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LACK OF STEROID-BINDING BY "PREGNANCY ZONE" PROTEIN

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SUMMARY

The "pregnancy zone" protein (PZ) is a serum factor of unknown origin and function. The formation of this α_2-globulin is stimulated by steroids and a carrier function has been postulated by most authors. The purified PZ protein was investigated *in vitro* using a new two-phase system for equilibrium partition. The association constant for PZ to six different steroids (oestriol, oestradiol, oestrone, progesterone, testosterone, cortisol) was low and there was no evidence for steroid-binding properties.

INTRODUCTION

THE OCCURRENCE in plasma of pregnant women of an extra protein associated with the α_2-globulin fraction was first demonstrated by Smithies[1] by starch-gel electrophoresis, and the presence of this globulin, referred to as the "pregnancy-zone" protein (PZ), has been reported by several authors[2–19]. "Pregnancy Zone" occurs in increasing amounts as pregnancy progresses and disappears during the puerperium. The same protein is also found in the plasma of women taking oral contraceptives[7, 17, 20, 21] and in plasma of men treated with oestrogen for prostatic cancer[6, 22]. PZ has been obtained in a highly purified state, and has a molecular weight of 326000 and an isoelectric point of 4·7[16]. The protein contains about 10 per cent carbohydrate and is built up of two polypeptide chains.

The stimulation of the formation of this protein by steroids suggests a carrier function for oestrogen or other steroids. In fact most authors postulate that PZ has steroid-binding properties[2, 3, 8–13, 20–22]. Recently, Hofman *et al.*[23] using absorbed antipregnancy sera and radioimmunological methods were unable to show binding of [^{3}H]-oestriol and [^{3}H]-oestradiol *in vitro* for an α_2-glycoprotein, probably identical with PZ.

A new method, theoretically analogous to equilibrium dialysis but simpler and more rapid, has been developed for the determination of the binding of steroids and other ligands to plasma proteins[24]. This method is based on partition equilibrium between two aqueous phases obtained by mixing concentrated solutions of the two polymers, dextran and poly(ethylene glycol)[25]. The phase system can also be used to study the binding properties of isolated serum proteins.

The postulated binding properties of PZ were investigated *in vitro* with respect to its binding affinity for oestrone, oestriol, oestradiol, progesterone, testosterone and cortisol by means of the equilibrium partition method using purified PZ protein.

EXPERIMENTAL

PZ protein

"Pregnancy Zone" was isolated from retroplacental blood [16], and the protein content was determined according to Lowry [26]. A solution of 53·5 μg/ml PZ in 30 mM sodium phosphate buffer pH 7·4 was prepared and stored at −15°C.

Materials. [1,2-^{3}H]-Progesterone (30 Ci/mmol) and [6,7-^{3}H]-oestriol (53·1 Ci/mmol) were obtained from New England Nuclear Corp., U.S.A. [2,4,6,7-^{3}H]-Oestradiol (100 Ci/mmol), [2,4,6,7-^{3}H]-oestrone (100 Ci/mmol), [1,2-^{3}H]-testosterone (51 Ci/mmol) and [1,2-^{3}H]-cortisol (12 Ci/mmol) were supplied by The Radiochemical Centre, Amersham, England. The steroids were purified by paper chromatography in an appropriate Bush system [25]. Corrections were made for the decrease in specific activity of ^{3}H-steroids which had occurred since the date of their production.

Dextran T40 (DT40), $M_w = 4 \times 10^4$, was purchased from Pharmacia Fine Chemicals, Sweden and poly(ethylene glycol) (PEG), grade Carbowax 6000, $M_n = 6 \times 10^3$, from Union Carbide Corp., U.S.A. All other chemicals were of analytical grade and water was distilled twice in quartz equipment.

The basis of the method for the determination of binding

A detailed description of the theoretical basis for the partition equilibrium method used has been given earlier [24] and will be only briefly presented here.

When a steroid and a plasma protein with affinity for that steroid are partitioned in the two-phase system described, the lower phase contains bound and unbound steroid in equilibrium with the protein, while the upper phase contains only unbound steroid. From the concentration of the steroid in the upper phase, $[S_F]_U$, the total concentration of the steroid in the system, [S], and the partition coefficient, K_s, of the steroid determined separately in the absence of the protein, it is possible to calculate the concentration of the bound and unbound steroid in equilibrium with the protein. While K_s is a constant for a given steroid, the effective partition coefficient, $K_s' =$(steroid concentration in the upper phase/steroid concentration in the lower phase), is a function of the concentration of the protein in the system as well as of the association constant, K_a, for the binding and the number of binding sites, n. Thus,

$$K_s' = [S_F]_U/[S_L] = [S_F]_U/[S_F]_L + [S_B]_L \quad (1)$$

where $[S_F]_U$ is concentration of the unbound (free) steroid in the upper phase and $[S_L]$, $[S_F]_L$ and $[S_B]_L$ are the concentrations in the lower phase of the total, free and bound steroid respectively.

In the present case the Scatchard equation [30] can be written as

$$[S_B]_L/[S_F]_L = K_a n [P]_L/(1 + K_a[P]_L) \quad (2)$$

where $[P]_L$ is the concentration of the protein in the lower phase. From equation (2) one obtains on simplification and approximation [24]

$$[S_B]_L = K_a[P]_L[S]_L\, n/(1 + K_a[S]_L + [P]_L\, n). \quad (3)$$

Thus $[S_B]$ can be calculated corresponding to a given value of $[S]_L$, $[P]_L$, K_a and n, and from the value so obtained it is possible to calculate $[S_F]_L$, since $[S_F]_L =$

$[S]_L$–$[S_B]_L$. Knowing K_s, $[S_F]_U$ can be calculated. Subsequently using equation (1), it is possible to calculate K_s' corresponding to a given set of values of K_a, n, $[P]_L$ and [S], the total concentration of the steroid in the system. Here, K_s' has been calculated as a function of K_a at fixed values of $n(n = 1)$ and $[P]_L$.

When K_s' is determined experimentally at the same values of $[P]_L$ and [S], a comparison with the theoretical curve gives information of the amount of binding of the steroid concerned.

Experimental procedure. A phase mixture was prepared by mixing, by weight, the required quantities of 20 per cent (w/w) DT40 solution, 40 per cent (w/w) PEG solution, 2 M KSCN and 0·1 M Na_2HPO_4-KH_2PO_4 buffer (pH 7·4), so that the addition of 0·3 g PZ solution to 2·2 g of the homogenized phase mixture would yield a system with the final composition: 10 per cent (w/w) DT40, 7 per cent (w/w) PEG, 0·1 M KSCN and 0·005 M buffer. The phase mixture was cooled to 4°C, the temperature at which the partition equilibrium experiments were performed.

The partition equilibrium experiments were performed as follows: To test tubes (100 × 10 mm) was added 20000 d.p.m. ^{3}H-steroid dissolved in ethanol. The solvent was removed at 40°C under a stream of nitrogen. 0·3 g PZ solution was added to each tube and incubated for 30 min at 4°C. Phase systems were similarly prepared by adding 0·3 ml water instead of the PZ solution in order to determine K_s. After incubation, 2·2 g of the well homogenized phase mixture were added to each tube by a calibrated automatic Cornwall syringe. The test tubes were fitted with neoprene stoppers and the contents mixed by inverting the tubes 50 times. The tubes were then centrifuged for 15 min at 2000 *g* to speed up the separation of the phases. Samples, 1·0 ml from the upper phase and 0·5 ml from the lower phase, were withdrawn with a disposable tuberculin syringe to which a glass capillary had been fused, and added to scintillation vials containing 15 ml scintillation mixture (5 per cent POP, 0·3 per cent dimethyl-POPOP in toluene). The vials were vigorously shaken and left at 4°C for 10 h before counting. Radioactivity was determined by counting each sample for 10 min in a Packard Tricarb model 3310 or long enough to keep the counting error below 5 per cent. Quench corrections were made using an external standard. The counting efficiency was about 33 per cent.

RESULTS

The experimentally determined values of K_s and K_s' for the different steroids are recorded in Table 1. These correspond to a concentration of 20,000 d.p.m. labelled steroid and 16·05 μg PZ in 2·5 g phase system.

An example of the theoretical plots of $[S]_F$, the total concentration of steroid, in this case oestrone, in the lower phase, as a function of [S], the total concentration of steroid in the system, at different values of K_a is shown in Fig. 1. These plots were constructed from equation (3), using the values 0·052 μM and 2·23 for $[P]_L$ and K_s respectively. The plots for each of the values of K_a are straight lines and from the slopes one can calculate the effective partition coefficient, K_s', for oestrone at the particular value of [S] used in the partition equilibrium binding experiments. [S] was calculated from the known specific activity of the labelled steroid.

The theoretical plots of K_s' vs K_a for six different steroids are shown in Figs. 2a–2f, in which experimentally determined values of K_s' for the respective steroid,

Table 1. The partition coefficients ± S.E.M. of steroids in the two-phase system in the absence (K_s) and presence (K_s') of PZ

Steroid	K_s	K_s'
Testosterone	1·850±0·018 (n = 4)	1·783±0·016 (n = 3)
Cortisol	1·233±0·010 (n = 4)	1·253±0·011 (n = 3)
Progesterone	1·788±0·043 (n = 4)	1·817±0·034 (n = 3)
Oestrone	2·262±0·039 (n = 4)	2·233±0·003 (n = 3)
Oestradiol	2·052±0·007 (n = 4)	2·110±0·004 (n = 3)
Oestriol	0·823±0·016 (n = 3)	0·827±0·014 (n = 3)

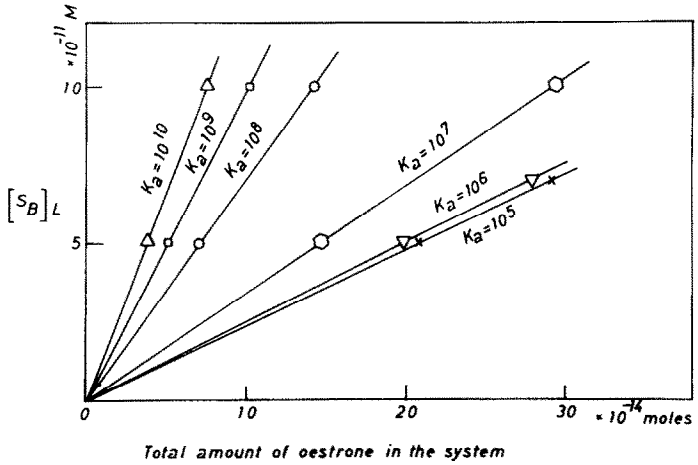

Fig. 1. Plots of the total concentration, $[S_B]_L$ (in mol/l), of oestrone in the lower phase as a function of the total quantity, [S], (in mol) of the steroid in the system, for different values of the association constant, K_a, for a steroid–protein complex. The plots were calculated from equation (3) (see text), using a value of 0·052 μM for $[P]_L$, the concentration of the binding protein in the lower phase; 2·23 for K_s, the partition coefficient of oestrone in the two-phase system in the absence of the protein, and with $n = 1$.

indicated by circles, are also included. The experimental value of K_s' differs very little from the experimental value of K_s (Table 1). The determination of K_a from the theoretical curves (Figs. 2a–2f) may involve a large error because of the position of K_s' on the curves.

DISCUSSION

All the steroids studied in this work are known to bind non-specifically to human serum albumin with an association constant of approximately 10^4–10^5 M^{-1}. A protein with a specific binding function for a particular steroid would bind that steroid with K_a of the order of 10^6 M^{-1} or higher. Thus transcortin and testosterone–oestradiol-binding globulin for example specifically bind cortisol and testosterone respectively with K_a approximately 10^8. It is evident from plots of K_s' vs K_a that for $K_a > 10^5$ there will be a measurable difference between K_s' and K_s. From Table 1 it is seen that values of K_s' for each of the steroids studied are,

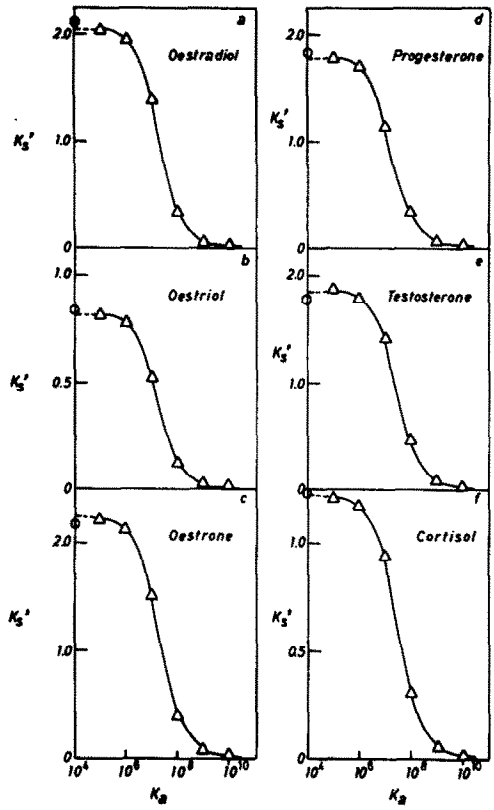

Fig. 2. Theoretical curves representing the variation of K_s', the apparent partition coefficient of steroids in a two-phase system containing a binding protein, as a function of K_a, the association constant for the binding. Curves were calculated from the plots of the type shown in Fig. 1. The plots represent: a. oestradiol; b. oestriol; c. oestrone; d. progesterone; e. testosterone; and f. cortisol.

within the limits of experimental error, equal to the respective value of K_s (the partition coefficient in the absence of PZ). Hence there is no evidence for specific binding of these steroids to the pregnancy zone α_2-globulin.

The techniques commonly used to identify a steroid carrier in a mixture of proteins are usually based on electrophoresis or gel filtration, which may give rise to artefacts due to dissociation of the steroid-protein complex. Use of the isolated PZ protein *in vitro* in a two phase system permits quantitative determination of binding capacity. The association constant for PZ for six different steroids (oestriol, oestradiol, oestrone, progesterone, testosterone, cortisol) is very low.

It could be argued that a delicate function like steroid binding could have been disturbed during the purification procedure or by freezing the protein or by the polymers in the phase system. However, throughout the isolation procedure [16] mild techniques were used. The pH varied only between 7·4–8·1, and the salt concentration did not exceed 0·3 M NaCl. No techniques involving precipitation with organic solvents were used. Also properties of PZ like antigenicity, electrophoretic mobility and immunoprecipitation reactions, are unaffected by repeated freezings and thawings [20]. Hofman *et al.*[23] obtained similar results for

[^{3}H]-oestriol and [^{3}H]-oestradiol using radioimmunodiffusion and radioimmune electrophoresis. This supports the conclusion that, although being steroid 'inducible', PZ is not a steroid carrier.

The organ of origin and the biological role of PZ is still unknown. It is characterized by Bohn[27] as an 'acute phase' reactant. However, another α_2-globulin, the C-reactive protein (CRP), a well known "acute phase" reactant, is generally seen in cases of spontaneous abortions while PZ is usually absent[19]. Furthermore CRP is produced by the liver[28, 29] but preliminary results indicate that PZ is not. The possibility that PZ is involved in the complex immunological reactions during pregnancy seems more probable.

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REFERENCES

1. Smithies O.: *Adv. Protein Chem.* **14** (1959) 65.
2. Afonso J. F. and Farnham N. G.: *Am. J. Obstet. Gynec.* **84** (1962) 199.
3. Afonso J. F. and de Alvarez R. R.: *Am. J. Obstet. Gynec.* **86** (1963) 815.
4. Afonso J. F. and de Alvarez R. R.: *Am. J. Obstet. Gynec.* **89** (1964) 204.
5. Beaton G. H., Arroyave G. and Flores M.: *Am. J. clin. Nutr.* **14** (1964) 269.
6. Cooper D. W.: *Nature* **200** (1963) 892.
7. Margolis J. and Kenrick K. G.: *Aust. J. exp. Biol. med. Sci.* **47** (1969) 637.
8. Studd J. W., Blainey J. D. and Bailey D. E.: *J. Obstet. Gynaec. Br. Commonw.* **77** (1970) 42.
9. Bohn H.: *Arch. Gynaek.* **210** (1971) 440.
10. Bayer H. and Kadach D.: *Zentbl. Gynäk.* **92** (1970) 1201.
11. Bayer H. and Kadach D.: *Zentbl. Gynäk.* **92** (1970) 1264.
12. Kadach D.: *Zentbl. Gynaek.* **92** (1970) 1321.
13. Wilken H.: *Arch. Gynaek.* **199** (1963) 243.
14. Hofman R., Straube W., Klaush B., Friemel H. and Günther J.: *Arch. Gynaek.* **212** (1972) 246.
15. Beckman L., Beckman G. and Stigbrand T.: *Hum. Hered.* **20** (1970) 530.
16. von Schoultz B. and Stigbrand T.; *Acta obstet. gynec. scand.* **52** (1973) 51.
17. Beckman L., von Schoultz B. and Stigbrand T.: *Acta obstet. gynec. scand.* **52** (1973) 157.
18. Beckman G., von Schoultz B. and Stigbrand T.: *Acta obstet. gynec. scand.* (1973) in press.
19. Beckman G., Beckman L., Magnusson S. S. and von Schoultz B.: *Acta obstet. gynec. scand.* (1973) in press.
20. Afonso J. F. and de Alvarez R. R.: *Penn. med. J.* **70** (1967) 43.
21. Beckman L., von Schoultz B. and Stigbrand T.: *Acta obstet. gynec. scand.* **50** (1971) 369.
22. Beckman L., von Schoultz B. and Stigbrand T.: *Urol. Res.* **1** (1973) 67.
23. Hofman R., Schulze H.-A., Straube W. and Klausch B.: *Arch. Gynaek.* **213** (1972) 86.
24. Shanbhag V. P., Södergård R., Carstensen H. and Albertsson P.-Å.: *J. steroid Biochem.* **4** (1973) 545.
25. Albertsson P.-Å.: *Partition of Cell Particles and Macromolecules.* 2nd Edition. Almquist & Wiksell. Stockholm. Wiley, New York (1971).
26. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265.
27. Bohn H.: *Arch. Gynaek.* **213** (1972) 54.
28. Hurliman J., Thorbecke E. J. and Hochwald G. M.: *J. exp. Med.* **123** (1966) 365.
29. Conell E. B. and Conell J. T.: *Am. J. Obstet. Gynec.* **110** (1971) 633.
30. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660.