A Specific Progesterone-binding Component of Human Breast Cyst Fluid*

William H. Pearlman, Jean L. Guériguian, and Mary E. Sawyer

SUMMARY

The steroid hormone-binding properties of human breast cyst fluid were investigated and compared to those of female serum. Breast cyst fluid exhibited a high binding affinity for progesterone and pregnenolone, but very little affinity for cortisol, 17α-hydroxyprogesterone, and deoxycorticosterone. Serum exhibited a high binding affinity for progesterone, and also for cortisol, 17α-hydroxyprogesterone, and deoxycorticosterone; but only little affinity for pregnenolone. It thus appears that the progesterone-binding component (or components) of breast cyst fluid is unique, since its steroid-binding properties are distinctly different from those of the progesterone-binding component of human serum, i.e. corticosteroid-binding globulin. Breast cyst fluid, in contrast to female serum, exhibited a low binding affinity for 17β-estradiol and testosterone.

Column chromatography of breast cyst fluid on hydroxyapatite and DEAE-cellulose afforded considerable purification of the steroid-binding component (or components). Although the progesterone- and pregnenolone-binding component (or components) were eluted simultaneously from the chromatographic columns, similarity of the antigens is not presumed. According to a gel filtration study, the Stokes radius of the steroid-binding component (or components) appears to be about 38 Å, corresponding to a molecular weight of about 72,000 for a globular protein. Of more general interest are the other proteins in breast cyst fluid which are present in high concentration, at least one-fourth of that of serum; the proteins of cyst fluid do not, however, resemble those of serum on polyacrylamide gel electrophoresis.

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There seems to be a paucity of information regarding the precise nature and origin of the proteins in human breast cyst fluid although the protein concentration in cyst fluid is appreciable (as shown in the present study) and cystic disease is a common benign lesion of the human breast (1). Cyst formation and fluid accumulation appear to be an expression of abnormal ovarian function, according to the discussion of Haagensen (1). Whether this is an excess of estrogen, as has been suggested on the basis of some clinical observations, has not been clearly established; in early studies of the effects of estrogen on mice, it became evident that estrogen not only stimulates the mammary epithelium to proliferate, but that it also causes dilatation of the mammary ducts and cyst formation (cited in Ref. 1). Although the relationship of cystic disease to breast carcinoma in women is debatable, a common etiological factor and hormonal milieu may exist. To gain a better understanding of the biochemical mechanisms underlying hormonal influences in the normal and abnormal development of the breast, the steroid hormone-binding properties of human breast cyst fluid were investigated and compared to those of peripheral blood serum (2–4). The results of an exploratory survey are now presented.

EXPERIMENTAL PROCEDURES

Materials

Clinical Material—Breast cyst fluid was aspirated from women with cystic disease at the North Carolina Memorial Hospital in Chapel Hill. Fresh specimens were immediately chilled to 5°C and assayed for steroid binding within 2 days, when convenient. Occasionally, cyst fluid specimens were frozen (and labeled f) as noted in the text) and stored at −15°C: on thawing, there was no appreciable change in steroid-binding activity. Peripheral blood serum was obtained from nonpregnant women with gynecological complications at the North Carolina Memorial Hospital. The specimens were stored at 5°C and assayed for steroid binding within a few days.

Radioactive Steroids—The labeled steroids and the respective specific activities are listed: [1α,2α-3H]progesterone, 53 Ci per mmole; [1,2-3H]cortisol, 42 Ci per mmole; [7α-3H]pregnenolone, 18 Ci per mmole; 17α-hydroxy[7α-3H]progesterone, 10.2 Ci per mmole; [1α,2α-3H]deoxycorticosterone, 58 Ci per mmole; [1,2,4-3H]testosterone, 45 Ci per mmole; and 17β-[6,7-3H]estradiol, 40 Ci per mmole. The last two steroids were obtained from New England Nuclear; and the other steroids, from Amersham-Searle. The precise value for the specific activity was noted in each instance so that the amount (moles) of radioactive steroid added to the assay system would satisfy standard conditions of assay.

1 Trivial names (not included in the listing by IUPAC) for the following steroids are defined: pregnenolone, 3β-hydroxy-5-en-20-one; 17α-hydroxyprogesterone, 17α-hydroxyprog-4-en-3,20-dione.
as defined below. Radiochemical purity of labeled progesterone and cortisol was confirmed on occasion by diluting the radioactive steroid with the corresponding radioinert steroid and determining the specific activity before and after thin layer chromatography on Silica Gel G (Brinkman); a radiochromatogram scanner (Packard model 7201) was useful in locating the major radioactive area for elution. The solvent systems for chromatography of progesterone and cortisol were benzene-ethanol (v/v), 90:10 and 155:45, respectively, and are comparable to Solvent Systems XI and X, respectively, used by Lisboa (5). The radiochemical purity of labeled pregnenolone was confirmed by addition of radioinert pregnenolone and recrystallization from ethanol to constant specific activity. Other labeled steroids were obtained in fresh batches and used without further purification.

Other Materials—Sephadex G-25 (fine) and G-100 (superfine), and Blue Dextran 2000 were obtained from Pharmacia; bovine serum albumin (crystalline) from Penetex; α-chymotrypsin (three times crystallized, type II) and cytochrome c (type VI) from Sigma; Norit-A from Fisher Scientific; hydroxylapatite (Hypapatie C) from Clarkson Chemical Co., Williamsport, Pa.; and DEAE-cellulose (Whatman DE-52) from II. Reeve Angel.

Protein Determination
The protein concentration in breast cyst fluid and serum was determined before and after treatment with Norit-A, by the method of Lowry et al. (6) employing bovine serum albumin as a standard protein.

Tritium Determination
Aqueous solutions of tritium labeled steroids were counted by liquid scintillation in a dioctane-xylene-ethanol solution of phosphor as previously described (2); nonaqueous radioactive samples were similarly counted but in a toluene-phosphor.

Measurement of Steroid Binding, $S_b$, $S_a$
The method for measuring steroid binding by batchwise use of Sephadex G-25 was essentially that previously described (2) for measuring testosterone binding in serum. The technique is comparable in principle to that of equilibrium dialysis. Protein, $P$, and steroid bound to protein, $S_b$, is excluded by the gel (200 mg of Sephadex G-25 in a final volume of 2 ml of 0.155 M phosphate buffer, pH 7.4) at 3°; unbound steroid, $S_a$, partitions freely between the external phase ($V_0 = 1.5$ ml) and the internal gel phase ($v_i = 0.5$ ml). About 0.13 ng (instead of 1 ng) of radioactive steroid was added to each Sephadex assay tube. Certain advantages are gained by reducing thus the amount (mass) of radioactive steroid in the assay system and by lowering the temperature. The assay for specific steroid binding is thereby rendered more sensitive, i.e. the $S_b:S_a$ value is increased, whereas the $S_b:S_a$ value for nonspecific binding appears to be largely unaffected, remaining very low. The steroid is to some extent reversibly adsorbed to the gel matrix. Adsorption is taken into account when routinely determining the partition factor, $K'$, i.e. the ratio of the total amount of steroid in the phase external to the gel to that in the internal gel phase in the absence of protein. Mean values ± S.D. (n = number of assays) for $K'$ were 1.11 ± 0.03 (n = 10) for progesterone; 1.66 ± 0.06 (n = 10), cortisol; 0.97 ± 0.10 (n = 10), pregnenolone; 1.21 ± 0.08 (n = 4), deoxycortisol; 1.20 ± 0.02 (n = 4), 17α-hydroxyprogesterone; 1.16 ± 0.05 (n = 6), testosterone; and 0.58 ± 0.08 (n = 5), 17β estradiol. The theoretical value for $K'$ would be 3 if adsorption were nil since $k_rV_i - 3$.

The apparent value for $S_b:S_a$ will be reduced if competing endogenous steroid is present in the assay system, i.e. radioinert (endogenous) steroid may compete with the radioactive steroid for binding to the same site. To obviate this complication, specimens of breast cyst fluid (and also peripheral blood serum) were routinely treated with Norit-A in a manner similar to that described by Heyns et al. (7) for removing endogenous steroid from blood plasma. About 0.7 mg of powdered Norit-A were added per mg of protein in solution; the suspension, after gentle shaking for 30 min at 3° was centrifuged at 105,000 × g for 30 min. The clear supernatant was filtered through a tiny plug of glass wool and fat-free cotton in a transfer (Pasteur) pipette. The protein concentration of the filtrate was determined; the assay for steroid binding was then performed.

Some pigment is present in breast cyst fluid, the amount is usually reduced on treatment of the specimen with Norit-A, see above. The pigment (light yellow, or sometimes greenish green) is not visible in the assay system because the cyst fluid is highly diluted, 200-fold or more, in the Sephadex assay tube; it does not affect the assay. The pigment may be hemoglobin, according to the discussion of Haagen (1).

Estimation of Steroid-binding Activity as $1/P$ Value
A fixed amount (∼0.15 ng) of radioactive steroid and variable amounts of protein were added to the assay system at 3°. The ratio of unbound to bound radioactivity, $S_b:S_a$, is plotted against the reciprocal of the total protein concentration (liters per g), $1/P$, in the phase external to the gel; typical plots are shown in Figs. 1 and 2. Steroid-binding activity is defined as the $1/P$ value at 50% steroid binding (i.e. $S_b:S_a = 1$) and when $S_b = 1$ × 10−10 M, i.e. under standard conditions. The $1/P$ value is the total volume (liters) of external phase ($v_i$) which would be furnished by 1 g of total protein under standard conditions of assay. Thus, the method for estimating $1/P$ is essentially the same as that originally described (2) for estimating the testosterone-binding activity of serum at 25°.

Scanning Procedure for $S_b:S_a$
A rapid scanning procedure was used, as previously described (8), for estimating the relative level of steroid binding in the effluent from chromatographic columns. A constant aliquot volume (e.g. 0.1 ml) from individual chromatographic fractions was added to Sephadex assay tubes in duplicate and the mean value for $S_b:S_a$ determined.

Electrophoresis
Polyacrylamide gel electrophoresis was carried out in an Ortec apparatus (rectangular vertical cells) as described in the Ortec operating manual. A discontinuous buffer ion system was used. The buffer in the tank was Tris-borate, pH 9.0; and that in the separating gel, Tris-sulfate, pH 9.0. The zone-sharpening procedure is based on the principle described by Hjertén et al. (9). SDS-gel electrophoresis was performed with the same apparatus; the procedure was that described by Weber and Osborn (10), who used vertical tubes, however.

Column Chromatography
Chromatography of breast cyst fluid on columns of hydroxylapatite, DEAE-cellulose, and Sephadex G-100 (superfine) was performed in a manner similar to that previously described (8) but in connection with serum.

The abbreviation used is: SDS, sodium dodecyl sulfate.
TABLE I
Comparison of progestosterone- and cortisol-binding activity of breast cyst fluid and peripheral blood serum

<table>
<thead>
<tr>
<th>Tissue specimen No. (patient)</th>
<th>Protein concentration</th>
<th>Cyst fluid</th>
<th>Serum</th>
<th>Cyst fluid</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyst fluid specimen No.</td>
<td>Protein conc. mg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (VM)</td>
<td>73 (MMG)</td>
<td>24</td>
<td>77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (MB)</td>
<td>74 (CPT)</td>
<td>23</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (VM)</td>
<td>75 (GCS)</td>
<td>25</td>
<td>82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (MV)</td>
<td>70 (TMH)</td>
<td>24</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 (DDM)</td>
<td>80 (HBM)</td>
<td>21</td>
<td>81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 (NJ)</td>
<td>82 (EOW)</td>
<td>28</td>
<td>81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 (LSB)</td>
<td>83 (VL)</td>
<td>32</td>
<td>77</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td><strong>95</strong></td>
<td><strong>80</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S.E. (μ)</strong></td>
<td></td>
<td>± 1.1 (7)</td>
<td>± 0.8 (7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Serum was obtained from nonpregnant women with gynecological complications.
* The binding activity was too low to allow estimation at 50% steroid binding, i.e. when \( S_r/S_s = 1 \); the binding activity is expressed in this instance as \( (S_r/S_s)(1/P) \), corresponding to a single value for \( P \).

TABLE II
Binding of related C_11 steroids to protein of breast cyst fluid and pregnancy serum: comparative survey

<table>
<thead>
<tr>
<th>Steroid tested for binding</th>
<th>Steroid binding activity, 1/P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyst fluid specimen No.</td>
</tr>
<tr>
<td></td>
<td>of 12f specimen No. 15 16 167 168 + 169</td>
</tr>
<tr>
<td>Progesterone</td>
<td>10.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>~1.3</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>1.1</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>0.3</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>21.4</td>
</tr>
</tbody>
</table>

* The protein concentration was 27, 21, 28, and 32 mg per ml for cyst fluid specimens, Nos. 8f, 12f, 15, and 16, respectively; and similarly, 27 and 4 mg per ml for serum specimen Nos. 15 and 16, respectively. When \( y = 1 \) (i.e., at 50% steroid binding), \( 1/P = 15.1 \) liters per g of protein in the above assay.

RESULTS

According to Table I, breast cyst fluid exhibits considerable progesterone-binding activity (1/P) when compared to peripheral blood serum: the mean 1/P values were 14.9 and 9.5 liters per g of total protein, respectively. But unlike serum, breast cyst fluid exhibits little or no cortisol-binding activity, see Table I. The progesterone-binding activity of human serum may be attributed to corticosteroid-binding globulin since it strongly binds progesterone as well as cortisol (3, 4). It would thus appear that a unique progesterone-binding component is present in breast cyst fluid. Moreover, this component appears to have a high degree of steroid ligand specificity: cyst fluid exhibits very little binding activity with respect to certain other related C_11 steroids, notably 17α-hydroxyprogesterone and deoxycorticosterone, whereas serum shows high binding activity (Table II) in this respect, due to the presence of corticosteroid-binding globulin (11). On the other hand, the pregnenolone-binding activity of cyst fluid is high, whereas that of serum is low, see Table II. This suggests that the progesterone-binding component of cyst fluid may also bind pregnenolone, but this remains to be established; it is noteworthy that serum corticosteroid-binding globulin does not bind pregnenolone (11). The steroid
Fig. 2. Estimation of pregnenolone-binding activity, $1/P$, in breast cyst fluid (Specimen No. 8). The procedure was similar to that described above (see Fig. 1).

![Graph showing the regression equation $y = 0.022x - 0.115$ with a coefficient of determination $R^2 = 0.014$. The x-axis represents $1/P$ in liters of standard steroid binding per gram of protein, and the y-axis represents the fraction of unbound steroid. The graph shows a linear relationship with a slope of 0.022 and an intercept of -0.115.](http://www.jbc.org/)

![Graph showing the estimation of pregnenolone-binding activity, $1/P$, in breast cyst fluid. The x-axis represents $1/P$ in liters of standard steroid binding per gram of protein, and the y-axis represents the fraction of unbound steroid. The graph shows a linear relationship with a slope of 0.022 and an intercept of -0.115.](http://www.jbc.org/)

Fig. 3. Hydroxyapatite chromatography of breast cyst fluid at 3°C. The Norit-treated cyst fluid (2.0 ml containing 40 mg of protein), Specimen No. 17, was dialyzed against 0.0175 M sodium phosphate buffer, pH 6.4, and then introduced into a column (1.5 x 20.4 cm) of DEAE-cellulose previously equilibrated with the same buffer. Stepwise elution was performed with the following sodium phosphate buffers: I (0.0175 M, pH 6.4), II (0.04 M, pH 5.9), III (0.10 M, pH 5.8), IV (0.40 M, pH 4.4), and V (0.40 M, pH 4.4, in 2 M sodium chloride), see top panel; the protein concentration is represented as the absorbance of the effluent monitored at 280 nm (light path, 0.3 cm). Effluent fractions (each 0.5 ml) were collected for rapid scanning of relative steroid-binding activity, $S_b/S_o$, see lower panel; 0.2-ml aliquot volumes were taken to estimate progesterone ($\bullet$), pregnenolone (□), and cortisol (○) binding.

hormone-binding properties of breast cyst fluid differ from those of peripheral blood serum in other important respects: there is very little testosterone-binding activity ($1/P$ values, range 0.1 to 1.5) and low $17\beta$-estradiol-binding activity ($1/P$ values, range 1.4 to 2.0) in cyst fluid, specimen numbers 4, 5, 6f, 12f, and 15, whereas the concentration of testosterone-binding globulin (12) in the serum of women is similar to that of corticosteroid-binding globulin (3, 4).

Typical plots for the assay of progesterone- and pregnenolone-binding activities ($1/P$) in breast cyst fluid are shown in Figs. 1 and 2, respectively. The high values for $1/P$ suggest the presence of macromolecular components with high binding affinity for the respective steroid hormones, a view which gains support from preliminary experiments entailing Scatchard plots similar to those which we have described for serum testosterone-binding globulin (12). Because precise measurement of the binding parameters of purified cyst fluid protein is desirable, we are diligently collecting specimens of breast cyst fluid. Only a small amount, e.g. 2 to 5 ml, of fluid may be aspirated at infrequent intervals from patients with cystic disease of the breast; however, the specimens may be stored frozen, as noted above, without appreciable loss of steroid binding activity.

Considerable purification of the steroid-binding component (or components) of breast cyst fluid may be achieved on column chromatography, particularly on hydroxyapatite, see Fig. 3. The progesterone- and pregnenolone-binding component (or components) were eluted simultaneously with 0.01 M sodium phosphate buffer, pH 6.8, see Fractions 19 to 30 (Fig. 3); about
Steroid-binding curves, $S_b/S_u$

Pregnenolone 0.9 -0.4 E c

Progesterone u 22 -0.3 N

2 -0.2 g

5 -0.1 E

::

-1 0.0 %

50 60 70 80 90 100 110 120

EFFLUENT FRACTION

FIG. 5. Ascending Sephadex G-100 chromatography of breast cyst fluid at 3°. The Norit-treated cyst fluid (1.5 ml containing 50.4 mg of protein), Specimen No. 12f (see Tables I and II), was introduced into the chromatographic column (2.5 X 30.3 cm). The buffer system (for column equilibration and elution) was 0.155 M sodium phosphate, pH 7.4, containing 50 mM 2-mercaptoethanol and 20% (v/v) glycerol. The flow rate was 4.1 ml per hour. Effluent fractions (each 1 ml) were collected; 0.1-ml aliquots were assayed (scanning procedure) for pregnenolone (0-0-0) and progesterone (O---O)-binding activity, $S_b/S_u$ (left ordinate). No steroid-binding activity was detectable in Fractions 76 to 130. The protein concentration (right ordinate) of cyst fluid protein (---) and reference proteins (.. *..) are represented as the absorbance of the effluent monitored at 280 nm (light path, 0.3 cm). The reference proteins, bovine serum albumin (BSA) and cytochrome c, were introduced in a separate run in amounts of 15 and 5.2 mg, respectively, each in 1.5 ml of the above buffer; resolution of the bovine serum albumin preparation into bovine serum albumin monomer (see arrow, Peak Fraction 60) and bovine serum albumin polymers (Peak Fraction 50) may be seen. The hold-up volume ($V_H = 46.4$ ml, see arrow) was determined with 0.2% blue dextran (1.5 ml in the above buffer); 50 to 65% of the applied steroid-binding activity and 27% of the applied protein were recovered in this area. The recovery of steroid-binding activity in the total column effluent was practically quantitative; hence, the degree of purification of the steroid-binding component (or components) in Fractions 19 to 30 may be estimated to be about 4-fold. The elution behavior of serum corticosteroid-binding globulin in a parallel run (not shown) was similar to that of the binding proteins in cyst fluid (Fig. 3); but serum testosterone-binding globulin is eluted much later, i.e. with 0.1 M of the same buffer, according to a previous study (8). Another specimen of breast cyst fluid was subjected to chromatography on a column of DEAE-cellulose (see Fig. 4); the stepwise elution scheme was that previously (8) used for separating testosterone- from corticosteroid-binding globulin in serum. Breast cyst fluid afforded minor (A) and major (B) areas of steroid-binding activity (see Fig. 4). About 20% of the steroid-binding activity was recovered in Fractions 48 to 60 (A) and about 60% in Fractions 68 to 80 (B); and since 50% of the applied protein was recovered in Area B, the degree of purification of the binding components in Area B may be estimated to be 2-fold. The steroid-binding component (or components) in Area A clearly does not resemble serum corticosteroid-binding globulin in elution behavior but may not be identical to corticosteroid-binding globulin. The progesterone-binding component in Area B resembles serum corticosteroid-binding globulin in elution behavior but may not be identical to corticosteroid-binding globulin. Although the major progesterone- and pregnenolone-binding components of breast cyst fluid were eluted simultaneously, both on hydroxylapatite and DEAE-cellulose, the internal volume ($V_I = 108.1$ ml) was determined with the aid of tritiated water.

Fig. 6. Disc gel electrophoresis (left panel): about 140 and 200 $\mu$g of protein were applied for serum and breast cyst fluid, respectively. SDS-gel electrophoresis (right panel): about 3, 12, and 10 $\mu$g of protein were applied for bovine serum albumin (BSA), $\alpha$-chymotrypsin, and breast cyst fluid, respectively; the location of the electrophoretic bands is shown and the estimated molecular weight is stated. The origin (0) and direction of migration (+ pole to + pole) are shown.
chromatography, identity may not be presumed; further study is required.

Whole breast cyst fluid was chromatographed on a short column of Sephadex G-100 in an exploratory run, see Fig. 5. The elution curves for the progesterone- and pregnenolone-binding component (or components) overlap. The Stokes molecular radius of the binding components appears to be about 38 Å, corresponding to a molecular weight of about 72,000 for a globular protein.

Of more general interest are the other proteins in breast cyst fluid which are present in remarkably high concentration, about one-fourth to almost one-half of that of serum, see Table I. The proteins of cyst fluid bear no resemblance, however, to those of serum. On disc gel electrophoresis (see Fig. 6), the cyst fluid proteins give rise to two broadly diffuse and faintly staining bands, i.e. representing a complex mixture of proteins of widely different electrophoretic mobility. However, distinct bands were obtained on SDS-gel electrophoresis (Fig. 6) of breast cyst fluid, indicating the presence of only a few classes of proteins (or subunits) of molecular weights of about 74,000 and 32,000 (a major class) and 51,000 and 18,000 (a minor class). From the results obtained on Sephadex G-100 chromatography, see Fig. 5, most of the proteins of cyst fluid appear to have molecular weights ranging above 70,000.

**DISCUSSION**

What physiological role, if any, can be ascribed to the progesterone-binding component of breast cyst fluid? In a somewhat analogous situation, corticosteroid- and testosterone-binding globulin (plasma proteins which bind strongly progesterone and cortisol, and testosterone and 17β-estradiol (13), respectively) apparently regulate the circulating level of physiologically effective hormone, generally regarded to be the unbound form (4). In general, estrogens are responsible for the growth of the mammary ducts in animals with intact pituitary, whereas progesterone is necessary for complete lobule-alveolar growth (14). However, from the present biochemical study of breast cystic disease, no conclusions can be drawn regarding the origin or pathophysiological significance of the steroid-binding component (or components) of the cyst fluid.

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