ANTIBODY COMPETITION FOR PLASMA PROTEIN-BOUND ESTRIOL

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1. Introduction

Plasma unconjugated estriol (E_3) is now a more generally accepted indicator of feto-placental function than urinary E_3-metabolite excretion [1,2]. Of the numerous methods which have been used to measure estrogens only radioreceptor assay (RRA) and especially radioimmune assay (RIA) easily quantitate estriol. Most current methods utilize ether extracts of non-polar plasma estrogens. Assays performed with non-specific antisera or steroid binding plasma protein (SBP) require chromatography of plasma extracts to separate estriol (E_3) from estradiol (E_2) and estrone (E_1) [2,3].

We have described specific azoestriol antiserum for direct plasma E_3 assay which reliably measures E_3 even in the presence of E_1, E_2 and E_3 metabolites [4,5]. It had previously been thought that direct plasma E_3 assay might not accurately quantitate serum E_3 because most plasma estriol is protein bound [6,10]. The present report describes quantitative experiments in which high affinity specific estriol antibody overwhelms binding of E_3 by non-specific serum proteins, notably steroid binding globulin (SBG).

2. Materials and methods

Materials were obtained from the following sources: E_3, E_2 and testosterone (T) (Calbiochem. Corp.): tritiated estriol ([^{3}H]E_3) (spec. act. = 53 Ci/mM), tritiated testosterone ([^{3}H]T) (spec. act. = 25 Ci/mM) and Aquasol (New England Nuclear); DEAE Sephadex (Pharmacia); Biogel Al. 5 (Bio-Rad Corporation); and human serum albumin (Sigma).

2.1. *Pregnancy sera*

Sera obtained from 36–40 week pregnant women were pooled and dialysed against daily changes of 6 liters 0.01 M sodium phosphate, 0.85% NaCl pH 7.4 (PBS) for 2 weeks at 4°C. Previous work has shown that dialysis for 48 h is sufficient to remove endogenous steroids from plasma [7].

2.2. *Antibodies (IgG)*

The antiserum used in these experiments has been described elsewhere [5]. IgG was prepared from serum obtained from a simple bleeding by $(NH_4)_2SO_4$ precipitation followed by DEAE Sephadex chromatography [8].

2.3. *Chromatography*

Sephadex G-200 (fine) and Biogel Al. 5 were defined with H_2O, equilibrated with PBS and deaerated under vacuum. Column (45 × 1.5 cm) were prepared with each gel and equilibrated with 500 ml PBS. The columns were operated at 8–20 cm pressure using a large diameter 2 liter glass reservoir to insure minimal variation in flow during each run. A 0.2 ml/min flow rate was maintained by slight changes in reservoir height as necessary, and 0.5 ml fractions were collected in graduated conical polystyrene test tubes (Curtin Scientific) with the aid of a Technicon time/flow fraction collector. Aliquots (0.1 ml) were saved for radioactivity measurements and the rest used for optical density (O. D.) determinations. Calibration for molecular weight of eluent fraction utilized the elution profiles of Rabbit IgM and IgG, and human serum albumin.

400 pg of [^{3}H]E_3 was incubated with 0.5 ml preg-

nancy serum (PS) for 12 h at 4°C and subsequently chromatographed to measure [^{3}H]E_3 binding to serum proteins. For antibody competition experiments, 0.1 ml of antiestriol IgG (10 mg/ml PBS) was mixed with [^{3}H]E_3 + PS, incubated as above, and chromatographed after 0 to 60 min at room temperature.

Antibody IgG was also incubated with [^{3}H]E_3 in the absence of serum and chromatographed in identical fashion to measure antibody binding to estriol.

2.4. *Equilibrium dialysis*

0.5 ml PS (undiluted or diluted 1/5) was placed in cellulose sausage casing (VWR Scientific, average pore diameter 24 Å) and dialyzed against 3.0 ml of PBS or 1% HSA/PBS in 5.0 ml disposable glass culture tubes (Curtin Scientific) sealed with parafilm. To each tube a given amount of tritiated steroid (350–400 pg) was added and the incubation was continued for 72 h at 4°C. Varying amounts of unlabeled steroids (0–100 ng) were also added to the tubes in inhibition experiments. Duplicate results were averaged. No absorption of label occurred to glass or casing and recovery of radioactivity was 90–100%.

Antibody competition for PS bound [^{3}H]E_3 was next studied by placing dialysis tubing containing 0.5 ml undiluted PS and 360 pg [^{3}H]E_3 into 2.9 ml PBS to which were added various dilutions of antisera (0.1 ml). Binding of [^{3}H]E_3 to antibody was determined by precipitation with 50% $(NH_4)_2SO_4$ [5].

2.5. *Instrumentation*

Sample aliquots (0.1 ml) in 10 ml Aquasol were counted for ^{3}H radioactivity using a Nuclear Chicago Mark II Liquid Scintillation counter. O. D. was measured with a Zeiss spectrophotometer using matched quartz cells.

3. Results

3.1. *SBG affinity and capacity*

The affinity and capacity of the SBG in PS which potentially competes with antibody for E_3 were determined by equilibrium dialysis. Dialysis was performed against 1% albumin/PBS and binding was studied by addition of from 0 to 100 ng unlabeled steroid to tubes containing 0.5 ml PS (diluted 1/5) and 360 pg of the corresponding tritiated steroid in cellulose casing. Correction was made for binding to albumin and Scatchard plots constructed according to the method of Vermeulen and Verdan [9] (fig.1).

Testosterone more accurately delineates SBG binding parameters because of its higher affinity for the receptor and hence its binding data was used to estimate more precisely the binding capacity of SBG. The association constants (K_a) for SBG binding of E_3 and T (fig.1) were $K_aT = 9 \times 10^9$ M^{-1} and $K_aE_3 = 4 \times 10^7$ M^{-1}. K_aT is in agreement with values from other laboratories [7,9]. However, K_aE_3 as determined from equilibrium dialysis experiments is not entirely reliable. Weaker binding of E_3 by SBG yields Scatchard plots whose intercepts on the ordinate and abscissa are not as accurately determined as those of testosterone. SBG bound 8.5×10^{-9} M testosterone (12.5 ng steroid/ml PS), which is in the range reported for PS [9,10]. Thus there is sufficient concentration of SBG binding sites in PS to compete with antibody for the 400 pg of [^{3}H]E_3 in experiments described below.

3.2. *Antibody competition for SBG bound estriol*

A typical elution profile of 0.5 ml PS incubated for 12 h with 400 pg [^{3}H]E_3 is shown in fig.2. In all runs, 94–97% total radioactivity was recovered. As previously shown, SBG bound radioactivity elutes with the albumin fraction due to their similar mol. wt and physical characteristics [7,9]. Although 0.5 ml

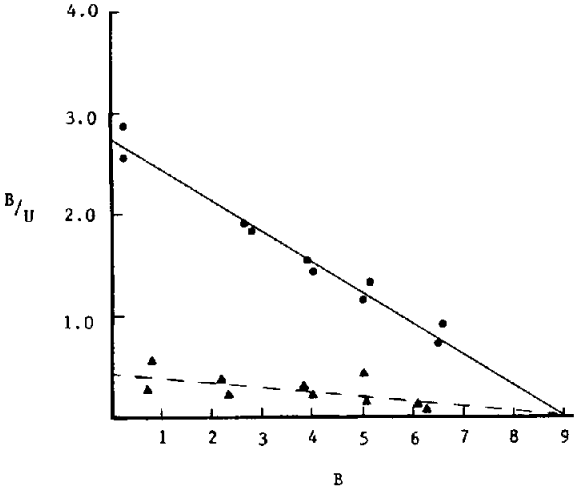

Fig.1. Scatchard plots of steroid binding to PS. Plasma (1 ml) diluted 1:5 was dialysed against 1% albumin (3 ml) testosterone (● - ●), or estriol (▲ - ▲).

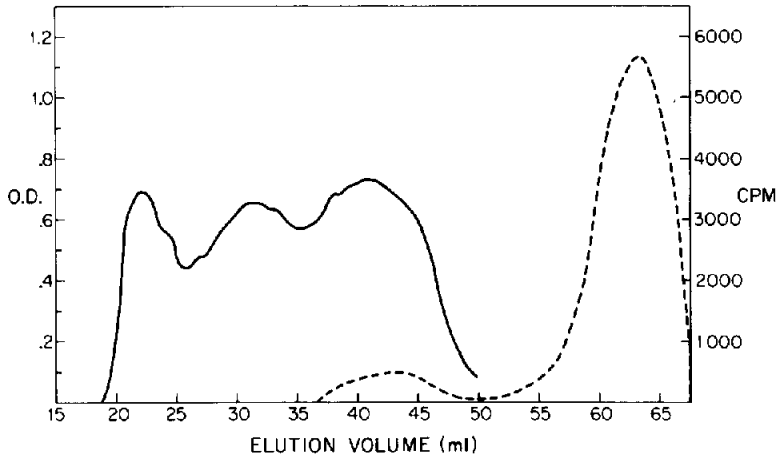

Fig.2. Sephadex G-200 separation of 0.5 ml pregnancy serum (PS) incubated 12 hours with 400 pg $^3HE_3$. (————) optical density (OD). (----------) Counts per minute/0.1 ml sample (CPM). 10% of total counts elutes in the mol. wt range of albumin while 86% is recovered unbound (see table 1).

PS bound only 10% of [^{3}H]E_3 after chromatography, 41.5% of [^{3}H]E_3 was bound in equilibrium dialysis experiments (table 1). This apparent discrepancy results from the more rapid disassociation during chromatography of that portion of serum estriol weakly bound to albumin, as compared to the stability of SBG-E_3 complexes under similar conditions [7].

Antibody permitted to directly compete for SBG-bound [^{3}H]E_3 (fig.3) effectively stripped protein-bound estriol during a 60 min incubation (table 1). Similar data for 0 incubation time showed that antiboby did not remove all SBG-bound estriol upon mixing, although dimunition in 'receptor'-bound [^{3}H]E_3 was significant. In addition, when [^{3}H]E_3 and PS were incubated for 60 min with antibody, a portion of the added radioactivity (1%) was eluted earlier than either IgG or IgM (fig.4).

Indirect competition of estriol antibody with SBG for

Table 1
Antibody competition for protein-bound estriol

Binding study	[^{3}H]E_3 bound[a] Antibody	Serum protein	% $^3HE_3$ free[a]
Equilibrium dialysis:			
Pregnancy serum	–	41.5	56
Antibody	85	–	14
Chromatography:			
Pregnancy serum	–	10	86
Antibody	84	–	15
Antibody + Pregnancy serum	82	<1[b]	14

[a] Binding in chromatography studies was determined by counting pooled samples from graphically assigned peaks in fig.1 and 2, and a similar figure for antibody binding [^{3}H]E_3.

[b] No measurable difference from the elution profile of antibody and [^{3}H]E_3 is detectable in this region.

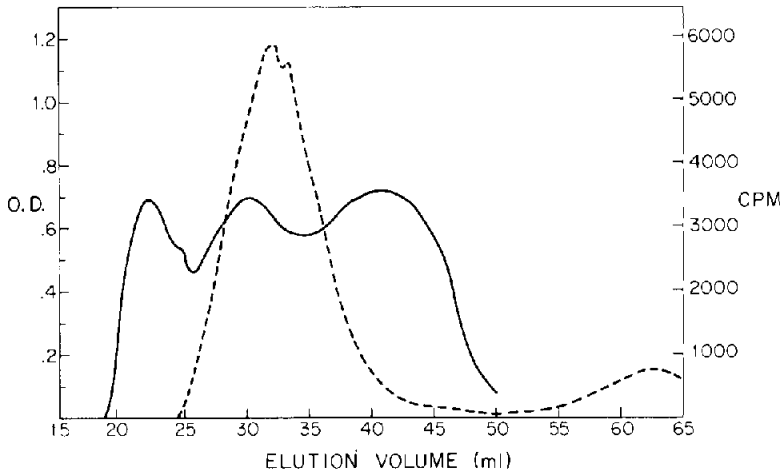

Fig.3. Antibody competition for serum protein-bound estriol (Sephadex G-200). 0.1 ml antiestriol IgG (1% solution) added to 0.5 ml PS preincubated with 400 pg [^{3}H]E_3 and applied to Sephadex G-200 after 1 h. (————) OD. (------) = Cpm. 84% of the [^{3}H]E_3 added is recovered in the IgG cut. Counts in the albumin peak closely approximate those observed with antibody and [^{3}H]E_3 alone.

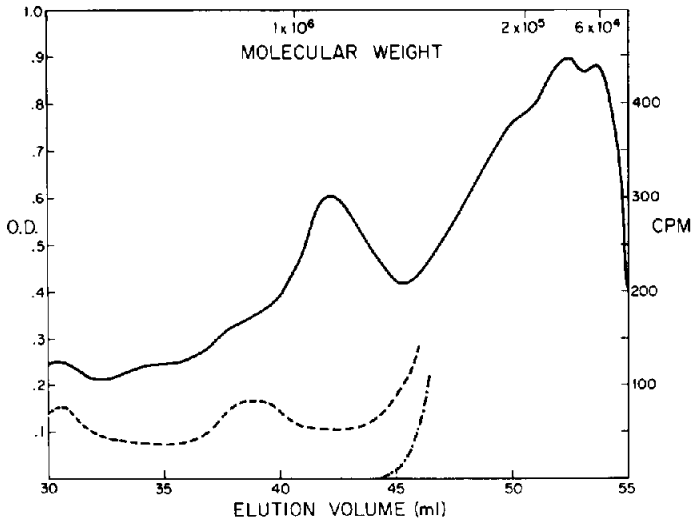

Fig.4. Biogel Al. 5 chromatography of anti-estriol IgG mixed with preincubated [^{3}H]E_3 + PS (————) OD. (--------) = Cpm antibody + [^{3}H]E_3 + PS. (– . – . –) Cpm antibody + [^{3}H]E_3. Radioactivity appears early (> 1 × 10^6 mol. wt) only when incubated with serum containing antibody and SBG-[^{3}H]E_3.

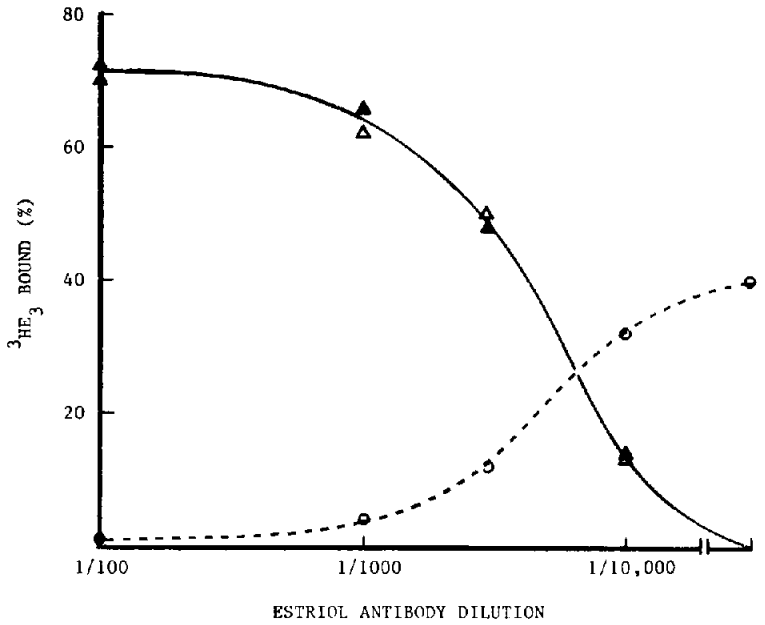

Fig.5. Antibody competition for serum protein bound estriol (equilibrium dialysis). 360 pg [^{3}H]E_3 was mixed with 0.5 ml undiluted PS or 0.5 ml PBS and dialysed against 0.1 ml E_3 antiserum (above dilutions) in 2.9 ml PBS. AB binds [^{3}H]E_3 (▲ - ▲) at the expense of PS (○ - ○) and in the same amount as when dialyzed against buffer (△ - △). Antibody preferentially strips E_3 from PS.

[^{3}H]E_3 was studied by equilibrium dialysis experiments in which IgG was separated from PS or PBS by a semipermiable membrane (fig.5). Various dilutions of antiserum bound identical [^{3}H]E_3 whether dialysed against PS or PBS. PS however bound 40% of label in the absence of antibody but bound successively less [^{3}H]E_3 in competition with increasing antibody concentration. High affinity azoestriol IgG thus preferentially bound estriol at the expense of albumin and SBG.

4. Discussion

Human serum contains several glycoproteins of mol. wt 52 000 [7,10] which bind small mol. wt non-polypeptide hormones with moderate affinity [11,12]. The increase of such 'receptor' proteins in certain hyperestrogenic states e.g. pregnancy [13] suggests their importance as hormone buffers for regulation of 'active' (unbound) hormone levels [14]. Albumin also binds these hormones but with demonstrably less affinity. These serum 'receptors' are relatively non-specific, binding molecules which vary widely in structure and biological activity. Corticosterone-binding globulin (CBG) binds cortisol, corticosterone and progesterone [15]. Thyroxine-binding globulin has strong affinity with several organic dyes in addition to thyroxine and triiodothyronine [18]. Likewise, in contradistinction to fixed tissue receptors, SBG reacts with several different steroids possessing 17β hydroxyl groups and coplanar A and B rings [17]. Other functional groups, which serve as determinants of the immune response to steroids [18] are of vastly less importance as evidenced by the relatively high association of SBG for both testosterone and 17β-estradiol [19]. The semispecific nature of serum binding proteins contrasts markedly with the exquisite specificity of the immune response [18].

Potentially an interesting way to dissociate estriol-SBG might be the addition of testosterone to assay solutions since the two steroids are bound competitively by SBG and the K_a is much higher for the latter, in marked contrast to E_3-specific antibody [8]. 8-Anilino

sulphonic acid serves an analogous role in displacing protein-bound thyroxine for serum RIA [20]. Similar considerations would influence crucial experiments with biologically active estradiol (E_2) since more avidly bound testosterone also displaces E_2 from their shared serum 'receptor', SBG. Such experiments have been performed (JDG, unpublished, and [21]). It would seem reasonable that substrate functional groups concerned with SBG binding do not include all haptenic determinants required for antibody recognition.

The observation of high mol. wt complexes (fig.4) is in harmony with earlier observations (SJG, unpublished) of rapidly formed precipitin bands when pregnancy urine samples are layed on appropriate estriol/estradiol antisera. Whether these complexes represent binding of estriol antibody to SBG-E_3 is not determined. Ultracentrifuge studies utilizing purified SBG, ED, and specific antiestradiol antibody are now in progress.

The current work demonstrates that a specific, high affinity antibody competes effectively with less specific, lower affinity serum protein(s) for binding of E_3 substrate. SBG and other serum binding proteins rapidly disassociate from E_3 during gel chromatography (table 1) thus giving further evidence to the buffering nature of serum binding proteins. Antibodies [22] and tissue receptors [23] however, characteristically have low back molecular rate constants, thus accounting for their high K_a values for ligands. Therefore specific anti-estriol IgG maintains [^{3}H]E_3 binding against the concentration gradient effected by chromatography (table 1). Equilibrium dialysis and chromatography experiments show that high affinity estriol antibody efficiently strips PS proteins of E_3. If serum binding proteins exist for solubility and transport of these relatively insoluble hormones, it would therefore seem reasonable that the latter be released to antibody or tissue receptor possessing higher inherent affinity.

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