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BINDING OF PROGESTERONE, OESTRADIOL AND TESTOSTERONE TO HUMAN A, C AND S ERYTHROCYTES AND HAEMOGLOBINS

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SUMMARY

Binding characteristics of progesterone, testosterone and oestradiol to human erythrocytes and haemoglobins were studied by equilibrium dialysis in order to investigate the possible mechanism whereby steroids prevent sickling of haemoglobin-S erythrocytes. Both the erythrocytes and haemoglobin solutions of Hb-A had lower binding constants compared to those of Hb-C and Hb-S. With progesterone and testosterone, the values for Hb-S erythrocytes were highest. There was however no significant variation between the binding constants of the steroids for the different haemoglobins, thus suggesting that these could not be correlated with any of their properties.

INTRODUCTION

The uptake of steroids and their metabolism by normal erythrocytes have been previously studied [1–3]. Also, it has been reported that the sickling phenomenon of erythrocytes with haemoglobin-S could be inhibited *in vitro* and *in vivo* by progesterone and testosterone [4–6]. Deoxyhaemoglobin-S is less soluble than deoxyhaemoglobin-A and the sickling of haemoglobin-S most likely results from hydrophobic interaction between the region of the B-chain altered by the mutation ($^{B}6$ glutamic acid–valine) and the complementary sites on the neighbouring molecule [7].

Whilst possible mechanisms whereby steroids inhibit sickling have been suggested (4 and 5), these are yet not known. It is not unlikely that steroids bind to haemoglobin-S and thereby prevent the structural change that leads to the sickling phenomenon.

In this paper, we have investigated the binding of progesterone, testosterone and oestradiol to erythrocytes and haemoglobins from normal erythrocytes (Human A) and the other variants (Human S and Human C) with the aim of elucidating the possible mechanism whereby steroids affect the sickling phenomenon.

MATERIALS AND METHOD

Non-radioactive progesterone and testosterone were from Koch–Light Laboratories Limited, Colnbrook, Bucks, England. Radioactive progesterone, testosterone and oestradiol were from the Radiochemical Centre, Amersham, England. The following radioactive steroids were used. [1β,2β-^{3}H]-testosterone—55 Ci/mmol; [^{3}H]-progesterone—750 mCi/mmol; [2,4,6,7-^{3}H]-oestradiol—100 Ci/mmol. All chemicals were of analar grade. For the equilibrium dialysis, visking dialysis tubing was used and unless otherwise stated, the buffer used was 0·1 M phosphate pH 7·4.

Biological material

Blood samples containing haemoglobins Human-A or Human-C were obtained from the Blood Bank of the University College Hospital, Ibadan and had been characterized as such by paper electrophoresis by the method of Aronsson and Gronwall[8] in the routine screening of blood taken from donors. Blood samples containing haemoglobin Human-S were obtained directly from patients who had also been characterized as such by paper electrophoresis. They were all in remission at the time of the study.

Experimental procedure

Red cells were prepared from individual blood samples by centrifuging at 10,000 rev./min for 20 min at 5°C. The supernatant was discarded by aspirating with a water pump. The red cells were washed seven times with sodium chloride solution (9·5 g/l) at 5°C and centrifuged between washings at 8000 rev./min for 15 min. The packed red cells were then suspended in phosphate buffer pH 7·4 and the haemoglobin content was determined for each experiment.

Part of the cells prepared above were used for preparing the haemoglobin solution. The cells were lysed by shaking well with a mixture of an equal volume of ice cold distilled water and about half this volume of ice cold peroxide-free ether. The solution was then centrifuged at 8000 rev./min for 20 min. Excess ether

The following trivial names have been used in the text: Testosterone: 17β-hydroxy-4-androsten-3-one. Oestradiol: 1,3,5(10)-oestratriene-3,17β-diol. Progesterone: 4-pregnene-3, 20-dione.

was first removed at the water pump and the remaining traces were evaporated by gently blowing air into the centrifuge tube. The haemoglobin solution was decanted from beneath the cake of cell debris. The haemoglobin solution was then dialysed at 5°C against 0·001 M phosphate buffer pH 7·4 until the smell of ether was no longer detectable. About 3 changes at intervals of 2 h were usually sufficient. The haemoglobin solution was finally dialysed against phosphate buffer pH 7·4 overnight at 5°C or passed through Dintzis Column [9] to strip it of ions including 2,3-diphosphoglyceric acid. When the haemoglobin was passed through Dintzis Column an appropriate amount of 0·2 M phosphate buffer was added to it to make it 0·1 M phosphate.

The haemoglobin concentration of red cells or the haemoglobin solution was determined by its conversion to cyano-methaemoglobin complex and using molar extinction coefficient of $10{\cdot}9 \times 10^{-3}$ at 540 nm for the cyanomethaemoglobin complex.

For the determination of the binding parameters for non-radioactive steroids, these were dissolved in ethanol and an appropriate amount of 0·2 M phosphate buffer and distilled water was added to make the solution 0·1 M phosphate buffer and 10% ethanol (v/v). 1 ml of red cell or haemoglobin solution (in 0·1 M phosphate buffer pH 7·4) was put in visking dialysis tubing ($\frac{8}{32}$) and dialysed against 10 ml of steroid solution in a 15 ml test tube. Before use, the visking tubing was boiled for 10 min in distilled water and then rinsed again in distilled water. This procedure was repeated three times.

The dialysis tube was suspended from the mouth of the test tube using a rubber band and the test tube was covered with parafilm. The same steroid solution used above was put in another test tube and the test tube was covered with parafilm. This was later used for the determination of the concentration of the original steroid solution. On each sample, five experiments were performed using steroid solutions of different concentrations. A control blank had no steroid but only the buffer solution (0·1 M phosphate, 10% ethanol (v/v). The test tubes were then placed in a rack which was placed inside a metabolic incubator provided with a shaker at controlled temperature. After about twelve hours the optical densities of the steroid solutions against which no red cell or haemoglobin was dialysed was determined using buffer (0·1 M phosphate and 10% ethanol) as blank. The optical densities of the steroid solutions against which red cell or haemoglobin was dialysed were determined using as blank for red cell, buffer solution (0·1 M phosphate and 10% ethanol), against which red cell was dialysed, and for haemoglobin that against which haemoglobin was dialysed.

Radioactive steroids

Benzene in which the steroids were formerly dissolved was evaporated off and the steroid was dissolved in phosphate buffer pH 7·4. 1 ml of red cell or haemoglobin solution (in phosphate buffer pH 7·4) was put in visking dialysis tubing ($\frac{8}{32}$) and dialysed against 10 ml of steroid solution in 15 ml test tube for about twelve hours. The amount of radioactivity in 1 ml of the steroid solution against which red cell or haemoglobin was dialysed was determined before and after dialysis, using a Packard Tricarb Liquid Scintillation Spectrometer Model 3004 and counting for at least 5 min. The counting efficiency was 15%. Five experiments were also performed using steroid solutions of different concentrations.

Calculation—binding constant

The apparent binding constant K^1/mol of haemoglobin tetramer for the steroids to red cells and haemoglobin were calculated using the formula:

$$K^1 = \frac{D_0 \times 10 - Df \times 11}{Df(\mathrm{Hb})}$$

where D_0 is the optical density at 249 nm of the original steroid solution, Df that of the same solution after dialysis and Hb the haemoglobin concentration in mol/tetramer. With the radioactive steroid studies, the 5-min radioactivity counts before (Co) and after (Cf) dialysis were substituted for the optical densities.

In all experiments with non-radioactive steroids the concentration used was between $1{\cdot}0 \times 10^{-5}$ and $1{\cdot}3 \times 10^{-4}$ μm/l; and for the radioactive steroids between $1{\cdot}0 \times 10^{-9}$ and $1{\cdot}0 \times 10^{-7}$. The haemoglobin concentration in red cells and haemoglobin solutions was between 5×10^{-4} and $1{\cdot}5 \times 10^{-4}$ moles of haemoglobin/l.

RESULTS

Table 1 gives a typical result of the readings for the binding at one temperature of non-radioactive steroids using progesterone and Hb-A erythrocytes and Table 2 those for radioactive oestradiol. There was no difference in the apparent binding constants over a wide range of steroid concentrations. Similar observations were also made for Hb-S and Hb-C erythrocytes over a wide range of steroid concentrations. Tables 3 and 4 compare the average apparent binding constants for Hb-A, Hb-C and Hb-S for progesterone and radioactive progesterone and oestradiol, respectively. Whilst with increasing temperature the values for progesterone binding by Hb-A and Hb-S erythrocytes fell, those for Hb-C remained unchanged. Between 23–25°C, Hb-A and Hb-C erythrocytes had a lower binding than Hb-S erythrocytes Table 3. This higher binding shown by Hb-S erythrocytes at this temperature was also evident with radioactive progesterone (Table 4) and non-radioactive testosterone (Table 3). However with oestradiol, both Hb-S and Hb-C erythrocytes had higher binding constants than Hb-A erythrocytes (Table 4).

The typical results for the binding of steroids to haemoglobin solutions at one temperature are shown in Table 5 for progesterone and Hb-S, and Table 6 for testosterone and Hb-A. As previously observed

Table 1. Progesterone binding to human Hb-A red cell at 24·5°C and pH 7·4

Number of Experiment	'D_o' optical density of steroid solution at 249m before dialysis	'D_f' optical density of steroid solution at 249m after dialysis	Apparent binding constant K^1 per mole of haemoglobin tetramer x 10^3
1	1.138	0.904	2.21
2	1.031	0.812	2.38
3	0.915	0.730	2.09
4	0.925	0.747	1.94
5	0.768	0.598	2.55

Optical density of 1.138 is equivalent to concentration of 7.78×10^{-5} moles per litre.

Average $K^1 = (2.30 \pm 0.10) \times 10^3$ per mole.

Concentration of haemoglobin in red cells is 7.20×10^{-4} moles of haemoglobin tetramer per litre.

with the different erythrocytes, no differences in the apparent binding constants were also observed over a wide range of steroid concentrations with the haemoglobin solutions. Table 7 gives the results for the binding of the various haemoglobins to progesterone and testosterone respectively. Both Hb-C and Hb-S haemoglobin solutions showed higher binding than Hb-A haemoglobin solutions. This was also the case for radioactive progesterone and radioactive oestradiol (Table 4).

DISCUSSION

In order to assess the binding of steroids by human erythrocytes and haemoglobin solutions in a quantitative way, we have expressed the binding constant/mol of haemoglobin tetramer. The results indicate

Table 2. Binding of oestradiol-^{3}H to Human-A red cell at 23·5°C and pH 7·4

Number of Experiment	Counts per minute before dialysis, 'C_o'	Counts per minute after dialysis 'C_f'	K^1 x 10^3
1	93232	66371	2.54
2	38312	25667	3.28
3	17393	12949	2.08
4	9236	6581	2.55
5	3787	2748	2.32

Average $K^1 = (2.60 \pm 0.20)\ 10^3$

Table 3. Steroid binding to red cell

Steroid	Genotype - temp. °C	K x 10^{3*}
Progesterone	Human A -24.5	2.30 ± 0.10
	-30.5	1.50 ± 0.10
	-36.0	0.47 ± 0.50
	" C -23.0	2.10 ± 0.10
	-30.0	2.30 ± 0.20
	" S -25.0	3.50 ± 0.40
	-30.5	1.45 ± 0.06
Testosterone	Human A -24.0	0.74 ± 0.09
	30.5	0.60 ± 0.04
	" C -23.0	0.79 ± 0.09
	30.0	0.96 ± 0.09
	" S -23.0	0.91 ± 0.06
	28.5	1.10 ± 0.04

* Mean of five experiments ± SD.

Table 4. Steroid binding at 23·5°C

Sample	Mean K^1 x 10^{3*}	
	Oestradiol ^{3}H	Progesterone ^{3}H
Erythrocyte - A	2.60 ± 0.20	1.40 ± 0.10
- C	3.50 ± 0.20	1.60 ± 0.30
- S	3.50 ± 0.30	4.30 ± 0.60
Haemoglobin Solution -A	2.60 ± 0.30	1.60 ± 0.10
C	4.70 ± 0.70	3.0 ± 0.40
S	6.0 ± 0.50	3.0 ± 0.70

* See footnote Table III

Table 5. Progesterone binding to Human-S Haemoglobin at 25°C and pH 7·4

Number of Experiment	'D_o'	'D_f'	K^1 x 10^3
1	1.052	0.844	1.63
2	0.836	0.668	1.65
3	0.732	0.585	1.65
4	0.632	0.509	1.55
5	0.532	0.431	1.50

Concentration of haemoglobin is 9.13×10^{-4} moles of haemoglobin tetramer per litre.

Average $K^1 = (1.59 \pm 0.02)\ 10^3$ per mole.

Table 6. Testerone binding to Human-A haemoglobin at 30·5°C and pH 7·4

Number of Experiment	'D_o'	'D_f'	$K^1 \times 10^3$
1	0.772	0.642	0.83
2	0.680	0.565	0.82
3	0.590	0.488	0.87
4	0.493	0.411	0.81
5	0.406	0.338	0.81

Average $K^1 = (0.82 \pm 0.03)10^3$ per mole of haemoglobin tetramer.

Table 7. Steroid binding to haemoglobin

Steroid	Genotype - Temp. °C	$K^1 \times 10^{3*}$
Progesterone	Human A - 23.0	1.50 ± 0.10
	- 30.0	1.40 ± 0.10
	- 36.0	0.75 ± 0.03
	" C - 23.0	1.60 ± 0.09
	- 30.0	2.10 ± 0.10
	" S - 25.0	1.59 ± 0.02
	31.0	1.72 ± 0.06
	38.5	2.40 ± 0.10
Testosterone	Human A - 23.0	0.93 ± 0.30
	30.5	0.82 ± 0.03
	" C - 23.0	2.50 ± 0.30
	30.2	1.36 ± 0.07
	" S - 22.0	2.10 ± 0.10
	28.5	1.90 ± 0.20

that human haemoglobins and erythrocytes bind to steroids. The binding constants of testosterone, oestradiol and progesterone to Human A, C and S erythrocytes and haemoglobin solutions were however of the same order of magnitude even though the results suggest that the Human S erythrocyte and haemoglobin solution bind more to all the steroids than the others. The binding of C haemoglobin to the steroids also seems to be more than that of A haemoglobins.

The binding constants observed in the present studies are similar to those reported for the binding of human erythrocytes to progesterone and testosterone [10] and rat erythrocytes to progesterone [11]. They are also of the same order of magnitude as those reported for the binding of albumin to progesterone and testosterone [12].

It is interesting to note that the variation between the binding constants of the various steroids for the different haemoglobins and erythrocytes was little. No clear cut correlation could be made between the binding properties and the haemoglobin status of the erythrocytes. Thus, the role which the binding of steroids, especially those already reported to reverse sickling [4–6], play in this phenomenon is not obvious from these studies. On the other hand, it is interesting that with progesterone and testosterone, the two steroids which have been shown to reverse sickling [4–6], the highest binding was seen with the Hb-S erythrocytes. Oestradiol to which both Hb-S and Hb-C erythrocytes and haemoglobin solutions bind much more than Hb-A has not been reported to affect the sickling phenomenon. Further work is in progress including the effect of ions on these binding parameters in order to understand their mechanism and possible physiological or other biological importance.

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