase activity. The binding

of testosterone by this enzyme fraction was investigated and is shown in Fig. 4. It is evident from the Scatchard curve that there are two types of binding by this fraction: a specific one (with a limited number of high affinity binding sites) and a non-specific one (with a large number of low affinity binding sites). The apparent association constant for the high affinity sites was in the order of magnitude of 10^8 M^{-1}.

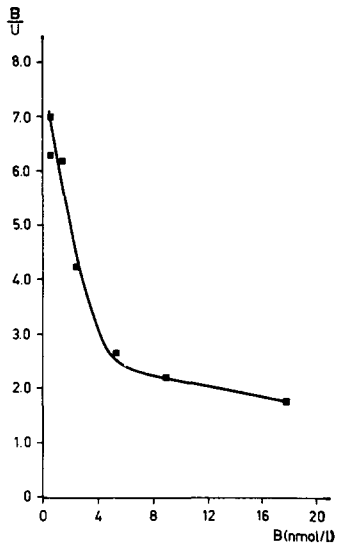

Fig. 4. Binding of testosterone by an erythrocyte fraction with steroid dehydrogenase activity ("17β OHSD-fraction"). This protein fraction was dialysed at 4 °C against the following testosterone concentrations: 0.058, 0.116, 0.232, 0.694, 2.373, 4.687 and 11.631 nM. Protein concentration: 45 mg/ml.

We have considered that this binding might result from plasma proteins "trapped" by the packed cells and not removed during three saline washings and during isolation and purification of the 17β-hydroxysteroid dehydrogenase. With [^{131}I]albumin, added to the heparinized blood as a marker, the amount of "trapped" plasma in the cells was found to be 200 μl per 600 ml packed cells after three saline washings. Therefore two enzyme fractions were prepared: the first one from 600 ml packed cells to which 200 μl plasma were added and the second one also from 600 ml packed cells but without further additions. According to the results in Table II no increase in the number of high affinity binding sites for 5α-dihydrotestosterone was observed in the first preparation as compared with the second preparation after equilibrium dialysis at 4 °C.

An explanation for the specific binding of testosterone by this enzyme preparation might be specific binding to active sites of the enzyme itself. Therefore attempts were made to separate 17β-hydroxysteroid dehydrogenase activity from binding activity by gel chromatography on a Sephadex G-150 column. The collection of the fractions during this separation was based on the 17β-hydroxysteroid dehydrogenase distribution pattern and on the protein distribution pattern. After gel chromatography of the enzyme preparation four protein fractions were collected: A, B, C and D (see Fig. 5).

TABLE II

COMPARISON OF THE BINDING OF 5α-DIHYDROTESTOSTERONE BY A "17β OHSD-FRACTION" PREPARED FROM 600 ml SALINE-WASHED PACKED CELLS WITHOUT ADDED PLASMA WITH THE BINDING OF 5α-DIHYDROTESTOSTERONE BY A "17β OHSD-FRACTION" PREPARED FROM 600 ml SALINE-WASHED PACKED CELLS CONTAINING AN ADDITIONAL AMOUNT OF 200 μl PLASMA

Binding was estimated by equilibrium dialysis at 4 °C and binding data are expressed as bound steroid/unbound steroid. Protein concentration of both fractions: 36 mg/ml.

Steroid concn in dialysis system (nM)	*Bound steroid / Unbound steroid*	
	No additions	*200 μl plasma added*
0.058	7.40	6.69
0.058	7.70	6.69
2.373	1.72	1.54
2.373	1.64	1.82

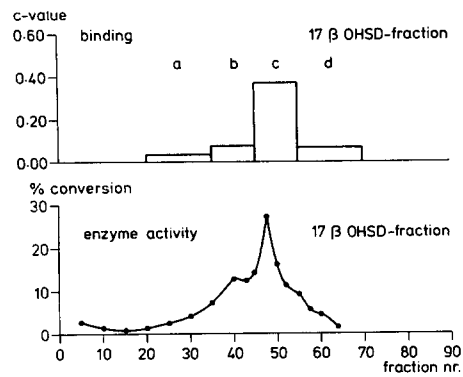

Fig. 5. Gel chromatography elution pattern on Sephadex G-150 of the erythrocyte fraction with hydroxysteroid dehydrogenase activity (17β OHSD-fraction). 4 ml of a protein sample (10–25 mg/ml) were applied to a 25 mm × 1000 mm column of Sephadex G-150 equilibrated at 4 °C with 0.05 M phosphate buffer (pH 7.0), 0.1 M KCl, 1 mM EDTA, 1 mM mercaptoethanol, 0.02 mM NADP. Elution was performed at 4 °C with the same buffer and using an upward constant flow (15–20 ml/h) obtained by use of a Mariotte flask. Fractions of 6 ml each were collected. Binding of testosterone after equilibrium dialysis was expressed as the combining affinity[12] (upper part). Enzyme activity was expressed as % testosterone converted to androstenedione by 1 ml of the collected fractions (see Materials and Methods) (lower part).

Fractions A and B contained the major amount of protein with molecular weights > 100000. Fraction C contained the 17β-hydroxysteroid dehydrogenase (molecular weight: 70000)[15] while Fraction D mainly contained haemoglobin. From each fraction the 50% saturated $(NH_4)_2SO_4$ precipitate was prepared. The four different protein precipitates were dissolved to the same protein concentration and the binding of testosterone by these fractions was estimated using equilibrium dialysis. Binding was expressed as the combining affinity (*C* value). Specific binding of testosterone could only be demonstrated with Fraction C, the fraction with the highest 17β-hydroxysteroid dehydrogenase activity.

Binding of [1,2-$^3H_2$]testosterone to the erythrocyte enzyme preparation during gel filtration could not be demonstrated (Fig. 6). All the radioactive testosterone applied on the Sephadex column was recovered as unbound steroid. Testost-

erone binding during gel filtration could be demonstrated, however, with the testosterone-binding globulin fraction isolated from plasma and the 20–50 % saturated $(NH_4)_2SO_4$ precipitate of plasma. The testosterone-binding globulin was eluted between fraction number 35 and 45, while the testosterone radioactivity bound to proteins of the 20–50 % saturated $(NH_4)_2SO_4$ precipitate of plasma was eluted between fraction number 35 and 50 (see Fig. 6).

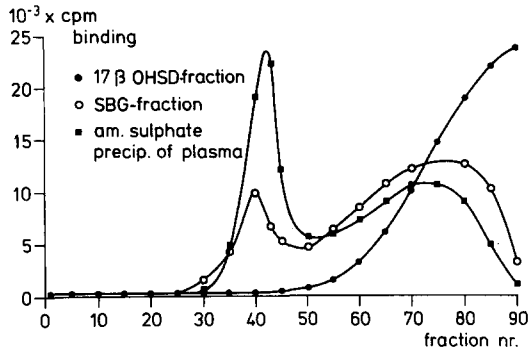

Fig. 6. Gel chromatography elution patterns on Sephadex G-150 of the erythrocyte fraction with hydroxysteroid dehydrogenase activity (17β OHSD-fraction), a sex steroid-binding globulin (SBG) fraction and the 20–50% saturated $(NH_4)_2SO_4$ precipitate of plasma. Binding of testosterone by the three protein fractions during gel chromatography was estimated as follows: 4-ml protein samples with [1,2-$^3H_2$]testosterone to a final steroid concentration of $2.8 \cdot 10^{-8}$ M were applied to the column. Chromatography conditions were the same as described in Fig. 5. 0.5 ml of the collected fractions was analysed for radioactivity.

DISCUSSION

In current concepts on mechanism of action of steroid hormones it is generally assumed that the steroid has to be taken up by the tissue cells followed by binding of the steroid to a specific cytoplasmic receptor and finally the transport of the steroid to the nucleus[1]. In which way the steroid is taken up by the target cell is not precisely known. There are indications that the target cell membrane is freely permeable for steroids and that the steroids will enter the cell by a simple diffusion process[2]. Only Gross *et al.*[16] postulated an active transport of cortisol through the membrane of mouse fibroblasts. It is generally accepted that during the passage of the cell membrane the steroid is not bound to a specific protein, although Keller *et al.*[17] postulated an important role for transcortin in the passage of cortisol through the liver cell membrane. In this respect the presence of a transcortin-like protein in the rat uterus cytosol may also be important[19].

From our previous studies concerning the binding of steroids by intact erythrocytes, we could not conclude where the bound steroid was localized[4]. The present binding studies with the membranes and the membrane-free haemolysate showed the highest combining affinity of testosterone by the membranes (Fig. 1). This might be explained by a better solubility of testosterone in the lipid-rich membrane phase compared with the solubility of the steroid in the protein-rich membrane-free haemolysate. The interaction of testosterone with intact erythrocytes might therefore partly be explained in terms of non-specific uptake by the membranes with

a large capacity for the steroid. DeVenuto *et al.*[14] have in fact demonstrated the strong interaction of progesterone with haemoglobin-free membranes.

The binding of steroids by haemoglobin-free membranes was investigated for six steroids of which Δ^5-pregnenolone showed the highest combining affinity (Fig. 2). Similar binding values were obtained for binding of Δ^5-pregnenolone by a comparable suspension of intact erythrocytes[4]. It is rather unlikely, therefore, that Δ^5-pregnenolone will penetrate the intact erythrocyte. It may be completely bound in the membrane. Combining affinity values for progesterone, 20α-dihydroprogesterone and testosterone by haemoglobin-free membranes were lower than those obtained with a comparable suspension of intact erythrocytes[4]. Binding of these steroids to intact erythrocytes *in vitro* may therefore be explained in terms of an interaction with both the membrane, and binding sites inside the cell.

The exact nature of the interaction between steroids and membranes has not yet been elucidated. DeVenuto *et al.*[14] concluded that the membrane protein from erythrocytes can contribute to the binding of progesterone by haemoglobin-free membranes. Our binding studies at 4 °C with a membrane-protein preparation showed relatively high binding of testosterone and progesterone by the membrane protein as compared to the binding by haemoglobin-free membranes (Fig. 3 and Table I). Our finding that testosterone and progesterone showed a higher combining affinity for a membrane-protein preparation compared with haemoglobin-free membranes might be explained as a result of an interaction with binding sites which are not exposed to the steroids in the intact membrane structure. In this respect, the studies of Metcalfe[19] concerning the haemolytic action of benzyl alcohol on erythrocytes may be relevant. By adding increasing amounts of alcohol to intact erythrocytes Metcalfe demonstrated that the partition of the alcohol between the membranes and the water phase increased in favour of the membranes with increasing degradation of the membranes. This was explained by the possible exposure of new binding sites for the alcohol which were inaccessible or protected before the degradation process started. A comparison of the binding of progesterone by haemoglobin-free membranes and membrane protein at 4 °C and 37 °C also showed a difference in binding behaviour between the membrane protein and the haemoglobin-free membranes (Table I). The membrane structure may be less rigid at 37 °C than at 4 °C, thus allowing a better insertion of progesterone into the membrane and resulting in a higher combining affinity at 37 °C. In contrast the combining affinity of progesterone for the membrane protein at 37 °C is smaller than at 4 °C, possibly due to a dissociation of the steroid protein complex at the higher temperature. Based on these temperature effects it can therefore be concluded that progesterone in haemoglobin-free membranes is not bound or only to a small extent to membrane protein and that other membrane constituents in an intact membrane structure might be involved in progesterone binding.

The binding of testosterone by the membrane-free protein fractions of human erythrocytes reflected a specific binding of the steroid with the 20–50 % $(NH_4)_2SO_4$ precipitate of the membrane-free haemolysate (Fig. 4). The magnitude of the affinity constant for testosterone was found to be of the same order as that of the testosterone-binding plasma protein (10^8 M^{-1}). For intact erythrocytes an apparent association constant of much lower value was estimated. The order of magnitude of this constant was 10^3 M^{-1} (ref. 4).

The exact nature of the binding protein in the 20–50 % saturated $(NH_4)_2SO_4$ precipitate of the membrane-free haemolysate could not be determined. The $(NH_4)_2SO_4$ precipitate of the membrane-free haemolysate contained a relatively large amount of 17β-hydroxysteroid dehydrogenase activity. A separation of binding activity and 17β-hydroxysteroid dehydrogenase activity, however, could not be achieved by gel chromatography on Sephadex G-150 (Fig. 5). The specific binding of testosterone by this fraction in equilibrium dialysis experiments could therefore reflect binding of testosterone by the enzyme. The possibility of a contamination due to "trapped" plasma could be ruled out (Table II).

Gel chromatography of the $(NH_4)_2SO_4$ precipitate of the membrane-free haemolysate previously incubated with [1,2-$^3H_2$]testosterone did not show any association of radioactivity with macromolecules (Fig. 6). Dissociation of the steroid protein complex on the column is probably the reason. This dissociation, which to a certain extent is unavoidable under non-equilibrium conditions, might imply that the number of high affinity binding sites for testosterone in the erythrocyte fraction with hydroxysteroid hydrogenase activity is very small.

Because both testosterone and 5α-dihydrotestosterone were bound specifically, the possibility cannot be excluded that the sex steroid-binding globulin, which has been shown to bind specifically testosterone, is present in the erythrocyte cytosol. There are a few indications in the literature that specific steroid-binding plasma proteins might be present in target tissue cytosols[18,20,21]. From the elution volumes of the fraction with hydroxysteroid dehydrogenase activity, the testosterone-binding globulin fraction from plasma and the 20–50 % saturated $(NH_4)_2SO_4$ precipitate of plasma it can be concluded that the presence of testosterone-binding plasma proteins in the fraction with hydroxysteroid dehydrogenase activity is very unlikely (Figs 5 and 6). From the data in Fig. 4 it may be calculated that the number of specific binding sites per erythrocyte is in the order of 10. In the cytosol of a target tissue (*e.g.* uterus) the number of specific binding sites for oestradiol per cell is in the order of several thousands[22]. It is not surprising therefore that specific binding of testosterone by intact erythrocytes could not be detected[4]. The occurrence of this binding principle in the erythrocyte cytosol may be of limited physiological significance.

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