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SOME STUDIES ON THE BINDING OF ANDROSTENEDIOL BY HUMAN PLASMA PROTEINS*

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SUMMARY

Proteins from late pregnancy plasma were found to bind androstenediol (5-androstene-3β, 17β-diol). Electrophoretic separation of the proteins indicated that the binding fractions were: albumin, alpha globulins and beta globulin. Equilibrium dialysis experiments with plasma at several dilutions, and with albumin as reference, demonstrated the presence in plasma of a binding component with a greater affinity than albumin.

From the electrophoresis data, beta globulin was found to be the fraction with the highest specific activity in terms of steroid bound per amount of protein. Also, the beta globulin electrophoretically isolated showed a greater affinity for androstenediol than albumin when subjected to equilibrium dialysis. The results from the equilibrium dialysis experiments indicate that at steroid concentrations of 0·2 and $2{\cdot}0 \times 10^{-8}$ M, 96–99% of the androstenediol is bound to plasma proteins. The equilibrium constant, *nk*, for the complex albumin–androstenediol was found to be $1{\cdot}7 \times 10^{3}$ l/mole.

IN THE course of our studies on the biological significance of androstenediol (5-androstene-3β, 17β-diol) we became interested in its binding to plasma proteins.

The formation of steroid–protein complexes has profound implications, since, in most cases, it renders the steroid biologically inactive without destroying it [1–5].

Androstenediol has been reported as being an important intermediate in the biosynthesis of testosterone by testis [6–8] and also as having high androgenic activity of its own [9]. It is an important estrogen precursor in the normal ovary as well as in the postmenopausal ovaries secreting excess estrogen [10]. Its presence in human plasma has been demonstrated [11, 12]. Previous studies suggest that its peripheral production may be increased in idiopathic hirsutism [13].

In addition, it has also been shown that audrostenediol can displace testosterone bound to plasma proteins [16] and estradiol bound to a β globulin fraction, with the same affinity as testosterone [14].

Some results regarding the binding of androstenediol by different proteins from late pregnancy plasma are reported. The percentage binding of androstenediol to proteins in diluted plasma samples and purified globulin fractions is also studied.

MATERIALS AND METHODS

Plasma was obtained from healthy women in the third trimester of pregnancy.

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Blood was received in heparinized syringes and centrifuged immediately. The plasma was stored at −15°C in 1 ml aliquots. Prior to use, endogenous steroids were removed by shaking the plasma with activated charcoal (Norit A), 50 mg/ml, for 30 min at room temperature [17].

A partially purified globulin fraction was obtained by precipitation of whole plasma with an equal volume of saturated $(NH_4)_2\ SO_4$ solution at pH 7. The precipitate was dissolved in a small volume of 0·2 M phosphate buffer pH 7·4, and dialyzed at 4°C against several changes of the same buffer.

Electrophoresis of plasma mixed with radioactive Δ5 diol was performed on cellulose acetate strips, 4 × 15 cm, using Veronal buffer pH 8·6, 0·07 M, and applying a current of 0·5 mA/cm. A small amount of bromophenol blue was added to the plasma and used as an indicator. The runs were carried out at 4°C for approximately 4 hr.

To achieve a better electrophoretic separation of globulins on a larger scale, agar gel plates, 20 × 20 cm, were employed. The same Veronal buffer was used and a current of 1·5 mA/cm was applied. Protein bands were located by staining with a Nigrosin 0·01% solution in 2% acetic acid.

To determine the radioactivity associated with the protein fractions separated on cellulose acetate strips, the corresponding zones were cut off and extracted with 3 ml of a mixture methanol : chloroform 1 : 2 (v/v). The extracts were transferred to counting vials and evaporated.

The procedure used to extract protein–steroid complexes from agar plates was homogenization of the gel with water, which was then extracted twice with 10 ml of a mixture ether : chloroform 3 : 1 (v/v). The organic layer was evaporated to dryness in counting vials.

Purified beta and gamma globulins were obtained by agar gel electrophoresis of the crude globulin fraction prepared by $(NH_4)_2\ SO_4$ precipitation of whole plasma. The corresponding zones were eluted by homogenizing the gel with a minimal volume of phosphate buffer pH 7·4, 0·2 M.

Androstenediol-7α-^{3}H (specific activity: 12·9 Ci/mmole) was prepared by the sodium borohydride reduction of dehydroepiandrosterone-7α-^{3}H. It was purified by thin layer chromatography and the purity was checked by isotope dilution prior to use. Nonradioactive androstenediol was obtained from Mann Research Lab.

Equilibrium dialysis was performed following the general outline of Rivarola *et al.* [18]. Briefly, a 1 ml aliquot of the test solution was pipetted inside a cellophane dialysis tube (½ in. dia.), previously soaked in water and carefully dried, and placed in a 50 ml Erlenmeyer flask with 10 ml of phosphate buffer containing ^{3}H-androstenediol at concentrations of $0{\cdot}2 \times 10^{-9}$ M and $2{\cdot}0 \times 10^{-9}$ M. The tightly stoppered flasks were put in a shaking water bath at 37°C and the dialysis allowed to proceed for 18 hr. The contents of the tubes and aliquots of the outside solution were extracted with 10 ml of a mixture ether : chloroform 3 : 1 (v/v) and the organic solvents were transferred to counting vials and evaporated.

The percentage of steroid bound was calculated according to the formula proposed by Slaunwhite [20]: % steroid bound =

$$100\left(1 - \frac{\text{cpm outside tubing} \times \text{volume inside tubing}}{\text{cpm inside tubing} \times \text{volume outside tubing}}\right).$$

Radioactivity was measured in a Packard model 3003 scintillation counter, using 10 ml of toluene-based scintillation fluid per sample.

Protein determinations were carried out by the method of Lowry[19], using crystalline bovine albumin as standard.

RESULTS

Undiluted plasma, previously depleted of endogenous steroids by the charcoal method, was mixed with ^{3}H-androstenediol, 30,000 dpm representing 0·3 ng of steroid, and subjected to electrophoresis in cellulose acetate strips. The distribution of radioactivity in the different fractions obtained was: prealbumin 8%; albumin 12%; alpha globulins 11%; beta globulin 21%; gamma globulin (overlapping with application point) 48%. In the absence of protein, the free steroid did not move from the starting point (Fig. 1).

In order to elucidate whether the radioactivity remaining at the origin was bound or free steroid, agar gel electrophoresis was employed to obtain migration of the gamma globulin fraction. The distribution of ^{3}H-androstenediol in whole pregnancy plasma was: albumin 12%; alpha globulins 13%; beta globulin 42%; gamma globulin 25%. A zone 1 cm cathodal from the gamma globulin area contained 14% of the radioactivity. In similar conditions, but in the absence of protein, the free steroid migrated towards the negative poles, finding the total amount applied in a broad band comprising the gamma globulin zone and an area 1 cm cathodal from the gamma globulin zone (Fig 2).

Since later experiments showed that gamma globulin did not bind androstenediol to a significant extent, it became apparent that the steroid–protein complex was partially dissociated during the electrophoretic run. Furthermore, it was

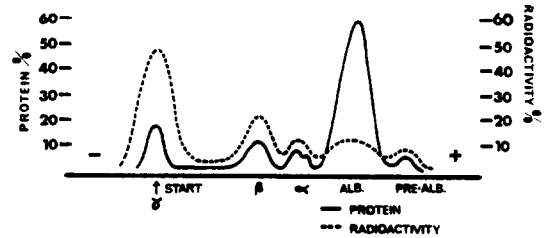

Fig. 1. Cellulose acetate electrophoresis of pregnancy plasma mixed with ^{3}H-androstenediol.

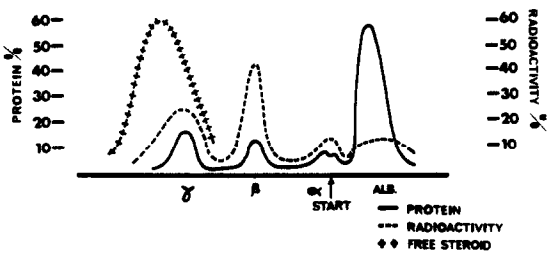

Fig. 2. Agar gel electrophoresis of pregnancy plasma mixed with ^{3}H-androstenediol. +++ represents the migration of free ^{3}H-androstenediol in the absence of protein and is due to electroendosmosis.

established that the migration of free androstenediol was due to electroendosmosis and not to the presence of a positive charge in the steroid molecule.

The percentage binding of androstenediol by diluted plasma, albumin, beta and gamma globulins, obtained by equilibrium dialysis is shown in Table 1.

The results clearly showed that at equal protein concentrations plasma had a greater affinity for androstenediol than albumin. It can also be seen that beta globulin had a marked affinity for the steroid, while gamma globulin did not bind any androstenediol.

From this data, the equilibrium constant, nk, for the complex albumin–androstenediol was estimated.

According to Scatchard[30], $Sb/Su \times 1/P = k(n - Sb/P)$, where Sb and Su are the molar concentrations of bound and unbound steroid respectively, and P the concentration of protein. k is the intrinsic association constant of a binding site and n the number of binding sites per molecule of protein. Therefore, by plotting our values for $Sb/Su \times 1/P$ (ordinate) vs. Sb/P (abscissa) we could obtain nk as the interception of the regression line on the ordinate. The value obtained was: $1{\cdot}7 \times 10^3$ l/mole.

DISCUSSION

The distribution of radioactive androstenediol in the plasma fractions obtained by electrophoresis resembled in some aspects that of testosterone. If we accept that the radioactive peak overlapping with the gamma globulin area is dissociated free steroid, then it becomes evident that the principal binding fraction is beta globulin, as is the case for testosterone[21–23].

When the data from the electrophoresis experiments is expressed as "specific activity" of each protein fraction, calculated by the ratio cpm/ug of protein, the results from the cellulose acetate runs are: albumin 2·5; alpha globulins 12 and beta globulin 21. From the agar gel runs the following specific activities were calculated: albumin 2·5; alpha globulins 15 and beta globulin 44.

The results obtained by equilibrium dialysis demonstrated the presence in plasma of a binding component with greater affinity than albumin. In each and every case, at equal protein concentrations, the percentage of steroid bound by plasma is greater than that bound by albumin. In a previous study[24] we reported the combining affinity of a partially purified globulin fraction for androstenediol (6·45–9·25 l/g) as being higher than that of whole pregnancy plasma (3·49–4·38 l/g). The present results add to the evidence that the beta globulin may be the fraction that accounts for higher affinity of plasma as compared to albumin.

Furthermore, competition experiments demonstrated that androstenediol and testosterone had similar affinities for plasma proteins, while that of 17β estradiol was slightly lower[24].

These results are in close agreement with those in the literature, recently reviewed by Murphy[25], and reinforce the concept that androstenediol is bound by the same protein moiety known to bind testosterone and estradiol, as are several other 17β-hydroxylated steroids[14, 15].

Alpha globulins bind some androstenediol. Dialysis experiments (not shown) rendered a percentage binding close to that of albumin. This binding activity is thought to be due to the presence of transcortin for which a certain affinity for some C_{19} steroids has been demonstrated[26–29].

Table 1. Percentage binding of androstenediol (Δ_5-diol) by different proteins, determined by equilibrium dialysis

	Albumin* (mg/ml)	% bound	Plasma* (mg/ml)	% bound	Beta Globulin* (mg/ml)	% bound	Gamma-globulin* (mg/ml)	% bound
(Δ_5-diol)†								
$0{\cdot}2 \times 10^{-9}$ M	16	94 ($n = 3$)‡	16	98 ($n = 6$)	—	—	—	—
	10	86 ($n = 3$)	10	96 ($n = 4$)	—	—	—	—
	1·5	72 ($n = 3$)	1·5	78 ($n = 3$)	—	—	1·5	0 ($n = 4$)
	1·0	60 ($n = 3$)	1·0	61 ($n = 3$)	1·0	81 ($n = 4$)	—	—
	0·8	54 ($n = 3$)	0·8	70 ($n = 4$)	—	—	—	—
	0·35	7 ($n = 3$)	0·35	18 ($n = 3$)	0·35	51 ($n = 4$)	0·35	0 ($n = 4$)
(Δ_5-diol)†								
$2{\cdot}0 \times 10^{-9}$ M	16	94 ($n = 4$)	16	96 ($n = 4$)	—	—	—	—
	10	86 ($n = 3$)	10	95 ($n = 4$)	—	—	—	—
	0·8	49 ($n = 3$)	0·8	67 ($n = 4$)	—	—	0·8	3 ($n = 3$)

*Protein concentrations.

†Molar concentration of androstenediol present in the medium.

‡n = Number of experiences.

The values presented in Table 1 indicate that in our *in vitro* conditions and at the molar concentrations of steroid employed more than 98% of the androstenediol is bound to plasma proteins. Although this calculation may not necessarily reflect the *in vivo* conditions, at present we do not have a better method to calculate this value.

Regarding the binding affinity of the albumin–steroid complex, we could demonstrate that androstenediol had an equilibrium constant of the order of $1{\cdot}7 \times 10^3$ l/mole, which is significantly lower than those reported for testosterone by Pearlman and Crepy [31], $4{\cdot}19 \times 10^4$ l/mole, and by Vermeulen and Verdonck [32], $2{\cdot}5 \times 10^4$ l/mole.

It is known from the work of Eberlein [11] and Vihko [12] that androstenediol is present in human plasma mostly in the form of sulfoconjugate. Yamaji [6] measured free androstenediol in the dog, and found that the concentration in spermatic vein blood rose manyfold upon adminstration of LH. Therefore, at a certain time the steroid circulates as the free compound.

We have not studied the binding of androstenediol sulfate, although it may be assumed, following Plager [33] and Wang and Bulbrook [34], that it would be bound non-specifically to albumin.

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