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MODEL STUDIES WITH ERYTHROCYTES ON THE INITIAL STEPS OF CELLULAR UPTAKE AND BINDING OF STEROIDS

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SUMMARY

The uptake of steroids by human red blood cells was investigated with an incubation technique and with equilibrium dialysis, and the following observations were made:

(1) In the sequence pregnenolone, 20α-dihydroprogesterone, testosterone, androstenedione a decrease in binding affinity of erythrocytes suspended in Krebs–Ringer-bicarbonate buffer was observed. The ratio of bound to unbound steroid did not vary significantly with the steroid concentration. Most of the bound steroid was removed by washing the red blood cells several times with isotonic buffer, indicating that steroids might be adsorbed to the cell surface.

(2) The percentage of progesterone bound to erythrocytes in a 5% (v/v) plasma solution increased with increasing concentrations (0–75 ng/ml) of progesterone. With progesterone concentrations > 75 ng/ml the distribution of progesterone between the plasma proteins and erythrocytes remained constant. In studies with the whole blood 25–30% of added [^{3}H]-pregnenolone was bound to the red blood cells, but only 0–10% [^{3}H]-progesterone was bound to the erythrocytes.

(3) Experiments on the reduction rate of DHA and DHA sulfate, and of estrone and estrone sulfate, by intact and hemolysed human erythrocytes indicate a difference in permeability of the intact cell membrane for steroid sulfates and the corresponding free steroids.

(4) In binding studies with a total hemolysate, most of the steroid was associated with proteins of the membrane-free hemolysate and only a small fraction was bound to the membrane. The combining affinity, expressed as the ratio of bound to unbound steroid per unit amount of protein was higher for hemoglobin-free membranes than for a membrane-free hemolysate. The binding of testosterone and progesterone by a soluble protein fraction from erythrocyte membranes is higher than could be expected on the basis of dialysis studies with intact membranes. Testosterone was bound in a specific way by a fraction from a membrane-free hemolysate, 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 l (mol protein)$^{-1}$. The testosterone-binding fraction isolated from the membrane-free hemolysate was further characterized with gel chromatography.

The results of these studies suggest that the uptake of steroids by intact erythrocytes can be explained in terms of (a) adsorption and/or binding to the membrane, (b) diffusion into the cell, and (c) binding inside the cell.

INTRODUCTION

THE UPTAKE of steroid hormones by target tissue cells may be regulated by extracellular plasma proteins and intracellular receptors[1]. Current ideas about the passage of steroids through cell membranes assume that the steroid is taken up by

Trivial names and abbreviations: Androstenediol; 5-androstene-3β,17β-diol; Androstenediol sulfate: 17β-hydroxy-5-androsten-3β-yl sulfate; Androstenedione: 4-androstene-3,17-dione; Corticosterone: 11β,21-dihydroxy-4-pregnene-3,20-dione; Dehydroepiandrosterone (DHA): 3β-hydroxy-5-androsten-17-one; Dehydroepiandrosterone sulfate (DHA-S): 17-oxo-5-androsten-3β-yl sulfate; 20α-Dihydroprogesterone, 20α-hydroxy-4-pregnen-3-one; Estrone: 3-hydroxy-1,3,5(10)-estratrien-17-one; Estrone sulfate: 17-oxo-1,3,5(10)-estratrien-3-yl sulfate; Estradiol: 1,3,5(10)-estratriene-3,17-diol; Estradiol sulfate: 17β-hydroxy-1,3,5(10)-estratrien-3-yl sulfate; Pregnenolone: 3β-hydroxy-5-pregnen-20-one; Progesterone: 4-pregnene-3,20-dione; S.B.G., Sex Steroid Binding Globulin; Testosterone: 17β-hydroxy-4-androsten-3-one.

a simple diffusion process[2, 3]. During this process the steroid is considered to be in the unconjugated form and not bound to proteins. Relatively little is known about the role of the plasma membrane of the target cell in this process. In order to obtain more information about the uptake of steroids by cells, the human erythrocyte was chosen as a model system. The human erythrocyte is a relatively uncomplicated cell type, which contains no nucleus and mitochondria and can easily be isolated. We have tried to study the following aspects:

(a) the uptake of different steroids by intact erythrocytes suspended in Krebs–Ringer buffer,
(b) the influence of the presence of plasma proteins in the buffer medium on the uptake of steroids,
(c) the effect of conjugation of steroids on the conversion of steroids by the 17β-hydroxysteroid dehydrogenase present inside the erythrocyte and
(d) the binding of steroids by isolated cell fractions.

MATERIALS AND METHODS

The binding studies were performed using equilibrium dialysis and an incubation technique[4]. All binding experiments were carried out at 4°C. At this temperature little metabolism of the steroids occurs. Temperature variations between 4 and 45°C had little effect on the binding of steroids by erythrocytes[4].

For incubations 4 ml of an erythrocyte suspension in Krebs–Ringer buffer were incubated with an appropriate amount of radioactive and non-radioactive steroid. After incubation the suspension was centrifugated for separation of bound and unbound steroid. Steroids were extracted with ethyl acetate from the total suspension and from the supernatant after incubation. The percentage binding was calculated as:

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

Ht = hematocrit value (Vol. %).

For proper comparison of the amount of radioactivity in the supernatant after incubation with the amount of radioactivity in the original suspension a correction was made for the volume of the erythrocytes. This volume was estimated as the hematocrit value.

Equilibrium dialysis was also performed at 4°C. An erythrocyte suspension (1 ml) was placed inside the dialysis sac and was dialysed against a Krebs–Ringer buffer containing the steroid. Radioactivity in the outside solution was counted directly; radioactivity in the inside solution was counted after ethyl acetate extraction. The percentage binding was calculated after correction for the volume of the erythrocytes. Equilibrium during incubation was reached within 2 min. Equilibrium during dialysis was reached within 24 h[4]. The binding curves for incubation and dialysis after proper equilibrium indicated almost the same percentage of binding[4]. On the basis of these results it was concluded that data from incubation and dialysis experiments could be compared with each other.

The binding of steroids by erythrocytes was evaluated quantitatively in order to permit a comparison with the binding of steroids by proteins, such as albumin

and transcortin. The amount of bound over unbound steroid (B/U) was plotted as a function of the amount of bound steroid (B)[5].

RESULTS AND DISCUSSION

1. *Uptake of steroids by intact erythrocytes*

In order to study some characteristics of the binding of steroids by erythrocytes we have tried to saturate the binding sites by dialysing or incubating erythrocytes with increasing amounts of steroid. The results in Fig. 1 show the difference in binding affinity for the 4 steroids studied. In the concentration range studied no significant change in binding was observed. This may reflect a high binding capacity. A Scatchard curve for the binding of steroids by human serum albumin has the same shape as the curves in Fig. 1, which may reflect that erythrocytes and albumin both have a large capacity for binding of steroids.

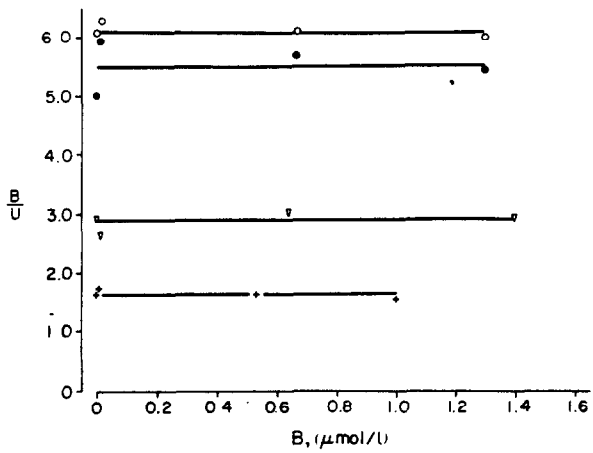

Fig. 1. Effect of steroid concentration on the binding of steroids by erythrocytes in Krebs–Ringer buffer. Binding was estimated by incubation. The ratio bound to unbound steroid (B/U) is plotted as a function of the amount of bound steroid (B). ○ progesterone; ● 20α-dihydroprogesterone; ▽ testosterone; + androstenedione; Haematocrit = 37–42 Vol%; Hemoglobin = 140–150 g/l.

The differences in binding affinity of erythrocytes for the four steroids cannot be explained by differences in polarity only. Although progesterone is bound more than 20α-dihydroprogesterone, androstenedione is bound less than testosterone.

If steroids are transported through the erythrocyte membrane, such transport may be time and temperature dependent. With the incubation technique it was not possible to detect an influence of time and temperature on the binding. However, the possibility exists that only a minor fraction of the bound steroid is transported. From other studies we know that interconversion of androstenedione and testosterone and of progesterone and 20α-dihydroprogesterone most likely occurs inside the erythrocytes[6]. This indicates that transport may occur prior to the conversion of the steroid. Separation of the steroid fraction inside the erythrocyte from the steroid adsorbed onto the erythrocyte can be achieved by washing the cells after incubation. Therefore, the following experiment was carried out. [^{3}H] labelled androstenedione and [^{14}C] labelled testosterone were incubated at 37°C for 3 h with an erythrocyte suspension in a Krebs–Ringer glucose buffer. Under these conditions [^{3}H] labelled testosterone is formed from androstenedione.

After incubation the cells were washed 8 times with isotonic buffer. The combined washes were extracted with ethyl acetate and the residues of the ethyl acetate extracts were chromatographed on thinlayer plates to separate androstenedione from testosterone. A semi-logarithmic plot of the bound fraction of the original amount of steroid against the number of washings gave a straight line (Fig. 2).

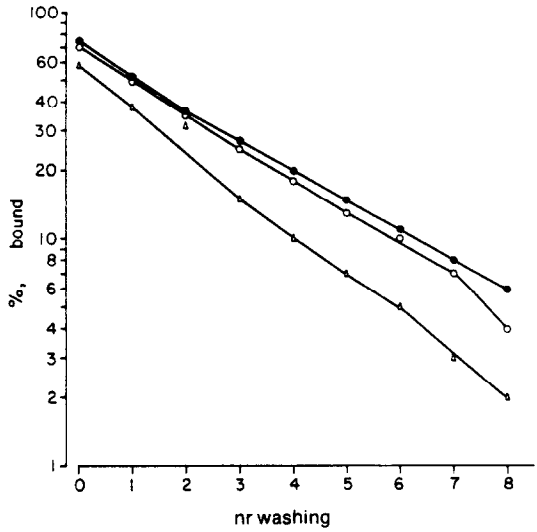

Fig. 2. Binding of steroids by erythrocytes after successive washings. Erythrocytes were incubated with [1,2-^{3}H]-androstenedione and [4-^{14}C]-testosterone in Krebs–Ringer buffer containing glucose (10^{-2} M). After 3 h of incubation the erythrocytes were washed (8 times) with Krebs–Ringer buffer.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

Because no difference was found between the slopes of two testosterone curves we have concluded that [^{3}H] testosterone and [^{14}C] testosterone behave in the same way during the washing procedure. The curves in Fig. 2 show that the bound steroids can be removed almost completely from the cells by repeated washing. This could indicate that the steroids were loosely bound by the membrane and that androstenedione might have been converted to testosterone in the membrane.

In whole blood steroids are mainly bound to plasma proteins and it is accepted that only a small fraction is associated with erythrocytes [5, 7]. In order to obtain more information about the influence of plasma proteins on the uptake of steroids by cells, the competition between plasma proteins and erythrocytes for binding of progesterone and pregnenolone was studied by incubation.

The binding of progesterone by erythrocytes suspended in 5% plasma increased with increasing steroid concentrations (see Fig. 3). This increase in binding of progesterone may be explained if it is assumed that specific progesterone binding proteins in plasma with a high affinity and a limited capacity are first saturated and that thereafter at progesterone concentrations of 25 ng/ml suspension or higher, a competition for binding exists between albumin and erythrocytes. The binding of pregnenolone by erythrocytes in 5% plasma was not significantly dependent on the steroid concentrations that were used. This is to be expected if there is only

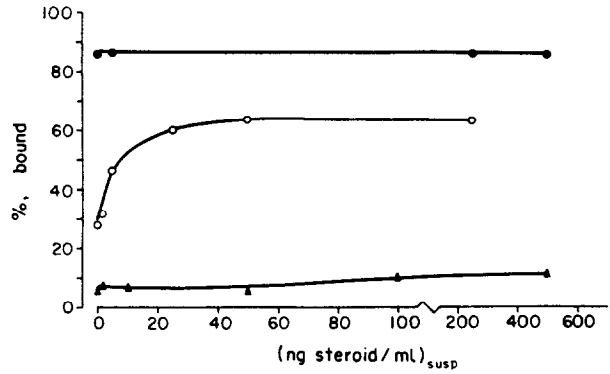

Fig. 3. Comparison of the binding of progesterone by erythrocytes in Krebs–Ringer buffer, in 5% (v/v) plasma and in whole blood at different steroid concentrations. Binding was estimated by incubation. ● Krebs–Ringer buffer; ○ 5% (v/v) plasma; ▲ whole blood.

competition between the aspecific binding of pregnenolone by erythrocytes and by albumin.

In whole blood the binding of progesterone by erythrocytes varied from 5 to 12% depending on the steroid concentration (Fig. 4). Because of the high affinity of progesterone-binding proteins in the plasma little progesterone is available for the erythrocytes. Pregnenolone, however, not bound to specific plasma proteins, was 30% bound by erythrocytes in whole blood (Fig. 4). Summarizing the results of the uptake studies with intact erythrocytes we have concluded that the capacity of erythrocytes for binding of steroids is high, because binding is independent of the steroid concentration.

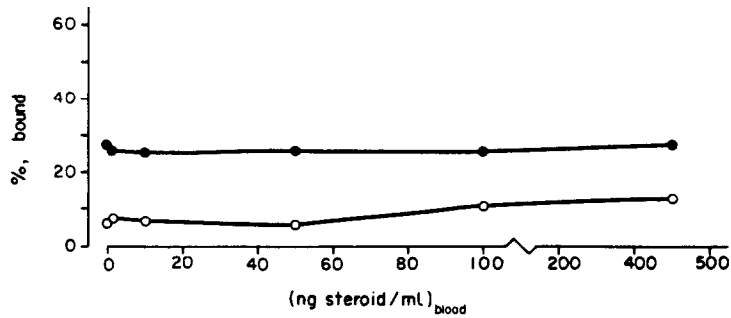

Fig. 4. Binding of progesterone and pregnenolone by erythrocytes in whole blood at different steroid concentrations. Binding was estimated by incubation. ● pregnenolone; ○ progesterone; Ht = 45/47 Vol_; Hb = 155–160 g/l.

Therefore, the uptake of steroids by intact erythrocytes can be considered as a distribution of steroids between a lipid-rich phase (erythrocytes) and a water phase. This distribution depends on the amount of erythrocytes, on the kind of steroid that is studied and on the presence of plasma proteins in the buffer medium. The uptake is independent of time and temperature within the ranges used.

2. *Transport of free and conjugated steroids through the erythrocyte membrane*

It has already been established that erythrocytes contain a 17β-hydroxysteroid

dehydrogenase[8–10]. This NADP-requiring enzyme has a broad specificity for steroids with a 17-oxo or 17β-hydroxyl-group and acts on both estrone and dehydroepiandrosterone as well as on their sulfates[6]. We investigated the relative rates of reduction of dehydroepiandrosterone and dehydroepiandrosterone sulfate, and of estrone and estrone sulfate by the 17β-hydroxysteroid dehydrogenase during incubation with intact and hemolysed erythrocytes. The dehydrogenase is located inside the cell, and is not bound to the membrane: therefore the reduction rates may be considered to be affected by the relative rates of permeation of steroids through the erythrocyte membrane.

Intact human erythrocytes incubated in a medium containing glucose showed marked differences in the reduction rate of the conjugated and non-conjugated steroids (Table 1). During a 5 h incubation 33–46% of the DHA was converted to androstenediol. In contrast, the amount of DHA sulfate converted to androstenediol sulfate (1·0–1·1%) did not differ significantly from control incubations.

Table 1. Reduction of dehydroepiandrosterone and dehydroepiandrosterone-sulfate, estrone and estrone-sulfate by intact and hemolysed human erythrocytes.

$$\text{Ratio (1)} = \frac{\%\ \text{DHA reduced}}{\%\ \text{DHA-S reduced}};\quad \text{Ratio (2)} = \frac{\%\ \text{E reduced}}{\%\ \text{E-S reduced}}$$

	Substrate concentration nmol/ml	% reduction		Ratio (1)	% reduction		Ratio (2)
		DHA	DHA-S		E	E-S	
Intact erythrocytes	1·75	33	1·1	30	16	7·5	2·1
	0·035	46	1·0	46	21	7·0	3·0
Hemolysed erythrocytes	1·75	18	10·9	1·6	6·1	10·4	0·59
	0·035	25	12·2	2·0	11·0	14·7	0·75
Purified enzyme from erythrocytes	1·75	38	33	1·2	22	40	0·55
Control incubation without erythrocyte preparation	1·75	0·3	0·8	—	1·0	0·7	—
	0·035	0·5	0·7	—	1·1	1·4	—

Conversion of DHA to androstenediol by lysed erythrocytes was about half that by intact cells. The amount of DHA sulfate converted to androstenediol sulfate by the hemolysate, however, was at least ten times higher than the amount converted by intact cells. After lysis of the erythrocytes the ratio expressing the percent reduction of DHA vs. the percent reduction of DHA sulfate was at least ten times smaller. For estrone and estrone sulfate a comparable, but much less pronounced effect was observed and the difference between the reduction of estrone sulfate by intact and lysed cells was smaller (see Table 1). Regardless of the exact intracellular location of the steroid dehydrogenase enzyme, our data suggest that the intact erythrocyte membrane is a greater barrier for the conjugated steroids than for the non-conjugated steroids to reach the enzyme. If the steroid dehydrogenase is present in the cytoplasm of the erythrocyte the differences between results of metabolism of steroids by intact and lysed cells may reflect differences between the rate of transport of the steroids to the enzyme. The data in Table 1 would then indicate that the rate of transport through the membrane of the conjugated steroids is smaller than the rate of transport of the free steroids.

3. *Localization and characterization of steroid binding sites in erythrocytes*

Although the results of conversions of steroids by erythrocytes may reflect that the steroid penetrates the erythrocyte, from our studies concerning the binding of steroids by intact erythrocytes it could not be concluded where the bound steroid was localized. We therefore attempted to determine the site of localization and also to characterize the possible binding sites for steroids in the erythrocyte. For these studies cell membranes and membrane-free fractions were used. Fractionations were carried out after hemolysis of intact erythrocytes with 2·5 volumes of hypotonic phosphate buffer. The resulting total hemolysate was separated by high speed centrifugation into membranes, and a membrane-free hemolysate. Each fraction was diluted with buffer to the same protein concentration. The membrane fraction still contained a considerable amount of hemoglobin, while the protein in the membrane-free hemolysate consisted almost exclusively of hemoglobin. Binding studies with these three fractions showed a high affinity of the membrane fraction for testosterone (Fig. 5). For the three fractions which we have isolated, it was not possible to saturate the binding sites with steroid within the

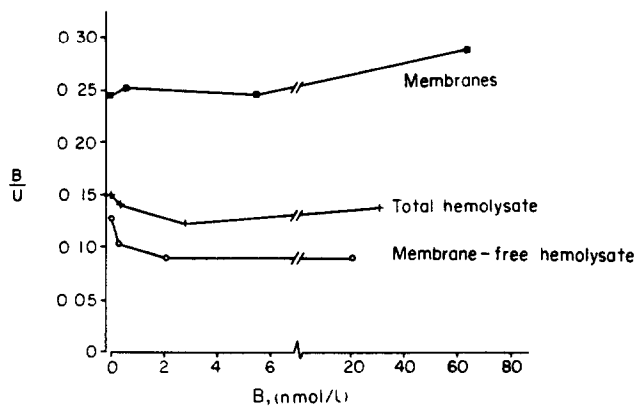

Fig. 5. Binding of testosterone by different erythrocyte fractions. Binding was estimated by equilibrium dialysis at 4°C. Protein concentration of each fraction: 20 mg/ml.

concentration range used. This indicates a rather non-specific type of steroid binding. The membrane fraction was further investigated in binding studies with membranes containing only non-hemoglobin-proteins. These membranes were prepared by washing the membrane fraction to free it of hemoglobin, according to the method of Dodge[11]. The membranes 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 obtained with this membrane suspension with binding data from erythrocytes suspended in an equal volume of isotonic buffer. Binding studies with this membrane suspension were carried out with six steroids and are shown in Fig. 6. Pregnenolone showed the highest affinity. Similar binding values were obtained for binding of pregnenolone by a comparable suspension of intact erythrocytes. It is rather unlikely, therefore, that pregnenolone will penetrate the intact erythrocyte. It may be completely bound in the membrane. This cannot be said for progesterone, 20α-dihydroprogesterone or testosterone. Binding values for these steroids by membranes were lower than those obtained with a comparable suspension of intact erythrocytes. Binding of

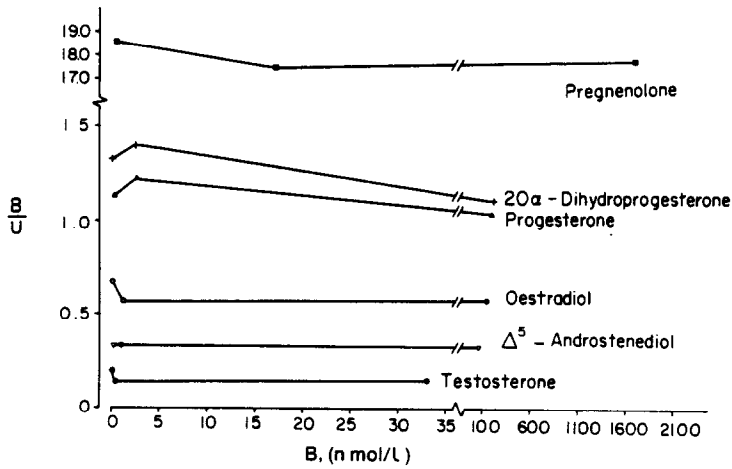

Fig. 6. Effect of steroid concentration on the binding of steroids by hemoglobin-free membranes. Binding was estimated by equilibrium dialysis at 4°C. Protein concentration: 1,6 mg/ml.

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. These results might give a possible explanation for the non-specific binding of steroids by target tissues. It is still not clear with which part of the membrane structure the steroids interacted.

DeVenuto stated that only the membrane protein is responsible for this interaction[12]. Therefore, the hemoglobin-free membranes were further fractionated by a butanol-extraction procedure according to Maddy[13]. It is possible to obtain in this way a lipid-free protein fraction which contains almost all the membrane proteins. The binding of testosterone by this membrane protein fraction and by the total membrane is shown in Fig. 7. Both preparations were diluted to the same protein concentration. The affinity of testosterone for the membrane protein was found to be significantly higher than for the total membrane. This higher binding by the isolated membrane protein may be a result of an interaction

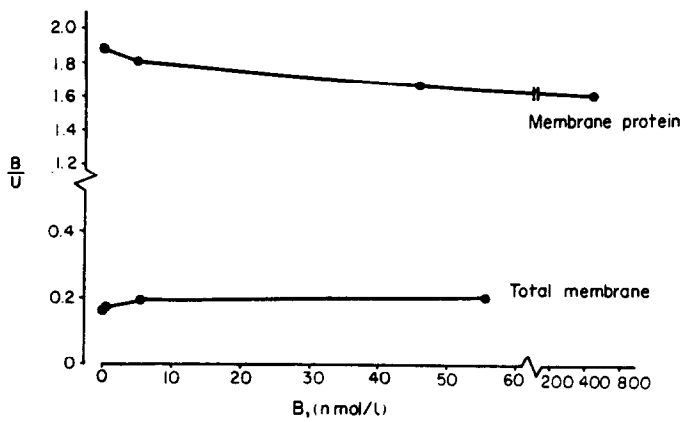

Fig. 7. Binding of testosterone by hemoglobin-free membranes and by a membrane protein fraction. Binding was estimated by equilibrium dialysis at 4°C. Protein concentration: 2 mg/ml.

of testosterone with binding sites which are not exposed to the steroid in the intact membrane structure. The binding sites of both preparations could not be saturated within the steroid concentration range used. This indicates again a non-specific type of binding. DeVenuto *et al.* reported a small difference between the binding of progesterone by total membranes and by the membrane protein fraction at 37°C, and they also found a positive temperature effect on the binding of progesterone by the total membranes[12]. As a result of our data obtained for the binding of testosterone by membranes and by a membrane protein fraction and the positive temperature effect on the progesterone binding by membranes found by DeVenuto, we investigated the temperature effect on the binding of progesterone by both the membranes and the membrane protein fraction. The results of these studies are presented in Table 2. Binding data are expressed in terms of the combining affinity, according to Westphal[14]. Together with the positive temperature effect for the total membrane, we found a significant negative temperature effect for the binding of progesterone by the membrane protein fraction.

At 37°C the binding of progesterone by the total membrane, expressed as the combining affinity, is higher than at 4°C. The membrane structure may be less restricted at 37°C than at 4°C, allowing a better insertion of the steroid into the

Table 2. Binding of progesterone by hemoglobin-free membranes and by a membrane-protein fraction at 4°C and at 37°C. Binding was estimated by equilibrium dialysis.
Binding data are expressed as:

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

	4°C	37°C
Total membrane	0·83	1·05
Membrane-protein	4·45	1·63

membrane. However, at 37°C the combining affinity of progesterone for the membrane proteins is smaller than at 4°C due to a possible dissociation of the steroid-protein complex at the higher temperature. Therefore, considering the positive temperature effect on the binding of progesterone by the total membrane and assuming that the membrane protein is responsible for this binding, we have to deal with the summation of two temperature effects, a positive and a negative one, of which the positive one at 37°C prevails.

A 17β-hydroxysteroid dehydrogenase activity was detectable only in the total hemolysate, and in the membrane-free hemolysate. In order to correlate binding with enzyme activity, the membrane-free hemolysate was further fractionated by ammonium sulphate precipitation. Another two fractions were obtained: the 25–50% saturated ammonium sulphate precipitate, containing all the dehydrogenase activity, and the residue after precipitation of the enzyme fraction.

We have investigated the binding of testosterone by this enzyme fraction (Fig. 8). An apparent association constant could be calculated from the slope of the Scatchard curve. It is evident from this curve that there are more binding proteins in this fraction. The magnitude of the affinity constant for testosterone was found to be of the same order as that of the testosterone-binding plasma–protein. For intact erythrocytes we estimated an apparent association constant of much lower

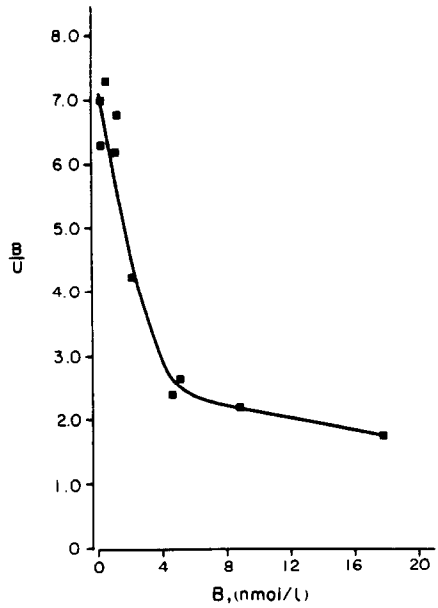

Fig. 8. Binding of testosterone by an erythrocyte fraction with steroid dehydrogenase activity ("17β-OHSD-fraction"). Binding was estimated by equilibrium dialysis at 4°C. Protein concentration: 45 mg/ml.

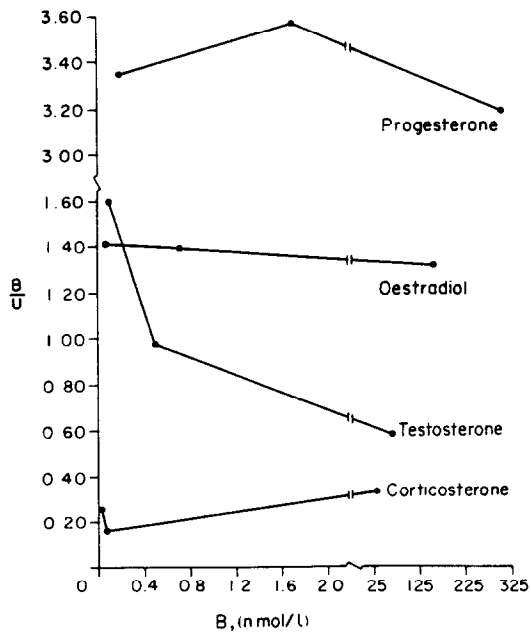

Fig. 9. Effect of steroid concentration on the binding of steroids by the "17β-OHSD-fraction". Binding was estimated by equilibrium dialysis at 4°C. Protein concentration: 30 mg/ml.

value. The order of magnitude of this constant, per mole of hemoglobin, was 10^3.

We have considered that this binding might result from the presence of a plasma protein (transcortin or the testosterone-binding globulin), from the presence of the 17β-hydroxysteroid dehydrogenase in this fraction or from a still unknown factor. To see whether a plasma protein could be present, equilibrium dialysis experiments have been performed with progesterone, oestradiol, testosterone and corticosterone. The results of these binding experiments only showed specific binding of testosterone (Fig. 9). Therefore, the presence of transcortin in this erythrocyte fraction is very unlikely. Equilibrium dialysis experiments with 5α-dihydrotestosterone and testosterone resulted in a higher binding of 5α-dihydrotestosterone. This might indicate that the testosterone-binding globulin could be present in this fraction.

After gel filtration on Sephadex G-150 four protein fractions were collected (see Fig. 10). This separation was based on the 17β-hydroxysteroid dehydrogenase distribution pattern and on the protein distribution pattern. From each fraction the 50% saturated ammonium sulphate precipitate was prepared. The

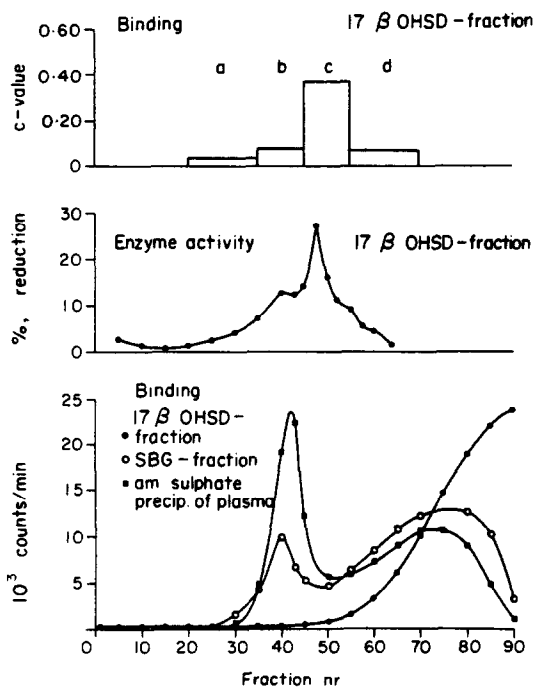

Fig. 10. Gel filtration elution patterns on Sephadex G-150 of the "17β-OHSD-fraction", an SBG fraction and the 25–50% saturated ammonium sulfate precipitate of plasma. 4 ml protein samples (10–25 mg/ml) were applied to a 420 ml column of Sephadex G-150 equilibrated with 0·05 M phosphate buffer pH: 7·0, 10^{-1} M KCl, 10^{-3} M EDTA, 10^{-3} M mercaptoethanol, $1{\cdot}6 \times 10^{-5}$ M NADP. Elution was performed at 4°C with the same buffer and using an upward constant flow (15–20 ml/h) obtained by the use of a Mariotte flask. Six ml fractions were collected. Binding after equilibrium dialysis is expressed as C-value according to Westphal[14]. Binding during gel chromatography was estimated as follows: 4 ml protein samples with [1,2-^{3}H]-testosterone to a final steroid concentration of $2{\cdot}8 \times 10^{-8}$ M were applied to the column and 0·5 ml of the collected fractions were counted. Enzyme activity was expressed as % androstenedione reduced by 1 ml of the collected fractions.

four different fractions were diluted to the same protein concentration, and the binding of testosterone by these fractions was estimated by equilibrium dialysis.

Specific testosterone binding could be demonstrated with fraction C, the fraction with the highest 17β-hydroxysteroid dehydrogenase activity. It was not possible to demonstrate binding of testosterone to the erythrocyte protein fraction during gel filtration. All the radioactive testosterone applied on the Sephadex column was recovered as unbound steroid. An explanation for this could be the dissociation of the steroid protein complex during the gel filtration procedure. Testestosterone binding could be demonstrated with the testosterone-binding globulin fraction and the 25–50% saturated ammonium sulphate precipitate of plasma. The testosterone-binding globulin had the same elution volume as fraction B. The testosterone radioactivity in the 25–50% saturated ammonium sulphate precipitate of plasma was eluted between that of the B and C fraction. It may be concluded, therefore, that it is impossible to separate binding activity from enzyme activity and that fraction C may contain plasma proteins that will bind testosterone although the concentration is too small to give a radioactivity peak in the gel filtration experiment.

CONCLUSION

A comparison of the binding affinities of the isolated erythrocyte fractions for testosterone on basis of the same protein and steroid concentration showed a high bound/unbound value for the 17β-hydroxysteroid dehydrogenase fraction. The character of the protein that specifically binds testosterone in this fraction is still obscure. The possibility of a plasma protein cannot be completely ruled out. The contribution of the enzyme fraction to the binding of testosterone by intact erythrocytes, however, can be neglected.

In general the membrane or possible membrane proteins may play the major role in the total uptake of steroids by erythrocytes. This was indicated by the results of washing the red blood cells several times with isotonic buffer during which procedure most of the bound steroid was removed, indicating that steroids might be adsorbed to the cell surface. This uptake of steroids by intact erythrocytes in Krebs–Ringer buffer, therefore, can be considered as a partition between a lipid-rich membrane phase and an aqueous buffer phase. The presence of plasma proteins in the buffer medium, however, dramatically decreases the uptake of steroids by erythrocytes. The observed differences in reduction rate between steroid sulfates and the corresponding free steroid suggest a difference in permeability of the intact erythrocyte cell membrane for these steroids.

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DISCUSSION

Jensen: Dr. Brinkmann, you have shown that erythrocytes have certain enzymatic systems which transform the steroids and that these erythrocytes are capable of binding certain steroids. Now I wonder, is there any evidence that the steroid hormones have an effect on the processes in the erythrocytes themselves; in other words, is there any biological action of the hormones on the erythrocytes?

Brinkmann: The purpose of this investigation was not to study the biological action of steroid hormones on processes in the erythrocyte. The human erythrocyte was chosen only as a model system for studying steroid-cell interactions. It is known from *in vitro* studies that pharmacological high doses of steroids can protect erythrocytes against mechanical induced haemolysis. Also relevant to your question may be the inhibitory action of certain steroids on the enzyme glucose-6-phosphate dehydrogenase in erythrocytes *in vitro*. How significant this inhibitory role is *in vivo* is not known.

Wira: I was wondering if you've had an opportunity to look at the binding of steroids by nucleated erythrocytes, and contrast that binding with the anucleated ones?

Brinkmann: We have only used mature human erythrocytes which contain no nucleus and we did not investigate the binding of steroids by avian erythrocyte or nucleated blood cells from other species.

Wira: When Dr. Munck and I were just starting to look for intracellular glucocorticoid receptors in various tissues, it struck us that one interesting potential target tissue might be the erythrocyte. When we looked at mature rat erythrocytes and distinguished between specific vs. non-specific binding by comparing the dissociation of cortisol, a glucocorticoid which binds specifically against cortisone, a non-glucocorticoid which does not bind specifically, we found no evidence for specific binding. However, when we studied the chicken erythrocyte, which is nucleated, we did find specific binding.

Brinkmann: Did you isolate a specific corticosteroid binding protein?

Wira: No, we didn't follow this up. As soon as we had this evidence—it was just a bit of suggestive evidence—that the glucocorticoid receptors were located in the nucleus, we immediately turned to the thymus system and isolated the nuclei by the magnesium chloride procedure that Dr. Munck spoke about yesterday.

Brinkmann: How did you estimate the specific binding of cortisol by the nucleated erythrocytes?

Wira: We washed the nucleated cells, made a suspension, and incubated them in the same way which Dr. Munck described yesterday, with 10^{-8} M cortisol or cortisone. After equilibrium was reached, we did the 50-fold dilution into Krebs-Ringer bicarbonate at 37°C and looked at the dissociation of both cortisol and

cortisone. We found a slowly dissociating cortisol fraction with a time constant that was very similar to that found by Dr. Munck with the thymus cells. In contrast, cortisone, an inactive steroid *in vitro*, dissociated rapidly.

Holzbauer: I would like to draw the attention of the meeting once more to the possibility, that the steroid binding capacity of an erythrocyte in the blood stream might be different from that of an erythrocyte which has been washed and is suspended in an artificial medium in the test tube. I hope that, in the near future, some biochemists or biophysicists might divert their attention from the steroid binding plasma proteins to the erythrocytes.

Brinkmann: I think a rather important point in this respect is to be sure that you are not dealing with plasma "trapped" by the erythrocytes after centrifugation of the blood. We used erythrocytes which were washed at least three times with saline to eliminate any possible plasma contamination.

Holzbauer: The amount of plasma trapped between the erythrocytes after centrifugation at 2000 *g* for 20 min is only a very small proportion of the total amount of plasma in a given blood sample. Let us take as an example a blood sample in which 50% of the total amount of corticosterone could be extracted from the plasma and 50% from the cells. If you assume that the corticosterone extracted from the "cells" was only contained in the plasma trapped between the cells, then the corticosterone concentration in this plasma would have to be very much higher than the corticosterone concentration in the plasma which was separated off by centrifugation.

Van der Molen: If I am permitted to make a comment, I think Dr. Holzbauer's statement may be relevant, but I'm still a bit worried about what you consider *in vivo*, because the moment you have isolated your erythrocytes, whether after washing or without washing, I think you're operating an *in vitro* system, and I believe that probably the most relevant information in this respect comes from the studies which Dr. Brinkmann mentioned by Little and co-workers, who have compared metabolic clearance rates of steroids, where they estimated the steroid concentration either in the plasma fraction or in the total blood. I think there were only one or two steroids, and most strikingly pregnenolone, where they found a significant discrepancy between these metabolic clearance rates. I think there, you have an *in vivo* argument, that there may be an important uptake of steroids by erythrocytes. But I think also that for aldosterone, even when you have shown that in the isolated erythrocyte fraction aldosterone is bound to a significant extent, Dr. Tait and Dr. Little as far as I know, did not find a discrepancy between the metabolic clearance rates whether they used blood or plasma for their isolation of aldosterone. So I think in general I would completely agree with you that there may be a difference between *in vivo* uptake and *in vitro* uptake, but still the actual information is fairly limited if it exists at all, and I would still argue, in agreement with Dr. Brinkmann, that even if we consider the system *in vivo*, it still may be *in vitro* but a different *in vitro* system than Dr. Brinkmann may have used after washing erythrocytes.

Oertel: With respect to this subject we have compared the penetration rates of labeled steroids under *in vitro* conditions, where isolated erythrocytes were incubated, and under *in vivo* conditions. Here, the penetration of steroids was determined after intravenous injection of labeled material and isolation of erythrocytes. There was little difference between *in vitro* and *in vivo* penetration rates.

Brinkmann: Did you use conjugated steroids?

Oertel: Conjugated and free steroids.
Brinkmann: Which conjugated steroids did you use?
Oertel: Steroid sulphates and steroid sulphatides.
Holzbauer: The difficulties involved in the quantitative extraction of steroids from packed red cells may also account for some of the discrepancies in the literature. I find it difficult to understand how it is possible to obtain correct values for the metabolic clearance rate of a steroid from the blood if only the plasma concentration of this steroid is taken into account. After infusing radioactively labelled or unlabelled cortisol i.v. into dogs we found about 50% of the total blood cortisol in the cell fraction. Similar observations were made by de Moor P. and Steeno O. (*J. Endocr.* **26**, (1963) 301) on human subjects.
Van der Molen: I agree again completely with what you say, but I think it's fairly simple to wash your erythrocytes with physiological saline. In studies which we have done over the years with Professor Eik-Nes, we have always worked with dog plasma, and I think it's essential that in isolating steroids from dog plasma, you wash your erythrocytes. If you do not, I agree completely that part of your steroids are adsorbed onto the erythrocytes. Therefore I would consider part of that binding which you may indicate as "erythrocyte-bound steroids" may still be some contamination. I do not have any relevant data as to which part of the fraction in plasma is bound to the erythrocytes, but again it's not too difficult to remove the steroid from the erythrocytes by washing.
Holzbauer: I agree with that. However, as I showed yesterday, it is not always possible to remove steroids quantitatively from the erythrocytes by repeated washing with saline. In one case, e.g. 95% of the cortisol originally present in the packed cells remained attached to the cells after repeated washing with saline.