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2-IODOESTRADIOL BINDS WITH HIGH AFFINITY TO HUMAN SEX HORMONE BINDING GLOBULIN (SHBG)

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Summary—Human sex hormone binding globulin (SHBG) binds a set of steroids that differ slightly from each other in structure. Dihydrotestosterone and testosterone are bound with high affinity by SHBG whereas estradiol is bound with a lower affinity. In this work we have studied the binding to human SHBG of the derivatives obtained by substituting iodine in the aromatic A-ring of estradiol.

Three A-ring iodinated estradiol derivatives, 2-iodoestradiol, 4-iodoestradiol and 2,4-di-iodoestradiol, were obtained by treating 17β-estradiol with NaI and Chloramine T and separating the reaction products by HPLC. Their structures were confirmed by mass spectrometry and ^{1}H-NMR. The corresponding radioactive compounds were obtained with use of Na[^{125}I] in the same synthesizing procedure.

Incubation of whole serum, serum albumin and purified SHBG with each of the three [^{125}I]iodoestradiols followed by agarose gel electrophoresis showed only 2-iodoestradiol to have a strong binding to SHBG. This steroid was also bound to albumin, but with a lower affinity. Besides SHBG and albumin, there were no other binders of 2-iodoestradiol in human serum.

The affinity constant for the binding of 2-iodoestradiol to purified human SHBG at 37°C and physiological pH was determined by a dextran-coated charcoal method to be $2.4 \times 10^9\ M^{-1}$ (i.e. exceeding that of dihydrotestosterone). It was found that 0.9 mol of 2-iodoestradiol was bound per mol of SHBG dimer (93 kDa) at saturation, and that 2-iodoestradiol competed with dihydrotestosterone for the same binding site of SHBG.

It was concluded that 2-iodoestradiol has a remarkably high affinity for human SHBG, and that its γ-emitting ^{125}I-analog is useful for binding studies of human SHBG.

INTRODUCTION

Sex hormone binding globulin (SHBG) is a plasma glycoprotein that binds a number of different sex steroid hormones [1–3]. Of the steroids occurring *in vivo* human SHBG has the highest affinity for the highly active androgens, dihydrotestosterone and testosterone, and for some weaker androgens such as androstanediol [4]. The affinity constants for these steroids are $>10^9\ M^{-1}$. Human SHBG also binds estradiol, though its affinity for this estrogen (about $5 \times 10^8\ M^{-1}$) is lower than for the androgens, dihydrotestosterone and testosterone [4].

The molecular mass of purified human SHBG is about 93 kDa [3] which is in agreement with the apparent molecular size of SHBG in plasma as estimated by gel chromatography [5]. The protein has been shown to be a homodimer of two essentially identical non-covalently associated subunits [6, 7] with molecular masses ranging from 45 to 52 kDa [7–10]. The sequence of the monomer is known both from analysis of the isolated protein [11] and from cDNA sequencing [12–14].

At saturation, SHBG binds 1 mol of steroid per dimeric protein [15]. Why SHBG, composed of two essentially identical subunits, should only bind one molecule of steroid has not yet been explained.

We have previously shown human SHBG to have a high affinity for estradiol iodinated with ^{125}I in its A-ring [16]. The iodinated estradiol seems to have a higher affinity for SHBG than estradiol itself, and a knowledge of the structural changes associated with the increased affinity might shed light on the steroid binding mechanisms of SHBG. Furthermore, radioactive steroids with high affinity for SHBG and favourable radiation properties, such as those of γ-emitting ^{125}I and ^{131}I, should be useful tools in the study of SHBG steroid binding.

There are two positions in the estradiol A-ring that would accept an iodine atom under the reaction conditions that were used [17], namely the carbons in an *ortho*-position relative to the 3-OH group. The

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HPLC, high-performance liquid chromatography; SHBG, sex hormone binding globulin.

iodination might thus have resulted in three different estradiol derivatives: 2-monoiodoestradiol, 4-monoiodoestradiol and 2,4-diiodoestradiol. In order to elucidate the nature of the high affinity of A-ring iodinated estradiols for human SHBG, we have synthesized the three A-ring substituted iodoestradiols and studied their binding to SHBG.

EXPERIMENTAL

Materials

SHBG was purified from pooled human serum as described previously [18]. Na[^{125}I] (carrier free) was obtained from The Radiochemical Centre, Amersham, Bucks, England; Coomassie Brilliant Blue R 250 protein stain from British Drug Houses, Poole, England; human serum albumin from Kabi Vitrum AB, Stockholm, Sweden; NaI (analytical reagent grade), Chloramine T and gelatin from KEBO Lab AB, Stockholm, Sweden; agarose (SeaKem LE, lot No. 6639) from Marine Colloids Div., FMC Corp., Rockland, Maine; charcoal (activated, GR) from Merck AG, Darmstadt, West Germany; [1,2-^{3}H]dihydrotestosterone (50.6 Ci/mmol) from NEN Chemicals, Dreieich, West Germany; dextran T70 from Pharmacia, Uppsala, Sweden; Luma Gel scintillation fluid from Lumac/3M, Schaesberg, The Netherlands; estradiol (1,3,5(10)-estriene-3,17β-diol) from Sigma, St Louis, Mo; non-radioactive dihydrotestosterone from Steraloids Inc., Wilton, N.H. Water was purified with a Milli-Q system (Millipore Corporation, Bedford, Mass). All other chemicals used were of analytical reagent grade and obtained from British Drug Houses, Poole, England or Merck AG, Darmstadt, West Germany.

Methods

Preparation of iodoestradiols. 272 mg (1 mmol) of estradiol was dissolved in 10 ml of 1,4-dioxane and 2 ml of a NaI-solution (1.0 mmol, 150 mg in 50 mM sodium phosphate buffer, pH 7.5) was then added followed by 5 ml of Chloramine T solution (1 mmol, 280 mg in the same buffer). The reaction was allowed to proceed at 20°C for 5 min with constant mixing, before being quenched by the addition of 5 ml of a solution of sodium metabisulfite (1 mmol, 190 mg in the same buffer). Water (20 ml) was then added and the mixture extracted twice with 50 ml of ethyl acetate. The pooled extracts were evaporated to dryness and the reside obtained (0.61 g) dissolved in 5 ml of 70% (v/v) methanol in water.

The dissolved reaction products (in 1 ml portions) were separated by preparative HPLC on a 0.78 × 30 cm μ-Bondapak C_{18} reversed phase column (Waters Associates Inc., Milford, Mass), using 70% (v/v) methanol in water as the eluant. The elution rate was 2 ml/min and the effluent was continuously monitored at 280 nm (Waters model 440). Separate peaks were collected, the corresponding fractions from the five runs were pooled, the eluant was evaporated, and the residues obtained were crystallized from methanol.

Iodoestradiol was also synthesized with a method said to yield predominantly 2-iodoestradiol [19].

The corresponding ^{125}I-labeled iodoestradiols were prepared as described elsewhere [17], i.e. essentially as in the first method described above but on a smaller scale and with estradiol in excess. The radioactive reaction products were separated by HPLC as described above for the non-radioactive reaction products.

Radioactivity measurements. ^{125}I was measured in an NE1600 (Nuclear Electronics, Ltd) 16-well gamma spectrometer with 70% efficiency. ^{3}H was measured in 10 ml of Luma Gel scintillation fluid in a beta counter (LKB-Wallac AB, Stockholm, Sweden) with a counting efficiency of 34%. The external standard ratio method was used for quench correction.

Mass spectrometry. Samples were silylated with N,O-*bis*(trimethylsilyl)acetamide [20] and analyzed by gas chromatography–mass spectrometry in a Varian model 3700 gas chromatograph fitted with a 3% OV1 column and connected to a JEOL JMS D300 mass spectrometer. Injection and column temperatures were, respectively, 290 and 270°C for monoiodinated derivatives and 320 and 300°C for diiodinated derivatives.

^{1}H-NMR spectrometry. Proton NMR spectra were recorded on a Nicolet WB360 spectrometer in deuterated chloroform with $Si(CH_3)_4$ as internal standard.

Collection of serum. Blood samples were obtained by venipuncture from healthy subjects: a male (age 31 yr), a non-pregnant female (age 29 yr) and a pregnant female (age 29 yr). The blood was allowed to clot for 60 min at room temperature or overnight at 4°C, and was then centrifuged at 2000 ***g*** for 20 min. The serum samples were stored at −20°C.

Electrophoresis. Agarose slab–gel electrophoresis was carried out as described by Johansson [21] but with a 25 mM sodium phosphate buffer, pH 7.1 and using 200 V for 1 h. Prior to electrophoresis the samples were incubated for 30 min at 20°C with [^{125}I]iodoestradiol (1 μl of an ethanol solution containing 32,500 cpm was mixed with 25 μl of the sample). After electrophoresis the gel was sliced into 2 mm sections and the radioactivity of each section measured. An unsectioned gel used as reference was stained with Coomassie Brilliant Blue [21].

Equilibrium binding studies. A solution of purified SHBG (about 1 g/l) in 50 mM Tris HCl buffer was treated with charcoal (1.25 g/l) overnight at room temperature. The charcoal was removed by centrifugation at 10,000 ***g*** for 20 min. The concentration of SHBG in the solution after the charcoal treatment was determined by 280 nm absorbance measurement [11]. The molar concentration was calculated with use of 93 kDa as the molecular mass of SHBG [3].

Charcoal treated SHBG was incubated in 11 × 55 mm borosilicate glass tubes in 0.5 ml of

10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl, 1 mM $CaCl_2$, 0.1% (w/v) gelatin, and 0.05% (w/v) NaN_3 for 30 min at 37°C with 20,000 cpm of [^{125}I]iodoestradiol and various concentrations of the corresponding unlabeled iodoestradiol. Before use the Hepes buffer had been treated with charcoal (2.5 g/l) overnight at room temperature and the charcoal removed by centrifugation followed by ultrafiltration through a filter with 0.45 μm pore size (HAWP filter, Millipore Corporation, Bedford, Mass).

After incubation, the bound and the free fractions of steroid were separated by adding 0.5 ml of ice-cold dextran-coated charcoal (2.5 g/l charcoal and 0.25 g/l dextran T70 in the Hepes buffer), mixing, incubating for 20 min at 0°C and sedimenting the charcoal at 6000 *g* and 4°C for 20 min. The radioactivity of the decanted supernate (bound fraction) was measured.

The concentrations of free and of bound steroid were calculated from the fraction of bound radioactivity and the known total concentration of steroid in the incubate. Unspecific binding was corrected for by subtracting the radioactivity in the bound fraction when the concentration of unlabeled steroid was 1 μM. Control experiments were performed by varying the incubation time and the time the charcoal was in contact with the incubation mixture.

Analysis of data. The binding model:

$$Y = B_{max}/(1 + K_d/X)$$

(derived from the law of mass action), where Y is the concentration of bound steroid, B_{max} the maximal binding, K_d the dissociation constant, and X the concentration of free steroid, was fitted to the experimental data by non-linear regression analysis using the NDF derivative-free program in the BMDP statistical program package (Biomedical Computer Programs, P-series, University of California, 1977). The data were also analyzed by Scatchard plots [22].

RESULTS

Synthesis and characterization of iodoestradiols

Three different iodine derivatives of estradiol were obtained by treating estradiol with NaI and Chloramine T followed by separation of the reaction products by preparative HPLC. The separation of the three iodoestradiols by HPLC under analytical conditions (employing the same chromatographic system as was used in the preparative separations) is shown in Fig. 1. In this experiment equimolar amounts of estradiol and the crystalline synthetic products were mixed before chromatography. The chromatograms of the individual iodoestradiols (not shown) showed them all to be more than 95% pure.

The structures of the three iodoestradiols were determined from the results of mass spectrometry after trimethylsilylation (M^+ ions at m/e 542, 542 and 668, respectively) and proton NMR (Fig. 2) as 2-iodoestradiol, 4-iodoestradiol and 2,4-diiodoestradiol.

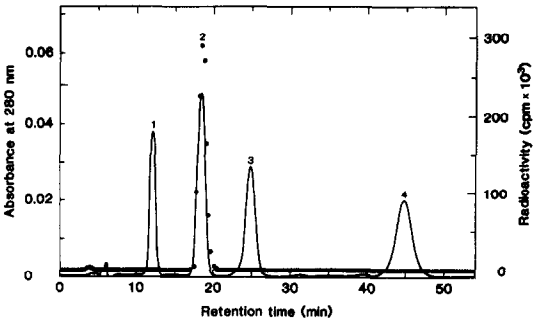

Fig. 1. Analytical HPLC of a synthetic mixture comprising 50 nmol each of crystalline estradiol (1), 2-iodoestradiol (2), 4-iodoestradiol (3), and 2,4-diiodoestradiol (4). The mixture also contained 1.2×10^6 cpm of 2-[^{125}I]iodoestradiol. Column: Waters C_{18} μ-Bondapak, 0.78 × 30 cm. Elution: methanol/water (70/30, v/v), 2 ml/min. Temperature: 20°C. The effluent was monitored at 280 nm (—) and collected in 0.6 ml fractions for radioactivity measurement (●).

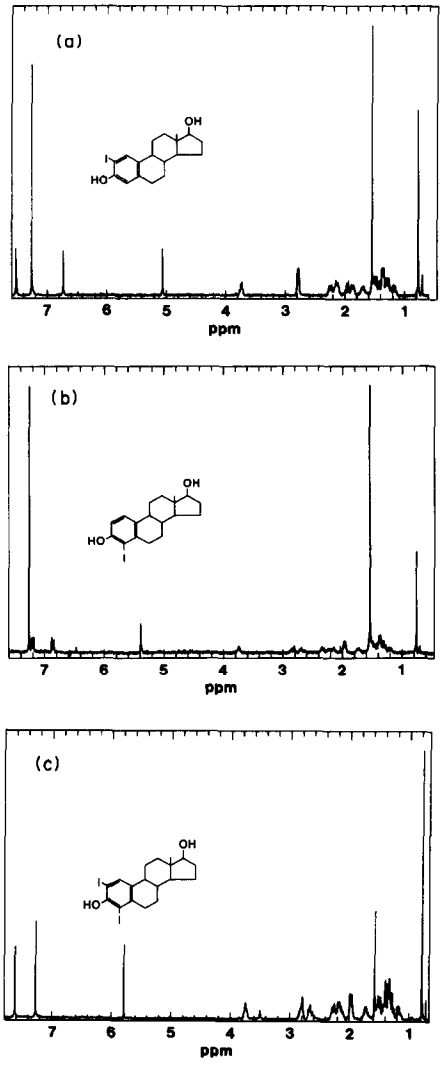

Fig. 2. ^{1}H-NMR spectra of iodoestradiol derivatives isolated by HPLC. (a) Compound corresponding to peak 2 in Fig. 1; (b) compound corresponding to peak 3 in Fig. 1; (c) compound corresponding to peak 4 in Fig. 1. The chemical structure in accordance with the spectrum is shown for each compound.

The yields after the preparative HPLC step, determined by u.v. spectroscopy with use of published spectral data [23] and expressed as the percentage of invested estradiol, were 37, 11 and 36% for 2-iodoestradiol, 4-iodoestradiol and 2,4-diiodoestradiol, respectively. Recrystallization gave 64, 7.6 and 56 mg, respectively. The predominant product obtained by an alternative synthesis [19], claimed to yield predominantly 2-iodoestradiol, was indeed the compound here identified as 2-iodoestradiol.

The corresponding radioactive iodoestradiols were obtained by substituting the non-radioactive NaI used in the synthesis with Na[^{125}I] and separating the products by HPLC as in the non-radioactive synthesis. The yields were high; more than 80% of the radioactivity was incorporated into the three iodoestradiols, and they were obtained in approximately the same relative proportions as in the non-radioactive synthesis (see above).

The [^{125}I]iodoestradiols all had a high specific radioactivity and a high degree of radiopurity. The specific radioactivity of 2-[^{125}I]iodoestradiol, estimated from the radioactivity and the 280 nm absorbance in the HPLC peak, was 7×10^{16} Bq/mol which is 90% of the theoretical value, though the figure is uncertain due to the low absorbance value obtained. The high degree of radiopurity of 2-[^{125}I]iodoestradiol is shown in Fig. 1. In this experiment the radioactive 2-iodoestradiol had been added to the mixture of estradiol and the three crystalline non-radioactive iodoestradiols before HPLC. As can be seen in the figure, the radioactive tracer coeluted exactly with the corresponding unlabeled compound and it contained almost no detectable radioimpurities. The peak included 97% of the radioactivity applied to the HPLC column.

Binding of iodoestradiols to plasma proteins

The binding of the three iodoestradiols to plasma proteins was tested by incubation of human serum, human serum albumin and purified human SHBG with each radioactive steroid, followed by agarose gel electrophoresis. Only 2-iodoestradiol showed a significant binding to SHBG, and only the results with this steroid are shown (Fig. 3). As seen in the figure, 2-iodoestradiol binds to both albumin (Fig. 3d) and SHBG (Fig. 3e). The distribution of the steroid between albumin and SHBG in serum depends on the concentration of SHBG as demonstrated by the results obtained with serum samples with different concentrations of SHBG (Fig. 3a–c). The results with serum (Fig. 3a–c) suggest that no other major binders for 2-iodoestradiol than albumin and SHBG are present in serum and that SHBG has a much higher affinity for the steroid than does albumin (considering the difference in concentration of the two proteins).

Affinity of 2-iodoestradiol for SHBG

In order to quantify the binding of 2-iodoestradiol to SHBG, purified SHBG was equilibrated at physiological pH and 37°C with various concentrations of 2-iodoestradiol and a constant amount of 2-[^{125}I]iodoestradiol followed by separation of free and bound steroid with dextran-coated charcoal. The unspecific binding was between 1 and 2%. The incubation time used was 30 min because control experiments showed that equilibrium was reached within this time. Another control experiment, in which the incubation mixture was left in contact with the dextran-coated charcoal for various time periods before the centrifugation and decantation, demonstrated that the procedure for separation of free and bound steroid caused a dissociation of steroid from SHBG of less than 3% within the duration of the binding experiments.

The results of the binding experiments, performed at three different SHBG concentrations, are shown in Fig. 4. The data suggest that SHBG at saturation

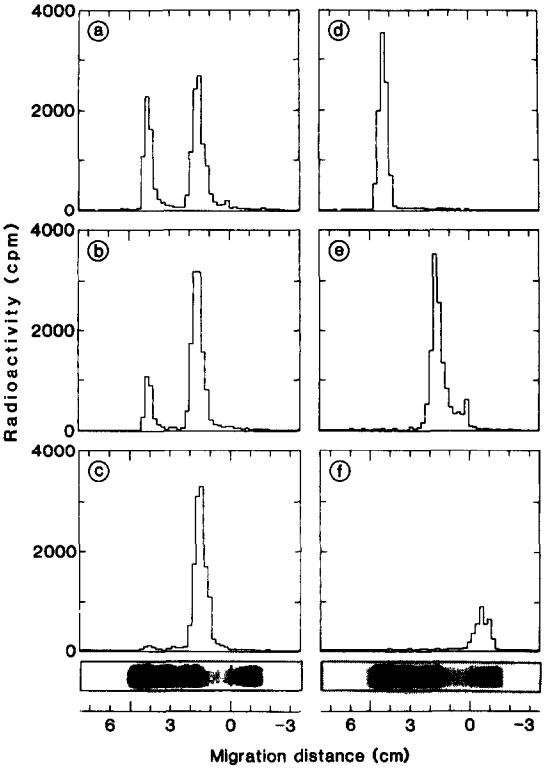

Fig. 3. Electrophoretic analysis of the binding of 2-iodoestradiol to plasma proteins. Serum samples from a male (a), a non-pregnant female (b), and a pregnant female (c), solution (40 g/l) of human serum albumin (d), solution (100 mg/l) of purified human SHBG (e), and buffer (f) were incubated with 2-[^{125}I]iodoestradiol (10^5 cpm) and then subjected to agarose slab-gel electrophoresis at pH 7.1 (the pH 7.1 buffer was used in order to obtain a sufficient separation of free steroid from the binders in serum). The anode is to the left. A plasma sample stained for protein after electrophoresis is shown as a reference at the foot of each column.

binds slightly less than one mol of steroid per mol of dimeric SHBG (93 kDa), and that half saturation occurs at a free steroid concentration of about 0.5 nM. These visual estimates agree well with the results obtained by fitting the binding model derived from the law of mass action to the experimental data. The constants giving the best fit were 0.89 mol/mol for the maximal binding (B_{max}) and 0.41 nM for the dissociation constant (K_d). Essentially the same estimates of the binding constants were obtained from a Scatchard plot of the data.

Competition of dihydrotestosterone and 2-iodoestradiol in binding to SHBG

To compare the binding of 2-iodoestradiol with that of the more commonly studied steroid, dihydrotestosterone, SHBG was incubated with a trace amount of 2-[^{125}I]iodoestradiol together with either dihydrotestosterone or 2-iodoestradiol at various concentrations (Fig. 5). With both steroids, a sufficiently high concentration displaced the tracer completely from SHBG. The displacement of the 2-[^{125}I]iodoestradiol tracer by dihydrotestosterone suggests a common binding site on SHBG for the two steroids. Dihydrotestosterone required a 2.7-fold higher concentration for a 50% displacement than did 2-iodoestradiol (Fig. 5). Essentially the same results were obtained when [^{3}H]dihydrotestosterone was used instead of 2-[^{125}I]iodoestradiol as the tracer. In this case the concentration required for a 50% displacement was 6.0-fold higher for dihydrotestosterone than for 2-iodoestradiol.

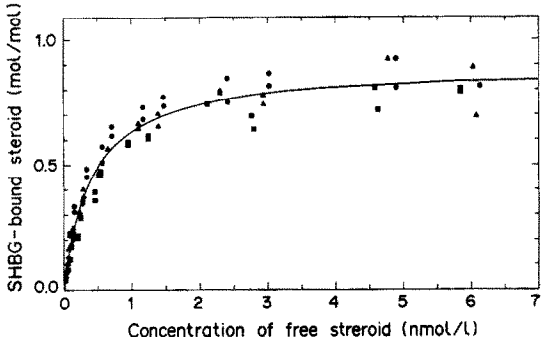

Fig. 4. Binding of 2-iodoestradiol to human SHBG at 37°C. Purified human SHBG was incubated for 30 min with various concentrations of 2-iodoestradiol containing 20,000 cpm of 2-[^{125}I]iodoestradiol. The bound fraction was separated from the free fraction with dextran-coated charcoal, and the concentration of free and bound steroid calculated from the radioactivity in the bound fraction. The experiment was performed with three different concentrations of SHBG in the incubation mixture: 0.13 nM (circles), 0.26 nM (triangles), and 0.52 nM (squares). The binding was expressed as mole steroid bound per mole SHBG dimer (93 kDa molecular mass). The best fit (solid line) of the binding model $Y = B_{max}/(1 + K_d/X)$ to the experimental data was obtained with a dissociation constant (K_d) of 0.41 nM and a maximal binding (B_{max}) of 0.89 mol/mol.

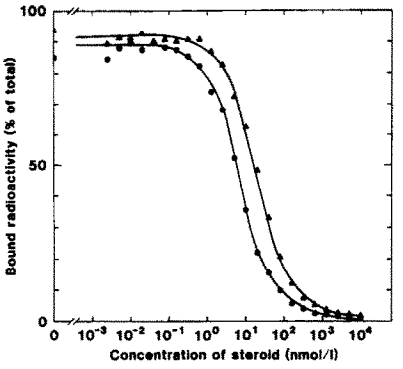

Fig. 5. Competition between dihydrotestosterone and 2-iodoestradiol in binding to human SHBG. Purified human SHBG (4 nM) was incubated in a total volume of 0.5 ml for 2 h at 37°C with 2-[^{125}I]iodoestradiol (20,000 cpm) and various concentrations of non-radioactive steriod, either 2-iodoestradiol (circles) or dihydrotestosterone (triangles). Dextran-coated charcoal was then added, the mixture centrifuged, and the radioactivity of the supernate measured. The total concentration of the non-radioactive steroid in the incubation mixture is given on the abscissa.

DISCUSSION

In a previous work it was shown that the radioactive derivative (or derivatives) obtained by direct iodination of estradiol with ^{125}I has a high affinity for human SHBG [16]. It might be inferred that under the reaction conditions used, which were those of a classic procedure for labeling proteins with radioactive iodine [24], iodine was substituted in the aromatic A-ring of estradiol and that the substitution gave rise to several different iodoestradiols. The reaction products were not separated, however, and it was not determined which derivatives were obtained or which of them has a high affinity for SHBG.

Iodoestradiols have previously been synthezised by several different methods [19, 23, 25–27]. In this work we chose to follow the protocol that was used for iodination with ^{125}I when the high affinity for human SHBG of A-ring iodinated estradiol was discovered [17]. The only change we made in the protocol was to separate the reaction products by HPLC which resulted in a complete separation of the three iodinated estradiols from each other and from unreacted estradiol. The analytical data for the crystalline compounds obtained were in agreement with published data [19, 23, 25–27], and unambiguously identified the three different iodoestradiols. Electrophoresis experiments with human serum and with purified human SHBG then showed that of the three different iodoestradiols only 2-iodoestradiol had a high affinity for SHBG.

The affinity constant and the maximal binding capacity for the binding of 2-iodoestradiol to SHBG were estimated by a method employing dextran-coated charcoal. This method is experimentally simple but not without drawbacks. Among other things it is based on the assumption that equilibrium is not

significantly perturbed during the adsorption of free steroid to the dextran-coated charcoal. Although the control experiment indicated that dissociation of steroid from SHBG was slow, it cannot be excluded that some dissociation might have taken place during the charcoal adsorption step. As this would result in underestimation of the bound fraction and overestimation of the free concentration, systematic low values would be obtained for both the affinity constant and the binding capacity. The values of the binding constants obtained in this work for the binding of 2-iodoestradiol to SHBG must therefore be regarded as rough estimates only, and it would be desirable to detemine them with a more stringent method, such as equilibrium dialysis.

There is no doubt, however, that the affinity of 2-iodoestradiol for human SHBG is high, the estimated affinity constant being at least $2.4 \times 10^9\ M^{-1}$ which is higher than that of dihydrotestosterone, a steroid regarded as having one of the highest affinities for human SHBG [4]. That the affinity of 2-iododoestradiol is higher than that of dihydrotestosterone was confirmed by the competition experiments which also showed that the two steroids bind to a common site on the SHBG molecule.

The stereochemical requirements for the binding of steroids to human SHBG have previously been defined by systematic testing of the binding of a large number of steroids with differing structures [28]. From these studies it was concluded that the three most important structural requirements for a steroid to bind with high affinity to human SHBG are an equatorial electronegative group on the C_3-carbon, a planar junction of the steroid A- and B-rings, and the presence of a 17-OH group in the β-configuration [28]. Although estradiol fulfills these requirements, its affinity for human SHBG is lower than that of androgens, such as dihydrotestosterone and testosterone, fullfilling the same requirements [4, 28]. The difference in affinity may be due to the fact that, in contrast to the case with the androgens, the A-ring in estradiol is aromatic. Estradiol, with its single equatorial hydrogen on the C_2-carbon, occupies less space in the vicinity of the C_2-carbon on the β-side of the steroid than do the high-affinity steroids with non-aromatic A-rings. This fact might explain the increased affinity of SHBG for estradiol after A-ring iodination of the latter. Substitution with a bulky chemical group on the C_2-carbon, as has been done in 2-iodoestradiol, makes the molecule more space filling around the C_2-carbon and structurally more similar to the high-affinity androgens. In agreement with this explanation is the earlier observation that 2-methoxyestradiol, which also has a bulky group on the C_2-carbon, has a higher affinity for human SHBG than testosterone [29].

The catabolism of estrogens in the body follows two main routes: C_{16} and C_2 hydroxylation [30]. In the second route, 2-methoxy derivatives of estrogens are formed via catecholestrogens, the latter being suggested to mediate the effects of estrogens in the central nervous system [31]. One might speculate, therefore, that SHBG is also involved in the transport of estrogen metabolites, and that the high affinity for methoxyestradiol and consequently for the "unnatural" 2-iodoestradiol represents an adaptation of SHBG for this purpose.

In an earlier work [25], in which the interaction of iodoestrogens with uterine receptors was studied, it was found that 2-iodoestradiol does not bind to the intracellular estrogen receptor. 2-Iodoestradiol is thus a steroid that binds specifically to the main extracellular sex steroid binder (SHBG) but not to intracellular binders (steroid receptors). These binding characteristics contrast with those of 16-iodoestradiol, another iodine substituted estradiol which has been reported to bind specifically to the intracellular estrogen receptor but not to SHBG in plasma [32]. Both these steroids can easily be made radioactive with isotopes having favourable measuring properties and may complement each other in studies of intra and extracellular steroid binding.

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