CELLULAR UPTAKE OF STEROIDS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE

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OP GEZAG VAN DE DEKAAN DR. J. MOLL,

HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE,

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UIT DE FACULTEIT DER GENEESKUNDE TE VERDEDIGEN OP

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PROMOTOR : PROF. DR. H.J. VAN DER MOLEN

CO-REFERENTEN: PROF. DR. K.B. EIK-NES

PROF. DR. W.C. HÜLSMANN

Aan mijn ouders,

aan Truus,

Jasperina en Rutger

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LIST OF TRIVIAL NAMES

Δ^5-androstenediol	- 5-androstene-3β,17β-diol
androstenedione	- 4-androstene-3,17-dione
cholesterol	- 5-cholesten-3β-ol
corticosterone	- 11β,21-dihydroxy-4-pregnene-3,20-dione
cortisol	- 11β,17,21-trihydroxy-4-pregnene-3,20-dione
dehydroepiandrosterone (DHEA)	- 3β-hydroxy-5-androsten-17-one
dehydroepiandrosterone sulphate (DHEA-sulphate)	- 17-oxo-5-androsten-3β-yl sulphate
deoxycorticosterone	- 21-hydroxy-4-pregnene-3,20-dione
20α-dihydroprogesterone	- 20α-hydroxy-4-pregnen-3-one
5α-dihydrotestosterone	- 17β-hydroxy-5α-androstan-3-one
17α-hydroxy-Δ^5-pregnenolone	- 3β,17α-dihydroxy-5-pregnen-20-one
17α-hydroxyprogesterone	- 17α-hydroxy-4-pregnene-3,20-dione
oestradiol	- 1,3,5(10)-oestratriene-3,17β-diol
oestrone	- 3-hydroxy-1,3,5(10)-oestratrien-17-one
oestrone sulphate	- 17-oxo-1,3,5(10)-oestratrien-3-yl sulphate
ouabain	- 1β,3β,5β,11α,14,19-hexahydroxy-5β-card-20(22)-enolide-3L-rhamnoside
Δ^5-pregnenolone	- 3β-hydroxy-5-pregnen-20-one
progesterone	- 4-pregnene-3,20-dione
testosterone	- 17β-hydroxy-4-androsten-3-one

LIST OF ABBREVIATIONS

ADH - alcohol dehydrogenase
ATP - adenosine-5'-triphosphate
B - concentration of bound steroid
BPB - bromophenol blue
BSA - bovine serum albumin
CBG - corticosteroid binding globulin (transcortin)
DNA - deoxyribonucleic acid
DPM - disintegrations per minute
EDTA - ethylenediaminetetraacetate
FSH - follicle-stimulating hormone
HSA - human serum albumin
ICSH - interstitial cell-stimulating hormone
K_a - association constant of steroid-protein complex
K_d - dissociation constant of steroid-protein complex
mRNA - messenger RNA
$NADP^+$ - nicotinamide-adenine dinucleotide phosphate (oxidized)
N_m - concentration of m identical binding sites
PBP - plasma progesterone binding protein
PCMB - p-chloromercuribenzoate
PCMBS - p-chloromercuri-benzene sulfonic-acid
POPOP - 1,4-bis-(5-phenyloxazolyl-2)-benzene
PPO - 2,5-diphenyloxazole
RNA - ribonucleic acid
rRNA - ribosomal RNA
S - Svedberg unit of sedimentation coefficient
$S_{20,w}$ - sedimentation coefficient in water at 20°C
SBG - sex steroid binding globulin
Tris - 2-amino-2-hydroxymethylpropane-1,3-diol
U - concentration of unbound steroid

CHAPTER 1

INTRODUCTION

The general concept concerning mechanism of steroid hormone action includes several steps at the molecular level about which very little is known. For several steroid responsive tissues it has been shown, that the steroid molecule after entering its target cell, becomes immediately bound by a "cytoplasmic receptor" which transfers the steroid into the nucleus. This translocation step is temperature dependent. Simultaneously the receptor part of the complex is transformed into a smaller subunit, the "nuclear receptor". In the nucleus the steroid receptor complex becomes attached to an acidic non histone protein, the "nuclear acceptor" site, and provokes an increase in RNA polymerase activity resulting in new RNA synthesis followed by increased protein synthesis.

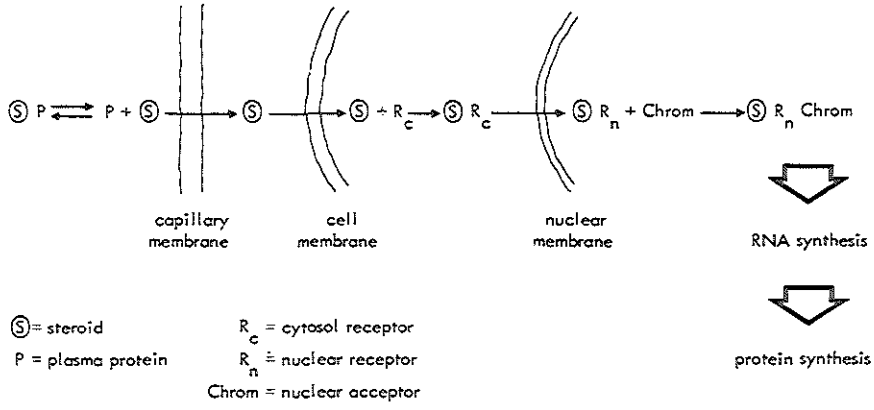

Fig. 1,1. Simplified representation of the sequence of biochemical events by which steroid hormones may exert their biological action in a target cell.

Most of the circulating steroid in the extracellular environment is bound to plasma proteins. Dissociation constants of the plasma protein-steroid complexes and the association constants of the intracellular protein-steroid complexes may determine whether or not a steroid molecule

is taken up by a cell.

Before the steroid reaches its hypothetical site of action in the cell nucleus it has to pass different membrane structures (capillary membrane, target cell membrane and nuclear membrane). The mechanism by which steroids can pass membrane structures is not precisely known. It was considered of interest, therefore, to investigate in more detail the factors which could possibly influence cellular uptake of steroids, at the extracellular level, at the plasma membrane level and at the intracellular level.

The human erythrocyte was chosen as a model system, because it is a rather simple cell type. It cannot be considered as a steroid target cell and contains neither a nucleus nor mitochondria. In contrast to steroid target tissue cells, cellular suspensions of human erythrocytes can easily be obtained. In addition much information is available on the structure, composition and permeability of the human erythrocyte membrane.

The influence of factors at the intracellular level were studied in a much more complicated model system: the testis. According to function the testis can be devided into two compartments: the interstitial tissue responsible for steroid production and the seminiferous tubules responsible for the development of spermatozoa. The seminiferous tubules can be considered as a target tissue for steroids, because testosterone is needed for maintenance of spermatogenesis. The mechanism by which testosterone can maintain spermatogenesis is, however, unknown. If the working hypothesis concerning the mechanism of action of steroid hormones is correct and valid for different steroid target tissues, including the seminiferous tubules, the testis should contain steroid receptors. To test this possibility a search for steroid binding macromolecules in the soluble fraction of testis tissue therefore seemed justified.

1.1 Mechanism of action of steroids

Most steroid responsive tissues show some time after exposure to the steroid an increase in protein synthesis which is preceded by RNA synthesis. In order to explain these observations several authors have considered cell regulation at the transcriptional level an attractive explanation for steroid action in target organs[1-5]. Specific retention of steroid hormones in their target organs is well documented[6]. Such retention had led to an intensive search for the localization, isolation and characterization of steroid binding components in steroid target tissues. These intracellular binding components are considered to transfer the steroid to its site of action in the nucleus where the steroid may act as derepressor of gene activity. Evidence for such a steroid transfer process has been obtained for *in vitro* experiments with oestradiol in uterine tissue[7], with 5α-dihydrotestosterone in prostatic tissue[8], with progesterone in the chicken oviduct[9] and with cortisol in the liver[10].

Specific steroid binding proteins have been isolated from the 105,000 x g supernatant of steroid target tissues. These proteins are called "cytosol receptors" and they are highly specific for binding of a particular steroid. The apparent association constants were found to be in the order of 10^9 M^{-1}. In "low salt" (< 0.1M) media a sedimentation coefficient of 8-10 S was obtained for the steroid-receptor complex, while in "high salt" (> 0.4M) media this value was 4 S. The 8-10 S form of the receptor is considered an aggregate of four 4 S subunits.

It is difficult to determine if and in which form the cytosol receptor really exists *in vivo*. For the uterus Reti and Erdos[11] found oestradiol bound to a fraction with a sedimentation coefficient of 6.5 S after ultracentrifugation of uterine cytosol on sucrose gradients prepared in uterus "press juice". This illustrates the difficulty to interpret the significance of an observed value for the sedimentation

coefficient *in vitro* with respect to the *in vivo* situation.

Indications for the existence and specificity of the steroid transfer to the nucleus, were obtained when it was shown by investigations *in vitro*, that only the 8-10 S form of the cytosol receptor can transfer the steroid to the nucleus. Because extraction of nuclei with "high salt" media revealed a 5 S receptor-steroid complex it was concluded that during this translocation a transformation of the steroid-receptor complex occurs. The translocation of the steroid from cytosol to nucleus has been demonstrated *in vivo* and occurs under certain conditions also *in vitro*. Under conditions *in vitro* a steroid 5 S receptor complex could only be extracted from the nuclei either after incubations of the steroid with whole tissue at 25-37°C or after incubations of the steroid with isolated nuclei provided the 8-10 S cytosol receptor was present. No translocation of the steroid could be observed at 0°C. At least two conclusions can be drawn from these observations, firstly the cytosol receptor appears to be required for the translocation step and secondly the translocation step is temperature dependent.

A binding component with an exceptionally high affinity for oestradiol (K_a : 10^{14} M^{-1}) has been isolated from the non histone chromatin protein fraction of nuclei obtained from rat uterus[12]. It was also demonstrated that the progesterone cytosol receptor complex from chicken oviduct can be bound *in vitro* by an acidic non histone protein fraction obtained from oviduct nuclei[13]. A similar nuclear binding exists in the prostate for the 5α-dihydrotestosterone cytosol receptor complex[14]. These steroid binding non histone proteins are thought to act as repressors of gene activity and it has been suggested that association of the steroid with this protein could provoke derepression of the gene and initiate DNA transcription[12].

Recently, synthesis of m-RNA in the uterus within 15 minutes after oestradiol administration has been reported by

Baulieu and coworkers[15]. This m-RNA initiates the synthesis of a protein which is detectable within 30 minutes after oestradiol administration. It is suggested that this protein could function as an activating protein in the synthesis of r-RNA, because the synthesis of this "key intermediary protein" (KIP) precedes the synthesis of r-RNA, which in the uterus can be determined 1-3 hours after administration of oestradiol.

1.2 Transport of steroids across membranes

Steroid hormones have to pass several biological membranes on their way from the steroid secreting cells (testis, adrenal, placenta and ovary) into the steroid target tissues. Binding of steroids to plasma proteins and intracellular receptors may play an important role during these processes. In this respect it is of importance to consider a possible role of cellular and subcellular membranes in more detail.

Willmer[16] postulated in 1961 that steroid hormones exert their molecular action in target tissues by altering the structure and permeability of the cell membrane. It was suggested that the steroid molecule is taken up by the membrane because the most lipophilic group of the steroid molecule would seek the aliphatic chains of the membrane phospholipids and the most hydrophylic end of the steroid molecule would seek the aqueous phase or any polar groups on the membrane. Experimental evidence for this hypothesis has not yet been presented. There are, however, indications that steroids can influence the structure and permeability of both artificial and biologically occurring membrane structures.

Weismann et al.[17] found that progesterone and deoxycorticosterone can cause leakage of anions and glucose from liposomes. These liposomes consisted of lecithin, cholesterol and dicetylphosphate and can be considered a useful model system for biological membranes. The increased

permeability of the liposomes was explained by structural rearrangement of the lipid layers caused by the steroids. Similar observations were made by Heap et al.[18] on the increased K^+ leakage from liposomes under the influence of steroids. The uptake of steroids by liposomes has been studied by Snart and Wilson[19]. They found an increased uptake with decreasing cholesterol content of the liposomes. An important aspect in the Willmer hypothesis, the exact orientation of the steroid molecules in the lipid structures of such liposomes is, however, still unknown.

Biological membranes do not exclusively consist of cholesterol and phospholipids but contain also proteins and polysaccharides. Therefore a role of these latter substances in the interactions of steroids with biological membranes should not be ruled out.

The red blood cell membrane has been used as model in many investigations concerning the influence of steroids on biological membranes. When high concentrations (10^{-4} M) of steroids were used, it was observed that steroids caused a destabilisation of the red blood cell membrane, resulting in increased permeability and osmotic fragility[20-22]. On the other hand stabilisation of the red blood cell membrane by steroids has been reported by De Venuto[23]. He tested the osmotic resistance of the red blood cells after prolonged storage in the presence of progesterone (5.10^{-7}M) and concluded that progesterone can protect red blood cells against haemolysis. Isaacs and Hayhoe[24] reported that progesterone and testosterone (3.10^{-6} M) could inhibit the development of sickling in sickle cell disease and suggested also a possible stabilisation of the red blood cell membrane by these steroids.

It is evident from these observations, that steroid hormones can interact with cell membranes, and can alter membrane structure and function possibly by affecting the lipid part of the membrane.

In terms of transport (diffusion, "active" transport,

passive transport and carrier-mediated transport) the steroid transfer across cell membranes can probably be considered as a simple diffusion process through a lipid rich layer without involvement of an energy source or a carrier.

Evidence for a diffusion process has been presented by several investigators. Giorgi et al.[25] found during superfusion studies of prostatic tissue that the entry of steroids into the tissue was independent of the steroid concentration in the superfusate which may exclude an "active" saturable transport mechanism. Munck[26] reported that thymus cells are freely permeable for cortisol. Baulieu and coworkers[27] observed that neither dinitrophenol, oubaïne nor oligomycine were effective in influencing the uptake of oestradiol by endometrium from castrated rats. It is very unlikely, therefore that "active" transport, a process that requires energy which can be supplied by the hydrolysis of ATP is involved in the uptake of oestradiol by castrated rat endometrium. Gross et al.[28], came to the conclusion that there might exist an energy-dependent outward transport of cortisol in mouse fibroblasts. They found that this process was saturable and temperature and glucose dependent. This is the only published evidence for an "active" transport mechanism of a steroid across a biological membrane. In a recent paper Milgrom et al.[29] postulated a protein dependent step at the cell membrane level, which plays a role during the uptake of oestradiol by uterine cells.

If transfer of the steroid across the cell membrane is supposed to occur when the steroid is in an unbound state, this would include dissociation of the steroid plasma protein complex, because 95-98% of steroids in the extracellular fluid is bound to proteins. Studies of Peterson and Spaziani[30] on the accumulation of albumin and α-globulin in the rat uterus after oestradiol administration showed however that plasma proteins can pass capillary and cell membranes. The presence of a CBG-like protein in the

cytosol fraction from rat uteri also might indicate a possible transfer of a plasma protein into an oestrogen target tissue[31]. Keller et al.[32] have suggested that transcortin may play an "active" role in the uptake of cortisol by the liver. A similar mechanism was suggested by Amaral et al.[33] for the uptake of cortisol by lymphocytes grown in culture. Thus plasma proteins may be involved to some extent in the transport of steroids across cell membranes although no direct proof nas ever been given for this contention.

In conclusion it appears difficult to give a general mechanism for the passage of steroids through biological membranes. There are strong indications for a simple diffusion process, although an "active" transport mechanism or a role of proteins as steroid carriers in certain cells should not be excluded.

1.3 Theory of steroid - macromolecule binding

Several quantitative approaches have been made to express steroid macromolecule interactions. The calculation of the affinity and the concentrations of binding sites are based on the general equation which follows the law of mass action when applied to the following reaction:

$$\text{Steroid} + \text{Protein} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{Steroid-Protein} \qquad [I]$$

The affinity is usually expressed by either the association constant: $K_a = \frac{k_{+1}}{k_{-1}}$ in M^{-1} or the dissociation constant $K_d = \frac{k_{-1}}{k_{+1}}$ in M.

In this thesis the association constant K_a has been used and is given by the following equation:

$$K_a = \frac{[B]}{[U].[N_m - B]} \qquad [II]$$

where [B] is the concentration of bound steroid, [U] the concentration of unbound steroid and $[N_m]$ the concentration of one set of m, independent identical binding sites. Equation [II] can be rewritten as:

$$[B] = \frac{[N_m] \cdot [U]}{\frac{1}{K_a} + [U]} \qquad [III]$$

or as:

$$\frac{[B]}{[U]} = K_a \cdot [N_m] - K_a \cdot [B] \qquad [IV]$$

When there are n sets of m identical binding sites equation [III] becomes:

$$[B] = \sum_{i=1}^{n} \frac{[N_{m_i}] \cdot [U]}{\frac{1}{K_{a_i}} + [U]} \qquad [V]$$

For a mathematical treatment of the binding of testosterone by blood plasma, equation [V] can be used as follows:

$$[B] = \frac{[N_{m_{CBG}}] \cdot [U]}{\frac{1}{K_{a_{CBG}}} + [U]} + \frac{[N_{m_{SBG}}] \cdot [U]}{\frac{1}{K_{a_{SBG}}} + [U]} + \frac{[N_{m_{HSA}}] \cdot [U]}{\frac{1}{K_{a_{HSA}}} + [U]} \qquad [VI]$$

where CBG is corticosteroid binding globulin, SBG is sex steroid binding globulin and HSA is human serum albumin. In most cases, however, the number of different binding sets and the number of binding sites per set are not known. In such situations equation [III] is the most practical formula for graphical representation and calculation of binding parameters.

Several graphical representations can be used for equation [III] [34]. In a logarithmic plot (Fig.1,2) where [B] is plotted against log [U], a wide range of steroid

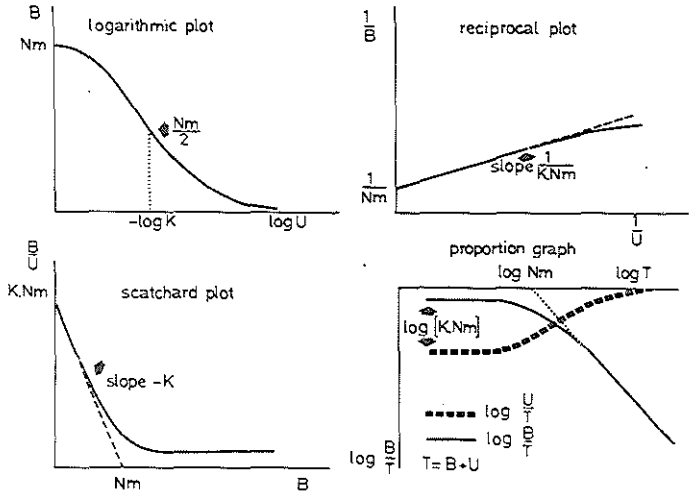

Fig. 1,2. Different graphical representations of steroid-macromolecule binding.

concentrations can be covered but this representation does not give a method for adequate determination of binding values in complex systems. In a reciprocal plot, $\frac{1}{[B]}$ is plotted against $\frac{1}{[U]}$ (Fig.1,2). This representation is essentially the same as the Lineweaver-Burk plot for representing enzymic activity. The reciprocal plot is useful as graphical representation of binding data in cases of simple binding systems. In more complex systems the binding values at high steroid concentrations will cluster close to the ordinate and binding sites of lower affinity cannot be accurately detected. In the graphical representation according to Scatchard[35] $\frac{[B]}{[U]}$ is plotted against [B]. For a single binding system a straight line is obtained (Fig.1,2). In contrast to the reciprocal plot the Scatchard plot is still useful for calculation of binding parameters by extrapolation if different classes of binding sites are present. Baulieu and Raynaud[36] introduced the proportion graph, where the logarithm of the proportion of bound steroid is plotted as a function of the logarithm of the total steroid

concentration (Fig.1,2). This representation will cover a wide range of steroid concentrations and can be adequately employed in the case of complex binding systems.

In this thesis the Scatchard plot has been used for graphical representation of binding data.

If $[N_m] >> [B]$ i.e. if the concentration of total binding sites is extremely large compared with the concentration of occupied binding sites, a straight line is obtained parallel to the $[B]$-axis in the Scatchard plot (See: equation IV). In this case the binding system has a large capacity. An apparent association constant can be calculated if it is assumed that m is unity and the molecular weight of N is known. This has been applied for the calculation of apparent association constants of steroids for intact erythrocytes as described in Chapter 2 and Appendix paper I. If the molecular weight of N is unknown, the protein concentration in grams per litre can be used, assuming that the binding system is a protein. The value calculated for K_a is then called the "combining affinity" according to Daughaday[37]. This parameter is very useful in comparing steroid binding by different protein samples. The combining affinity has been used as a binding parameter for comparison of the binding of testosterone by different erythrocyte fractions as described in Appendix paper II.

If $[N_m]$ is of the same order as $[B]$ a straight line is obtained with a negative slope in the Scatchard graph. The intercept at the $[B]$-axis gives the value for $[N_m]$. The value for K_a can be obtained from the slope of the line.

When more than one binding system is present a linear relationship no longer exists between $\frac{[B]}{[U]}$ and $[B]$. Values for association constants and concentrations of binding sites can, however, be obtained in such cases by extrapolation. The accuracy of these calculations is much less than in the case of one binding system. Calculations of apparent association constants are performed in this way for the binding of testosterone by an erythrocyte protein fraction

(Appendix paper II) and for the binding of oestradiol by a soluble protein fraction obtained from rat testis (Chapter 4).

1.4 Methods for studying steroid - macromolecule binding

For an experimental evaluation of the equilibrium of the binding between a steroid and a macromolecule it is theoretically possible to measure any of the three reactants in reaction [I]. In actual practice estimation of either the amount of unbound steroid or the amount of steroid-macromolecule complex is most frequently used. An extensive review of most of the techniques that have been used has recently been given by Westphal[34].

Several methods have been described for measuring the concentration of the steroid-macromolecule complex. These methods can be divided into two main types: in one type the equilibrium is maintained during the measurement, whereas in the other it is not. The main advantage of the first type method is that both high affinity and low affinity binding can be measured.

1.4.1 Equilibrium dialysis

A technique in which equilibrium is maintained during measurement is equilibrium dialysis. This method appeared to be very suitable for measuring low affinity binding of steroids by red blood cells and fractions of such cells.

The essence of the equilibrium dialysis method is to contain the binding system within one compartment separated from another compartment by a membrane permeable to the steroid. At equilibrium the unbound steroid concentration [U] is the same in both compartments. The concentration of bound steroid [B] can be obtained by subtracting [U] from the total steroid concentration in the compartment which contains the binding system. Steroid adsorption by the membrane and denaturation of the binding system due to the

long period required to reach equilibrium (24-40 hours) may be sources of errors which should be kept in mind. Experimental details of this method are described in Appendix paper I and II.

1.4.2 Charcoal adsorption

The charcoal adsorption technique is an example of a method in which equilibrium is not maintained during measurement. The unbound steroid is removed from the reaction mixture through adsorption by charcoal. The remaining steroid concentration in the reaction mixture is [B] . Only high affinity binding sites are detectable by this quick method which is very suitable for quantitative measurement of steroid-receptor interactions. Thus this method was used to measure the high affinity binding of oestradiol in testis cytosol as described in Chapter 4. Care should be taken that the binding system (protein) is not adsorbed by the charcoal, because this would lead to an underestimation of [B] . Since Murphy[38] reported the applicability of charcoal in competitive protein binding methods, this method has been used for the estimation of many steroids in biological samples.

Methods which include not only a separation of the bound from the free steroid, but also separate different binding entities, can be very useful for characterization of steroid-binding. Such methods are gel-chromatography, sucrose gradient centrifugation and acrylamide gel electrophoresis.

1.4.3 Gel Chromatography

Chromatography through a small column, containing Sephadex G-25, of a binding system (proteins) previously equilibrated with steroid provides a method for the separation of bound and unbound steroid. The macromolecules including the bound steroid are excluded from the

small pore Sephadex and are eluted without retention. This technique was introduced by De Moor et al.[39] for studying steroid-protein interactions. Possible dissociation of the steroid-macromolecule complex during the gel chromatographic step, however, prevents an accurate estimation of [B] . Sephadex G-100, G-150 or G-200 can be used when different binding macromolecules with different molecular weights are present in the binding system. In this way a qualitative picture is obtained of the different binding entities in the binding system under study. This application of gel chromatography is used in Appendix paper II.

1.4.4 Sucrose gradient centrifugation

In 1966 Toft and Gorski[40] introduced sucrose density gradient centrifugation as a technique for studying steroid-macromolecule interactions. The binding system (equilibrated with steroid) is layered on a linear sucrose gradient and is subsequently centrifuged. The sucrose gradient provides a linear migration of macromolecules with different sedimentation properties. In this way bound steroid is separated from unbound steroid which remains at the top of the gradient. Dissociation of the steroid-macromolecule complex occurs to some extent during centrifugation. The sucrose gradient centrifugation method is used in a qualitative manner in Chapter 3 and in Chapter 4 and in Appendix paper III.

1.4.5 Acrylamide gel electrophoresis

Recently Corvol et al.[41] published a method for the determination of steroid binding data by the use of polyacrylamide gel electrophoresis. One of the advantages of this technique is the high resolution which can be obtained of a sample containing different binding systems. The application and limitations of this technique in obtaining qualitative binding data are described and discussed in Chapter 3.

In this thesis the equilibrium dialysis technique and the charcoal adsorption method are used for calculation of binding parameters. The other methods discussed above are used as qualitative approaches for investigation of steroid binding by several protein samples.

1.5 Scope of this thesis

In the first two parts of this chapter several possibilities have been discussed for the mechanisms which can affect the uptake of steroids by their target cells. It was concluded, that this uptake can be influenced at the extracellular level by steroid binding plasma proteins, at the membrane level by membrane constituents and at the intracellular level by specific steroid receptors.

Several steroid binding proteins (transcortin, α-acid glycoprotein, sex steroid binding globulin and albumin) have been isolated from the extracellular environment. The binding of steroids by these proteins is mainly considered as a protection against metabolism by the liver and furnishes transport of the steroids in the circulation to their target organs. At the outerside of the target cell membrane the steroid plasma protein complex may dissociate and the unbound steroid is immediately taken up by the membrane. It is possible that at the inner side of the target cell membrane a cytosol receptor will pick up the steroid from the membrane and transfer it into the cell nucleus. Detailed information about the capacity and affinity of cell membranes for steroids and the nature of possible steroid binding components in cell membranes are however lacking. To study these phenomena a simple cell type without a nucleus and mitochondria only consisting of a functioning membrane, could be an excellent tool. The mature human red blood cell fulfills this condition, and was therefore chosen as a model system[42].

The uptake of different steroids by intact human erythrocytes and the influence of several factors at the

extracellular level on this uptake are described and discussed in Chapter 2 and in Appendix paper I.

The binding of steroids by cell fractions of human erythrocytes, in particular by haemoglobin-free membranes, membrane fractions and membrane-free fractions is described and discussed in Chapter 3 and Appendix paper II.

Since 1966, when Toft and Gorski[40] demonstrated the presence of an oestradiol binding protein in the immature rat uterus, similar steroid binding proteins have been isolated in several other steroid target tissues. The possible function of these binding proteins in the mechanism of action of steroid hormones has been discussed in the first part of this chapter. It is not known if a similar mechanism exists for the testosterone action in the seminiferous tubules as for the action of this steroid in the prostate. The demonstration of the presence of testosterone and 5α-dihydrotestosterone binding components in the seminiferous tubules might however be a first indication for such a mechanism. This prompted us to investigate the binding of testosterone and of 5α-dihydrotestosterone by the soluble protein fractions of whole testis tissue, and of seminiferous tubules of the rat. Also the binding of oestradiol and corticosterone by these tissues has been investigated and it has been found that there is a specific oestradiol binding component in the rat testis cytosol[43]. The preliminary characterization and localization of this oestradiol binder are described in Chapter 4 and in Appendix paper III.

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CHAPTER 2

UPTAKE OF STEROIDS BY INTACT HUMAN RED BLOOD CELLS

The binding of steroid hormones by cellular elements of blood has been the subject of many studies in the last 40 years since the early studies of Kemp and Pedersen-Bjergaard[1]. The main purpose of these studies was to obtain information about the distribution of steroid hormones among the different constituents of blood. Particularly the distribution of oestrogens and corticosteroids between plasma proteins and erythrocytes is well documented for blood of several species e.g. human[2-8], rat[9], dog[10-13], rabbit[11], guinea pig[11], horse[14] and mouse[15]. This distribution is influenced mainly by the concentration of specific steroid binding plasma proteins and the steroid concentration[2,7,8,10,16,17].

Several investigators (Wall and Migeon[3], Peterson et al.[18], Wu and Mason[19], Bajardi et al.[20] and De Moor and Steeno[10]) have shown that neither time nor temperature influences the distribution of steroids between plasma proteins and erythrocytes during incubation in vitro of blood with radioactive labelled steroids. Glucuronides of corticosteroids[4,5] and of oestrogens[6,21] and sulphates of DHEA and of androsterone[7] are less or not at all associated with erythrocytes in blood. In a recent paper Kornel et al.[22] reported that a significant amount of conjugated cortisol metabolites was found to be associated with erythrocytes in human blood.

The mechanism by which erythrocytes can take up steroids is not clear. Bush[23] has reported that fluoride ions decrease the rate of uptake of cortisol by erythrocytes. Holzbauer and Vogt[11], on the contrary, found no influence of sodium fluoride on the uptake of cortisol and corticosterone by erythrocytes in dog adrenal venous blood. Whether or not steroids penetrate the erythrocyte membrane during the uptake process is also

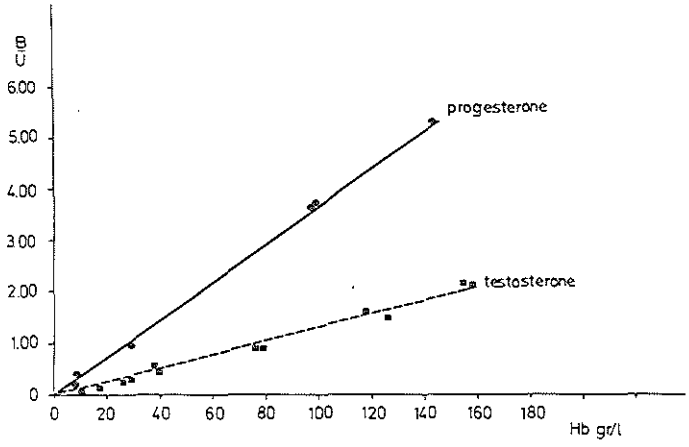

Fig. 2,1. The binding of testosterone (17 nM) and progesterone (17 nM) after incubation at 4°C by erythrocytes in suspensions with different haemoglobin concentrations. Hb = Haemoglobin concentration.(For experimental procedure,see:Methods section of Appendix paper I).

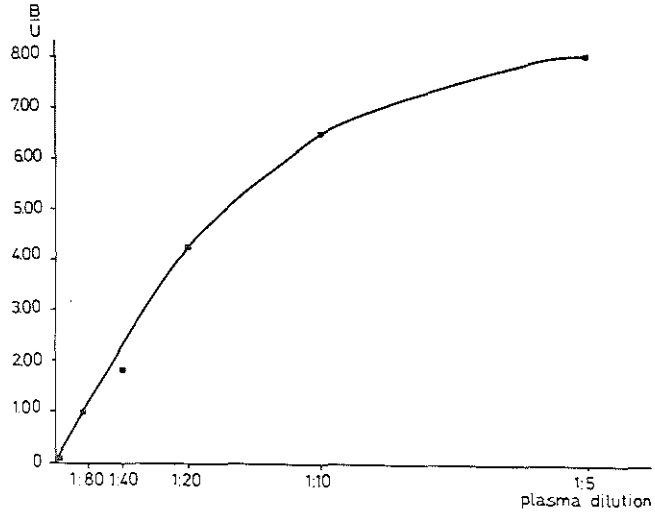

Fig. 2,2. The binding of progesterone by plasma proteins at different plasma dilutions.Binding was estimated by equilibrium dialysis at 4°C.Individual points represent the mean of duplicate estimations.(For experimental procedure,see:Methods section of Appendix paper I).

a matter of discussion. It is known that steroids taken up by erythrocytes can readily be removed by washing the cells with saline[3,5,11,24,25], suggesting an attachment of the steroids to the cell surface. This is, however, not generally applicable to all steroids. The presence of 20α- and 17β-hydroxysteroid dehydrogenases inside erythrocytes from different animal species and the interconversion of several steroids by these enzymes[26-31] may reflect that steroids pass the erythrocyte membrane.

The capacity for binding of steroids of erythrocytes suspended in isotonic media which contain no plasma proteins is very large. This has been shown for oestradiol, oestrone and cortisol by Fonzo et al.[32] and Goldzieher et al.[14]. In Appendix paper I (Figures 4-6) we have also reported for a series of other steroids a large capacity of erythrocytes for binding of steroids. The uptake of steroids by erythrocytes suspended in isotonic media should probably be considered as a distribution between a lipid-rich phase (erythrocytes) and a water-phase (medium). By changing the composition of this two phasic system, the distribution of the steroids will also be changed. This is shown in Figure 2,1. Data from incubation studies of progesterone and testosterone with erythrocyte suspensions, containing increasing amounts of erythrocytes, showed that there is a linear relationship between the ratio of bound steroid over unbound steroid (B/U) and the amount of haemoglobin present in the incubation system. These results confirm the aspecific character of the association of steroids with intact erythrocytes (see: Appendix paper I).

The binding of progesterone by plasma proteins does not give a linear relationship between B/U and the protein concentration which indicates an association of progesterone with saturable binding sites in plasma (Figure 2,2).

In Table I of Appendix paper I an apparent association constant is calculated in order to express the affinity of different steroids for erythrocytes. Differences in polarity

of the steroids studied can not completely explain the observed affinity differences. Agarwal and Carstensen[24] stated that in addition to the polarity, also certain configurational aspects of the steroids influence the uptake of steroids by erythrocytes.

In order to evaluate if the polarity of the steroid molecule plays an important role, binding studies were performed with another series of steroids. The results of these experiments are shown in Table 2,I.

Table 2,I

Binding of steroids by erythrocytes at 4°C.

Steroid	Association constant (M^{-1})
17α-hydroxy-Δ^5-pregnenolone	2.0×10^3
17α-hydroxy-progesterone	1.3×10^3
Corticosterone	0.7×10^3
Deoxycorticosterone	1.5×10^3
DHEA	1.1×10^3
DHEA-sulphate	0.5×10^3
Oestrone	1.9×10^3
Oestrone-sulphate	1.2×10^3

The 17α-hydroxy derivatives of pregnenolone and progesterone show definitively a lower affinity for erythrocytes than pregnenolone and progesterone. The affinity of corticosterone and deoxycorticosterone for erythrocytes was also investigated. Compared with less polar steroids (pregnenolone and progesterone) a lower uptake was noted. Conjugation of the steroid molecule decreased the affinity for erythrocytes as is shown in Table 2,I for the sulphates of DHEA and oestrone.

The introduction of hydroxyl- or sulfate-groups in steroid molecules increases the polarity of the molecule and therefore decreases the affinity of the steroid for

erythrocytes. The 3β-hydroxy-Δ^5-steroids show a higher affinity for erythrocytes as compared with their 3-oxo-Δ^4-derivatives (e.g. pregnenolone - progesterone; 17α-hydroxy Δ^5-pregnenolone - 17α-hydroxy progesterone). C_{21} steroids are less polar than their C_{19} derivatives and have therefore a higher affinity for erythrocytes (Δ^5-pregnenolone - DHEA; progesterone - androstenedione). The data published by Heap et al.[33] on the solubility of a variety of C_{21}, C_{19} and C_{18} steroids in buffered KCl and in liposomes support the idea that the uptake of steroids by erythrocytes suspended in isotonic media depends mainly on the polarity of the steroids.

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CHAPTER 3

CHARACTERIZATION OF A STEROID BINDING PROTEIN FRACTION FROM HUMAN RED BLOOD CELLS

3.1 Introduction

In studies on the localization and characterization of steroid binding sites of human erythrocytes a specific binding of testosterone by a protein fraction from the membrane-free haemolysate could be demonstrated. These results are described in Appendix paper II. This testosterone binding fraction showed a relatively high 17β-hydroxysteroid dehydrogenase activity which could not be separated from the testosterone binding activity by gel chromatography on Sephadex G-150. It was considered that the specific binding of testosterone by this fraction could be due either to plasma proteins or to binding by the enzyme protein. A contamination by plasma proteins of this fraction appears very unlikely (see Appendix paper II).

In order to investigate the nature of this specific testosterone binding fraction experiments were undertaken to characterize the binding protein. The results described in Appendix paper II were obtained with experiments performed with equilibrium dialysis and gel chromatography on Sephadex G-150. Only with equilibrium dialysis was it possible to demonstrate specific binding of testosterone by the erythrocyte protein fraction. For a better understanding of the character and nature of this protein fraction binding experiments were also performed using ultracentrifugation and polyacrylamide gel electrophoresis. These techniques are considered to be more specific for characterization of the protein fraction because in addition to the separation of bound and unbound steroid these techniques may result in a possible separation of the steroid binding protein from the other proteins present in this fraction. In these experi-

ments 17β-hydroxysteroid dehydrogenase activity was correlated with binding activity and steroid binding to plasma proteins was compared with steroid binding by this erythrocyte protein fraction.

3.2 Methods

3.2.1 Ultracentrifugation

Linear 5-20% (w/v) sucrose density gradients (total volumes 4.8 or 14 ml) in 50 mM Tris HCl buffer (pH 7.4) containing 3 mM $CaCl_2$, 1.5 mM EDTA and 2 mM mercaptoethanol were prepared in a gradient former. 400 µl of an erythrocyte protein fraction or a plasma dilution previously incubated for 2 h at 4°C in air with radioactive steroids, were layered on top of the gradients and centrifuged at 4°C for 18 h at 40,000 RPM in a S.W. 65 rotor or a S.W. 40 rotor in the Beckmann L2-65B ultracentrifuge. After completion of the centrifugation, the tubes were punctured and the contents of each gradient were collected in 28 fractions in counting vials. The individual fractions were counted after the addition of 1 ml distilled water and 15 ml of a dioxane solution containing 100 g naphtalene, 7 g PPO and 0.3 g POPOP per l dioxane. For estimation of 17ß-hydroxysteroid dehydrogenase activity each gradient was subdivided into 10 fractions. Corresponding fractions of two gradients were pooled and incubated with 0.9 ml 50 mM Tris buffer (pH 7.4) containing 3 mM $CaCl_2$, 1.5 mM EDTA, 2 mM mercaptoethanol, 0.3 mM $NADP^+$, 0.01 mM testosterone and 0.05 µCi 1,2 $^3H_2$ testosterone (specific activity: 37.0 Ci/mmole) during 3 h at 37°C in air. Analysis of the incubates was performed as described previously (see Appendix paper II).

3.2.2 Acrylamide gel electrophoresis

Electrophoresis was performed in glass tubes of 7 cm length and 0.6 cm internal diameter. 7% (w/v) acrylamide gels

with a gel volume of 1 ml were made in 0.4 M Tris HCl buffer pH 8.9. The electrode buffers consisted of 0.05 M Tris glycine buffer pH 8.3, as described by Maurer[1]. 40 µl of an erythrocyte protein fraction or a plasma dilution in 20% (w/v) sucrose, previously incubated for 2 h at 4°C in air with radioactive steroids were layered on top of the gels and a current of 1 mA/gel was applied for 5 min, followed by a current of 2 mA/gel. The lower buffer compartment was extensively cooled at 0-5°C. Bromophenol blue (BPB) was used as a marker of the migrating boundary during electrophoresis. Electrophoresis was stopped when BPB reached the end of the gel. After electrophoresis the gels were placed in a mould with a length of 50 mm provided with 25 notches. Subsequently the gels were sliced with the aid of a razor-blade into 2 mm sections. In binding studies both gels and buffers contained either ^{3}H-dihydrotestosterone (10^{-10}M) or ^{3}H-testosterone (10^{-10}M). 2 mm sections of the gels were placed in counting vials containing 1 ml 30% H_2O_2 and the vials were kept for 1 h at 60°C. After shaking for 1 h at room temperature the vials were cooled in ice and 15 ml of a Triton X 100-toluene mixture (3:2, v/v) containing 2.8 gram PPO per litre, was added and kept overnight at room temperature before counting. For estimating 17β-hydroxysteroid dehydrogenase activity 2 mm sections of the gels were minced and incubated with 0.9 ml Krebs-Ringer buffer pH 7.4 containing 0.15 µCi [7α ^{3}H] Δ^{5}-androstenediol (specific activity 16 Ci/mmole) and 0.3 mM $NADP^{+}$ for 3 h at 37°C in a 95% O_2 - 5% CO_2 atmosphere using a Dubnoff shaking incubator. Analysis of the incubates was performed as described previously (see Appendix paper II). Staining of the gels was performed with an Amido black solution (1 g in 100 ml 7% (v/v) acetic acid). Stained gels were stored in 7% (v/v) acetic acid.

3.2.3 Spectrophotometric determination of 17β - hydroxysteroid dehydrogenase activity

The activity of the enzyme (measured as the amount of reduced cofactor formed) was determined as described previously[2] in a Gilford model 2400 recording spectrophotometer at 37°C by measuring absorption at 340 nm. In a cuvette (10 x 4 mm) 0.1 ml of a 1.65 mM-testosterone solution in ethyl alcohol-benzene (1:9, v/v) was mixed with 0.1 ml of a mixture of propylene glycol and methanol (1:4, v/v) and the volatile solvents were evaporated in a nitrogen stream at 37°C. During this procedure the steroid was concentrated in a small drop of propylene glycol and could be readily solubilized in buffer solution. This amount of propylene glycol did not influence the enzymic reactions. To the steroid in the cuvette was then added 0.1 ml 0.4 M-Tris buffer pH 8.5; 0.1 ml 1.5 M-NaCl; 0.1 ml of an erythrocyte protein solution, containing 17β-hydroxysteroid dehydrogenase and 0.7 ml distilled water to make a total volume of 1 ml. After 5 min preincubation at 37°C in air, the reaction was started by the addition of 0.1 ml 3 mM-$NADP^+$ solution. The increase of reduced cofactor formed was linear in the time for periods up to 60 min . The optical density was recorded at 2 min intervals and the enzymic activity was defined as the average reaction rate during the first 30 min after the initiation of the reaction. The specific enzymic activity was calculated from these reaction rates and the protein content of the preparations.

The equilibrium dialysis technique and the isolation procedure for the erythrocyte protein fraction has been described in details in Appendix paper II.

Erythrocyte protein solutions were concentrated by adding batchwise dry Sephadex G-25 coarse[3]. The swollen gel was put in a column and the concentrated erythrocyte protein solution was eluted with a small amount of buffer.

3.3 Results

3.3.1 Correlation between binding and enzymic activity

In order to correlate the binding of testosterone by the erythrocyte protein fraction with the 17β-hydroxysteroid dehydrogenase activity present in this fraction experiments were performed in which both testosterone binding and the 17β-hydroxysteroid dehydrogenase activity were assayed in the same erythrocyte protein fraction.

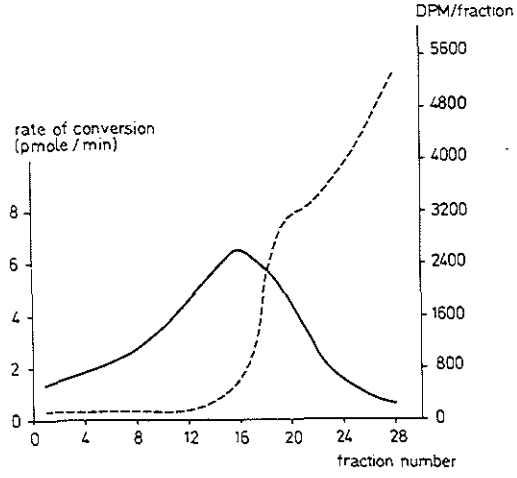

Fig. 3.1. Distribution of 17β-hydroxysteroid dehydrogenase activity after sucrose gradient centrifugation of an erythrocyte protein fraction and distribution of radioactivity after sucrose gradient centrifugation of an erythrocyte protein fraction previously incubated with ^{3}H-testosterone. The erythrocyte protein fractions (400 µl) were layered over linear 5-20% (w/v) sucrose density gradients and centrifuged at 40,000 rpm for 18 h in an SW 65 rotor of the Beckmann L2-65B ultracentrifuge.

——— 17β-hydroxysteroid dehydrogenase activity

------ radioactivity

Sucrose gradient centrifugation

When 400 µl of the erythrocyte protein fraction, previously incubated with [1,2 ^{3}H] testosterone at 4^{o}C, was centrifuged on a sucrose gradient a radioactivity pattern as shown in Figure 3.1, was obtained. The sedimentation profile of radioactivity showed a small shoulder around fraction 20 and an increase in radioactivity from fraction 22 until the top of the gradient. The latter part of the radioactivity pattern reflects unbound testosterone, while the shoulder around fraction 20 may reflect some testosterone binding to macromolecules. Fractions from another two gradients, also containing the erythrocyte protein fraction, not previously incubated with radioactive testosterone, were incubated with testosterone and $NADP^{+}$ for estimation of 17β-hydroxysteroid dehydrogenase activity. Because of a large decrease in enzymic activity after 18 h centrifugation at 4^{o}C and the inhibitory action of sucrose on the enzymic activity, corresponding fractions of two gradients had to be combined to obtain a reliable picture of the sedimentation pattern of the 17β-hydroxysteroid dehydrogenase. This is also shown in Figure 3.1. A very broad peak was obtained around fractions 14-18 for the enzymic activity pattern. It can be concluded that a direct correlation between testosterone binding and 17β-hydroxysteroid dehydrogenase activity does not exist.

Acrylamide gel electrophoresis

The polyacrylamide gel electrophoresis experiments were also performed to see whether or not binding activity correlated with enzymic activity. A 50% (v/v) human plasma solution and the erythrocyte protein fraction were incubated with [1,2 ^{3}H] testosterone and applied to the gels.

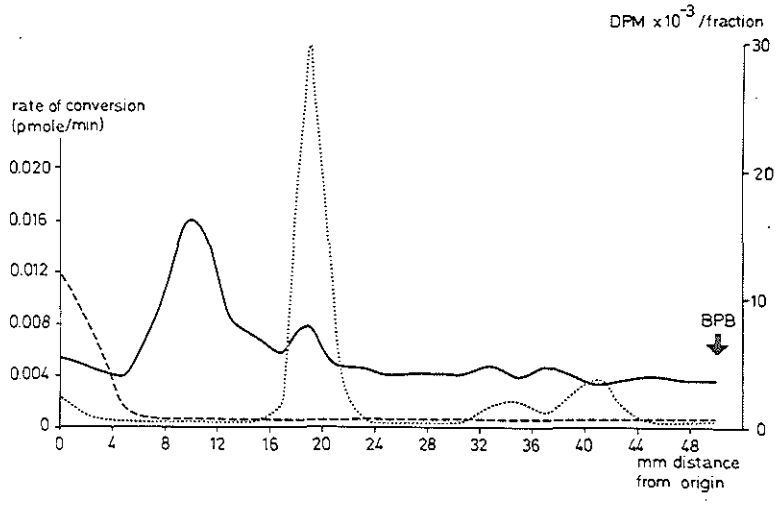

Fig. 3.2. Distribution of 17$_\beta$-hydroxysteroid dehydrogenase activity of an erythrocyte protein fraction after polyacrylamide gel electrophoresis ——— and distribution of radioactivity after polyacrylamide gel electrophoresis of a 50% (v/v) plasma solution previously incubated with ^{3}H-testosterone ····· and an erythrocyte protein fraction previously incubated with ^{3}H-testosterone - - - - - .Electrophoresis was performed on 7% gels with a current of 2 mA/gel. Broom phenol blue (BPB) was used as a marker for the migrating boundary.Estimation of radioactivity and 17$_\beta$-hydroxysteroid dehydrogenase activity in 2 mm gel sections after electrophoresis has been described under Methods.

In the plasma sample after electrophoresis radioactive peaks were observed at 19 mm, 34 mm and 41 mm from the origin (see Figure 3.2). The distribution pattern of radioactivity in the erythrocyte protein fraction only showed large amounts of radioactivity at the top of the gel. The 17β-hydroxysteroid dehydrogenase activity in 2 mm gel sections with testosterone as substrate was not measurable. Better conversions were ontained with Δ^5-androstenediol as substrate and $NADP^+$ as cofactor although the enzymic activity was very low compared with that of the erythrocyte protein fraction before electrophoresis. A peak of 17β-hydroxysteroid dehydrogenase activity was observed about 10 mm from the origin as is shown in Figure 3.2. It can be concluded from these electrophoretic experiments that testosterone binding cannot

Table 3.I

Effect of heat treatment on the 17β-hydroxysteroid dehydrogenase activity and the testosterone binding activity of an erythrocyte protein fraction. 17β-hydroxysteroid dehydrogenase activity was estimated spectrophotometrically as described under Methods. Binding was estimated by equilibrium dialysis at 4°C and expressed as $\frac{\text{Bound steroid}}{\text{Unbound steroid}}$ $(\frac{B}{U})$. Experiments 1-4 have been performed with 4 different erythrocyte protein preparations.

Exp. no.	Treatment	Protein (mg/ml)	Enzymic activity (nmole/min/mg protein)		$\frac{B}{U}$ Testosterone conc. (nM) in dialysis system				
			before dialysis	after dialysis	0.058	0.490	2.240	23.26	116.31
1a	none	21.4	0.100	-	0.976	1.028	-	-	0.616
1b	30' at 60°C	13.2	n.m.*	-	3.785	3.830	-	-	3.273
2a	none	26.3	0.280	-	4.952	4.000	-	-	0.858
2b	30' at 45°C	25.6	0.050	-	4.988	3.926	-	-	1.309
3a	none	17.1	0.090	0.077	0.845	0.890	-	-	0.706
3b	30' at 45°C	19.5	0.036	-	1.252	1.169	-	-	0.926
3c	60' at 45°C	17.6	0.017	-	1.188	1.288	-	-	1.036
4a	none	29.0	0.110	0.090	2.289	-	1.336	1.212	1.293
4b	60' at 45°C	35.0	0.040	-	3.975	-	2.533	2.649	2.436

* n.m. = not measurable

be correlated with 17ß-hydroxysteroid dehydrogenase activity.

Heat denaturation

When the erythrocyte protein fraction was heated for 30 or 60 min at 60° or 45° a decrease in 17ß-hydroxysteroid dehydrogenase activity was observed (Table 2.I). In one case (1b, Table 2.I) it was not possible to measure any enzymic activity after the heat treatment. Dialysis of the erythrocyte protein fraction against testosterone during 40 h at 4°C gave a small decrease in enzymic activity (experiments 3 and 4, Table 2.I). Equilibrium dialysis experiments of partially heat denaturated enzyme preparations with testosterone showed in all cases higher B/U values than for the corresponding untreated preparation of the erythrocyte protein fraction. Although the specific binding of testosterone by the untreated preparations (indicated by decreasing B/U values with increasing testosterone concentrations during dialysis) was less pronounced in experiments 1 and 3 (Table 2.I), the specific binding after the heat treatment was almost completely lost or decreased (experiments 2 and 4, Table 2.I).

3.3.2 Correlation between 5α-dihydrotestosterone binding in plasma and in the erythrocyte protein fraction

It was reported in Appendix paper II that both testosterone and 5α-dihydrotestosterone are specifically bound by the erythrocyte protein fraction in equilibrium dialysis experiments. In Figure 3.3 a Scatchard curve is plotted of the binding of both steroids by the same erythrocyte protein fraction. 5α-Dihydrotestosterone showed a higher affinity for this fraction when compared with testosterone. From the data in Figure 3.3 apparent association constants were calculated for 5α-dihydrotestosterone and testosterone as 3×10^9 M^{-1} and 0.9×10^9 M^{-1} respectively.

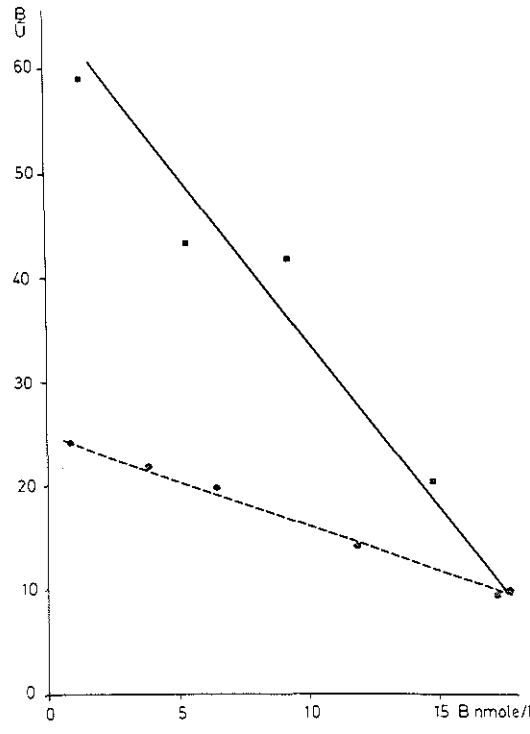

Fig. 3.3. Binding of testosterone and 5α-dihydrotestosterone by an erythrocyte protein fraction with steroid dehydrogenase activity. This protein fraction was dialysed at 4°C against the following steroid concentrations: 0.058 nM, 0.280 nM, 0.490 nM, 1.120 nM and 2.373 nM. Protein concentration: 58 mg/ml.
■ 5α-dihydrotestosterone
● testosterone

Subsequently the binding of 5α-dihydrotestosterone by a 2% (v/v) plasma preparation was compared with the binding of this steroid by the erythrocyte protein fraction. 5α-Dihydrotestosterone was used in these experiments because this steroid showed a higher affinity than testosterone for the erythrocyte protein fraction. The advantage of the higher affinity is that less dissociation occurs during sucrose gradient centrifugation or acrylamide gel electrophoresis.

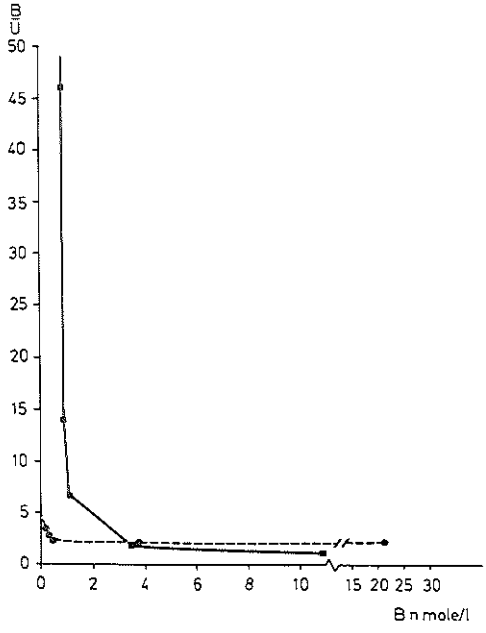

Fig. 3.4. Binding of 5α-dihydrotestosterone by 2% (v/v) plasma and an erythrocyte protein fraction. Both protein preparations were dialysed at 4°C against the following steroid concentrations: 0.058 nM, 1.120 nM, 2.373 nM, 23.730 nM and 116.31 nM.
■ 2% (v/v) plasma, protein concentration: 1.5 mg/ml
• erythrocyte protein fraction, protein concentration 50 mg/ml

Equilibrium dialysis

An equilibrium dialysis experiment of 5α-dihydrotestosterone was performed with these two protein preparations. The results are shown in Figure 3.4. The Scatchard plots show that part of the 5α-dihydrotestosterone is bound specifically in both protein preparations, although in the erythrocyte protein fraction, the specific binding part of the curve is not very clear.

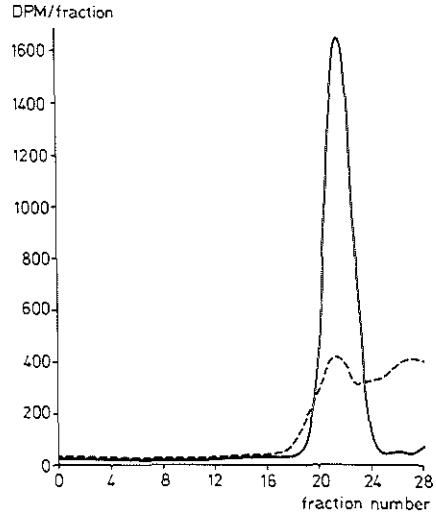

Fig. 3,5. Distribution of radioactivity after sucrose gradient centrifugation of an erythrocyte protein fraction previously incubated with ^{3}H-5α-dihydrotestosterone and of a 2% (v/v) plasma solution previously incubated with ^{3}H-5α-dihydrotestosterone.The gradients were centrifuged at 40,000 rpm for 18 h in an SW 40 rotor of the Beckmann L2-65B ultracentrifuge.For details see Methods section.

——— 2% (v/v) plasma,protein concentration: 1,5 mg/ml

------- erythrocyte protein fraction,protein concentration: 50 mg/ml

Sucrose gradient centrifugation

When 400 µl of both protein fractions, previously incubated at 4°C with [1,2 ^{3}H] 5α-dihydrotestosterone were layered on a sucrose gradient and centrifuged at 40,000 RPM during 18 h a sedimentation pattern was obtained as shown in Figure 3.5. A sharp peak of radioactivity was observed around fraction 22 of the 2% plasma sample and a much smaller peak was observed for the erythrocyte protein fraction. The plasma preparation and the erythrocyte protein fraction were the same as used in the preceding equilibrium dialysis experiment.

Acrylamide gel electrophoresis

Binding of 5α-dihydrotestosterone was also studied with

polyacrylamide gel electrophoresis. The electrophoretic profiles of radioactivity are shown in Figure 3.6. With 2% plasma a radioactive peak was observed about 19 mm from the origin. A much more complex picture was obtained with the erythrocyte protein fraction. No discrete radioactive peaks were observed, although there might be an indication for some steroid-protein association in material moving 10 and 19 mm from the origin. Electrophoresis of 40 µl buffer solution containing [1,2 ^{3}H] 5α-dihydrotestosterone without protein resulted in a radioactivity pattern which showed some electroendosmosis till 5 mm from the origin and a constant level of about 1500 dpm in the rest of the gel (see Figure 3.6).

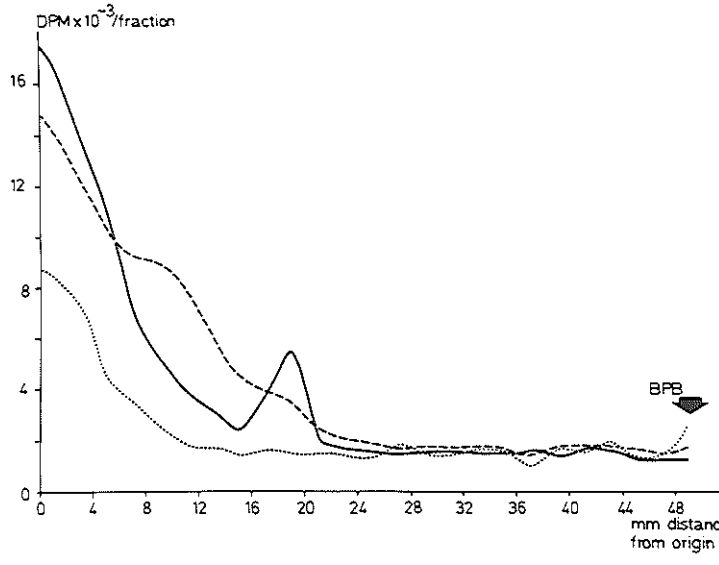

Fig. 3,6. Distribution of radioactivity after polyacrylamide gel electrophoresis of ^{3}H-5α-dihydrotestosterone incubated with 2% (v/v) plasma ———— , with an erythrocyte protein fraction --------, and with buffer containing no protein ····· . Electrophoresis was performed on 7% gels with a current of 2 mA/gel. Broom phenol blue (BPB) was used as a marker for the migrating boundary. Estimation of radioactivity in 2 mm gel sections after electrophoresis has been described under Methods. Protein concentration of the plasma sample was 1,5 mg/ml and of the erythrocyte protein fraction 50 mg/ml.

3.4 Discussion

The purpose of this investigation was to obtain more information about the character of a protein fraction from the membrane-free haemolysate of human erythrocytes that in equilibrium dialysis experiments reflects a specific binding of testosterone. Density gradient centrifugation and polyacrylamide gel electrophoresis together with equilibrium dialysis have been used to investigate the steroid binding. These techniques are widely accepted for demonstration of high affinity binding sites for steroids in several target tissues and in plasma[4,5]. Although some dissociation of the steroid from the high affinity binding sites occurs during the centrifugation process and during electrophoresis, a good separation can be obtained between unbound, aspecifically bound and specifically bound steroid.

3.4.1 Correlation between binding and enzymic activity

Sucrose gradient centrifugation

The sedimentation pattern of the erythrocyte protein fraction previously incubated with radioactive testosterone did not reflect an association of testosterone with any sedimenting macromolecules (see Figure 3.1). Because of the lack of separation between unbound steroid and a possible bound fraction gradients were run with protein preparations, which have previously been incubated with decreasing amounts of radioactive testosterone, but no improvement was obtained in the separation. The small shoulder around fraction 20, however, was always observed. This might indicate a low affinity association of a protein with testosterone, which readily dissociated during the centrifugation process. Binding of testosterone by binding sites of the 17β-hydroxysteroid dehydrogenase, the enzyme which is present in this erythrocyte protein fraction, could not be observed, because fraction 16, which contained the dehydrogenase activity in

parallel runs did not contain any radioactivity.

Acrylamide gel electrophoresis

Polyacrylamide gel electrophoresis can be used for studying steroid binding by proteins as is demonstrated radioactivity pattern after electrophoresis of the plasma sample. It was not possible, however, to compare the enzymic activity pattern with the binding activity pattern after electrophoresis, because after electrophoresis no association was observed between radioactive testosterone and macromolecules. The radioactivity at the top of the gel column might be due to dissociation of testosterone from any binding sites present in the erythrocyte protein fraction and to electroendosmosis of unbound testosterone.

Heat denaturation

Mulder et al.[2] showed that 17β-hydroxysteroid dehydrogenase from human erythrocytes is partially destructed after a short incubation period (15 min) at 43°C. It has also been shown that specific steroid binding by certain plasma proteins is abolished when the plasma is incubated for a short period at 60°C (ref. 6). The plasma progesterone binding protein (PBP) from the pregnant guinea pig, however, is thermostabile[7]. Heat treatment may therefore provide a way to discriminate between different steroid binding sites, particularly when these binding sites are present in the same protein sample. Heat denaturation of the 17β-hydroxysteroid dehydrogenase paralleled however the heat inactivation of specific testosterone binding sites. The increase in the B/U values of the heat treated samples compared with the B/U values of the corresponding untreated samples might result from the production of partially denatured proteins and an increase in non-specific low affinity binding sites. Increased B/U values has also been observed by Schaumburg

after heating specific cortisol binding fractions from thymocytes[8]. It is therefore concluded that it is impossible to discriminate between possible binding sites from the enzyme and binding sites from other proteins.

3.4.2 Correlation between 5α- dihydrotestosterone binding in plasma and in the erythrocyte protein fraction

Observations by Mulder et al.[2] showed that the 17β-hydroxysteroid dehydrogenase from human erythrocytes can also oxidize 5α-dihydrotestosterone. The erythrocyte protein fraction showed a higher affinity for 5α-dihydrotestosterone than for testosterone. Binding of 5α-dihydrotestosterone by the sex steroid binding globulin (SBG) also occurs with a higher affinity when compared with testosterone[9]. Therefore, it is still possible that binding by the erythrocyte protein fraction might be due to a plasma protein. A contamination by plasma proteins is very unlikely according to Table II of Appendix paper II. The possibility may exist, however, that intact erythrocytes contain very small quantities of plasma proteins and that these plasma proteins are present in the membrane-free haemolysate. Comparative studies in which 5α-dihydrotestosterone binding was studied by a 2% (v/v) plasma solution and by the erythrocyte protein fraction were performed using three different binding techniques. The protein preparations were the same throughout the three different methods.

Equilibrium dialysis

From the results of the equilibrium dialysis experiments it is evident that the concentration of high affinity binding sites in the erythrocyte protein fraction is very low as compared with plasma. Calculation of an apparent association constant from the Scatchard curve is possible but the value is very inaccurate and does not provide a basis for a comparison of the two protein preparations.

Sucrose gradient centrifugation

Density gradient centrifugation resulted in a radioactive peak in corresponding fractions for both protein preparations. Haemoglobin, however, sediments with the same rate as the peak of radioactivity found for the erythrocyte protein fraction previously incubated with ^{3}H-5α-dihydrotestosterone. The amount of haemoglobin in the erythrocyte protein fraction was about 20% of the total protein present in this prepartion. A contribution of haemoglobin to the binding of 5α-dihydrotestosterone could therefore not be excluded. Density gradient centrifugation of a haemoglobin solution previously incubated with ^{3}H-5α-dihydrotestosterone revealed a radioactivity sedimentation profile identical to the one obtained after centrifugation of ^{3}H-5α-dihydrotestosterone without any protein. Association of 5α-dihydrotestosterone with haemoglobin in the erythrocyte protein fraction is therefore very unlikely. The peak of radioactivity after gradient centrifugation obtained with plasma might be due to binding of 5α-dihydrotestosterone by both albumin and the sex steroid binding globulin. Our experimental conditions do not permit a discrimination between these two possible binding proteins.

Acrylamide gel electrophoresis

A good resolution of a protein mixture can be obtained by polyacrylamide gel electrophoresis. Binding of radioactive steroids by low affinity sites from a protein which migrates in front of a protein with high affinity sites may cause a high background level of radioactivity in the gel by dissociation of the steroid from the low affinity sites. This could be an explanation for the electrophoretic pattern obtained after electrophoresis of the erythrocyte protein fraction. The presence of low affinity sites, which bind

5α-dihydrotestosterone in a non-specific way was shown in equilibrium dialysis experiments.
Dissociation of 5α-dihydrotestosterone from these sites may interfere in the detection of binding of this steroid by high affinity sites in the electrophoresis experiment. Electrophoresis of radioactive 5α-dihydrotestosterone in buffer revealed a pattern of radioactivity where maximal background level is reached at 10 mm from the origin. It is therefore evident that the high level of radioactivity up to 20 mm distance from the origin in the electrophoretic run of the erythrocyte protein fraction reflects some kind of association of 5α-dihydrotestosterone with protein. Electrophoresis of 2% (v/v) plasma resulted in a peak of radioactivity at the same distance as the second small shoulder in the gel with the erythrocyte protein fraction. This might be an indication for the presence of the same binding entity in both samples.

Binding of progesterone by membrane-free erythrocyte protein fractions studied by DeVenuto et al.[10] resulted in a low value for the combining affinity for the non-haemoglobin proteins. Binding was not detectable by the membrane-free haemolysate and haemoglobin. The protein concentration of the fractions used by DeVenuto were low compared with the fractions used in the present investigation. This could explain the diverging results (see also Appendix paper II).

Because the concentration of binding sites in almost all preparations was very small, binding was hardly detectable with the techniques used. This can be explained by a low affinity of the binding sites, or by the small number of specific binding sites (see also Appendix paper II). In both cases it is very unlikely that these binding sites have any function or regulatory role in the uptake of steroids by intact erythrocytes and their presence in the erythrocyte is therefore of limited physiological significance.

3.5 References

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CHAPTER 4

BINDING OF STEROIDS BY RAT TESTICULAR TISSUE *

4.1 Introduction

One of the features of hormones in the male is the initiation and maintenance of spermatogenesis. The hormonal control of this process has been extensively reviewed by Steinberger[1]. In hypophysectomized animals daily injections of large amounts of testosterone propionate can partly restore and maintain spermatogenesis. The transitions gonocytes → type A spermatogonia and pachytene spermatocytes → secondary spermatocytes are considered to be under the influence of testosterone. The seminiferous tubules can therefore be considered as an androgen target tissue. It is also well known that in hypophysectomized rats only combinations of FSH and ICSH or of FSH and testosterone will restore spermatogenesis[2,3]. The obligatory requirement of FSH may imply that this hormone has a direct influence on the spermatogenic processes. Although hormonal control of spermatogenesis is obvious, the precise hormonal mechanisms in these processes are still unknown.

Specific retention of testosterone or its metabolites has been demonstrated in several androgen target organs and soluble proteins which show a high affinity for 5α-dihydrotestosterone and testosterone have been isolated. The occurrence of these "receptors" in different steroid target tissues of several animal species is summarized in Table 4.I. Association of a steroid with a target cell receptor may be a prerequisite for the action of that steroid on that target cell (see Chapter 1). Therefore, experiments were performed to investigate the possibility if a specific association of steroids with testicular soluble proteins

* Published in part in FEBS Lett. 26 (1972)

Table 4,I

The occurrence of receptors for testosterone and 5α-dihydrotestosterone in different tissues from several animal species.

Tissue	Species	References
Epididymis	Rat	Blaquier[9];Ritzen et al.[12]
Levator Ani Muscle	Rat	Jung and Baulieu[14]
Prostate	Calf	Jungblut et al.[8]
	Human	Hansson et al.[11]
	Rat	Unhjem et al.[4];Fang et al.[5] Mainwaring[6];Baulieu and Jung[7]
Seminal Vesicle	Pig	Jungblut et al.[8]
	Rat	Stern and Eisenfeld[13]
Uterus	Calf	Jungblut et al.[8]
	Rat	Giannopolous[10]

existed. Binding of testosterone, 5α-dihydrotestosterone, corticosterone and oestradiol by cytosol proteins from testis and prostate was studied by density gradient centrifugation. A comparison was made between the binding of steroids by testicular cytosol fractions obtained from immature rats, mature rats, hypophysectomized rats and from seminiferous tubules and interstitial tissue of mature rat testes.

4.2 Materials and methods

4.2.1 Steroids

[1,2,6,7 ^{3}H] Testosterone (specific activity:100 Ci/mmole); [2,4,6,7 ^{3}H] oestradiol (specific activity:100 Ci/mmole); [1,2,6,7 ^{3}H] corticosterone (specific activity:106 Ci/mmole) and [1,2 ^{3}H] 5α-dihydrotestosterone (specific activity: 49 Ci/mmole) were obtained from the Radiochemical Centre in Amersham (Great Britain). The radiochemical purity of these steroids was verified by paper and thin layer chromatography.

4.2.2 Preparation of subcellular fractions

Immature (26 day old) and mature (3 month old) Wistar rats were used in the experiments. After decapitation the testis was isolated and the tunica albuginea was removed. The testis tissue was homogenized in a motor driven Potter Elvehem homogenizer in 2 volumes 10 mM Tris buffer, (pH 7.4), containing 1.5 mM EDTA and 2 mM mercaptoethanol (TEM-buffer). Glycerol was added to make a final glycerol concentration of 10%. The homogenate was centrifuged at 105,000 x g for 60 min at 0^{o}C. The supernatant ("cytosol"), was incubated for 2 hours at 0^{o}C with steroid. Interstitial tissue and seminiferous tubules were obtained after wet dissection of the testis according to Christensen and Mason[15]. Any remaining small fragments of tubules were removed from the interstitial tissue with the aid of a dissecting microscope. The tubules were washed in Krebs-Ringer bicarbonate buffer (pH 7.4), containing glucose (10^{-2}M). Any residual interstitial tissue was removed from the tubular preparation. The 105,000 x g supernatants of interstitial tissue and tubules were prepared and incubated with steroid as described for whole testis. Ventral prostate lobes were obtained from rats 1 day after castration. The prostatic tissue was minced and homogenized. The 105,000 x g supernatant was prepared and incubated with steroid as described for whole testis. For

studies in vivo with immature rats 5µCi ^{3}H-oestradiol was injected subcutaneously 5 minutes before decapitation. Blood plasma was obtained by centrifuging heparinized rat blood for 10 minutes at 3000 x g. After dilution with 4 volumes TEM-buffer containing 10% of glycerol, the plasma mixture was incubated with steroid as described for testis cytosol.

4.2.3 Sucrose gradient centrifugation

Cytosols (200 µl, approximately 3 mg of protein) which had previously been incubated with steroid, were layered on linear 5-15% (w/v) sucrose gradients, prepared in TEM-buffer containing 10% of glycerol and centrifuged for 18 h at 49,000 RPM in a SW 65 rotor of a Beckmann L2-65B ultracentrifuge at 0°C. Yeast alcohol dehydrogenase ($S_{20,w}$:7.4 S) and bovine serum albumin ($S_{20,w}$:4.6 S) were used as markers for calculation of sedimentation coefficients[16]. After centrifugation, approximately 30 fractions were collected following piercing of the bottom of the centrifuge tube. The radioactivity in the individual fractions was estimated as described in Chapter 3.

4.2.4 Binding assay with dextran coated charcoal

^{3}H-Oestradiol (4 pg; 0.0015 µCi) dissolved in 100 µl of benzene was added to various amounts of unlabelled oestradiol and dried under nitrogen. Testis cytosol (200 µl) from immature rats was added and allowed to equilibrate with the steroid at 30°C for 1 h. After the equilibration period, a 50 µl aliquot was taken for counting and 100 µl was added to 200 µl of a 0,25% (w/v) charcoal suspension containing 0.025% (w/v) of dextran. After mixing for 5 s the solution was kept at 4°C for 15 min. Subsequently the solution was centrifuged at 1200 x g at 4°C for 10 min and 200 µl aliquots of supernatant were removed for counting. Results were expressed as B/U = f (B). B = concentration of bound steroid; U = concentration of unbound steroid.

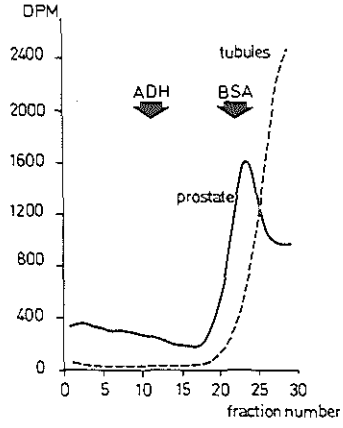

Fig. 4.1. Sucrose gradient analysis of 5α-dihydrotestosterone binding by the 105,000 x g cytosol fractions from prostate (full line) and seminiferous tubules (dotted line). The cytosol fractions were incubated with 5 x 10^{-10}M steroid. Yeast alcohol dehydrogenase (ADH) and bovine serum albumin (BSA) were used as sedimentation markers.

4.3 Results

4.3.1 Binding of 5α- dihydrotestosterone by cytosol fractions of prostate and of seminiferous tubules

The interaction of ^{3}H-5α-dihydrotestosterone with a soluble protein fraction of seminiferous tubules was studied by sucrose gradient centrifugation at 0°C. A sedimentation pattern of radioactive 5α-dihydrotestosterone, previously incubated with cytosol of tubules, is shown in Figure 4.1. Most of the radioactive steroid could be recovered at the top of the gradient and no distinct peaks of radioactivity were observed. Similar results were obtained when this testis fraction was incubated with testosterone prior to sucrose gradient centrifugation. Cytosol obtained from the ventral prostate incubated with radioactive 5α-dihydrotestosterone showed a sedimentation profile of radioactivity different from what was expected on the basis of published results of similar experiments[4,6,7]. A regular shaped peak of radioactivity in the 8-10 S sedimentation region which has been reported by others was, however, absent, only a broad

elevation of radioactivity towards the bottom of the gradient was found (Fig. 4.1). In several experiments of prostate cytosol with variable protein concentrations or steroid concentrations, the sedimentation pattern of radioactivity could not be improved. Cytosols obtained from prostates of rats 3 days after castration did not show any 5α-dihydrotestosterone binding in the 8-10 S area.

4.3.2 · Effect of hypophysectomy on testosterone binding

Endogenous steroid in the tubular cytosol preparation could possibly mask the presence of an androgen binding component. Therefore a comparison was made between the binding of testosterone by cytosols of testes obtained from hypophysectomized rats, and normal rats. Only the radioactivity sedimentation pattern obtained with cytosol from testis of rats 7 days after hypophysectomy resulted in a

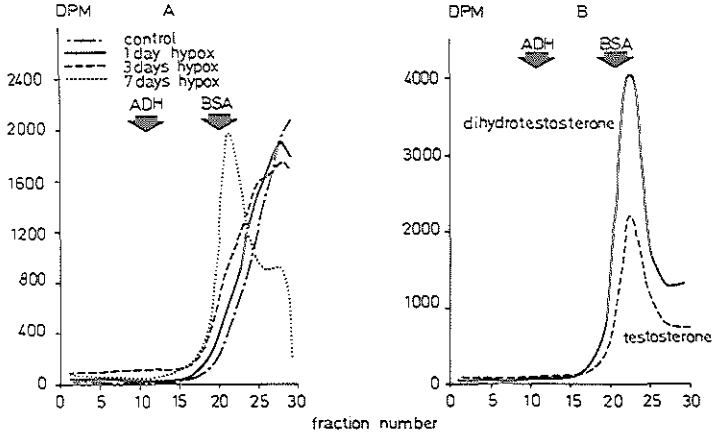

Fig. 4,2. A. Sucrose gradient analysis of testosterone binding by the 105,000 x g cytosol fractions of testes from normal rats (control) and from rats hypophysectomized for 1, 3 or 7 days (resp.: 1 day hypox, 3 days hypox, 7 days hypox). The cytosol fractions were incubated with 2.5×10^{-10}M steroid.

B. Sucrose gradient analysis of steroid binding by the 105,000 x g cytosol fraction of testis from immature (26 day old) rats. The cytosol fraction was incubated either with 3×10^{-9}M 5α-dihydrotestosterone (full line) or with 0.75×10^{-9}M testosterone (dotted line).

radioactivity peak in the 4 S region (Fig. 4.2A).

4.3.3 Binding of steroids by cytosol of testes from immature rats

In figure 4.2B the radioactivity sedimentation profiles are shown of cytosols obtained from immature rats previously incubated with either ^{3}H-5α-dihydrotestosterone or ^{3}H-testosterone. For both steroids binding macromolecules were observed with sedimentation coefficients of approximately 4 S. The binding of two other steroids, oestradiol and corticosterone, by testis cytosol from immature rats was also investigated by sucrose gradient centrifugation. The sedimentation profiles are shown in Figure 4.3B. Figure 4.3B shows that a binding component for oestradiol is present with a

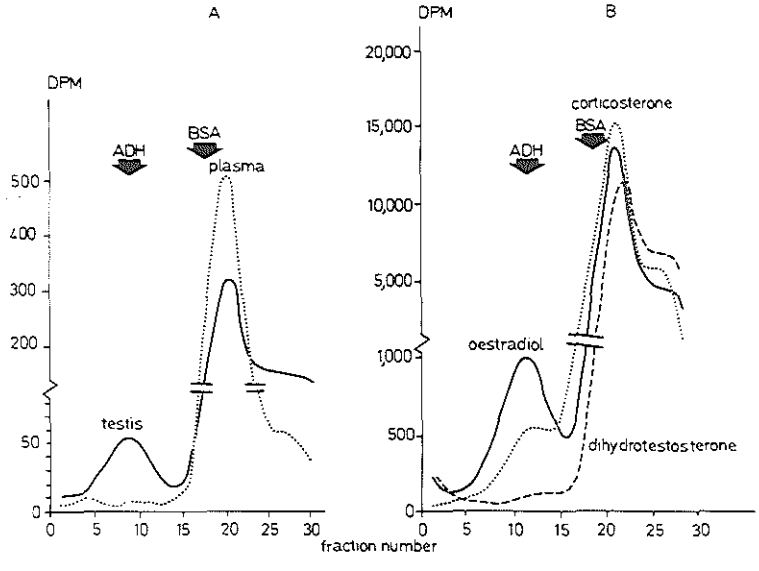

Fig. 4,3. A. Sucrose gradient analysis of oestradiol binding by the 105,000 x g cytosol fraction of testis and by blood plasma from immature (26 day old) rats. Plasma and cytosol fraction were incubated with 0.5 x 10^{-10}M oestradiol.

B. Sucrose gradient analysis of steroid binding by the 105,000 x g cytosol fraction of testis from immature (26 day old) rats. The cytosol fraction was incubated either with 4.5 x 10^{-9}M 5α-dihydrotestosterone, 4.5 x 10^{-9}M corticosterone or with 4.5 x 10^{-9}M oestradiol.

sedimentation coefficient of approximately 8 S. For corticosterone an elevation of radioactivity was observed in the 8 S region, while 5α-dihydrotestosterone was only associated with macromolecules located in the 4 S region (Fig. 4.2B and Fig. 4.3B).

4.3.4 Binding of oestradiol by plasma of immature rats

Plasma obtained from blood of immature rats was incubated with oestradiol and subsequently analyzed for the presence of oestradiol binding components by sucrose gradient centrifugation. The results of these experiments were compared with those of oestradiol binding by testis cytosol. As shown in Figure 4.3A only a peak of radioactivity was observed in the 4 S region with plasma, while analysis of

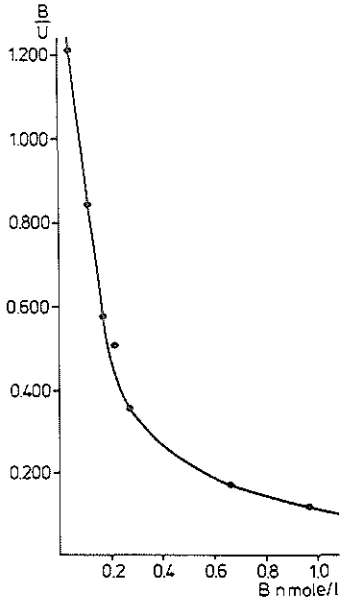

Fig. 4.4. Scatchard plot of oestradiol binding by the 105,000 x g cytosol fraction of testes from immature (26 day old) rats as determined by the dextran coated charcoal adsorption method (for details see: Methods section). B/U represents the amount of bound (B) over unbound (U) oestradiol at equilibrium. Protein concentration: 14.8 mg/ml.

testis cytosol resulted in a peak of radioactivity both in the 8 S and in the 4 S regions. Protein and steroid concentrations for the plasma preparation and for the testis cytosol preparation were identical.

4.3.5 Affinity of oestradiol binding sites

A quantitative approach to the problem of oestradiol binding in the testis cytosol of immature rats is shown in Figure 4.4. From the "Scatchard plot" of the binding of oestradiol by testis cytosol, values could be obtained for an apparent association constant and the concentration of binding sites. Binding data were obtained with a dextran-coated charcoal assay. From Figure 4.4 an apparent association constant of $4 \times 10^{9} M^{-1}$ could be calculated. The concentration of binding sites in the cytosol was found to be 2×10^{-14} mole/mg protein.

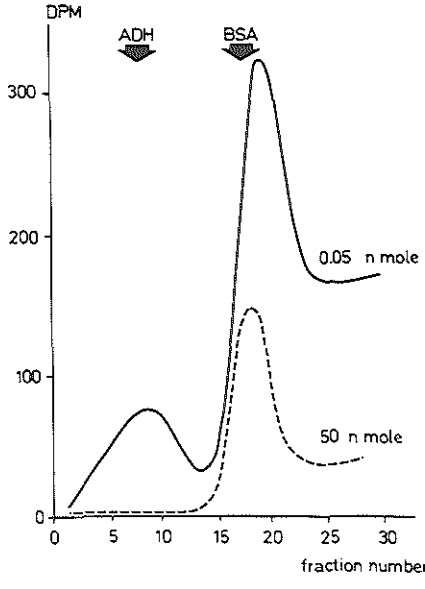

Fig. 4,5. Sucrose gradient analysis of oestradiol binding by the 105,000 x g cytosol fraction of testes from immature (26 day old) rats after subcutaneous administration of ^{3}H-oestradiol. Either 5×10^{-2} nmole ^{3}H-oestradiol (full line) or 5×10^{-2} nmole ^{3}H-oestradiol + 50 nmole oestradiol (dotted line) was injected per rat 5 min before decapitation.

4.3.6 In vivo experiments concerning oestradiol binding

When oestradiol binding by testis cytosol was analysed after labelling in vivo (subcutaneous injection) with ^{3}H-oestradiol a peak of radioactivity was observed in the 8 S region (Fig. 4.5). If a thousand fold excess of non radioactive oestradiol was injected together with ^{3}H-oestradiol no detectable radioactivity was observed in the 8 S region, indicating a limited number of high affinity binding sites for oestradiol.

4.3.7 Specificity of oestradiol binding sites

Competition for oestradiol binding sites between ^{3}H-oestradiol and a thousand fold excess of non radioactive oestradiol, 5α-dihydrotestosterone, corticosterone and

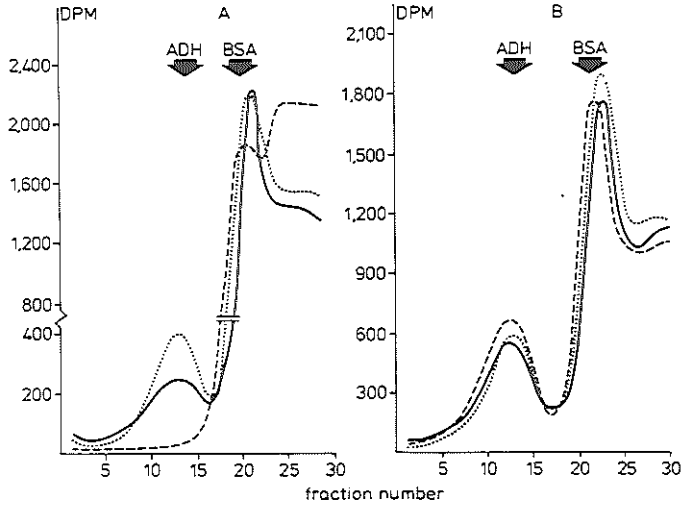

Fig. 4,6. A. Sucrose gradient analysis of oestradiol binding by the 105,000 x g cytosol fraction of testes from immature (26 day old) rats. The cytosol fraction was incubated either with 3 x 10^{-10}M ^{3}H-oestradiol ..... , 3 x 10^{-10}M ^{3}H-oestradiol + 3 x 10^{-7}M oestradiol ...., or with 3 x 10^{-10}M ^{3}H-oestradiol + 3 x 10^{-7}M 5α-dihydrotestosterone ———— .

B. Sucrose gradient analysis of oestradiol binding by the 105,000 x g cytosol fraction of testes from immature (26 day old) rats. The cytosol fraction was incubated either with 3 x 10^{-10}M ^{3}H-oestradiol ..... , 3 x 10^{-10}M ^{3}H-oestradiol + 3 x 10^{-7}M testosterone ---------, or with 3 x 10^{-10}M ^{3}H-oestradiol + 3 x 10^{-7}M corticosterone ———— .

testosterone respectively was investigated in vitro. As is shown in Figure 4.6A the binding of ^{3}H-oestradiol in the 8 S region was completely abolished by a large excess of non-radioactive oestradiol. The studies with 5α-dihydro-testosterone resulted in a slight decrease of ^{3}H-oestradiol binding in the 8 S region (Fig. 4.6A). Testosterone and corticosterone did also not compete for ^{3}H-oestradiol binding in the 8 S region as is shown in Figure 4.6B.

4.3.8 Binding of oestradiol in interstitial tissue and seminiferous tubules

Oestradiol binding by the cytosol of testis from mature rats (3 month old) is shown in Figure 4.7A. A peak of radioactivity was observed in the 8 S region similar to the results with testis cytosol from immature rats. In the 4 S region no regular shaped peak of radioactivity was detectable when the cytosol fraction of testes from

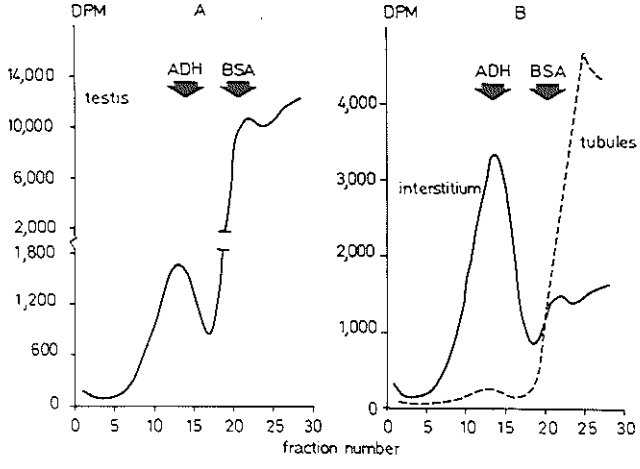

Fig. 4.7. A. Sucrose gradient analysis of oestradiol binding by the 105,000 x g cytosol fraction of testes from mature (3 month old) rats. The cytosol fraction was incubated with 3×10^{-10}M oestradiol.

B. Sucrose gradient analysis of oestradiol binding by the 105,000 x g cytosol fractions of interstitial tissue (full line) and seminiferous tubules (dotted line). The cytosol fractions were incubated with 0.7×10^{-10}M oestradiol.

mature rats was incubated with oestradiol and subjected to the standard technique of separation. In the next series of experiments rat testis was dissected into interstitial tissue and seminiferous tubules. The cytosols of both tissue preparations were incubated with ^{3}H-oestradiol and analyzed for binding components by sucrose gradient centrifugation. The results of these experiments are shown in Figure 4.7B. With the cytosol obtained from the interstitial tissue a sharp peak of radioactivity was observed in the 8 S region and only a small shoulder in the 4 S region. Analysis of the tubular preparation revealed only a very small elevation of radioactivity in the 8 S region.

4.4 Discussion

The mechanism by which androgens maintain spermatogenesis is unknown. The possibility may be considered that specific binding of the steroid by tubular tissue proteins has to occur. This hypothesis is mainly based on findings for oestrogens in uterine tissue and for androgens in prostatic tissue: specific binding of steroids by cytoplasmatic receptor proteins may be a prerequisite for their mode of action. Selective binding of steroids by tubular tissue has been suggested by Bell *et al.*[17] after incubation studies *in vitro* with progesterone and Δ^{5}-pregnenolone. Cooke *et al.*[18] have shown that a retention of testosterone by seminiferous tubules occurred after incubation *in vitro* of whole testis tissue. Uptake *in vivo* of radioactive steroids by seminiferous tubules has been studied by Parvinen *et al.*[19,20]. Although these authors observed that testosterone, Δ^{5}-pregnenolone and progesterone were readily taken up by tubular tissue *in vivo*, they did not demonstrate a specific binding of these steroids. Also small amounts of 5α-dihydrotestosterone were detectable in the seminiferous tubules[20]. In prostatic tissue 5α-dihydrotestosterone is probably the main biological active metabolite of

testosterone, because events characteristic of androgen action are preceeded by intranuclear binding of the 5α-reduced compound, rather than of testosterone[21]. In this respect the presence of a 3-oxosteroid-5α-reductase in testicular tissue may be relevant[22]. O'Malley and coworkers[23] found that chromatin from testis tissue is capable of binding prostatic cytosol labelled with 5α-dihydrotestosterone. This might indicate that testis chromatin has acceptor sites for a specific androgen-receptor complex. These observations prompted us to investigate the possible presence of androgen binding proteins in testicular tissue, particularly in the seminiferous tubules.

Sucrose gradient analysis of 5α-dihydrotestosterone binding by tubular cytosol gave no evidence for the presence of a binding component with a high affinity and low capacity with a sedimentation value of approximately 8 S (Fig. 4.1).

Prostatic cytosol from rats 1 day after castration showed binding of 5α-dihydrotestosterone in the 8-10 S region although no regular shaped peak was observed (Fig. 4.1). Partial denaturation of the 8 S androgen receptor may have occurred because this prostate protein is known to be very labile[6,7]. Interaction of the binding protein with coagulating mucopolysaccharides might also be an explanation[8].

Testosterone binding in mature rat testis cytosol could not be demonstrated with sucrose gradient centrifugation. Only in testes of immature or 7 days hypophysectomized rats testosterone binding macromolecules with a sedimentation coefficient of approximately 4 S were observed.

Steroid receptors in steroid target tissues can only be demonstrated in castrated or in immature animals. The explanation for this is the low level of endogenous steroids in the steroid target tissues of these animals. In normal mature animals the receptor binding sites are completely occupied by endogenous steroids, which are hardly exchangeable with labelled exogenous steroids. The absence of endogenous

steroid, however, can also cause a decrease in receptor sites in the tissue, as is demonstrated by Baulieu and Jung[7]. Endogenous testosterone and 5α-dihydrotestosterone may occupy the possible binding sites in the mature rat testis and may prevent therefore the demonstration of these sites with labelled testosterone or 5α-dihydrotestosterone. To eliminate a possible interfering role of endogenous steroids, binding by testis cytosols obtained from immature and hypophysectomized rats was studied. We have only observed binding of testosterone by 4 S macromolecules in testis cytosol of rats 7 days after hypophysectomy (Fig. 4,2A) and similar results were obtained with testis cytosol from immature rats for both testosterone and 5α-dihydrotestosterone (Fig. 4,2B). This observed binding in the 4 S region could also be partly due to plasma proteins which are present in testicular lymph and rete testis fluid[24,25]. A discrimination between binding by a 3-5 S receptor and a plasma protein contamination, however, is very difficult to determine under our experimental conditions. Centrifugation at a higher speed and for a longer period of time may possibly result in a better resolution of binders in the 3-5 S region of the sedimentation profile.

Mainwaring[31] has observed a 4 S testosterone binding component in the cytosol of testes from normal mature rats only after endogenous testosterone was removed from the cytosol by adsorption to charcoal. With an isoelectric focussing technique he was also able to demonstrate the presence of a testosterone binding protein in rat testis. Charcoal treatment of rat testis cytosol has also been used by Ritzen et al.[32] who demonstrated with gel electrophoresis the presence of an androgen binding protein with a high affinity for testosterone and 5α-dihydrotestosterone in rat testis cytosol. Recently Vernon et al.[33] reported the presence of two testosterone binding proteins in the cytosol fraction of the seminiferous tubules from 8 days hypophysectomized rats. Vernon and coworkers assayed the testosterone binding activity by a dextran coated charcoal adsorption

method, but their results do not exclude the possibility that one of the binding proteins may be a plasma protein. None of these recent reports on testosterone binding by rat testis cytosol, gives any indication about the possible presence of a specific "8 S" testosterone receptor in testis.

Binding studies with oestradiol and testis cytosol from immature rats resulted in the demonstration of an oestradiol binding component with a sedimentation coefficient of approximately 8 S, which was not present in plasma (Fig. 4.3A and 4.3B). Some corticosterone binding was also detectable in the 8 S region but a well defined peak of radioactivity was not observed.

The apparent association constant for oestradiol binding by testes cytosol is approximately 4×10^9 M^{-1}. For the oestrogen receptor complex from uteri of different species variable values for association constants have been published, ranging from 1.5×10^9 M^{-1} for calf uterus till 3×10^{10} M^{-1} for rabbit uterus respectively [26,27].

The number of oestradiol binding sites in testis tissue was limited as was shown by *in vivo* and *in vitro* labelling with ^{3}H-oestradiol in the presence of a thousand fold excess of non radioactive oestradiol. 5α-Dihydrotestosterone, testosterone and corticosterone did not compete with ^{3}H-oestradiol for the specific binding sites. These observations indicate that the oestradiol binding macromolecule has a specific affinity for oestradiol.

The oestradiol binding component was also present in cytosol from testis of mature rats (Fig. 4.7A). This oestradiol binding component in the mature rat testis is localized in the interstitial tissue (Fig. 4.7B). The small peak of radioactivity in the 8 S region observed with the tubular preparation may be due to a contamination by interstitial tissue, although the tubules were extensively washed after dissection.

Our finding that the oestradiol binding macromolecule is localized in the interstitial tissue and not in the seminiferous tubules is supported by the autoradiographic observations made by Stumpf et al.[28,29]. These authors demonstrated a nuclear localization of radioactivity in the interstitium of immature rat testis after in vivo administration of ^{3}H-oestradiol and suggested that the interstitial tissue could be considered as an oestrogen target tissue. The in vivo and in vitro demonstration of specific oestradiol binding sites in the mature rat testis with ^{3}H-oestradiol indicates that these sites are only partly occupied by endogenous oestradiol. Occupation of specific oestradiol binding sites by endogenous oestradiol could, however, be expected since de Jong and Van der Molen have demonstrated testicular oestradiol secretion in the rat[30]. Different localizations of testicular oestradiol synthesis and occurrence of specific receptors for this steroid hormone might explain our findings.

Data obtained in this study concerning the oestradiol binding in cytosol from rat testis strongly suggest the presence of an oestradiol receptor in the soluble protein fraction of the interstitial tissue of rat testis. This oestradiol receptor has a sedimentation coefficient of 8 S, an association constant of $4 \times 10^{9} M^{-1}$, a limited number of binding sites, an oestradiol specificity and a localization in the interstitial tissue and not in the seminiferous tubules. The occurrence of this oestradiol binding principle in the rat testis raises a series of questions about the role of an oestradiol receptor in testicular function:

- Does this binding component play a regulatory role in steroidogenesis or spermatogenesis?
- Do gonadotrophins regulate the concentration of this receptor?
- What is the relation between the oestradiol secretion by the testis and the presence of an oestradiol receptor in the cytosol fraction of the interstitial tissue?

- Can this binding principle provoke a translocation of oestradiol from cytosol to nucleus?
- Is the oestradiol binder needed for a particular function in the interstitial tissue which only produces and secretes steroids?
- Is it the same receptor as the uterine oestradiol receptor?

Further investigations are obviously necessary to answer these questions.

4.5 References

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SUMMARY AND CONCLUSIONS

Steroid hormones have to pass various membrane structures before they can interact with their hypothetical site of action in the target cell nucleus. It is, however, not precisely known by which mechanism steroids pass membranes. Steroid binding plasma proteins at the extracellular level and steroid receptors at the intracellular level may play a role in the uptake of steroids by cells, but the possible influence of factors at the membrane level is unknown.

This thesis deals with an investigation concerning some factors which may influence the uptake of steroids by cells. The human red blood cell was chosen as a model system to study such factors at the extracellular level and at the membrane level, while the rat testis was used as model for studying factors at the intracellular level.

In Chapter 1 the possible role of steroid receptors in the mechanism of action of steroid hormones is discussed. From a survey of the literature about the possible mechanisms of steroid transport through biological membranes, it is concluded that there is evidence which favours a simple diffusion process of steroids through membranes, although an "active" transport mechanism or a role of proteins as steroid carriers in certain cells should not be excluded. A summary of the theoretical background of steroid-macromolecule binding and a discussion of the different methods used in this thesis to study steroid binding are also included in Chapter 1.

In Chapter 2 and Appendix paper I results concerning the uptake of different steroids by intact human erythrocytes suspended in a Krebs-Ringer buffer and the influence of several factors at the extracellular level on this uptake are described and discussed.

The interaction of various steroids with human red

blood cells can be characterized as a non-specific association resulting from the large capacity and the relatively low affinity of erythrocytes for binding of steroids (K_a approximately 10^3 M^{-1}).

The small differences between the affinities of various steroids for erythrocytes are mainly determined by the differences in polarity between those steroids. The association of a steroid with erythrocytes suspended in an isotonic medium, can therefore be considered as a partition of the steroid between a lipid rich phase (erythrocytes) and a waterphase (medium).

The uptake of progesterone by erythrocytes is decreased by the presence of plasma proteins in the medium to a much greater extent than the uptake of Δ^5-pregnenolone. The uptake of progesterone under these conditions depends on the progesterone concentration in the medium. The absence of a specific Δ^5-pregnenolone binding protein in plasma could be an explanation for this difference.

The uptake of Δ^5-pregnenolone by erythrocytes suspended in Krebs-Ringer buffer is time- and temperature independent.

In Chapter 3 and in Appendix paper II the results of experiments on binding of steroids by cell fractions of human erythrocytes is described. In these experiments special attention has been paid to the binding of steroids by haemoglobin-free membranes, membrane fractions and membrane-free fractions of erythrocytes.

With the exception of Δ^5-pregnenolone, haemoglobin-free erythrocyte membranes have a lower affinity for various steroids, when compared with intact cells. The uptake of Δ^5-pregnenolone by intact erythrocytes can be considered as an association of this steroid with the membrane.

The affinity of haemoglobin-free membranes for progesterone is temperature dependent and is lower than the affinity of an isolated membrane protein preparation for this

steroid.

Specific binding of testosterone and 5α-dihydrotestosterone by a fraction of the membrane free haemolysate could be demonstrated by equilibrium dialysis (K_a approximately 10^8 M^{-1}). With other methods, such as gel chromatography, polyacrylamide gel electrophoresis and sucrose gradient centrifugation it was impossible to demonstrate specific binding sites for these steroids.

It is very unlikely that the binding sites for testosterone and 5α-dihydrotestosterone in a fraction of the membrane-free haemolysate originate from the 17β-hydroxysteroid dehydrogenase which is present in this fraction. The presence of steroid binding plasma proteins in this fraction could not be excluded as an explanation for the observed binding sites. The occurrence of this binding principle in the membrane-free haemolysate is, however, of limited physiological significance.

In Chapter 4 and in Appendix paper III the binding of steroids by macromolecules in the cytosol fraction from rat testes is described.

The 105,000 x g cytosol fraction of testes obtained from rats 7 days after hypophysectomy as well as from 26 day old rats contains a testosterone binding component with a sedimentation coefficient of 4 S. This binding component is not found in the cytosol fraction of testes from normal adult rats.

The 105,000 x g cytosol fraction of testes from 26 day old rats as well as from mature rats contains a macromolecule which specifically binds oestradiol. The apparent association constant for the oestradiol-macromolecule complex is 4×10^9 M^{-1}. This oestradiol binding component has a sedimentation coefficient of approximately 8 S and is present in the interstitial tissue, but not in the seminiferous tubules. The oestradiol binding component is not observed in plasma. The number of oestradiol binding sites is limited,

as demonstrated after labelling the 105,000 x g cytosol fraction *in vitro* as well as *in vivo* with ^{3}H-oestradiol in the presence of a large excess of non radioactive oestradiol. Competition for the oestradiol binding sites between oestradiol and 5α-dihydrotestosterone, corticosterone or testosterone respectively is not observed. The oestradiol binding component can also be demonstrated in the 105,000 x g cytosol fraction of testes from mature rats, indicating a partial occupation of the binding sites by endogenous oestradiol in the mature rat testis.

SAMENVATTING EN KONKLUSIES

Steroid hormonen moeten diverse membraan strukturen passeren alvorens ze in het doelwitorgaan een interaktie kunnen aangaan met hun hypotetische werkingsplaats in de celkern. Het biochemisch mechanisme van het transport van steroiden door membranen is niet nauwkeurig bekend. Op extracellulair niveau kunnen steroid bindende plasma eiwitten een rol vervullen bij de regulatie van de opname van steroiden door cellen, terwijl op intracellulair niveau deze opname mogelijk wordt beinvloed door steroid receptoren. De mogelijke invloed van faktoren op het niveau van de celmembraan is echter onbekend.

In dit proefschrift wordt een onderzoek beschreven dat tot doel had de invloed van een aantal faktoren bij de opname van steroiden door cellen te bestuderen. De menselijke rode bloed cel werd gekozen als een modelsysteem om faktoren op extracellulair niveau en membraan niveau te bestuderen, terwijl de rattetestis als modelsysteem diende voor het onderzoek naar faktoren op intracellulair niveau.

In Hoofdstuk 1 wordt de mogelijke funktie besproken, die steroid receptoren vervullen bij het mechanisme van werking van steroid hormonen. Uit een overzicht van mogelijke biochemische mechanismen voor het transport van steroiden door biologische membranen wordt gekonkludeerd dat er aanwijzingen zijn dat steroiden celmembranen passeren via een diffusie proces, hoewel in bepaalde celtypen een "actief" transport of een rol van eiwitten als "dragers" voor steroiden niet dient te worden uitgesloten. In Hoofdstuk 1 wordt tevens een samenvatting gegeven van de theoretische achtergrond van interakties tussen steroiden en makromolekulen en wordt een nadere toelichting gegeven bij een aantal methoden die in dit onderzoek zijn gebruikt voor de bestudering van deze interakties.

In Hoofdstuk 2 en in artikel I van de Appendix worden de resultaten beschreven van experimenten betreffende de opname van verschillende steroiden door intakte menselijke erythrocyten, gesuspendeerd in een Krebs-Ringer buffer en de invloed van diverse extracellulaire faktoren op deze opname.

De interaktie van diverse steroiden met menselijke rode bloedcellen wordt op grond van de grote kapaciteit en de relatief lage affiniteit van erythrocyten voor steroiden (K_a is in de orde van grootte van 10^3 M^{-1}) gekarakteriseerd als een niet specifieke associatie.

De geringe affiniteitsverschillen tussen diverse steroiden voor erythrocyten worden hoofdzakelijk bepaald door verschillen in polariteit tussen de steroiden. De associatie van een steroid met erythrocyten gesuspendeerd in een isotoon medium kan derhalve worden beschouwd als een verdeling van het steroid over een lipide rijke fase (erythrocyten) en een waterfase (medium).

De aanwezigheid van steroid bindende plasma eiwitten in het medium verlagen de opname van progesteron door erythrocyten in veel sterkere mate dan de opname van Δ^5-pregnenolon. De afwezigheid van een specifiek Δ^5-pregnenolon bindend eiwit in plasma kan hiervoor een verklaring zijn. De opname van progesteron door erythrocyten onder deze omstandigheden is afhankelijk van de progesteron koncentratie in het medium.

De opname van Δ^5-pregnenolon door erythrocyten gesuspendeerd in een Krebs-Ringer buffer is tijds- en temperatuur onafhankelijk.

Hoofdstuk 3 en artikel II van de Appendix bevatten de resultaten van experimenten die betrekking hebben op de binding van steroiden door geisoleerde frakties van menselijke erythrocyten. Bij deze experimenten werd in het bijzonder aandacht geschonken aan de binding van steroiden door hemoglobinevrije membranen, membraanfrakties en membraanvrije frakties van erythrocyten.

Hemoglobinevrije erythrocyten membranen hebben, in vergelijking met intakte cellen een lagere affiniteit voor verschillende steroiden met uitzondering echter van Δ^5-pregnenolon. De opname door intakte erythrocyten van dit steroid kan derhalve worden beschouwd als een associatie met de membraan.

De bindingsaffiniteit van hemoglobinevrije membranen voor progesteron is temperatuur afhankelijk en is lager dan de bindingsaffiniteit van een geisoleerde eiwitfraktie uit de membraan.

Specifieke binding van testosteron en 5α-dihydrotestosteron door een fraktie van het membraanvrije hemolysaat met 17β-hydroxysteroiddehydrogenase aktiviteit kon worden vastgesteld door middel van evenwichtsdialyse. Met behulp van andere technieken, zoals gelchromatografie, polyacrylamide gel elektroforese of ultracentrifugatie op sucrose gradienten, konden echter geen specifieke bindingsplaatsen voor deze steroiden worden aangetoond.

Het is onwaarschijnlijk dat de bindingsplaatsen voor testosteron en 5α-dihydrotestosteron in een fraktie van het membraanvrije hemolysaat afkomstig zijn van het enzym 17β-hydroxysteroiddehydrogenase. De aanwezigheid van steroidbindende plasma eiwitten in deze fraktie kon niet worden uitgesloten als een mogelijke verklaring voor de waargenomen bindingsplaatsen. Het voorkomen van deze bindingsplaatsen in de intakte erythrocyt in vivo is echter verwaarloosbaar in vergelijking met het aantal bindingsplaatsen voor steroiden in plasma.

In hoofdstuk 4 en in artikel III van de Appendix wordt de binding van steroiden door makromolekulen in de 105,000 x g cytosolfraktie van rattetestes beschreven.

Zowel de 105,000 x g cytosol fraktie uit testes van ratten 7 dagen na hypofysektomie als die uit testes van 26 dagen oude ratten, bevat een testosteron bindende komponent met een sedimentiekoefficient van 4 S. Deze bindende

komponent is niet waargenomen in de cytosol fraktie van testes van normale volwassen ratten.

De 105,000 x g cytosol fraktie van testes van 26 dagen oude ratten en van volwassen ratten bevat een specifiek oestradiol bindend makromolekuul. De schijnbare associatiekonstante van het oestradiol-makromolekuul komplex is 4×10^9 M^{-1}. De sedimentatie coefficient van het oestradiol-makromolekuul komplex bedraagt ongeveer 8 S. De oestradiol bindende komponent is gelokaliseerd in het interstitiele weefsel en niet in de seminifere tubuli. Deze oestradiol bindende komponent is niet waargenomen in plasma. Uit proeven in vivo en in vitro met getritieerd oestradiol in de aanwezigheid van een grote overmaat niet radioaktief oestradiol is gekonkludeerd, dat het aantal specifieke bindingsplaatsen voor oestradiol in de cytosol fraktie beperkt is. Er treedt geen kompetitie op tussen oestradiol en respektievelijk 5α-dihydrotestosteron, corticosteron en testosteron voor de bindingsplaatsen van oestradiol. De oestradiol bindende komponent is eveneens aangetoond in de 105,000 x g cytosol fraktie van testes van volwassen ratten. Dit wijst erop, dat de bindingsplaatsen in de testis van de volwassen rat slechts gedeeltelijk bezet zijn door endogeen oestradiol.

CURRICULUM VITAE

De schrijver van dit proefschrift behaalde in 1960 het diploma HBS-B aan de Christelijke HBS te Aalten. In hetzelfde jaar begon hij zijn scheikundestudie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen in de Wiskunde en Natuurwetenschappen (letter g) legde hij af in 1964. Het doktoraalexamen scheikunde (hoofdvak: organische scheikunde, bijvak: farmakologie) werd door hem in december 1967 afgelegd.

Gedurende de periode september 1964 - april 1967 was hij als student-assistent verbonden aan het Analytisch Chemisch Laboratorium der Rijksuniversiteit Utrecht. Sinds september 1967 is hij, aanvankelijk als student-assistent en vanaf januari 1968 als wetenschappelijk medewerker, verbonden aan de afdeling Biochemie II van de Medische Faculteit Rotterdam, waar het hier beschreven onderzoek werd verricht.

APPENDIX

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SOCIÉTÉ D'ENDOCRINOLOGIE

Séance du 27 septembre 1969

Interaction of steroids with human red blood cells

by

A. O. BRINKMANN, E. MULDER and H. J. van der MOLEN

Department of Biochemistry, Medical Faculty at Rotterdam, (The Netherlands)

SUMMARY

Binding characteristics of steroids to human red blood cells were studied by incubation and equilibrium dialysis. Differences in binding affinity of erythrocytes for steroids were found between pregnenolone (*), progesterone, 20α-dihydroprogesterone, testosterone, androstenedione. The ratio of bound to unbound steroid did not depend on the steroid concentration in the buffer solution and was independant of incubation time and temperature. In this respect the binding of steroids by erythrocytes resembled the interaction of steroids with human serum albumin. Addition of ouabain or p-chloromercuribenzene sulfonic-acid to the erythrocytes did not influence the binding of steroids by erythrocytes. Competition between steroids for binding sites of erythrocytes was not observed. Binding of steroids by erythrocytes in whole blood and by erythrocytes suspended in diluted plasma was influenced by binding of the steroids to proteins in the plasma. A fraction with high steroid dehydrogenase activity was isolated and purified from erythrocytes. It was found that this fraction bound testosterone in a specific way.

RÉSUMÉ

La liaison des stéroïdes aux érythrocytes

Les auteurs ont étudié la liaison aux érythrocytes des stéroïdes suivants : prégnénolone, progestérone, 20α-dihydroprogestérone, testostérone et androstènedione. Cette liaison est indépendante de la concentration des stéroïdes, du temps d'incubation et de la température. En outre, la liaison n'est pas influencée par l'addition de l'ouabaïne et du PCMBS. Une fraction contenant une 17β-hydroxystéroïde déhydrogénase et liant spécifiquement la testostérone, a été isolée.

(*) The following trivial names and abbreviations have been used throughout this paper :

androstenedione : androst-4-ene-3,17-dione ;
cholesterol : cholest-5-en-3β-ol ;
20α-dihydroprogesterone : 20α-hydroxypregn-4-en-3-one ;
ouabain : γ-Strophanthin ;
PCMBS : p-chloromercuri-benzene sulfonic-acid ;
Pregnenolone : 3β-hydroxypregn-5-en-20-one ;
Progesterone : pregn-4-ene-3,20-dione ;
Testosterone : 17β-hydroxy-androst-4-en-3-one.

Introduction

Most of the circulating steroid in blood is bound reversibly to specific plasma proteins and albumin. Only a small fraction appears to be associated with the erythrocytes (1, 2, 3, 4). *In vitro*, however, a high uptake of steroids by erythrocytes suspended in isotonic media differing from plasma has been shown (5, 6, 7, 8, 9, 10). The nature of this binding between steroids and erythrocytes is still not clear. Some experiments indicate that steroids may be adsorbed onto the erythrocyte membrane (11), whereas other results suggest that the steroid might penetrate the cell (12, 13). *In vitro* experiments have also shown that erythrocytes can catalyze conversion of steroids (14, 15, 16, 17, 18). The 17β-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase activities present in human erythrocytes (19) have a large capacity for interconversion of steroids.

In the present experiments, it was attempted to characterize and localize the binding sites of the erythrocyte for androstenedione, testosterone, 20α-dihydroprogesterone and progesterone, steroids known to be converted by erythrocytes. Binding of pregnenolone was also studied, because experiments of LITTLE and co-workers suggest that under *in vivo* conditions the erythrocytes may bind more pregnenolone than progesterone (20).

Materials and methods

The following radioactive steroids were employed :

androstenedione-1,2-^{3}H (50,0 C/mMol), testosterone-1,2-^{3}H (44,1 C/mMol) progesterone-1,2-^{3}H (55,0 C/mMol), 20α-dihydroprogesterone-1,2-^{3}H (32,7 C/mMol), Δ^{5}-pregnenolone-7-^{3}H (14,7 C/mMol) and testosterone-4-^{14}C (58,3 m C/mMol). All radioactive and non-radioactive steroids were obtained from commercial sources, and were purified by chromatography before use. Techniques for chromatography and incubation have been described previously (19).

Preparation of erythrocytes. Heparinized human blood was centrifuged at 1,200 × g for 15 minutes. Plasma and buffy coat were discarded and the cells were washed three times with two volumes 0,9 % NaCl solution. The washed erythrocytes were then suspended in a Krebs-Ringer-bicarbonate buffer *p*H 7,35, to a hematocrit value of 40-45 % (vol %) and a hemoglobin concentration of 150-160 g Hb/l suspension.

Extractions. For isolation of steroids 1 ml samples of the erythrocyte suspensions or of whole blood were extracted 4 times with 2 ml ethylacetate. Recoveries of added radioactive steroids were in the order of 98-100 % and 90 % respectively.

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 naphtalene, 7 g 2,5-diphenyloxazol (PPO) and 0.3 g 1,4-*bis*-2-(5-phenyloxazolyl)-benzene (POPOP) per liter dioxane. Ethylacetate extracts were evaporated under nitrogen, the residues were dissolved in 0,1 ml methanol and counted after addition of 15 ml of a toluene solution containing 4 g PPO and 0,4 g POPOP per liter toluene.

The hemoglobin content of suspensions was estimated by the cyanhemoglobin method (21).

Protein estimations were done by the method of Lowry (22).

Hematocrit values were estimated in micro hematocrit tubes in a Hawksley micro hematocrit centrifuge.

Equilibrium dialysis was performed using 1 ml of an erythrocyte suspension in a dialysis sac (Visking dialysis Tubing, type 8/32) placed in a vial with 14.5 ml Krebs-

Ringer-bicarbonate buffer *p*H 7,35 containing an appropriate amount of radioactive and non-radioactive steroid. Equilibrium was obtained within 24 hours. The percentage uptake was calculated as :

$$\% \text{ Uptake} = \frac{(\text{DPM/ml})_{inside} - \left[(\text{DPM/ml})_{outside} \times \frac{100 - \text{Ht}}{100}\right]}{(\text{DPM/ml})_{inside}} \times 100$$

Ht = hematocrit value in (vol %)

Incubations were done in 25 ml flasks with 4 ml of an erythrocyte suspension containing the steroid. For separation of bound and unbound steroid after incubation 2 ml of the suspension (susp.) were centrifuged at 1,200 × g for 15 minutes. The percentage uptake was calculated as :

$$\% \text{ Uptake} = \frac{(\text{DPM/ml})_{susp} - \left[(\text{DPM/ml})_{supernatant} \times \frac{100 - \text{Ht}}{100}\right]}{(\text{DPM/ml})_{susp}} \times 100$$

Affinity constants were calculated on basis of the relationship : $B/U = K \cdot (nP-B)$ (7, 23) . B = molar concentration of the bound steroid ; U = molar concentration of the unbound steroid ; K = association constant ; n = number of binding sites per mole of protein ; P = molar concentration of the protein.

Equilibrium dialysis was mainly used when the binding of steroids by erythrocyte suspensions was compared with the binding of steroids by protein solutions. Incubation of steroids with an erythrocyte suspension was used because separation of bound and

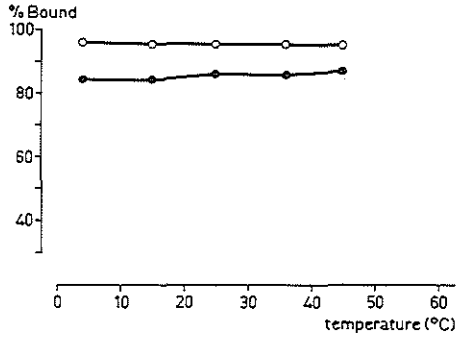

FIG. 1. — Effect of temperature on the binding of steroids by erythrocytes during incubation in Krebs-Ringer buffer. ○ pregnenolone ; ● progesterone ; Ht = 42-45 vol % ; Hb = 140-145 g/l.

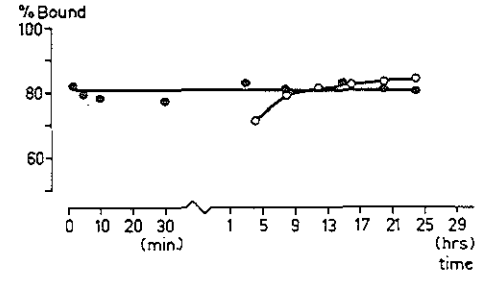

FIG. 2. — Effect of time of incubation and time of dialysis on the binding of steroids by erythrocytes in Krebs-Ringer buffer. ● incubation (100 ng progesterone/ml suspension) ; ○ equilibrium dialysis (100 ng progesterone/ml outside) ; Ht = 39-41 vol % ; Hb = 147-149 g/l.

unbound steroid could easily be achieved by centrifuging the suspension after incubation. All binding experiments were done at 4 °C. At this temperature little conversion of the steroids occurs. Temperature variations between 4 and 45 °C had little effect on the binding of steroids by erythrocytes (fig. 1). Figure 2 shows that binding takes place almost immediately after the steroid comes in contact with the cells. Even after incubation for 30 seconds binding of steroids to erythrocytes appears to be maximal. This short incubation time should, however, be considered with some reservation. Binding after 30 seconds could only be estimated by centrifuging the suspension immediately after the steroid came in contact with the cells and approximately 2 minutes centrifugation was required to obtain separation. The binding does not significantly change during longer incubation. An incubation time of 2 hours was used for all the incubation experiments. The curves

for binding of testosterone by erythrocytes (fig. 3) after incubation (for 2 hours) or after dialysis (for 24 hours) indicate almost the same percentage of binding. On basis of these

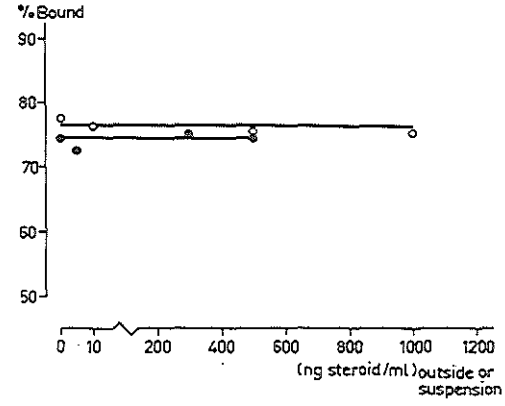

FIG. 3. — Incubation and equilibrium dialysis of erythrocytes in Krebs-Ringer buffer with testosterone. Effect of steroid concentration. ● incubation ; ○ equilibrium dialysis ; Ht = 42-47 vol % ; Hb = 140-150 g/l.

results it was concluded that data obtained under the described conditions for incubation and for dialysis could be compared with each other.

RESULTS

a) *Binding of different steroids by erythrocytes in Krebs-Ringer solution.* In order to study some of the binding characteristics of erythrocytes, saturation of the binding sites was tried by incubating or dialysing erythrocytes in Krebs-Ringer buffer with increasing amounts of steroid (fig. 4 and fig. 5). A difference in binding affinity was found for the 5 steroids used. No significant

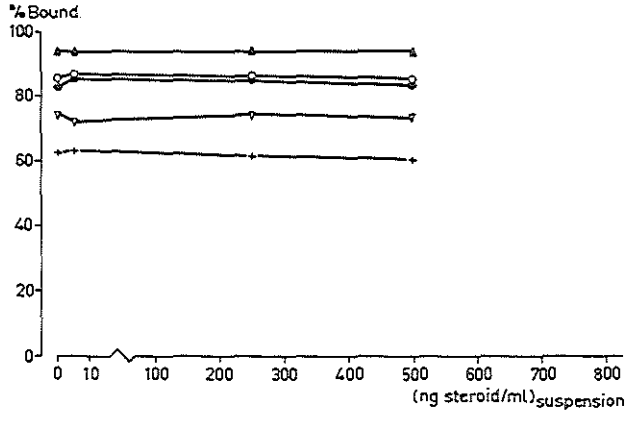

FIG. 4. — Effect of steroid concentration on the binding of steroids by erythrocytes in Krebs-Ringer buffer. Binding was estimated by incubation. ▲ pregnenolone ; ○ progesterone ; ● 20α-dihydroprogesterone ; ▽ testosterone ; + androstenedione ; Ht = 37-42 vol % ; Hb = 140-150 g/l.

change in binding was observed for the individual steroids over the concentration range studied. This may reflect a high binding capacity of erythrocytes. The binding of steroids by erythrocytes has been expressed in a quantitative way in order to permit a comparison with the binding of steroids by proteins,

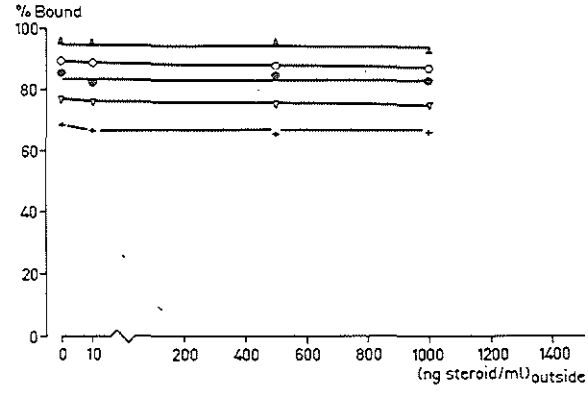

FIG. 5. — Effect of steroid concentration on the binding of steroids by erythrocytes in Krebs-Ringer buffer. Binding was estimated by equilibrium dialysis. ▲ pregnenolone ; ○ progesterone ; ● 20α-dihydroprogesterone ; ▽ testosterone ; + androstenedione ; Ht = 37-42 vol % ; Hb = 140-150 g/l.

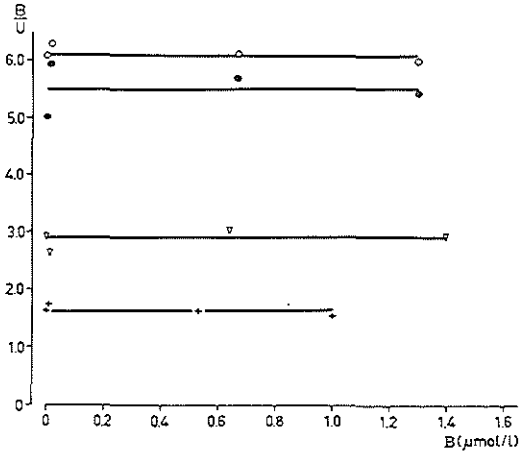

FIG. 6. — Effect of steroid concentration on the binding of steroids by erythrocytes in Krebs-Ringer buffer. Binding was estimated by incubation. The ratio bound to unbound steroid (B/U) is plotted as a function of the amount of bound steroid (B). ○ progesterone ; ● 20α-dihydroprogesterone ; ▽ testosterone ; + androstenedione ; Ht = 37-42 vol % ; Hb = 140-150 g/l.

such as albumin and transcortin. Therefore (fig. 6) the ratio of bound to unbound steroid (B/U) was plotted as a function of the amount of bound steroid (B) (23, 7). This « Scatchard plot » of the steroid binding by erythrocytes gives a straight line parallel to the abcissa. It is possible to calculate from this curve an apparent association constant for the binding between steroids and erythrocytes. On the assumption that all cellular protein may be represented by hemoglobin

and that this hemoglobin is responsible for the binding, an association constant can be expressed in liter per mole of hemoglobin (table I). Affinity constants of erythrocytes for steroids that were calculated in this way are of the same order of magnitude as affinity constants of albumin for steroids (table I).

TABLE I. — BINDING OF STEROIDS BY ERYTHROCYTES, HUMAN SERUM ALBUMIN AND TRANSCORTIN AT 4 °C.

	Steroid	Association constant (l/M)	References
Erythrocytes (Human)	Androstenedione	0.8×10^{3}*	Present investigation
	Testosterone	1.3×10^{3}*	
	20α-Dihydroprogesterone	2.6×10^{3}*	
	Progesterone	2.9×10^{3}*	
	Δ^{5}-Pregnenolone	8.5×10^{3}*	
Erythrocytes (Rat)	Progesterone	$2.9\text{-}4.3 \times 10^{3}$*	DE VENUTO (10)
H.S.A.	Progesterone	$4\text{-}6 \times 10^{4}$	SANDBERG et al. (6)
	Testosterone	3×10^{4}	SANDBERG et al. (6)
Transcortin	Progesterone	3×10^{8}	SEAL et al. (7)

(*) Affinity calculated per mole of hemoglobin.

b) *Binding of steroids by erythrocytes suspended in human plasma.* In order to obtain more information about the distribution of steroids in blood, the competition between plasma proteins and erythrocytes for binding of progesterone and pregnenolone was studied by incubation. Fig. 7 shows a Scatchard type plot of the binding of progesterone and pregnenolone to a 2 % (v/v) plasma solution.

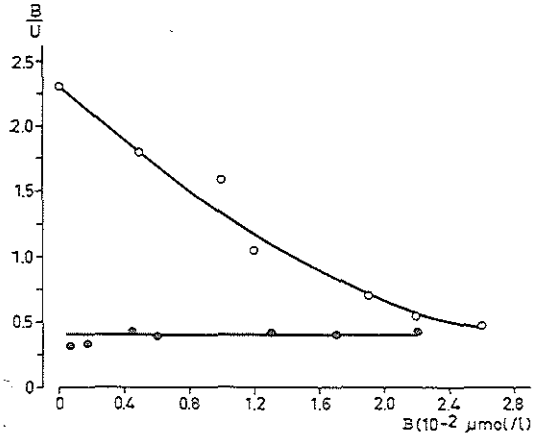

FIG. 7. — Binding of progesterone and pregnenolone to 2 % (v/v) plasma at different steroid concentrations. The unbound steroid was separated from the bound steroid by Florisil adsorption. ○ progesterone; ● pregnenolone.

Incubations of increasing amounts of progesterone with an erythrocyte suspension in 5 % (v/v) plasma resulted in an increase in the percentage of steroid bound by the erythrocytes (fig. 8). At steroid concentrations of 25 ng/ml suspension or higher the percentage binding remained constant. The binding of pregnenolone by

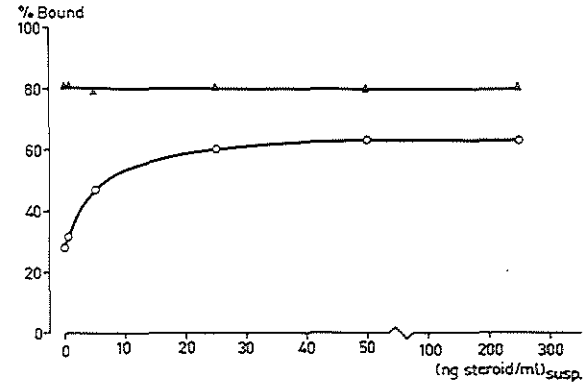

FIG. 8. — Binding of progesterone and pregnenolone by erythrocytes suspended in 5 % (v/v) plasma at different steroid concentrations. Binding was estimated by incubation. ◍ pregnenolone ; ◯ progesterone ; Ht = 37-40 vol % ; Hb = 130-140 g/l.

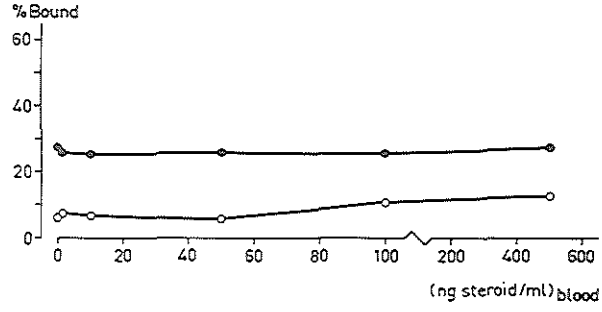

FIG. 9. — Binding of progesterone and pregnenolone by erythrocytes in whole blood at different steroid concentrations. Binding was estimated by incubation. ◍ pregnenolone ; ◯ progesterone ; Ht = 45-47 vol % ; Hb = 155-160 g/l.

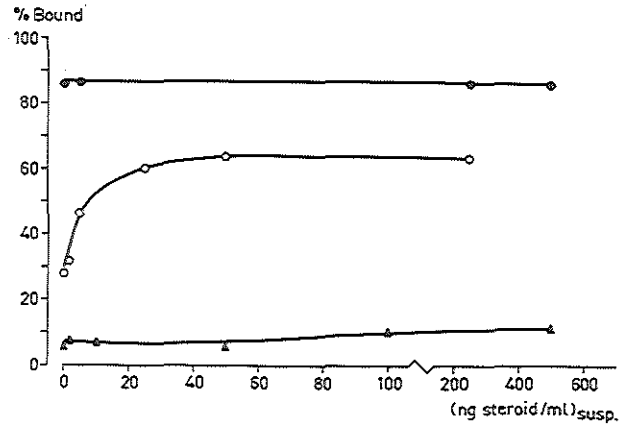

FIG. 10. — Comparison of the binding of progesterone by erythrocytes in Krebs-Ringer buffer, in 5 % (v/v) plasma and in whole blood at different steroid concentrations. Binding was estimated by incubation. ◍ Krebs-Ringer buffer ; ◯ 5 % (v/v) plasma ; ▲ whole blood.

erythrocytes in 5 % (v/v) plasma was not significantly dependent on the steroid concentrations that were used (fig. 8). In whole blood the binding of progesterone by erythrocytes varied from 5 to 12 % depending on the steroid concentration (fig. 9). Pregnenolone was bound for 30 % by erythrocytes in whole blood (fig. 9). In fig. 10, the binding of progesterone by erythrocytes suspended in Krebs-Ringer buffer, by erythrocytes in 5 % (v/v) plasma and by erythrocytes in whole blood are compared.

c) *Washing of erythrocytes after binding and conversion of steroids.* From other studies (24), it is known that interconversion of androstenedione and testosterone and of progesterone and 20α-dihydroprogesterone most likely occurs inside the erythrocytes. This indicates that transport through the erythrocyte membrane may occur prior to the conversion of the steroid. Separation of the steroid fraction inside the erythrocyte from the steroid fraction adsorbed

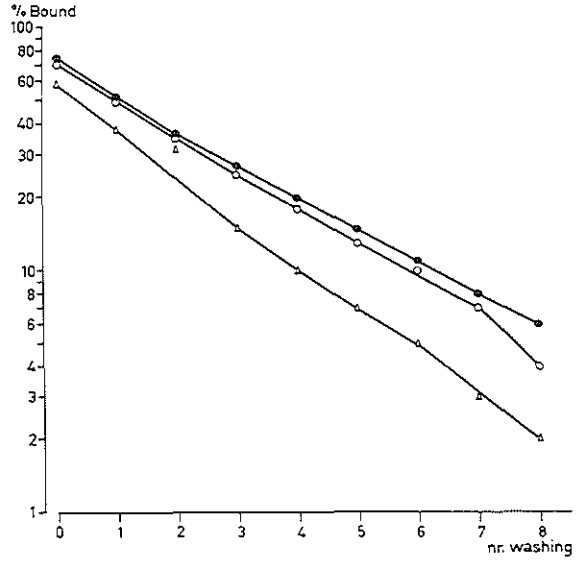

FIG. 11. — Binding of steroids by erythrocytes after successive washings. Erythrocytes were incubated with androstenedione-1,2-^{3}H and testosterone-4-^{14}C in Krebs-Ringer buffer containing glucose (10^{-2} molar). After 3 hours of incubation the erythrocytes were washed (8 times) with Krebs-Ringer buffer. % bound is the percentage bound of the original amount of steroid. ● testosterone-1,2-^{3}H formed ; ○ testosterone-4-^{14}C incubated ; △ androstenedione-1,2-^{3}H incubated.

onto the erythrocyte membrane may possibly be achieved by washing the cells after incubation. 10 μg androstenedione-1,2-^{3}H and 10 μg testosterone-4-^{14}C were incubated for 3 hours at 37 °C with 14 ml of an erythrocyte suspension in Krebs-Ringer-bicarbonate buffer containing glucose (10^{-2} M). After incubation, the suspension was centrifuged at 1,200 × g for 15 minutes. The supernatant was discarded and stored. The cells were suspended in an equal volume of cold isotonic buffer and were carefully shaken. After centrifugation the supernatant was stored. This whole procedure was repeated 8 times. The first supernatant and the 8 wash layers were extracted 4 times with 10 ml ethylacetate. After

evaporation of the extract androstenedione and testosterone were separated by thin-layer chromatography in benzene-ethylacetate 3 : 2. Fig. 11 represents a semi-logarithmic plot of the percentage of the original steroid bound to the erythrocytes as a function of the number of washings. These data show that it is possible to remove the bound steroid almost completely from the cells by repeated washing. The straight line suggests that during successive washings the testosterone-^{3}H formed from androstenedione-^{3}H and the unconverted testosterone-^{14}C were similarly distributed between the cells and the corresponding supernatant.

d) *Effect of ouabain, PCMBS, cholesterol and pregnenolone on the binding of androstenedione by erythrocytes at* 37 °C. In order to study the transport of steroids through the erythrocyte membrane and in order to obtain information about the localisation of binding sites, erythrocyte suspensions in Krebs-Ringer solution were pre-incubated for 30 minutes at 37 °C with different amounts or ouabain of p-chloromercuri-benzene sulfonic-acid.

Neither compounds altered the binding of androstenedione (table 2). With a 10^{-2} Molar concentration of PCMBS strong hemolysis was observed. Furthermore, pre-incubation of erythrocytes with several concentrations of cholesterol or pregnenolone did not influence the binding of androstenedione (table 2).

TABLE 2. — EFFECT OF PREINCUBATION OF ERYTHROCYTES WITH OUABAIN, PCMBS, CHOLESTEROL AND PREGNENOLONE ON THE BINDING OF ANDROSTENEDIONE BY ERYTHROCYTES IN KREBS-RINGER BUFFER. BINDING IS EXPRESSED AS PERCENT OF THE ORIGINAL AMOUNT OF INCUBATED ANDROSTENEDIONE.

Molarity	Erythrocytes + preincubated (30 mn) with :			
	Ouabain	PCMBS	Cholesterol	Δ5-Pregnenolone
10^{-2}	66.7 %	61.2 %*		
10^{-3}	66.5 %	63.6 %		
10^{-4}	66.7 %	64.5 %		
10^{-5}	66.0 %	65.5 %		
3.3×10^{-6}			63.4 %	66.5 %
3.3×10^{-5}			65.8 %	63.6 %
Saturated			65.9 %	66.2 %
Control	66.3 %	66.3 %	63.3 %	63.3 %

(*) Strong hemolysis was observed ; > Hb = 147-150 g/l.

e) *The binding of testosterone to a 17β-hydroxysteroid dehydrogenase activity containing erythrocyte protein fraction.* Preliminary experiments have been performed with a protein fraction isolated from hemolysed erythrocytes (24). This fraction was prepared from the hemolysate after removal of the membranes by high speed centrifugation. Ammonium sulfate was added to the supernatant and the fraction precipitating between 25 % and 50 % saturation of the

solution was collected. The specific activity of the 17β-hydroxysteroid dehydrogenase activity in this fraction was increased approximately 100 times as

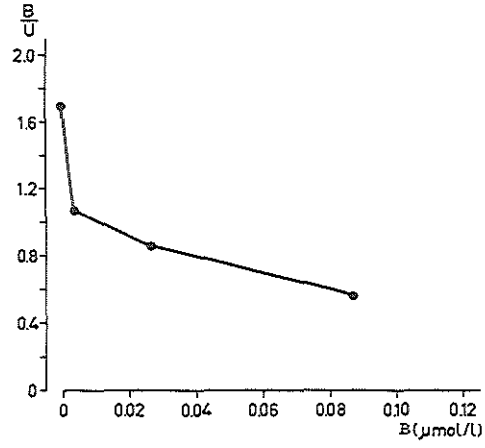

FIG. 12. — Binding of testosterone by an erythrocyte fraction with steroid-dehydrogenase activity (see : text). Binding was estimated by equilibrium dialysis.

compared with the activity in erythrocytes. This relative increase in activity was mainly achieved by removing the bulk of the hemoglobin. Equilibrium dialysis of this fraction with testosterone showed a decrease in percentage binding with increasing amounts of testosterone (fig. 12).

DISCUSSION

The present investigation confirms that human erythrocytes have a large capacity for binding of steroids. Differences in binding were observed between the five steroids studied (fig. 4, 5, 6). In the sequence pregnenolone, progesterone, 20α-dihydroprogesterone, testosterone, androstenedione, a decrease of the percentage bound steroid was observed. A direct correlation between the polarity of the steroid and the amount bound by the erythrocyte, however, can not be made. Progesterone is better bound than 20α-dihydroprogesterone, but androstenedione is less bound than testosterone.

In order to express the binding of steroids by erythrocytes in a quantitative way, we attempted to express the affinity per mole of protein. It was assumed that the erythrocyte may be represented by its hemoglobin protein. On the basis of this crude approximation of the composition of the erythrocyte only the order of magnitude of the apparent association constant should be compared. From Table 1 it is evident that the affinity constants of erythrocytes for steroids are small compared with those of transcortin but that they are of the same order of magnitude as those of albumin. From binding data of DE VENUTO (10), obtained by dialysis studies of progesterone with rat erythrocytes, an association constant (2,9-4,3 × 10^3 l/mol) can be calculated. This value is in good agreement with the constant (2,9 × 10^3 l/mol) of human erythrocytes for progesterone obtained in the present study.

The presence of specific progesterone binding proteins in plasma was confirmed in experiments with a 2 % plasma dilution (fig. 7). For this discussion « specific » binding of steroids is considered to represent binding by proteins with a high affinity and a limited capacity for steroids. When erythrocytes in 5 % (v/v) plasma were incubated with increasing amounts of progesterone, an increase in the percentage steroid (fig. 8) bound to erythrocytes was observed, in contrast to the incubation studies with erythrocytes in Krebs-Ringer solutions. This rise in binding percentage may be explained if it is assumed that specific progesterone binding proteins in plasma are first saturated, followed by a competition for binding between albumin and erythrocytes. This is in agreement with the observations of FARESE and PLAGER (25) who showed that the binding of cortisol by erythrocytes depends on the amount and the character of plasma proteins present in the cell suspension. In whole blood, the binding of progesterone by erythrocytes varied from 5 to 12 % depending on the steroid concentration (fig. 9, 10). Sufficient progesterone binding plasma proteins with a high affinity constant are available in whole blood to bind most of the progesterone. Binding experiments with pregnenolone in 2 % plasma solution show (fig. 7), that this steroid is bound in a non-specific way, probably only by albumin. For pregnenolone the binding by erythrocytes in 5 % plasma was not significantly dependent on the steroid concentrations that were used (fig. 8). This could be expected if there is only competition between a non-specific binding of pregnenolone by erythrocytes and by albumin. In whole blood, (fig. 9) a large percentage (30 %) of the pregnenolone was attached to the red cells, an observation that may be in agreement with the observations of LITTLE (20). LITTLE (20) found no difference in the metabolic clearance rates (MCR) of progesterone estimated on basis of plasma or blood concentrations. For pregnenolone, however, the MCR from plasma was found to be higher than from total blood. This may indicate that a rather large percentage of pregnenolone was associated with the erythrocytes in blood.

In order to determine the location of the steroid bound to the red cell it may be considered that binding could occur as a result of adsorption to the membrane, and transport into the cell. Such transport might be time and temperature dependent. With the incubation technique it was not possible to detect an influence of time (fig. 2) and temperature (fig. 1) on the binding. It could be possible, however, that only a minor fraction of the bound steroid was transported. In order to investigate this problem further, erythrocytes were incubated with androstenedione-^{3}H and testosterone-^{14}C. It was presumed that conversion of androstenedione to testosterone occurs inside the erythrocyte. If after incubation, therefore, testosterone-^{3}H and testosterone ^{14}C behaved different, this might possibly reflect differences between the behaviour of newly formed testosterone-^{3}H inside the erythrocyte and of testosterone-^{14}C adsorbed to the membrane of the erythrocyte. Washing the cells after incubation might achieve a separation of the adsorbed and the transported steroid. From the results in (fig. 11) it is clear that the testosterone-^{3}H and testosterone-^{14}C behave similarly during washing, which may indicate that both the newly formed and the adsorbed testosterone are similarly distributed between the erythrocytes and the washing fluid. This could indicate that the steroids are loosely bound in the membrane. DE MOOR et al. (13) suggest that there might be an agent in erythrocytes for binding of corti-

costeroids. They called this agent intracortin. In later studies (29), however, they could not confirm their previous results. Conversion of estrone to estradiol by an erythrocyte hemolysate as observed by BISCHOFF et al. (12) suggests a penetration of this steroid into the red blood cell. VERMEULEN, however, concluded that corticosteroids are adsorbed at the red cell surface (11).

A competition for binding sites by different steroids was not observed. Simultaneous incubations of testosterone and androstenedione with erythrocytes did not influence the binding of each steroid individually (fig. 11). Also preincubations of erythrocytes with several concentrations of cholesterol or pregnenolone did not change the extent of binding of androstenedione (table 2). These experiments again demonstrate the large capacity of the red cells for binding of steroids.

Experiments of AGARWAL et al. (27) have shown an inhibitory action of steroids on the hemolysis of erythrocytes caused by p-chloromercuribenzoate (PCMB). This might suggest that the erythrocyte membrane can be stabilized and protected by steroids against the hemolytic action of PCMB. It is also known that PCMBS can block SH-groups in membranes and that after several hours of incubation PCMBS can penetrate the erythrocyte (28). Preincubation of erythrocytes with PCMBS (table 2) did not affect the binding of androstenedione. It may therefore be unlikely that androstenedione is directly bound to SH-groups in the membrane. From studies of SKOU (26) it is known that ouabain inhibits a Na^+ and K^+ dependent ATP-ase with a half maximal inhibition concentration of 10^{-7} molar. The preincubation studies (table 2) with ouabain suggest that a possible steroid transport, is not influenced by this ATP-ase.

In a total hemolysate of red cells, the steroids are mainly bound to the proteins (8, 12). This might suggest the presence of steroid binding proteins in the hemolysate. Preliminary studies with a membrane free hemolysate fraction showed a « specific » binding of testosterone (fig. 12). The 17β-hydroxysteroid dehydrogenase specific activity of this purified fraction was 100 times higher than the specific activity of the lysed cells. Although the steroid dehydrogenase content of this isolated fraction is increased, this fraction also contains almost all non-hemoglobin proteins of the erythrocyte. Little can therefore be said about the nature of the proteins involved in the binding of testosterone to this purified fraction.

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

A. O. BRINKMANN AND H. J. VAN DER MOLEN

Department of Biochemistry, Division of Chemical Endocrinology, Medical Faculty at Rotterdam, Rotterdam (The Netherlands)

(Received March 23rd, 1972)

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.

* Part of this paper has been presented during a symposium on "The interaction of steroids with macromolecules" in Paris, September 13–15th, 1971.

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: $B/U = K(nP - B)$. 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 $B/U = K(nP - B)$ 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 $C = B/U \cdot 1/nP$ 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

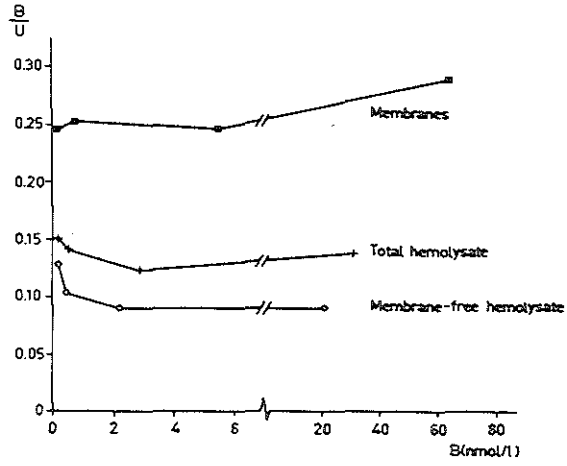

Fig. 1. Binding of testosterone by different erythrocyte fractions. Each protein fraction was dialysed at 4 °C against 0.058, 2.373, 23.73 and 237.3 nM testosterone, respectively. Protein concentration of each fraction: 20 mg/ml.

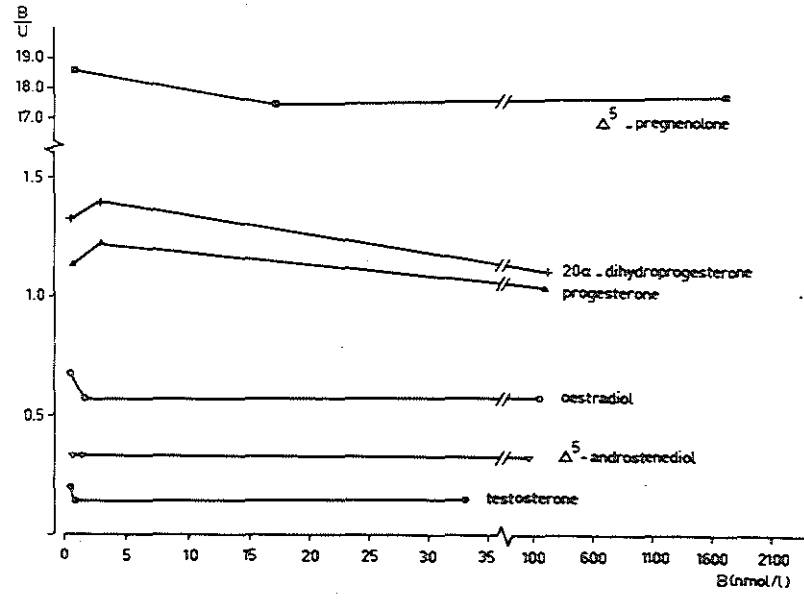

Fig. 2. Binding of different steroids by haemoglobin-free membranes. The membranes were dialysed at 4 °C against the following steroid concentrations: 0.095, 2.205 and 211.1 nM Δ^5-pregnenolone; 0.170, 2.293 and 212.5 nM progesterone; 0.690, 2.989 and 230.6 nM Δ^5-androstenediol; 0.042, 2.493 and 245.1 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

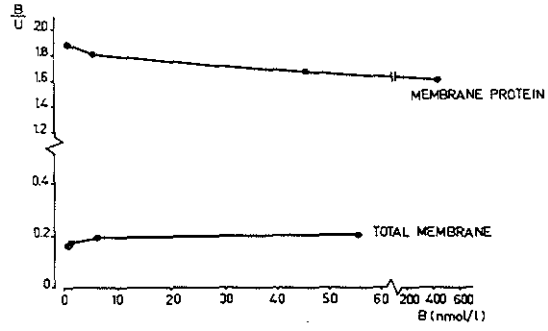

Fig. 3. Binding of testosterone by haemoglobin-free membranes (total membrane) and by a membrane protein fraction. Each fraction was dialysed at 4 °C against 0.058, 2.373, 23.73 and 237.3 nM testosterone, respectively at 4 °C. Protein concentration of each fraction: 2 mg/ml.

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}.

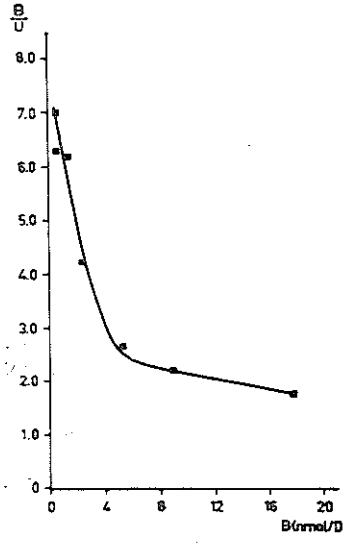

Fig. 4. Binding of testosterone by an erythrocyte fraction with steroid dehydrogenase activity ("17β OHSD-fraction"). This protein fraction was dialysed at 4 °C against the following testosterone concentrations: 0.058, 0.116, 0.232, 0.694, 2.373, 4.687 and 11.631 nM. Protein concentration: 45 mg/ml.

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

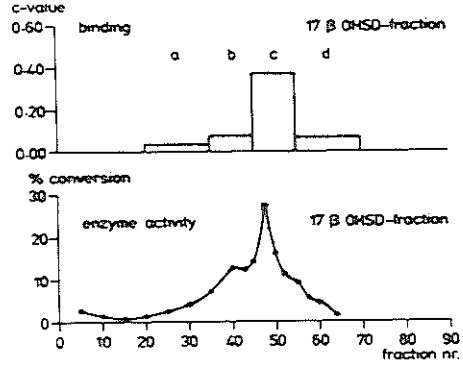

Fig. 5. Gel chromatography elution pattern on Sephadex G-150 of the erythrocyte fraction with hydroxysteroid dehydrogenase activity (17β OHSD-fraction). 4 ml of a protein sample (10–25 mg/ml) were applied to a 25 mm × 1000 mm column of Sephadex G-150 equilibrated at 4 °C with 0.05 M phosphate buffer (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).

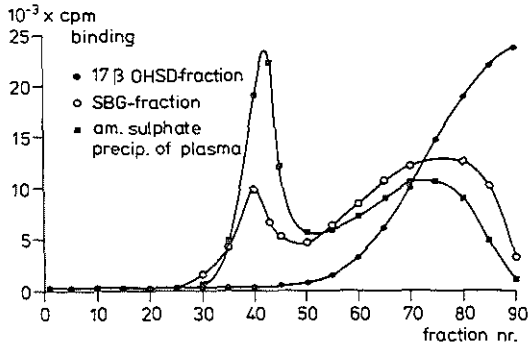

Fig. 6. Gel chromatography elution patterns on Sephadex G-150 of the erythrocyte fraction with hydroxysteroid dehydrogenase activity (17β OHSD-fraction), a sex steroid-binding globulin (SBG) fraction and the 20–50% saturated $(NH_4)_2SO_4$ precipitate of plasma. Binding of testosterone by the three protein 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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ENDOCRINOLOGIE. — *Récepteur testiculaire de l'œstradiol chez le Rat.* Note (*) de MM. **Albert O. Brinkmann, Eppo Mulder** et **Henk J. Van der Molen,** présentée par M. Robert Courrier.

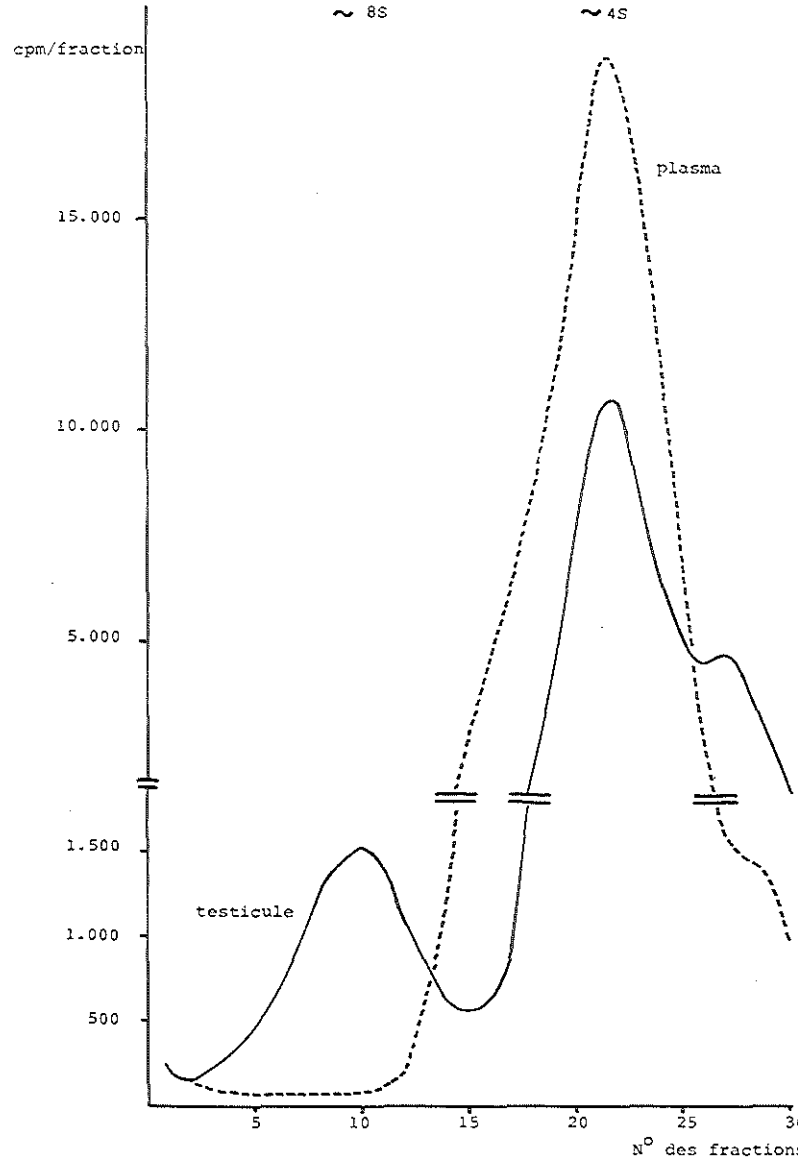
Après marquage *in vivo* et *in vitro* par l'œstradiol tritié, un récepteur soluble de l'œstradiol est mis en évidence dans le tissu testiculaire chez le Rat. Le coefficient de sédimentation obtenu par ultracentrifugation en gradient de saccharose est de l'ordre de 8 S. Les résultats préliminaires indiquent la localisation du récepteur au niveau des cellules du tissu interstitiel.

INTRODUCTION. — Les récepteurs des androgènes ont été beaucoup étudiés au niveau des organes cibles sexuels chez le mâle [(1), (2)]. Récemment, Jungblut et coll. (3) ont indiqué que les récepteurs spécifiques des œstrogènes et des androgènes n'existent pas uniquement dans ce qu'on considère généralement comme les tissus cibles respectifs des œstrogènes et des androgènes. Par exemple un récepteur des androgènes existe dans l'utérus de génisse et un récepteur des œstrogènes peut être mis en évidence dans la prostate de veau et de porcelet. Ce dernier diffère du récepteur des androgènes (3).

A notre connaissance, aucun récepteur spécifique des stéroïdes n'a été observé dans les testicules. De Jong et Van der Molen (4) ont démontré que les testicules de rat sécrètent de l'œstradiol, dont la fonction *in situ* reste à élucider. C'est dans ces conditions que nous avons été amenés à rechercher l'existence de récepteurs cytosolubles de différents stéroïdes au niveau des tissus testiculaires chez le Rat. Dans cette Note, sont rapportées des expériences concernant la liaison de l'œstradiol *in vivo* et *in vitro*.

MÉTHODES. — On a utilisé les testicules entiers de rats Wistar prépubères (26 jours) et adultes. Après décapitation et prélèvement des testicules, l'albuginée est enlevée et le tissu homogénéisé dans un appareil de Potter Elvejhem, en 2 volumes de tampon Tris 10 mM, pH 7,4, contenant de l'EDTA 1,5 mM et du mercaptoethanol 2 mM (tampon TEM). On ajoute du glycérol à concurrence de 10 %. L'homogénat est ensuite centrifugé à 105 000 g pendant 60 mn. Le surnageant est alors incubé 2 h à 0 °C avec de l'œstradiol 2,4,6,7-^{3}H 3 nM (activité spécifique : 100 Ci/mmole). Dans les expériences *in vivo*, les rats prépubères ont reçu 5 µCi d'œstradiol radioactif par voie sous-cutanée 5 mn avant la décapitation, suivie de la préparation du surnageant 105 000 g. Les surnageants (« cytosol »), marqués *in vivo* et *in vitro*, ont été ensuite étudiés par ultracentrifugation en gradient de saccharose afin de détecter des protéines liant spécifiquement l'œstradiol. 200 µl de cytosol, contenant approximativement 3 mg de protéines ont été déposés sur un gradient de saccharose 5-15 % (poids/volume), préparé dans le tampon TEM contenant 10 % de glycérol, et l'ultracentrifugation a fait suite, à 49 000 tr/mn pendant 18 h dans un « rotor SW 65 » d'une centrifugeuse « Beckman L 2-65 B » à 0 °C. L'alcool-déshydrogénase de levure et la sérum-albumine bovine ont été utilisées comme protéines de référence pour calculer les coefficients de sédimentation (5). Après ultracentrifugation, environ 30 fractions ont été collectées en perçant le fond du tube et on a mesuré la radioactivité dans les différentes fractions par comptage dans un spectromètre à scintillation liquide. On a

procédé de même avec des échantillons de plasma marqué *in vivo* ou *in vitro*, que l'on a soumis à ultracentrifugation après dilution avec 4 volumes de tampon TEM-10 % glycérol.



Profils de sédimentation de l'œstradiol tritié après incubation avec du cytosol de testicule de rat prépubère (trait plein) et avec le plasma correspondant (pointillés)

RÉSULTATS. — 1. *Expériences* in vitro *avec des testicules de rat prépubères.* — La figure indique le profil de sédimentation de la radioactivité au cours d'une expérience typique utilisant du cytosol testiculaire incubé préalablement avec l'œstradiol

radioactif. On remarque 2 pics, un dans la région « 8 S » et un plus important dans la région « 4 S ». Le tracé en pointillés indique que l'expérience parallèle faite avec du plasma ne permet d'observer qu'un seul pic dans la région 4 S.

2. *Expériences* in vivo *avec des testicules de rats prépubères.* — A la suite de l'injection d'œstradiol radioactif *in vivo*, l'étude du cytosol a donné essentiellement les mêmes résultats qu'après le marquage *in vitro*. Les pics de radioactivité 8 S et 4 S étaient approximativement égaux.

3. *Expériences* in vitro *avec des rats adultes.* — Dans ce cas, des résultats quelque peu différents ont été observés. En effet un seul pic 8 S était observé. La majorité de la radioactivité se trouvait dans la partie supérieure du tube de gradient, indiquant qu'une grande partie du stéroïde n'était pas liée. Le pic 4 S n'était pas nettement séparé alors que dans le plasma correspondant il était visible.

DISCUSSION. — L'observation d'un pic de radioactivité dans la région « 8 S » après ultracentrifugation en gradient de densité du cytosol testiculaire, marqué par l'œstradiol radioactif, suggère fortement la présence d'une protéine liant spécifiquement l'œstradiol dans les testicules. En plus du profil de sédimentation sous la forme d'un agrégat 8 S, cette observation correspond aux caractéristiques attendues pour un « récepteur », car des résultats préliminaires ont indiqué que les sites de liaison de cette fraction avaient une grande affinité pour l'œstradiol et pouvaient être saturés *in vivo* comme *in vitro*. Les résultats obtenus *in vivo* indiquent aussi que l'œstradiol, dans des conditions physiologiques, peut être retenu par les testicules du Rat : Stumpf a d'ailleurs trouvé au cours de ses études autoradiographiques une localisation nucléaire de la radioactivité au niveau des cellules interstitielles du testicule de rat prépubère après administration d'œstradiol [(6), (7)].

De plus, des essais de microdissection testiculaire ont permis de localiser la protéine de liaison de l'œstradiol dans le cytosol des cellules du tissu interstitiel de rat adulte après incubation *in vitro*.

Il reste à attribuer un rôle physiologique à ce récepteur. On admet que les stéroïdes testiculaires influencent la spermatogenèse dans les tubes séminifères, et les récepteurs de l'œstradiol du tissu interstitiel pourraient jouer un rôle à cet égard.

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Department of Biochemistry,
Division of Chemical Endocrinology,
Medical Faculty at Rotterdam, Rotterdam, The Netherlands.