Partial Purification and Preliminary Characterization of Estrogen-Binding Globulins from Human Plasma*

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ABSTRACT: This report concerns the partial isolation and preliminary characterization of an estradiol-binding globulin from human plasma. Sequential fractionation with ammonium sulfate, DEAE-cellulose, and Sephadex G-200 yielded at least two proteins which bind estradiol, both of which have the mobility of β-globulins. The larger of the two globulins has a molecular weight of 420,000 and the smaller a weight of 135,000 as determined by chromatography on a calibrated column of Sephadex. The larger protein has two association constants for estradiol at 4C: 1.7×10^9 and 0.12×10^9 M^{-1}. The smaller protein has three association constants for estradiol at 4C: 4.0×10^9, 0.69×10^9, and 0.11×10^9 M^{-1}.

Previous work in this laboratory has provided evidence for the presence in human plasma of an estrogen-binding factor other than albumin (Tavernetti *et al.*, 1964, 1967). Rosenbaum *et al.* (1966) showed that this binding factor has the electrophoretic properties of a β-globulin and also that the plasma of women and of men with cirrhosis of the liver has a higher capacity to bind estradiol than does the plasma of normal men (Tavernetti *et al.*, 1967). Murphy (1968) also reported an increased binding capacity for estradiol in the plasma of pregnant women. All the evidence obtained to date suggests that this binding factor is a protein. In this report, we describe the partial purification of two estrogen-binding proteins, A, and C, together with their preliminary characterization. Factor A has a molecular weight estimated from gel filtration on a calibrated column to be *420,000* and two association constants. Factor C has a molecular weight of *135,000* and three association constants. The association constants are presented in Table III.

Experimental Section

Materials and General Methods. All procedures involving the manipulation of proteins were carried out at 4C unless otherwise indicated. The concentration of proteins in various solutions was measured by the microbiuret method of Itzhaki and Gill (1964). Chromatographic effluents were examined for the concentration of protein with an ultraviolet monitor (LKB Instrument), or aliquots of individual fractions were assayed in a spectrophotometer at 278 mμ. 17β-Estradiol-6,7-^{3}H, specific activity 42.4 Ci/mmole (New England Nuclear Corp.), was purified and its radiochemical homogeneity was established as previously described (Tavernetti *et al.*, 1967). Radioactivity was determined in a liquid scintillation spectrometer in toluene–phosphor solution (Tavernetti *et al.*, 1967) (55% efficiency) or aliquots of 0.5 ml of aqueous samples were counted in 10 ml of a dioxane–phosphor solution containing 100 g of naphthalene, 5.3 g of 2,5-diphenyloxazole, and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1000 ml of dioxane (33% efficiency). Crystallized human albumin was purchased from Pentex Co. (Philadelphia, Pa.). A crude preparation of estrogen-binding globulin was obtained from plasma by ammonium sulfate fractionation as described below. Florisil (100–200 mesh) obtained from Fisher Chemicals (Union, N. J.) was washed free of fines by repeated decantation with deionized water. The concentration of sodium ion was determined by flame photometry. Electrophoresis of proteins on paper with or without added estradiol-^{3}H was conducted according to a modification of a previously described method (Rosenbaum *et al.*, 1966). Estradiol-^{3}H and albumin were added to the protein to be examined and the mixture applied to Whatman No. 3MM filter paper measuring 30.5 × 3.8 cm. Electrophoresis was carried out in Durrum tanks (Beckman Instruments) in 0.055 M glycine–acetate buffer (pH 8.6) at 160 V for 18 hr at ambient temperature. The strips were dried in air and then cut into 0.5-cm portions perpendicular to the direction of migration of the proteins. The cut strips were placed in counting vials and wet with 0.2 ml of methanol before the addition of 10 ml of toluene–phosphor (Cayen and Anastisiades, 1966). The recovery of radioactivity was 75–90%. Discontinous gel electrophoresis was performed on slabs of polyacrylamide gel in the apparatus manufactured by the E-C Apparatus Corp. (Philadelphia, Pa.) and according to their instructions (Technical Bull. 140). Whole blood or plasma anticoagulated with acid–dextrose was obtained from the blood bank 1–2 months after collection. The cells were separated by centrifugation and the plasma was stored at −20° until used. All reagents were of analytical grade and deionized water was used for the making of buffers.

Method of Assay for Binding Activity. The method is based on the principle that the fraction of ligand bound, in a system containing the ligand and a macromolecule which binds it, is dependent upon both the concentration of ligand and the

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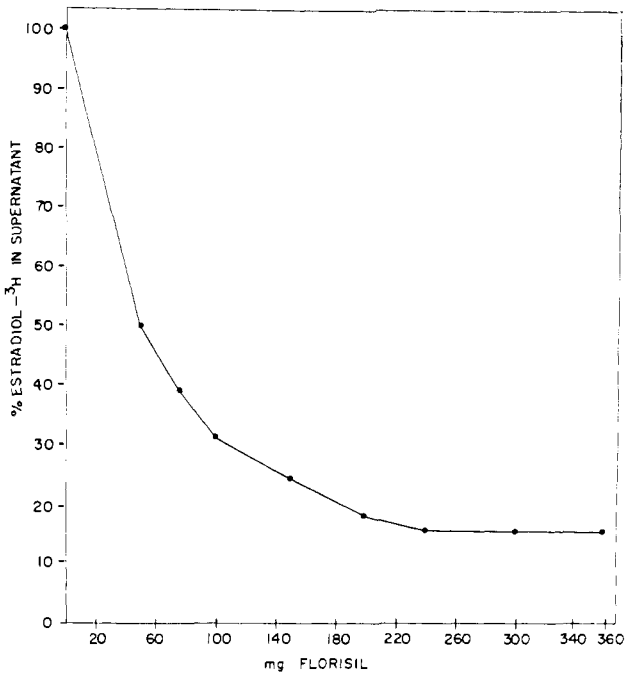

FIGURE 1: The effect of increasing weights of Florisil on the fraction of estradiol-^{3}H remaining in the supernatant of the assay tube. Each point is the mean of two experiments. See text for details of procedure.

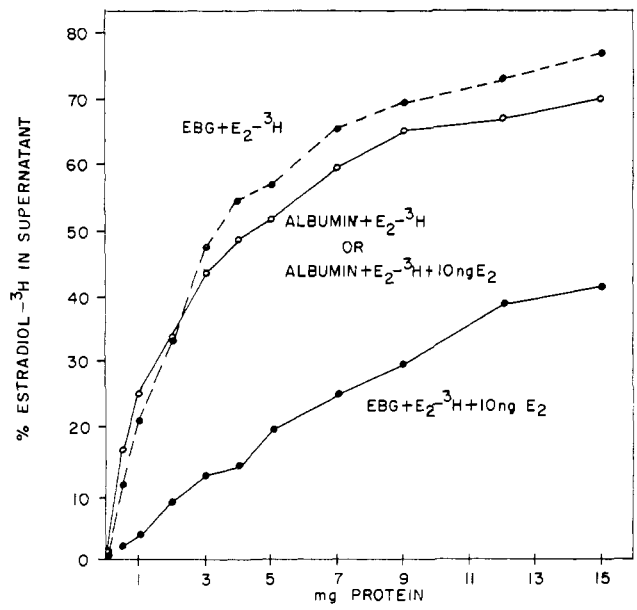

FIGURE 2: The effect of varying weights of estrogen-binding globulin (EBG) and albumin on the fraction of estradiol-^{3}H (E_2-^{3}H) remaining in the supernatant of the assay tube after separation with 280 mg of Florisil. Experiments were done with estradiol-^{3}H alone and then repeated with the addition of 10 ng of carrier estradiol (E_2). A single curve is shown for albumin with estradiol-^{3}H and albumin with estradiol-^{3}H plus carrier estradiol since the fraction of estradiol bound to albumin was found to be independent of the concentration of estradiol.

concentration of macromolecule. It is apparent that one can estimate the concentration of the macromolecule if the concentration of ligand is held constant. Florisil is used to separate bound ligand from that which is free. Free ligand is taken up irreversibly by the Florisil (Murphy, 1967).

Procedure for Assay. All assays were carried out in an ice bath in disposable test tubes measuring 10 × 75 mm. To each tube was added 16,000 cpm of estradiol-^{3}H (0.14 ng) in methanol. The solvent was evaporated to dryness under nitrogen and a solution of the appropriate binding factor in 1.5 ml of 0.05 M sodium phosphate buffer (pH 7.4) was added and followed by shaking on a Vortex mixture for 2–3 sec. After 15–30-min incubation 280 mg of Florisil was added to each of ten tubes and the tubes were shaken individually for precisely 15 sec on a Vortex mixer. The Florisil was allowed to settle, and aliquots of 0.5 ml were removed for counting from the supernatant in the same order as the Florisil had been added. The fraction of the radioactivity remaining in the supernatant is a function of the concentration of binding factor in the system. A standard curve of the percentage of radioactivity in the supernatant plotted against various known concentrations of binding factors was constructed. Concentration of binding factor in unknown samples could then be determined from the graph.

Validation of Assay. REMOVAL OF ESTRADIOL-^{3}H FROM BUFFER BY FLORISIL. The ability of varying weights of Florisil to remove estradiol-^{3}H from a solution of sodium phosphate buffer (pH 7.4, 0.05 M) is illustrated in Figure 1. The data were obtained by adding 16,000 cpm of estradiol-^{3}H (0.14 ng) in 1 ml of buffer to each tube, and then making the buffer up to 1.5 ml. Varying amounts of Florisil were then added to each tube and the tube was shaken for 15 sec on a Vortex mixer. After ten tubes had been processed, the supernatant fluid was separated from the Florisil in the same order as the Florisil was added. Aliquots (0.5 ml) were then taken for counting.

REMOVAL OF ESTRADIOL-^{3}H FROM SOLUTIONS OF PROTEIN BY FLORISIL. (1) The Effect of Varying Protein Concentration. The ability of both estrogen-binding globulin and albumin to compete with Florisil for estradiol was investigated at varying concentrations of protein. To each tube, containing 16,000 cpm of estradiol-^{3}H, was added 1.5 ml of the appropriate solution of protein (made up from the dried powder in buffer) and the assay carried out as described above. The data obtained are plotted in Figure 2, which shows that the fraction of estradiol bound is a curvilinear function of the concentration of binding factor.

The entire experiment was repeated with the addition of 10 ng of radioinert estradiol together with the tritiated material. The purpose of this experiment was to illustrate the difference in the type of binding which is a property of albumin but not of estrogen-binding globulin, namely, that carrier estradiol cannot displace labeled estradiol from albumin. This has been shown to be true in a system of paper electrophoresis (Rosenbaum *et al.*, 1966) and in equilibrium dialysis (Slaunwhite *et al.*, 1963). Figure 2 shows that the same is true in the system employed here. Consequently, when assaying binding activity with this system, it is necessary to determine first which portions of chromatographic effluents contain activity and then to repeat the assay after addition of carrier to the assay system in order to distinguish specific binding from nonspecific binding. We have found that the reassay of 3–5 well-chosen tubes in a peak containing 30–40 tubes is sufficient to furnish this type of information.

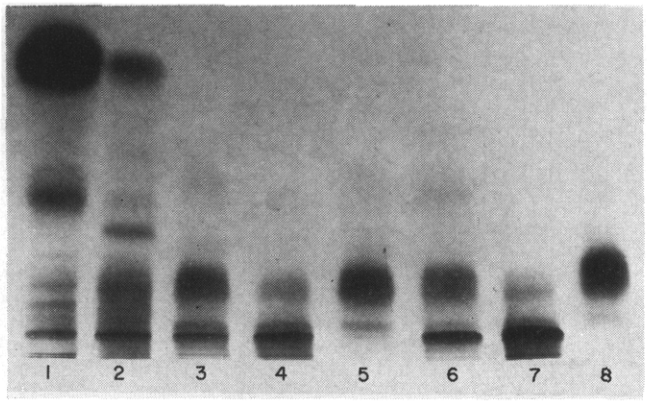

FIGURE 3: Flat plate polyacrylamide gel electrophoresis (7% running gel at pH 8.9) at various stages of purification of the binding proteins. The anode is at the top. (1) Plasma, (2) ammonium sulfate cut, (3) peak I from DEAE-cellulose (see Figures 4 and 5a), (4) peak IA from Sephadex G-200 (see Figure 7), (5) peak IC from Sephadex G-200 (see Figure 7), (6) peak II from DEAE-cellulose (see Figures 4 and 5b), (7) peak IA from Sephadex G-200 (see Figure 8b), and (8) peak IIC from Sephadex G-200 (see Figure 8c).

(2) The Effect of Varying pH. The effect of variations in pH on binding was determined using 0.05 M sodium phosphate buffers over the useful range of this system instead of the 0.05 M sodium phosphate buffer at pH 7.4. Table I shows that the binding is essentially independent of pH and that the magnitude of the displacement of estradiol-^{3}H with carrier shows a slight tendency to diminish above pH 6.5, *i.e.*, a greater fraction of radioactivity remains in the supernatant.

(3) The Effect of Varying Ionic Strength. We investigated the effect of varying concentrations of salts which were likely to be encountered in the chromatography systems used in isolating the protein. Accordingly we chose a pH 7.4, 0.017 M sodium phosphate buffer and increased the sodium concentration by adding NaCl. The assay was carried out as described above except for the change in ionic strength. The data in Table II show that the binding is essentially independent of ionic strength in the system tested. At sodium concentrations greater than 0.4 M, the relative decrease of radioactivity in the supernatant after addition of carrier was smaller.

TABLE I: The Effect of pH on Binding.[a]

	% of Estradiol-^{3}H in Supernatant	
pH	*Estradiol-^{3}H*	*Estradiol-^{3}H + 10 ng of Carrier Estradiol*
5.78	51	12
6.11	52	11
6.49	50	11
7.04	50	15
7.20	50	15
7.36	52	16
7.54	52	18
7.67	51	20

[a] The pH was determined after the protein was added to the buffer. See text for details.

TABLE II: The Effect of Ionic Strength on Binding.[a]

	% of Estradiol-^{3}H in Supernatant	
NaCl	Estradiol-^{3}H	Estradiol-^{3}H + 10 ng of Carrier Estradiol
0.000	52	17
0.020	51	17
0.030	52	16
0.040	52	16
0.720	51	14
0.102	49	14
0.122	54	12
0.362	53	12
0.432	54	16
0.702	54	19
0.982	58	27

[a] The buffer used was 0.017 M sodium phosphate (pH 7.4). The first column indicates the molarity of the solution as adjusted with NaCl. Experimental details are given in the text.

Isolation and Characterization of Estrogen-Binding Factors. GENERAL SCHEME OF PURIFICATION. The plasma was clarified by centrifugation for 15 min at 16,000*g* and sequentially submitted to ammonium sulfate precipitation, chromatography on microgranular DEAE-cellulose (DE 52, Reeve Angel Corp.), rechromatography on DEAE-cellulose, and repeated gel filtration on Sephadex G-200. Each of these steps is described in detail below.

AMMONIUM SULFATE PRECIPITATION. We slowly added 143 g of solid ammonium sulfate to 1 l. of plasma, stirred for 1.5–2 hr, removed the precipitate by centrifugation, and added an additional 88 g of ammonium sulfate to the supernatant. The resulting mixture was stirred overnight, after which the precipitate was recovered by centrifugation, washed once with a solution of ammonium sulfate (231 g/l.), and redissolved in a minimum amount of deionized water. Figure 3 (track 2) illustrates the composition of this fraction as indicated by gel electrophoresis.

CHROMATOGRAPHY ON DEAE-CELLULOSE. The redissolved precipitate was dialyzed against the starting buffer for chromatography (0.01 M sodium phosphate, pH 7.0, made 0.04 M in sodium by the addition of sodium chloride) for 28–30 hr. [Na^+] and pH were determined before application to DEAE-cellulose. Glass columns (4 × 70 cm) containing about 170 g of DEAE-cellulose were used for chromatography of approximately 11 g of protein. The solution of proteins was pumped onto the column at about 150 ml/hr and the column was then developed with starting buffer until the concentration of ultraviolet absorbing material in the effluent had decreased by about 25%. At that point a linear gradient in concentration of NaCl was begun. The starting buffer was as described above, and the limit buffer was 0.01 M sodium phosphate (pH 7.0) made 0.08 M in sodium by the addition of NaCl. The entire gradient was contained in 1500 ml. Upon com-

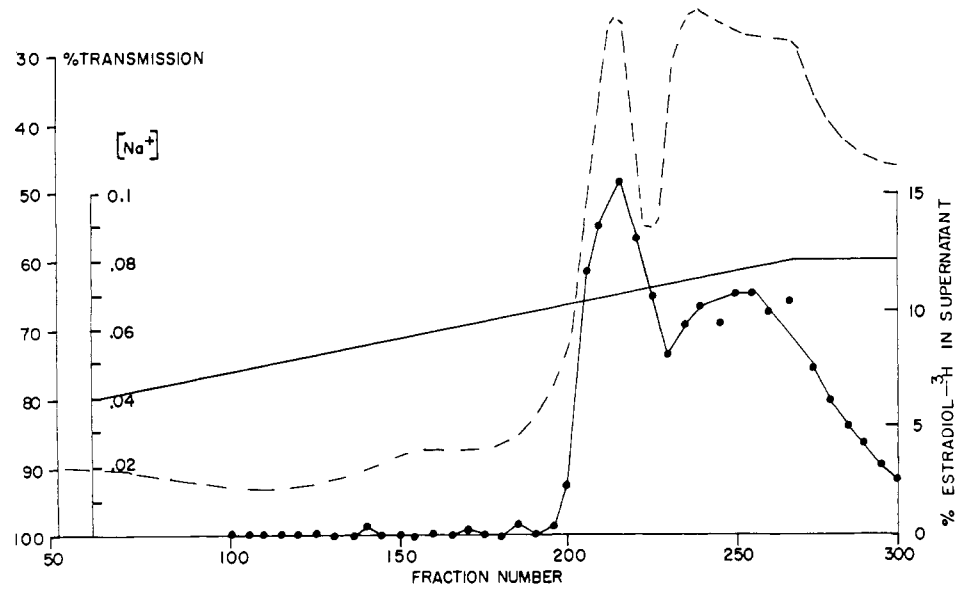

FIGURE 4: Chromatography of an ammonium sulfate cut of plasma on DEAE-cellulose. Protein concentration (- - - -) was monitored with an ultraviolet recording spectrometer at 280 mμ. [Na^+] was measured by flame photometry (———). Estradiol binding activity (●—●—●) was measured in every fifth fraction.

pletion of the gradient the column was further developed with about 1 l. of limit buffer and finally washed with 1 M NaCl prior to dismantling of the column and recovery of the DEAE-cellulose for reuse. All fractions from a given column were assayed for binding activity on the same day, as there is a variable loss of activity when the protein is in solution. Figure 4 illustrates the significant portion of a chromatogram developed in the system described. Neither the breakthrough peak nor the protein eluted with 1 M NaCl is illustrated. Most of the protein and little of the binding activity are present in the portions not illustrated. Although two major peaks of binding activity, termed I and II, were frequently eluted from the first chromatographic system, both the sodium concentration at which the active substances emerged and the relative areas under the peaks varied with different chromatograms. The data are insufficient to enable us to formulate an explanation for this phenomenon. A total of about 1 g of protein was recovered in the two peaks containing binding activity and this generally represented 8–12% of the protein applied to the column. The two active substances obtained from the large-scale columns were separately pooled, dialyzed against water, lyophilized, and stored at −20° until 3–4 g of each was accumulated. These pooled fractions were chromatographed again in the system described above except that the total volume of the gradient was increased to 1900 ml, thus providing a more slowly increasing concentration of sodium chloride. Figure 5A depicts the rechromatography of the earlier eluted active substances (peak I) from the first DEAE-cellulose chromatograms; and Figure 5B, the rechromatography of the active substances eluted later (peak II). Upon this rechromatography very little protein appeared at the solvent front, and only a small amount of protein appeared in the wash. Although one might expect each of the two active substances, I and II, partially resolved on the first DEAE cellulose column, to have distinctly different mobilities upon rechromatography on the second column, most of the binding activity emerged with the same volume of buffer regardless of what its mobility had been on the first column. As can be seen from Figure 5A a small amount of activity, possibly corresponding to another binding factor, was eluted before the major component. We have not further investigated this substance. The results of gel electrophoresis of the active substances eluted from the second two columns are shown in Figure 3, tracks 3 and 6. It is apparent that both fractions have essentially the same composition.

CHROMATOGRAPHY ON SEPHADEX G-200. Columns (3.0 × 80 cm) fitted with upward flow adaptors were packed with Sephadex G-200 by gravity and developed with 0.01 M sodium phosphate buffer (pH 7.0) made 0.03 M in sodium by the addition of NaCl. Flow rates from 15 to 18 ml per hr were maintained by the use of a mariotte flask. The columns were calibrated for the estimation of molecular weight by determining the mobilities of various proteins of known molecular weight applied to the column in a volume of 2 ml at a concentration of 5 mg/ml, as illustrated in Figure 6. The fractions containing active material eluted from the small-scale DEAE columns (illustrated in Figure 5) were separately pooled, dialyzed against water, lyophilized, taken up in the eluting buffer, and chromatographed on Sephadex G-200. The quality of the separations in this system was best when proteins were applied to the column at low concentrations in small volumes. Figure 7 illustrates the results of the chromatography on Sephadex of a portion of fractions 170–250 (peak I) obtained from the DEAE-cellulose chromatography (Figure 5a) and containing 130 mg of protein in 5 ml of buffer. Fractions 39–46 (IA) and 50–61 (IC) were separately pooled, dialyzed against water, lyophilized, and stored until used for the determination of equilibrium constants. Figure 3, tracks 4 and 5, illustrates the results of gel electrophoresis of these fractions. Pooled fractions 190–250, peak II, from the chromatogram illustrated in Figure 5B upon gel filtration on Sephadex were separated into two active components as illus-

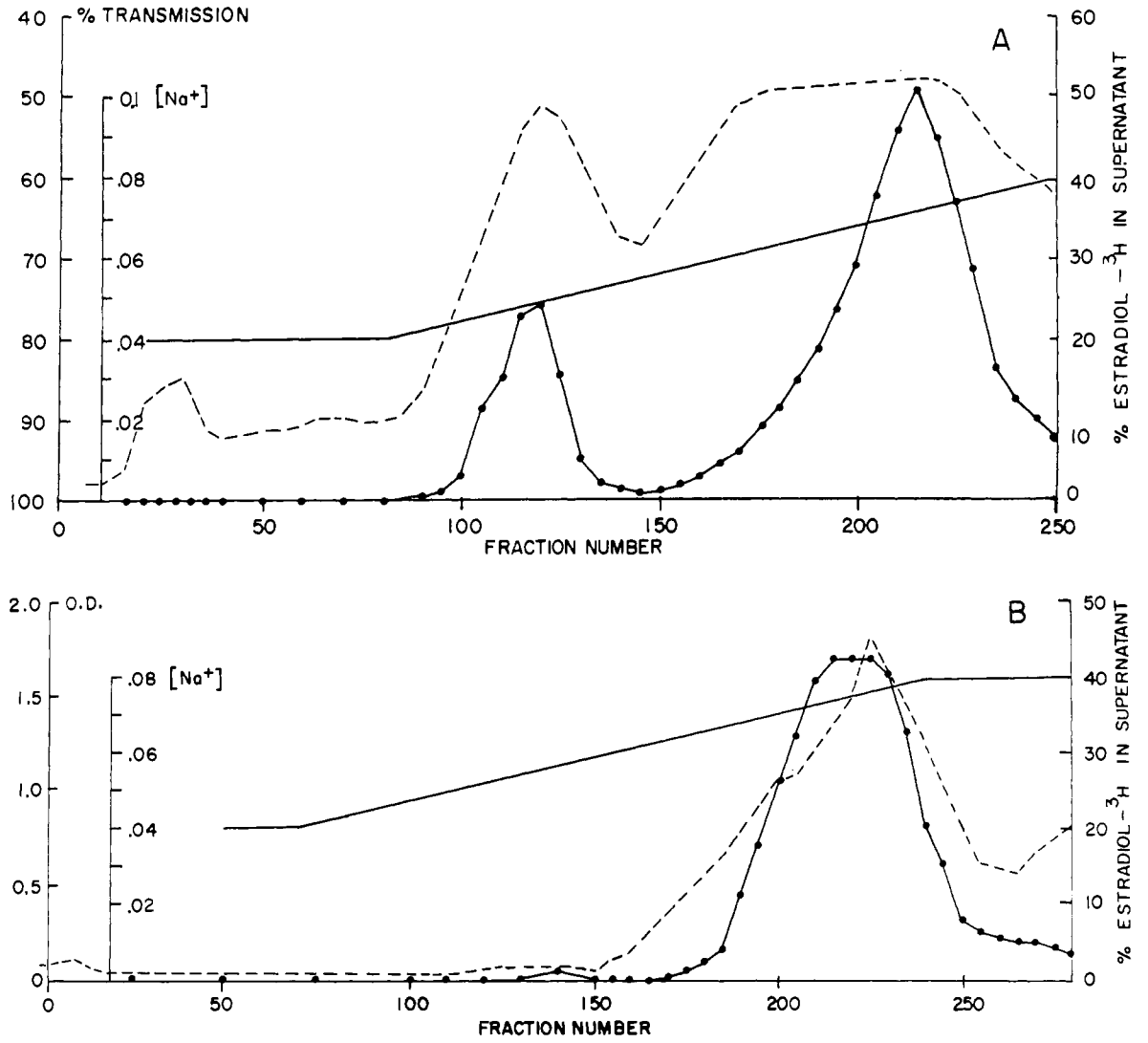

FIGURE 5: Symbols are as in Figure 2. (A) Rechromatography on DEAE-cellulose of peak I from the first DEAE-cellulose column. The conditions are described in the text. (B) Rechromatography on DEAE-cellulose of peak II from the first DEAE-cellulose column.

trated in Figure 8. Each of these components, comprising fractions 40–50 (IIA) and 57–76 (IIC), were separately rechromatographed on Sephadex. These chromatograms are depicted in Figure 8B,C. An almost complete separation of the material eluted from the DEAE-cellulose column into two components was achieved. The active substances from the last Sephadex columns were separately pooled, dialyzed, lyophilized, and stored until used for determination of equilibrium constants. Gel electrophoresis of these fractions was carried out (Figure 3, tracks 7 and 8). Three ml of plasma was also chromatographed on on a calibrated column of Sephadex G-200 in quadruplicate and the molecular weight of estrogen-binding globulin was estimated as described above.

PAPER ELECTROPHORESIS. In order to obtain evidence whether the purification procedure had altered the properties of the isolated material, it was subjected to electrophoresis on paper (Rosenbaum *et al.*, 1966). Human albumin at a concentration of 42 mg/ml was added to each of the four fractions (IA, IIA, IC, and IIC) from the chromatography on Sephadex. The results are illustrated in Figure 9 for the material from peak IIC (20 mg/ml) together with the results of a similar experiment using plasma from a normal man and albumin alone. At the same concentration IA, IIA, and IC gave results indistinguishable from those illustrated. The isolated material binds estradiol and has the same mobility as it does in plasma under the same conditions.

DETERMINATION OF EQUILIBRIUM CONSTANTS. The equilibrium constants of the four isolated active substances were determined by equilibrium dialysis. The binding factors in 1 ml of 0.05 M sodium phosphate buffer (pH 7.4) were dialyzed for 42 hr at 4° against 6 ml of buffer in the presence of various concentrations of unlabeled estradiol and a constant concentration of estradiol-^{3}H. Experiments were conducted in duplicate for each concentration of estradiol used. At the conclusion of the dialysis 0.5-ml aliquots taken from inside and outside the bag were assayed for tritium. From these data the concentrations of bound and unbound estradiol were calculated and these values were used to determine the association constants.

TABLE III: Association Constants, k, and Concentrations of Binding Sites, n[P], for the Four Isolated Proteins.[a]

	IA	IIA	IC	IIC
k_1 (M^{-1})	1.9×10^9	1.5×10^9	3.2×10^9	4.9×10^9
k_2 (M^{-1})	0.12×10^9	0.12×10^9	0.70×10^9	0.68×10^9
k_3 (M^{-1})			0.14×10^9	0.081×10^9
n_1[P]	1.1×10^{-3}	2.6×10^{-4}	4.7×10^{-4}	9.4×10^{-4}
n_2[P]	14×10^{-3}	50×10^{-4}	4.4×10^{-3}	9.0×10^{-3}
n_3[P]			1.2×10^{-2}	2.9×10^{-2}
n_1[P]/n_2[P]	0.0785	0.0520	0.106	0.104
n_1[P]/n_3[P]			0.039	0.032
n_2[P]/n_3[P]			0.366	0.310
n_1[P]/n_1[P]	1.0	1.0	1.0	1.0
n_2[P]/n_1[P]	12.7	19.2	9.4	9.6
n_3[P]/n_1[P]			25.5	30.9

[a] The constants were obtained by fitting the experimental data to a modified Scatchard equation (see text). A comparison of the experimental data with that predicted by these constants is shown in Figure 10.

Analysis of Data and Results

At the end of the fractionation four entities active in binding estradiol and having two different molecular weights were separated. The molecular weights were estimated to be *420,000* for the A fractions and *135,000* for the C fractions from the mobilities of these proteins on a calibrated column of Sephadex G-200. Figure 6 depicts the calibration curve for the column. When whole plasma was chromatographed on Sephadex G-200 only one binding factor other than albumin emerged. An accurate estimation of the molecular weight of this material was difficult to achieve because the descending limb of the estrogen-binding globulin overlapped the ascending limb of the albumin. In spite of the relative inaccuracy of measurements of binding activity under these circumstances it was important to have at least an approximate value for the molecular weight of the binding factor in plasma. In four experiments the molecular weight of estrogen-binding globulin was estimated to be 115,000. There was no binding ac-

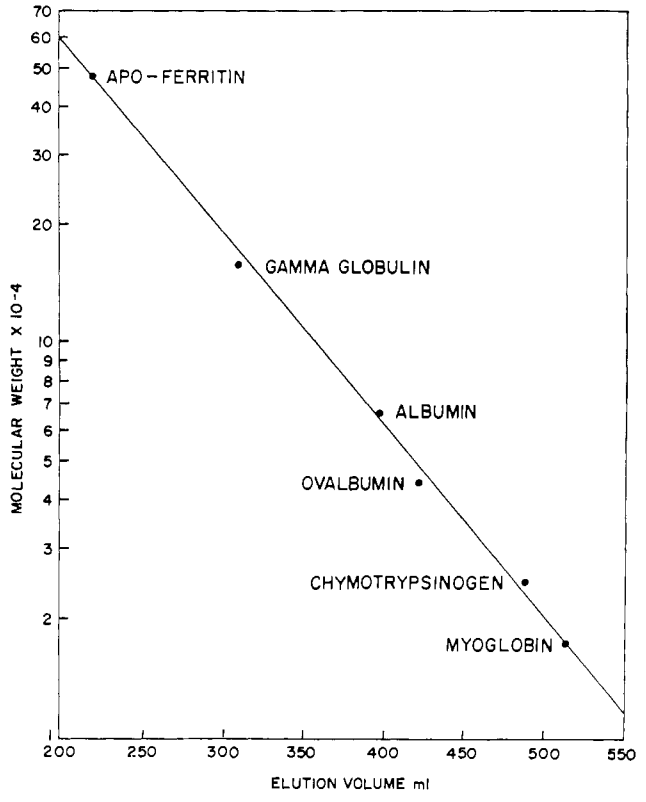

FIGURE 6: Calibration of Sephadex G-200 for molecular weight determinations. The column was 3.0 × 80 cm and the proteins were eluted by upward flow at 15–18 ml/hr.

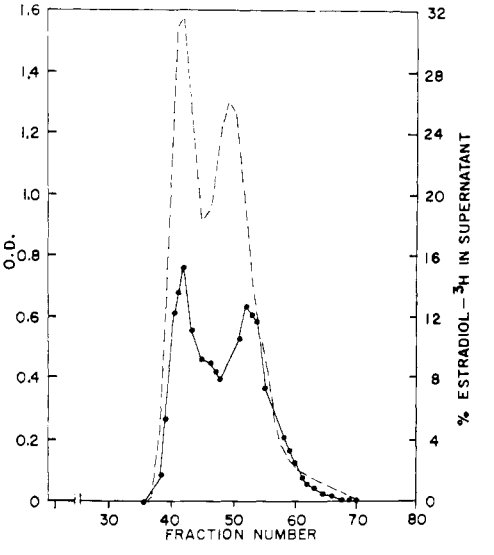

FIGURE 7: Chromatography on Sephadex G-200 of a portion of pooled fractions 170–250 from Figure 5A. Each fraction is 5.25 ml. The effluent was read at 278 mμ in a spectrophotometer (- - - - -) and each fraction was assayed for binding activity (●—●—●). Fractions 39–46 and 50–61 were separately pooled for determination of equilibrium constants.

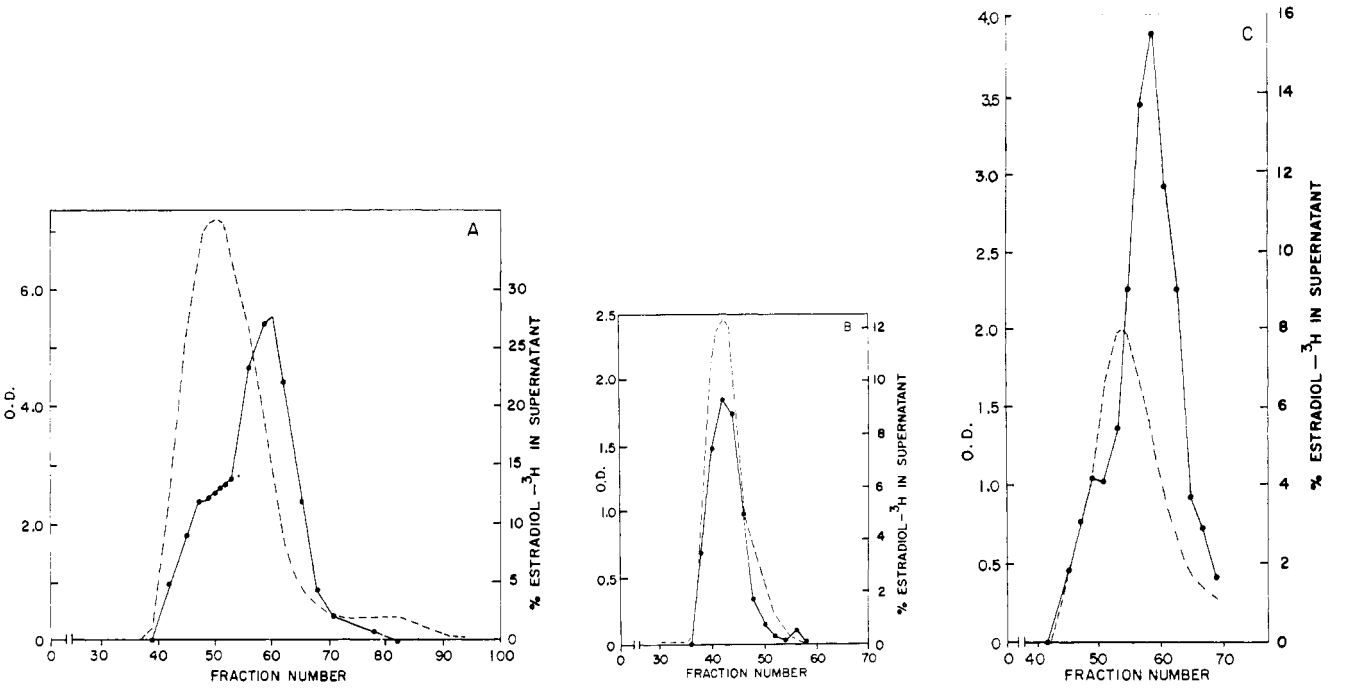

FIGURE 8: Symbols are as in Figure 7. (A) Chromatography on Sephadex G-200 of DEAE-cellulose peak II (fractions 190–250, Figure 5b). (B) Rechromatography of fractions 40–50 from part A on Sephadex G-200. (C) Rechromatography of fractions 57–76 from part A on Sephadex G-200.

tivity in the area of the chromatogram where substances having a molecular weight of 420,000 would be eluted.

The association constants for each fraction were determined. Since the materials were not obtained in pure form, the molar concentration of the binding protein and, therefore, also the number of binding sites per mole of protein could not be determined. The concentration of bound estradiol, b, was plotted against the ratio of bound to unbound estradiol, b/u, at equilibrium, according to the method of Scatchard as modified by Sandberg *et al.* (1966) for situations in which the molar concentration of binding factor is unknown. The equation used is

$$\frac{b}{u} = \sum_{i=1}^{m} \frac{n_i k_i [P]}{1 + k_i u}$$

where [P] is the molar concentration of protein and n is the number of sets of binding site per mole of protein with association constant, k. If b/u is a linear function of b, then there is a single set of independent binding sites with association constant, k. If b/u is not a linear function of b, the presence of two or more sets of binding sites each having a different association constant, k_1, k_2, ...k_m is indicated. Where a mixture of proteins is being dealt with, as in these experiments, it cannot be determined whether departure from linearity is due to several sets of binding sites on one protein or to a single set of binding sites on each of several proteins. The experimental values for b/u and $[b]$ were used to define the curve by the method of successive approximations with the assistance of a computer. The values for k and n[P] were calculated from the curve with the assumption that k is independent of both n[P] and b. Figure 10 shows the plots of b/u *vs.* b obtained for each of the isolated binding factors and the corresponding values for n[P] and k are given in Table III. As can be seen from Figure 10, the binding curves for all the fractions isolated were curvilinear. The data presented in Table III indicate that those fractions having a molecular weight of 420,000, IA and IIA, require the assumption of the presence of two sets of binding sites, whereas those fractions having a molecular weight of 135,000, IC and IIC, require the assumption that three sets of binding sites are present. The data in Table III also indicate the relative abundance of each class of binding site; that is, the products of the number of binding sites

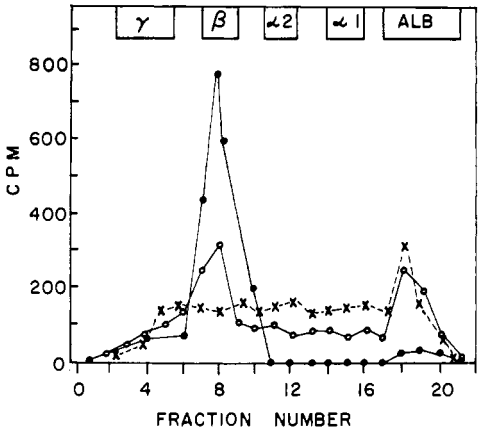

FIGURE 9: Paper electrophoresis of serum (○—○—○), human serum albumin (×—×—×), and pooled fractions 55–62 (●—●—●) from Figure 6 after prior incubation with estradiol-^{3}H. The fraction numbers represent 0.5 cm of the paper strip cut at right angles to the direction of protein migration. The mobility of the plasma proteins are represented at the top of the figure.

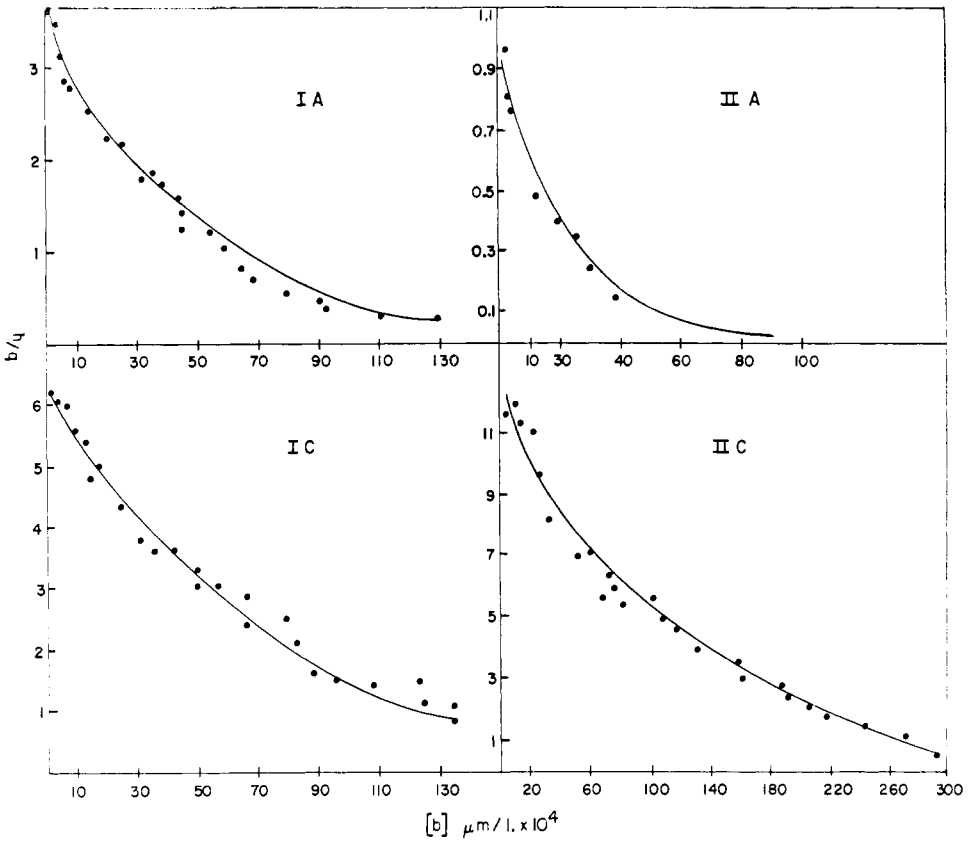

FIGURE 10: Scatchard plots used to determine equilibrium constants for each of the four isolated proteins. Note that the scale is not the same in each case. I refers to the more mobile substance and II to the less mobile substance on DEAE-cellulose. A and C refer, respectively, to the more and less mobile peaks on Sephadex G-200. The constants derived from these plots are shown in Table III.

with a given association constant and the molar concentration of the protein may be compared. Table III shows that the most abundant binding sites are associated with the lowest association constants.

Discussion

Method of Assay. We have described a method for the assay of an estrogen-binding protein one of whose important attributes is rapidity. The specificity of this type of method is determined by the choice of ligand and adsorbent. Several hundred samples can be assayed in the course of a working day. This is an especially important consideration in dealing with a protein whose activity changes with time. This change makes it difficult to compare assays done on different days. The main drawback of the procedure is the lack of linearity of activity as a function of the concentration of binding protein. This is to be expected in such a binding system. The fact that the experimentally determined data closely approximate a smooth curve (Figure 2) attests to the adequacy of the precision of the method for the intended purpose. On the steep portion of the curve (Figure 2), the increases in binding activity observed with increasing concentration of binding factor are large relative to experimental error of a single measurement. Since the less precise values are found at higher concentrations of binding factor, appropriate dilution of the sample with buffer affords a means of improving the precision. The method is sufficiently sensitive so that 5–15% of the chromatographic fraction suffices for determination of binding activity.

Isolation and Characterization of Estrogen-Binding Factors. The isolation from human plasma of four fractions which bind estradiol is described above. It is probable that two of these four active fractions are identical, since the separation of two active fractions upon chromatography on DEAE-cellulose seems to be an artifact. The evidence which supports this view is summarized as follows. (1) The separation was not reproducible and the individual fractions had similar mobilities when rechromatographed on DEAE-cellulose. (2) Gel filtration of each of the individual fractions gave rise to two proteins of different molecular weight. Thus each fraction obtained from the ion-exchange chromatography was further separated into the same fractions. (3) The data in Table III indicate that the materials obtained in fraction C, regardless of the initial mobility on DEAE-cellulose, are very similar with respect to the numbers of sets of binding sites, the association constants themselves, and the relative abundance of each class of binding site. Similarly, the same data pertaining to fraction A are substantially in agreement, although this agreement is not as striking as in the case of C. However, it must be pointed out that the quantity of active material in IIA was small and, therefore, the experimental data used to calculate k and $n[P]$ are less reliable than those for the other fractions. Nonetheless, it is apparent that IC

and IIC are identical and probable that IA and IIA are also identical. It is also apparent that fractions A and C are different with respect to both molecular weight and binding properties. The internal consistency of the relative abundance of binding sites in IC and IIC suggests further that a single molecular species bears the three classes of binding sites. A similar though not so convincing argument obtains in the case of IA and IIA. Consequently, all the evidence obtained so far suggests that two proteins active in binding estradiol have been isolated from human plasma. The association constants for these binding factors and estradiol are similar to those for transcortin and cortisol. However, the molecular weights of these binding factors are considerably higher than that of transcortin (Sandberg *et al.*, 1966).

Since only one binding factor (other than albumin), having a molecular weight of about 115,000, could be detected upon gel filtration of whole plasma, it appears probable that the binding factor (A) having a molecular weight of 420,000 arose during the process of isolation. Owing to the difficulties encountered in estimating the molecular weight of the binding factors in whole plasma, we consider the difference between the value of 115,000 estimated in plasma and the value of 135,000 for the purified material (C) to be within the probable experimental error.

The larger molecule could arise by a process of aggregation of the smaller molecule. The presence of only the smaller molecule in plasma and the fact that, within experimental error, the molecular weight of *A* is three times the molecular weight of *C* makes this hypothesis especially attractive. On the basis of a model for allosteric transitions proposed by Monod *et al.* (1965) one might expect such aggregation to influence the number and magnitude of the association constants for estradiol. Further, the model predicts that the tertiary, and possibly also the quaternary, structure would be influenced by the amount of estradiol bound. Our experiments afford some indirect evidence that the amount of estrogen might play such a role in the aggregation of the binding proteins. Chromatography of the binding factors on DEAE-cellulose results in nearly complete separation of both bound and unbound estradiol from the protein. The larger molecule was detected only subsequent to removal of most of the estradiol by chromatography on DEAE-cellulose. If the model of Monod *et al.* (1965) can be shown to describe the estradiol–estrogen-binding globulin system, estradiol might then be an allosteric determinant for its own binding. The physiological consequences of such a phenomenon might indeed be important.

In these experiments, the calculations of the parameters of binding are based on the assumption that all the binding sites are independent. The evidence that the binding factor can exist in both monomeric and trimeric forms suggests that this assumption may not be valid. However, it does not seem likely that this would result in large errors in the calculation of the association constants.

The evidence presented in this report shows that it is possible to isolate two estrogen-binding proteins from human plasma, and that the association constants of these binding factors are similar to those for other hormone-binding factors in plasma. Further investigation is required to determine the form in which the estrogen-binding factors are present in plasma and whether the estradiol–estrogen-binding globulin system undergoes allosteric transitions as described by the model proposed by Monod *et al.* (1965). However, further purification is necessary before an attempt at a more complete characterization of estrogen-binding globulin can be made.

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