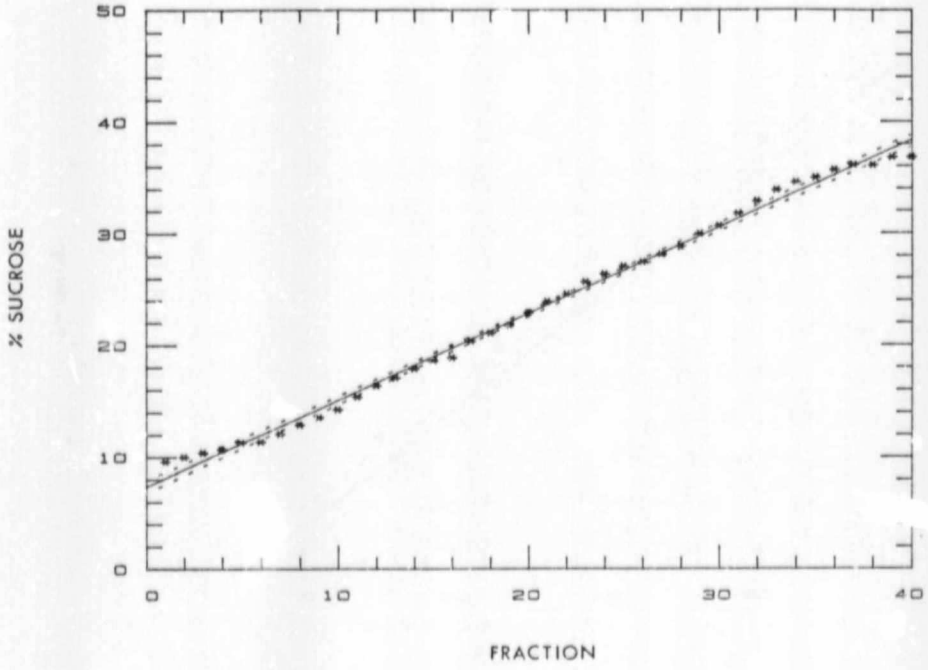


FIGURE 2.2

Linearity of continuous 10-35% sucrose density gradients as monitored by refractometry. Gradients were prepared by hand layering of 1,15 mℓ of each of four different concentrations (10%, 18,3%, 26,7% and 35%) of sucrose in TEDAG buffer. A minimum of 2 h of conditioning at room temperature was required to create continuous linear gradients. Sucrose concentration was monitored in the 40 manually fractionated samples by refractometry. The 99% confidence limits are indicated by the dotted lines.

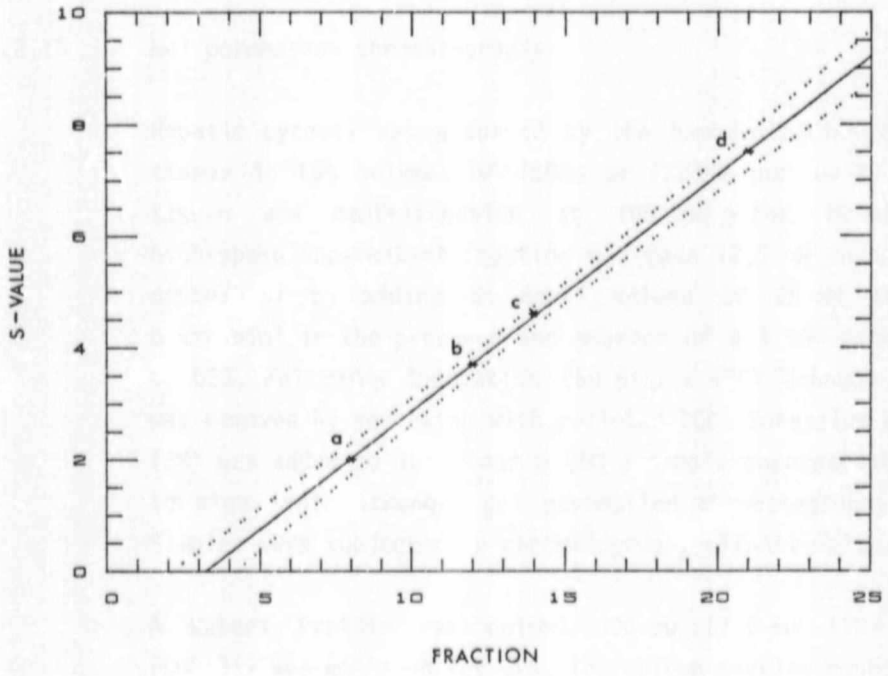


FIGURE 2.3

Calibration of sucrose density gradients. Gradients were prepared and samples layered as described in Section 2.2.14. The proteins employed were a) myoglobin (2,04S), b) ovalbumin (3,5S), c) bovine serum albumin (4,6S) and d) aldolase (7,35S). Gradients were centrifuged (520 000 *g* x 2 h x 4°C) and fractionated into 40, five drop fractions. The fractions were monitored for absorbance at 280 nm spectrophotometrically. The 99% confidence limits are indicated by the dotted lines.

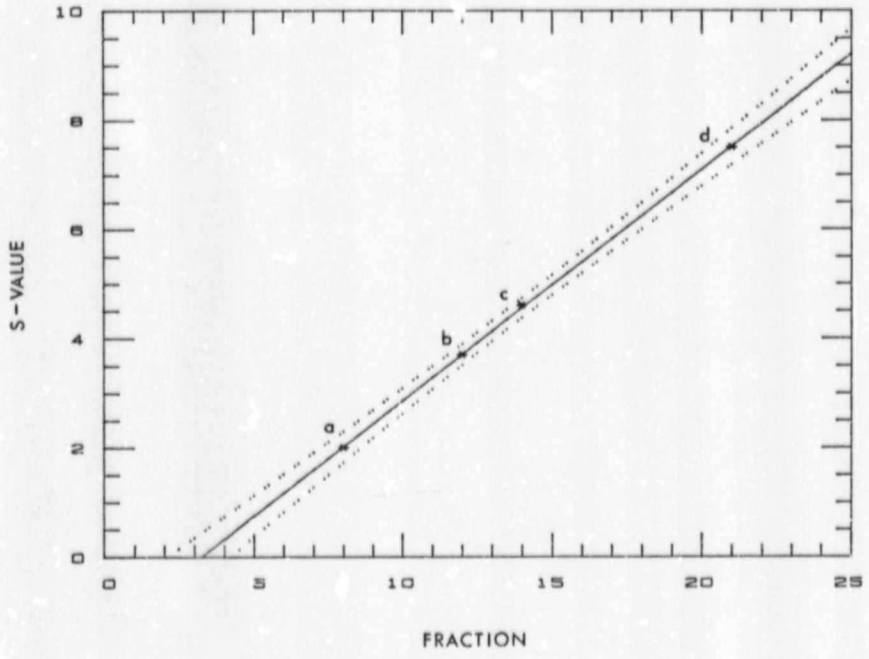


FIGURE 2.3

Calibration of sucrose density gradients. Gradients were prepared and samples layered as described in Section 2.2.14. The proteins employed were a) myoglobin (2,04S), b) ovalbumin (3,5S), c) bovine serum albumin (4,6S) and d) aldolase (7,35S). Gradients were centrifuged (520 000 *g* x 2 h x 4°C) and fractionated into 40, five drop fractions. The fractions were monitored for absorbance at 280 nm spectrophotometrically. The 99% confidence limits are indicated by the dotted lines.

fractionated manually into 5-drop fractions. [^{14}C]- Methylated albumin (4,6S) was run in separate gradients but simultaneously with experimental samples as a sedimentation marker.

2.2.15 Gel permeation chromatography

Hepatic cytosol was prepared by the homogenization of liver tissue in two volumes of TEAG or TEDAGM per unit mass of tissue and centrifugation at 105 000 *g* for 30 min. The high-speed supernatant fraction was made 12,5 nM in tritiated oestradiol by adding an equal volume of 25 nM tritiated oestradiol in the presence and absence of a 1 000-fold excess of DES. Following incubation (60 min. x 4°C) unbound steroid was removed by vortexing with pelleted DCC. Potassium chloride (2M) was added to the clear 2 000 *g* sample supernatants prior to high ionic strength gel permeation chromatography (GPC). Samples were subjected to chromatography without delay.

A Waters Protein Pak column 300 sw (7,5 mm (ID) x 30 cm (80013)) was employed for GPC. The column packing consisted of a silica base linked to a hydrophilic moiety. The column was calibrated with a standard calibration kit (Bio-rad) containing protein standards of molecular masses ranging from 1 350 - 670 000 daltons (Figure 2.4). A mixture of tritiated L-amino acids and Blue Dextran 2 000 were used to determine the total column volume (V_t) and the void volume (V_o) respectively.

2.3 RESULTS AND DISCUSSION

2.3.1 Protein determination

The protein concentration in hepatic cytosol was determined by employing the standard Bio-rad assay procedure for protein concentrations from 25 to 140 µg. The absorbance maximum for an acidic solution of Coomassie brilliant blue shifts from 465 nm to 595 nm when binding to protein occurs. The Bio-rad

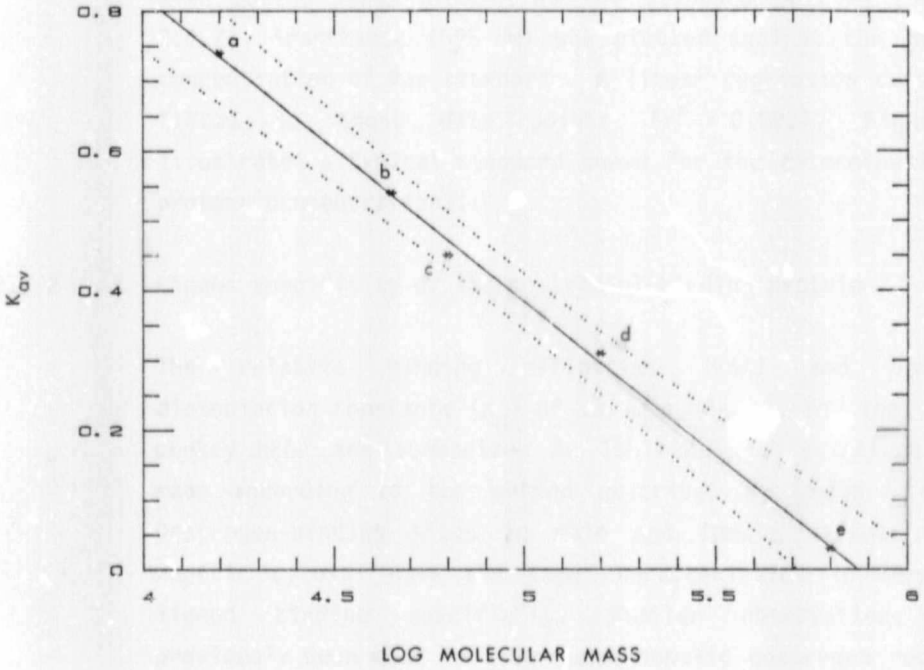


FIGURE 2.4

A representative calibration curve for the Waters Protein Pak column 300 sw, employed during gel permeation chromatography. The different marker proteins (Bio-rad) used were: a) myoglobulin (17 000 daltons), b) ovalbumin (43 000 daltons), c) albumin (67 000), d) gammaglobulin (163 000 daltons) and e) thyroglobulin (669 000 daltons). The column was eluted with PEDGP (0,15) or PEDGP (0,40) as mobile phases with corresponding results. The 99% confidence limits are indicated by the dotted lines.

protein assay is based on this principle which was first established by Bradford (152). A standard curve was determined with bovine serum albumin as the standard protein (Section 2.2.7). Absorbance (595 nm) was plotted against the protein concentration of the standards. A linear regression curve was fitted to these data points (r^2 = 0,993). Figure 2.1 illustrates a typical standard curve for the determination of protein concentrations.

2.3.2 Ligand specificity of the oestradiol-binding protein

The relative binding affinities (RBA) and apparent dissociation constants (K_d) of various ligands for the vervet monkey HEBP are summarized in Table 2.1. Calculations were made according to the method described by Rodbard (153). Oestrogen-binding sites in male and female vervet monkey hepatic cytosol have the same characteristics in terms of ligand binding specificity. Similar observations have previously been made for the human hepatic oestrogen receptor (146), the green monkey liver oestrogen receptor (102) and the rat liver oestrogen-binding component (114). The most effective competitors to the vervet monkey hepatic oestrogen-binding site were EE_2, E_2, and DES. Both E_3 and E_1 competed moderately well, while no affinity was observed for any of the other steroid hormone categories. These specificities were in agreement with ligand specificities published for the human (146), green monkey (102, 154), rat (103, 114, 154) and rabbit (154, 117) liver oestrogen-binding proteins. The HEBP of vervet monkey liver cytosol displayed much the same specificity towards steroids as the oestrogen receptor present in the vervet monkey uterine cytosol (151). Accordingly the hepatic oestrogen-binding sites appear to be proteins that are oestrogen specific.

TABLE 2.1 : RELATIVE BINDING AFFINITIES (RBA) AND APPARENT DISSOCIATION CONSTANTS (K_d) OF VARIOUS LIGANDS FOR THE HEBP FROM VERVET MONKEY.

LIGAND	RBA %	K_d (M)
EE_2	117,8	$2,0 \times 10^{-10}$
E_2	100,0	$2,4 \times 10^{-10}$
DES	88,3	$2,7 \times 10^{-10}$
E_3	26,6	$9,1 \times 10^{-10}$
E_1	14,1	$1,0 \times 10^{-9}$
NE	0,3	$7,7 \times 10^{-9}$
P	< 0,3	-
R5020	< 0,3	-
T	< 0,3	-
DHT	< 0,3	-
DHEA	< 0,3	-
HC	< 0,3	-

2.3.3 Oestradiol binding in liver cytosol prepared from tissue of different areas in the same liver

Cytosols were prepared from blocks of hepatic tissue taken from different areas in a single liver. The tissue samples more or less represented various lobes in the monkey liver.

Scatchard analyses were performed on the binding data obtained from multipoint titration experiments carried out on these cytosols. Results are summarized in Table 2.2. Statistical analyses executed on this data indicate no significant difference between oestradiol binding in the different areas showing that the HEBP is evenly distributed throughout the liver. Therefore, no further distinction was made between cytosols prepared from the three liver lobes. Not a great deal of attention is given in the literature to the distribution of oestrogen-binding proteins in the liver. Receptor autoradiography provides an effective method to unmask the biochemical organization of a variety of hormone receptors within an anatomical framework (36). In one study autoradiographic studies were executed on baboon liver following in vivo labelling with tritiated oestradiol (116). Labelling appeared to be absent in hepatic lobular components (hepatocytes, sinusoidal cells, Kupffer cells) but present in vascular smooth muscle and interstitial cells (connective tissue areas). The biochemical procedures for detecting receptors, however, rely upon complete homogenization of liver samples. These homogenates will therefore surely contain hepatic lobular components as well as vascular smooth muscle and interstitial cells. Our data clearly contradict the discussed autoradiographic study and without doubt illustrated the even distribution of HEBP throughout the whole liver.

TABLE 2.2 : A SUMMARY OF SCATCHARD ANALYSES PERFORMED ON THE BINDING DATA OBTAINED FROM MULTIPOINT TITRATION EXPERIMENTS ON VERVET MONKEY HEPATIC CYTOSOLS PREPARED FROM TISSUE OF DIFFERENT AREAS IN THE SAME LIVER

Lobe	[a]Dissociation constant (K_d) (10^{-10}M)	[a]Oestrogen receptor (B_{max}) ([b]fm/mg cytosolic [c]protein)
1	3,4 ± 1,1 (6)	107,5 ± 38,7 (6)
2	3,6 ± 2,3 (6)	92,6 ± 21,0 (6)
3	2,5 ± 0,4 (6)	98,7 ± 51,3 (6)

[a] Mean value ± SEM and the number of repetitions are given in parenthesis.

[b] fm/mg = femtomol per mg.

[c] The protein concentration of cytosols was determined by the method of Bradford (152).

2.3.4 Oestradiol binding in liver cytosol prepared at different protein concentrations

Scatchard analyses were performed on the binding data obtained from multipoint titration experiments carried out on vervet monkey cytosol prepared at different protein concentrations. The results are summarized in Table 2.3. The relationship between the dissociation constant (K_d), binding capacity (B_{max}) and cytosol protein concentration is illustrated in Figure 2.5. An increase in K_d and B_{max} values is revealed with an increase in protein concentration up to 10 mg/mℓ. At higher concentrations the K_d value increased and the B_{max} value decreased. Non-specific binding proteins may possibly play an important role in the tendency observed. As a consequence of deviations from linearity and poor reproducibility of Scatchard data at too low or too high concentrations, it was decided to execute all assays at cytosol protein concentrations of approximately 5 mg/mℓ.

2.3.5 Oestradiol binding in cytosol prepared from liver obtained from male and female vervet monkeys

Scatchard analyses were performed on the binding data obtained from multipoint titration experiments carried out on hepatic cytosols from male and female vervet monkeys. The results are summarized in Table 2.4 and depicted in Figure 2.6. When the specific binding (SB) of the cytosols was measured, no significant statistical difference could be observed between male (92,3 $\pm$ 25,9 femtomol/mg cytosolic protein; n = 11) and female (90,6 $\pm$ 33,2 femtomol/mg cytosolic protein; n = 9) livers. Multipoint titration analyses clearly reveal that male hepatic cytosols contain two distinctive binding components: a high affinity binder (K_d-1 = 2,9 $\pm$ 0,9 x 10^{-10}M) with low capacity (B_{max} = 92,3 $\pm$ 25,9 femtomol/mg cytosolic protein; n = 11) and a low affinity binder (K_d-2 = 1,5 $\pm$ 0,8 x 10^{-9}M) with high capacity (B_{max} = 152 $\pm$ 31,2 femtomol/mg cytosolic protein;n = 6). No sex related

TABLE 2.3 : A SUMMARY OF SCATCHARD ANALYSES PERFORMED ON THE BINDING DATA OBTAINED FROM MULTIPOINT TITRATION EXPERIMENTS ON HEPATIC CYTOSOLS PREPARED AT DIFFERENT PROTEIN CONCENTRATIONS.

[a]Protein concentration (mg/mℓ)	[b]Dissociation constant (K_d) (10^{-10}M)	[b]Oestrogen receptor (B_{max}) ([c]fm/mg cytosolic [a]protein)
5	2,7 ± 1,4 (10)	80,9 ± 38,6 (10)
10	7,1 ± 2,3 (10)	94,0 ± 29,1 (10)
18	40,2 ± 33,0 (10)	84,7 ± 93,5 (10)

[a] The protein concentration of cytosols was determined by the method of Bradford (152).

[b] Mean value ± SEM and the number of repetitions are given in parenthesis.

[c] fm/mg = femtomol per mg.

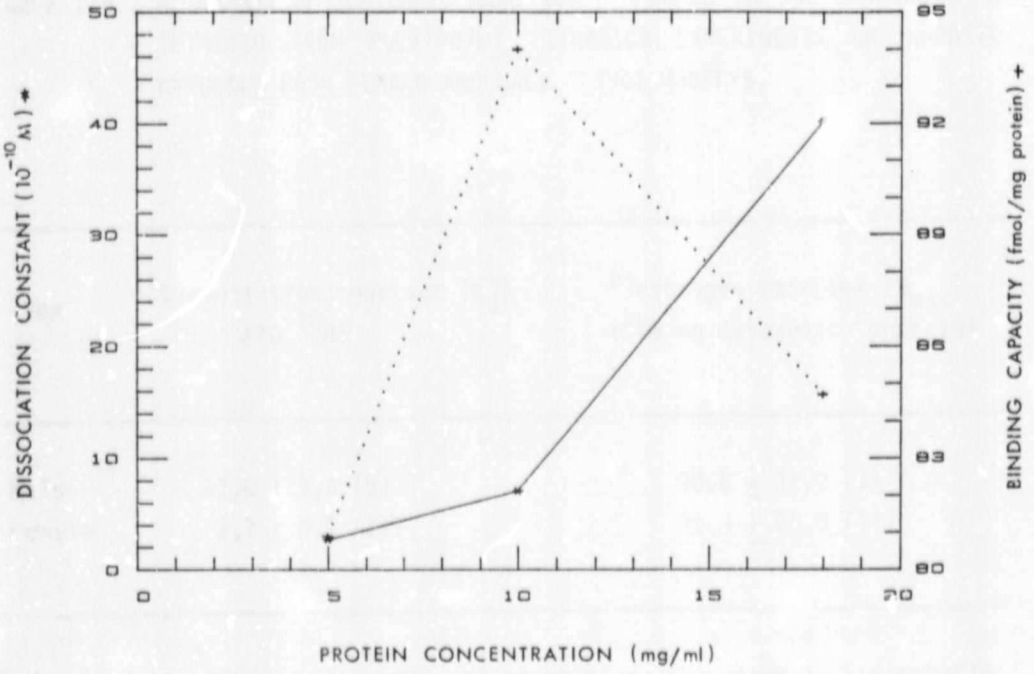


FIGURE 2.5

The relationship between the dissociation constant (K_d), binding capacity (B_{max}) and cytosol protein concentration. Multipoint titration experiments were performed on cytosols of increasing protein concentrations as described in Section 2.2.11. Scatchard analyses were performed on the binding data.

TABLE 2.4 : A SUMMARY OF SCATCHARD ANALYSES PERFORMED ON THE BINDING DATA OBTAINED FROM MULTIPOINT TITRATION EXPERIMENTS ON HEPATIC CYTOSOLS FROM FEMALE AND MALE VERVET MONKEYS.

Sex	[a]Dissociation constant (K_d) (10^{-10}M)	[a]Oestrogen receptor (B_{max}) ([b]fm/mg cytosolic [c]protein)
Male	3,0 ± 1,3 (9)	90,6 ± 33,2 (9)
Female	2,7 ± 0,9 (11)	92,3 ± 25,9 (11)

[a] Mean value ± SEM and the number of repetitions are given in parenthesis.

[b] fm/mg = femtomol per mg.

[c] The protein concentration of cytosols was determined by the method of Bradford (152).

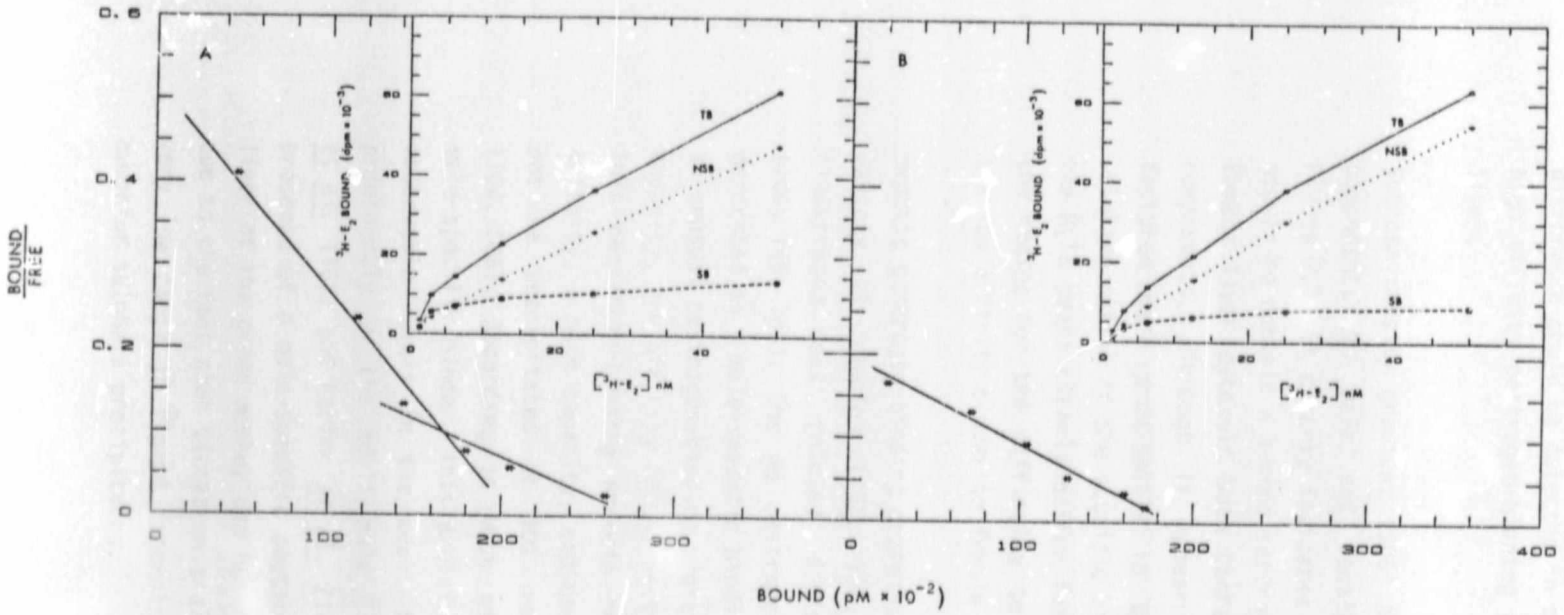


FIGURE 2.6

Scatchard and saturation curves for the HEBP from male (A) and female (B) vervet monkey. Aliquots (100 µℓ) of cytosol prepared in TEDAG buffer, were incubated with 17β-[^{3}H]-oestradiol in the absence (TB) or presence (NSB) of a 10^3-fold excess of DES at six different concentrations (0,78 - 50 nM); 16 h at 4°C. The reactions were terminated with the addition of DCC (500 µℓ) and aliquots (500 µℓ) of the 2 000 *g* supernatants monitored for TB and NSB.

difference could be detected in the B_{max} and K_d values of the high affinity oestrogen-binding protein in the vervet monkey liver.

Sucrose density gradient (SDG) profiles of oestrogen-binding components in male and female livers are depicted in Figure 2.7. It clearly indicates that the female livers also appear to contain a second oestrogen-binding protein. Male and female liver cytosols both contained 3,8S and 8,1S binding components, although it appeared that the 3,8S component featured more prominantly in the male monkey liver. The relative ratios of the specific binding in the 3,8S to that in the 8,1S peaks clearly differ for the two sexes. This may be the reason for the difficulty to illustrate the presence of the low affinity binder in female hepatic cytosols.

Hepatic oestrogen-binding proteins have been demonstrated in a variety of species (102, 103, 147). Some workers have illustrated sex related differences in some species (104, 146, 149). The 4S oestrogen-binding protein has been described as a male-specific binding protein (male-SBP) (104), an unusual oestrogen-binding protein (UEBP) (106) or as a high capacity, low affinity (HCLA) oestrogen-binding protein (112). This oestrogen-binding protein reportedly exhibits moderate affinity, a high capacity, sediments at 3-4S on SDG and can not be precipitated at 30% ammonium sulphate saturation (104, 106). According to most of the protagonists of the male-specific binder, this protein is present in very low if observable levels in the adult female liver, but features prominently in the adult male liver (104, 106, 112). Aten et al. (102) and Porter et al. (146) did not illustrate the presence of a male-specific oestrogen-binding protein in the liver of the green monkey and human respectively. This may be due to the fact that titration analyses were conducted with a very low range of ligand concentrations on redissolved 30% ammonium sulphate precipitates.

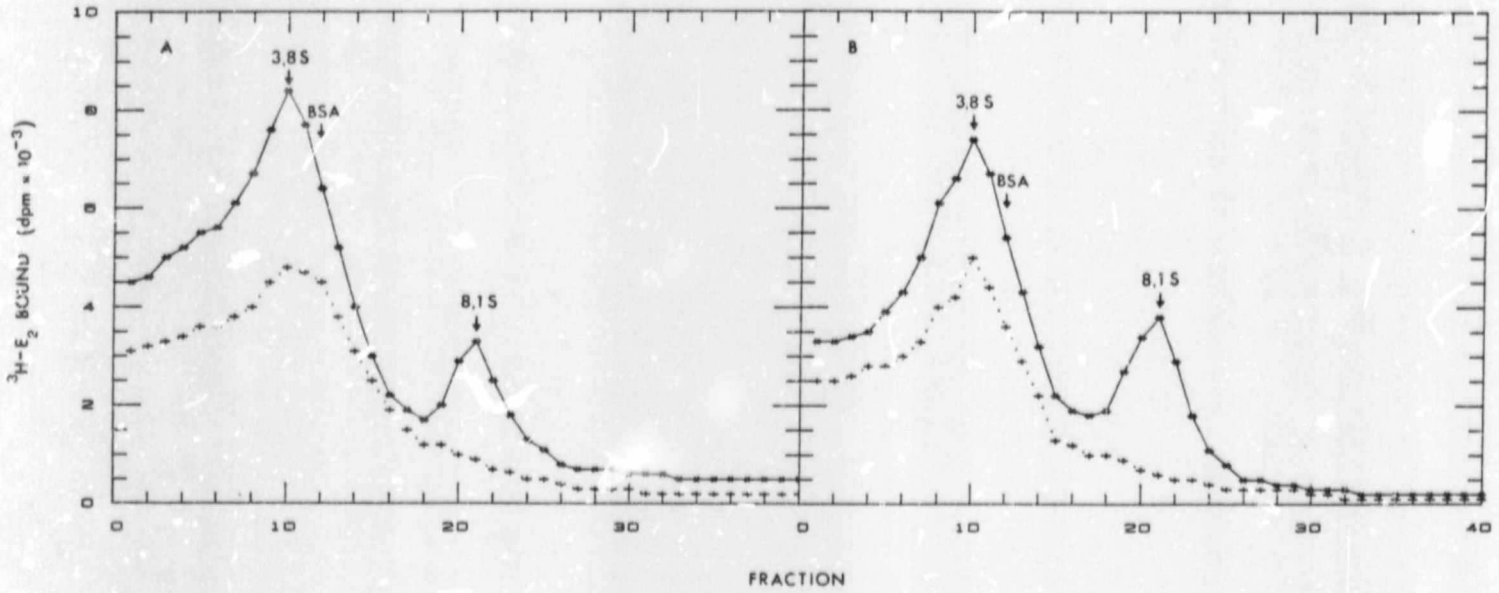


FIGURE 2.7

Sucrose density gradient (10-35%) profiles in low ionic strength buffer for the HEBP in male (A) and female (B) vervet monkey. Cytosols prepared in TEDAG buffer were incubated with 17β-[^{3}H]-oestradiol (10 nM) (●) and 17β-[^{3}H]-oestradiol (10 nM) plus a 10^3-fold excess of unlabelled DES (+) at 4°C for 16 h. Unbound ligand was removed by charcoal adsorption. Aliquots (200 µℓ) of cytosol were run in 10-35% SDG. Fractions were collected manually and monitored for TB and NSB. With each run a duplicate set of gradients with ^{14}C-methylated BSA was included as a marker. The position of the ^{14}C-labelled marker protein (BSA 4,6 S) is indicated by an arrow.

The observed levels of vervet monkey hepatic oestrogen-binding proteins in male and female animals appear to be higher than most reported levels (102, 104, 146, 150). It would be inappropriate to suggest that the low affinity oestrogen-binding protein observed in the male monkey liver is identical to the male-SBP, the UEBP or the HCLA oestrogen-binding protein, reported to be present in other species. Our data clearly shows that this binder is, however, not an exclusive property of the adult male liver.

2.3.6 Oestradiol binding in liver cytosol in the presence or absence of sodium molybdate and hydrocortisone

An illustration of the effect of sodium molybdate and hydrocortisone on oestradiol-binding in hepatic cytosols from the vervet monkey is given in Table 2.5. When hepatic cytosols were prepared in the presence of 10 mM sodium molybdate (TEDAGM), the levels of oestrogen-binding proteins were significantly lower ($p = 0,05$) compared to that of cytosols prepared in the absence of sodium molybdate (TEDAG). SDG profiles (Figure 2.8) indicated that the 8,1S binding component was stabilized in the presence of sodium molybdate while the 3,8S binder diminished significantly. No sex related differences were observed in the effect of molybdate on the levels and SDG profiles of the hepatic oestrogen-binding protein.

Over the last decade a considerable amount of research effort has gone into studies regarding the role of the molybdate oxyanion in the stabilization of steroid hormone receptors (155 – 157). The effect that this anion has on the molecular properties of the vervet monkey uterine receptor was previously investigated (158) as well as the reversibility of the molybdate effect (159). In these reports it is postulated that the interaction of the molybdate oxyanion with the receptor molecule might cause a positive co-operative effect

TABLE 2.5 : A SUMMARY OF THE EFFECT OF SODIUM MOLYBDATE (SM) AND HYDROCORTISONE (HC) ON OESTRADIOL BINDING IN HEPATIC CYTOSOLS FROM MALE AND FEMALE VERVET MONKEYS.

Buffer SM	Buffer HC	[a]Dissociation constant (K_d) (10^{-10}M)	[a]Oestrogen receptor (B_{max}) ([b]fm/mg cytosolic [c]protein)
-	-	2,8 ± 0,9 (10)	47,1 ± 22,1 (10)
+	-	3,0 ± 1,0 (7)	[d]24,7 ± 7,1 (7)
-	+	2,9 ± 0,6 (9)	44,5 ± 19,9 (9)

[a] Mean value ± SEM and the number of repetitions are given in parenthesis.

[b] fm/mg = femtomol per mg.

[c] The protein concentration of cytosols was determined by the method of Bradford (152).

[d] Significant difference ($p = 0,05$).

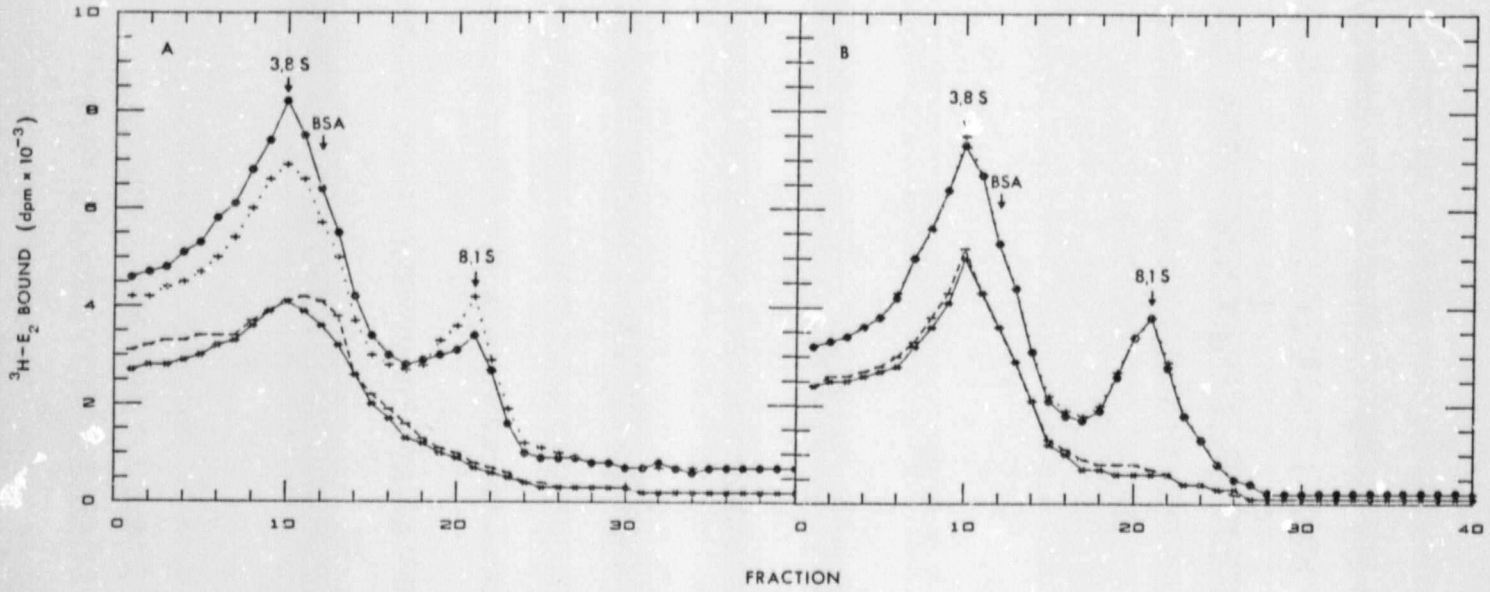


FIGURE 2.8

Sucrose density gradient (10-35%) profiles in low ionic strength buffer for the vervet monkey HEBP to show the effect of sodium molybdate (A) and hydrocortisone (B). Cytosols were prepared in TEDAG buffer either containing (+ , —) or not containing (● , ✱) sodium molybdate (A) and hydrocortisone (B) respectively. The cytosols were incubated with 17β-[^{3}H]-oestradiol (10 nM) (+ , ●) and 17β-[^{3}H]-oestradiol plus a 10^3-fold excess of DES (— , ✱) at 4°C for 16 h. Unbound ligand was removed by charcoal adsorption. Aliquots (200 µℓ) of cytosol were run in 10-35% SDG. Fractions were collected manually and monitored for TB and NSB. With each run a duplicate set of gradients with ^{14}C-methylated BSA was included as a marker. The position of the ^{14}C-labelled marker protein (BSA 4,6 S) is indicated by an arrow.

on the binding of oestradiol. In the case of the hepatic oestrogen-binding proteins the opposite effect was observed.

Cytosols, prepared in the presence of sodium molybdate, consistently displayed suppressed levels of tritiated oestradiol-binding (52,4% of non-molybdate controls) when compared to binding of this ligand by cytosols prepared in the absence of sodium molybdate. Whether this might be an example of negative co-operative binding caused by conformational changes in the oestrogen-binding proteins is an aspect that needs to be investigated. It might also be possible to conclude that differences in the molecular properties of the hepatic and uterine oestrogen-binding proteins might be the cause of this discrepancy. The exact function of the hepatic oestrogen-binding proteins is at present too vague t justify any speculation on the possible nature of differenc in the molecular structures of the hepatic and uterine oestrogen receptors. On SDG it would appear that the presence of molybdate effected a decrease in the 3,8S binding component (32%), while some apparent stabilization of the 8,1S component could be observed (Figure 2.8).

It is a known fact that liver tissue contains fairly high levels of the corticosteroid binding globulin (CBG) (160) and that oestrogen can bind to this steroid-binding protein (161). To make absolutely sure that high levels of CBG in the hepatic cytosols did not contribute to oestrogen-binding observed, assays were executed in the presence of a large excess (5 000-fold) of hydrocortisone. Results, summarized in Table 2.5 showed no contribution of CBG to oestrogen-binding in the hepatic cytosols. This finding was strongly corroborated by the SDG profiles demonstrated in Figure 2.8. No sex related differences were observed in the effect of hydrocortisone on the levels and SDG profiles of oestrogen-binding proteins in hepatic cytosols.

2.3.7 Gel permeation chromatagraphy

Gel permeation chromatography (GPC) of HEBP from male and female vervet monkeys was performed in either low or high ionic strength mobile phases. Cytosol was prepared in the presence or absence of molybdate. Columns were calibrated with a set of proteins of known molecular mass (Section 2.2.15). A typical calibration curve using high ionic strength conditions is illustrated in Figure 2.4. Calibration curves did not appear to be affected by the ionic strengtn of the mobile phase. Table 2.6 summarizes the results obtained for the GPC of hepatic oestrogen-binding proteins (male and female) under conditions described in Section 2.2.15. In Figures 2.9 and 2.10 representative gel permeation profiles of HEBPs from male and female liver are respectively illustrated. During GPC of male and female oestrogen-binding proteins (in the presence or absence of sodium molybdate) there was an overall decrease (27%) in the total specific binding under high ionic strength conditions. Two specific binding components were observed: a 4×10^5 (SB1) and a $4{,}2 \times 10^4$ (SB2) dalton component. The ratio of SB1 to SB2 was approximately 85% to 15% in the presence of 0,15 M potassium chloride but shifted to 56% to 44% in the presence of 0,4 M potassium chloride respectively. It therefore appears as if high ionic strength conditions tend to favour SB2. The only sex related difference observed was an increase in the level of SB2 of the female relative to that of the male under high ionic strength conditions. The peak area of the female SB2 component appeared to be double that of the male SB2. Sodium molybdate appeared to have no effect on GPC profiles of HEBP in cytosol. Gel permeation chromatography performed in a low ionic strength mobile phase appears to favour the formation of receptor aggregates ($\geq 4{,}0 \times 10^5$ daltons). The oestrogen-binding protein ($4{,}2 \times 10^4$ daltons), under high ionic strength conditions, appeared to display a lower molecular mass value than the values previously reported in the literature (162-164). This decreased size of the steroid-binding entity might be the

TABLE 2.6 : A SUMMARY OF THE RESULTS OBTAINED IN GEL PERMEATION CHROMATOGRAPHY OF MALE AND FEMALE HEPATIC OESTROGEN BINDING PROTEINS UNDER DIFFERENT CONDITIONS.

Sample buffer	Mobile phase (M KCl)	Molecular mass (% of total specific binding)	
Male hepatic oestrogen binding proteins			
TEDAG	PEDGP (0,15)	400 000 (80,7)	42 000 (19,3)
TEDAG	PEDGP (0,40)	400 000 (62,7)	42 000 (37,3)
TEDAGM	PEDGP (0,15)	400 000 (84,7)	42 000 (15,3)
TEDAGM	PEDGP (0,40)	400 000 (58,6)	42 000 (41,4)
Female hepatic oestrogen binding proteins			
TEDAG	PEDGP (0,15)	400 000 (87,8)	42 000 (12,2)
TEDAG	PEDGP (0,40)	400 000 (52,8)	42 000 (47,2)
TEDAGM	PEDGP (0,15)	400 000 (85,2)	42 000 (14,8)
TEDAGM	PEDGP (0,40)	400 000 (51,6)	42 000 (48,4)

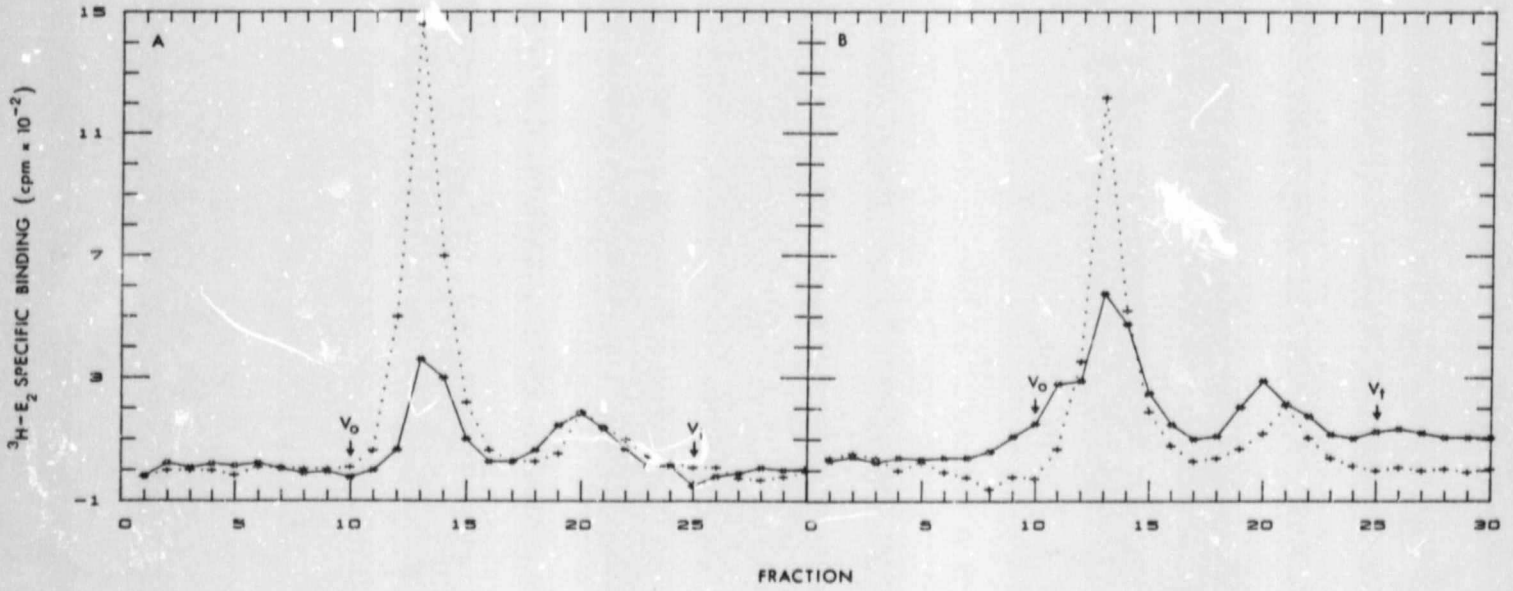


FIGURE 2.9

Gel permeation chromatography of male HEBPs in cytosol prepared in the presence (A) or absence (B) of sodium molybdate. The chromatography was executed under low (···+···) and high (—*—) ionic strength conditions as described in Section 2.2.15.

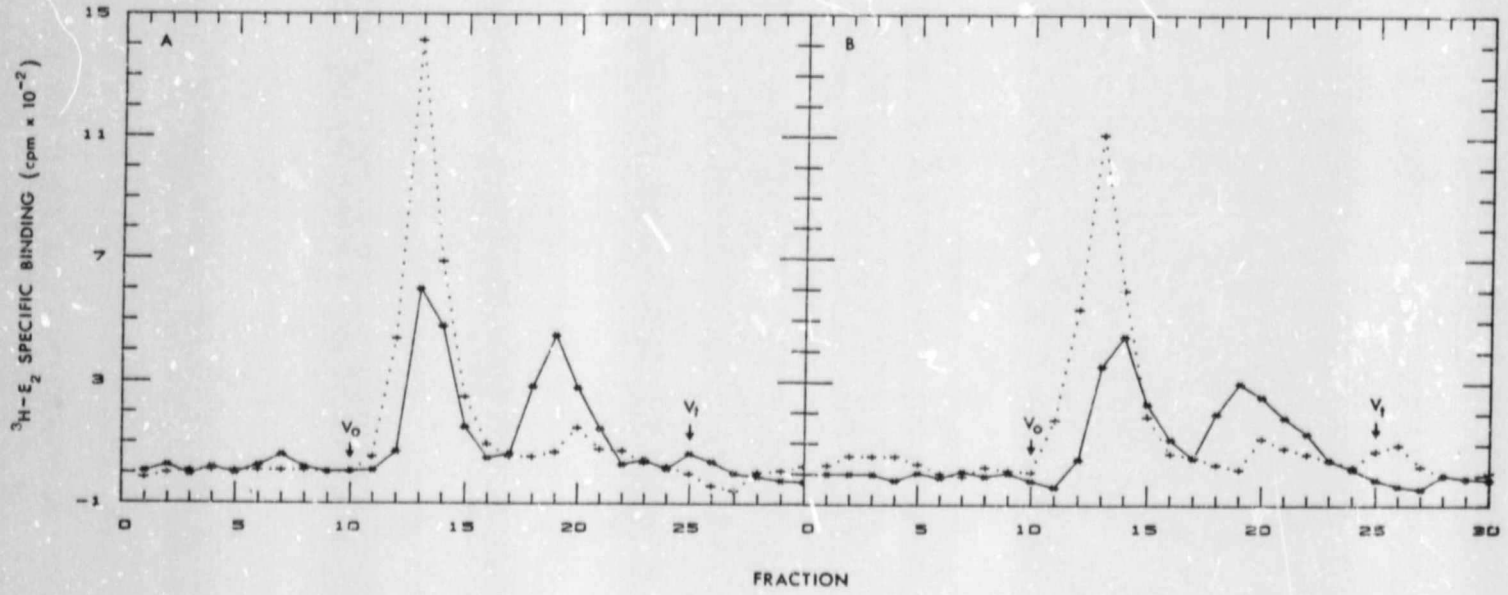


FIGURE 2.10

Gel permeation chromatography of female HEBPs in cytosol prepared in the presence (A) or absence (B) of sodium molybdate. The chromatography was executed under low (···+···) and high (—*—) ionic strength conditions as described in Section 2.2.15.

result of protease activity. Hendry and Danzo (65) reported on a receptor-active protease from the rabbit epididymis capable of decreasing the steroid-binding monomer (104 000 M_r) to a 42 000 M_r entity. Considering the fact that GPC was executed on cytosol prepared from the liver (the main protein production organ), protease activity can't be ignored.

2.4 SUMMARY

Hepatic cytosols from male and female vervet monkeys appeared to contain the same levels of high affinity oestrogen-binding proteins. Multipoint saturation analyses revealed that male liver cytosol contain two binding components (high affinity, low capacity and high capacity, low affinity), while SDG analyses established the presence of two binding components (3,8S and 8,1S) in both sexes. When sodium molybdate was included during cytosol preparation, a 50% lower level of specific oestrogen-binding was observed. SDG analyses indicated, however, that the 8,1S component was stabilized at the cost of the 3,8S component in the presence of sodium molybdate. Hydrocortisone did not have any effect on specific oestrogen binding in hepatic cytosols. GPC demonstrated oestrogen-binding components with molecular masses of 400 000 and 42 000 dalton in vervet monkey hepatic cytosol.

CHAPTER 3

PARTIAL PURIFICATION OF THE VERVET MONKEY HEPATIC OESTROGEN-BINDING PROTEIN

3.1 INTRODUCTION

The elucidation of the action mechanism of the ER in target tissues has been hindered by the need for a pure receptor. Early attempts to purify the ER from target tissues include standard methods such as ammonium sulphate precipitation, gel filtration, ion exchange chromatography and preparative isoelectric focussing (162, 165, 166). However, only modest purification has been achieved and the yields have been very poor (162, 165).

Biospecific adsorbents have been used extensively for the purification of ERs (167-171). Affinity chromatography allows high purification in a single step and is therefore suitable for biological samples containing very low concentrations of the macromolecule of interest. Reversible ligand-receptor interaction and specific stereochemical binding of high affinity are characteristics ideally suited for affinity chromatography and are distinctive of the ER. Highly purified ER have been isolated by affinity chromatography from the target tissues of various species (167, 169, 172). Affinity chromatography is, however, not without technical difficulties. Ligands linked to the supporting matrix by spacer arms containing ester or amide groups are susceptible to cleavage by hydrolytic enzymes (170). Therefore, stable spacer arms should be used to connect ligand and matrix in order to obtain a reliable steroid affinity adsorbent (173). The length of the spacer arm is another aspect influencing the efficiency of the affinity matrix. Greene and Press (164) have recently shown that a longer

linker arm gives rise to less non-specific adsorption of proteins to the adsorbent. Steroid ligand adsorbs non-covalently to the matrix during preparation and the affinity matrix should therefore be adequately washed before utilization (173). The insoluble support of the ligand has a relatively high ion-exchange capacity, which results in a considerable retention of non-specific proteins along with receptor (174). Moreover, only a fraction of the immobilized ligands may be accessible for binding to receptor molecules, due to the geometry of distribution of residues within the adsorbent (170). Finally, steroid-affinity chromatography is limited by the resistance of the bound receptor to elution under conditions compatible with its stability (164). This problem can be solved by including chaotropic salts and N,N-dimethylformamide together with oestradiol in the elution medium (175).

The unstable nature of the oestrogen receptor and the extremely low concentration present in the liver made purification of this macromolecule a troublesome task. Furthermore the oestrogen receptor has under various conditions, different molecular forms, particularly almost irreversible aggregates (176). Another problem to deal with is the decreased stability of the oestrogen receptor when it is highly purified (174).

Different groups have reported the use of an additional purification method in combination with affinity chromatography (165, 176, 177). Methods employed vary from ammonium sulphate precipitation and gel filtration to heparin-Sepharose chromatography. The latter appears to be the most popular because heparin-Sepharose reacts with complexed as well as uncomplexed receptor and adsorbed receptor can be recovered in good yield (177).

In this chapter the methods investigated for the partial purification of the vervet monkey HEBP are described. Some of

the molecular properties of the partially purified HEBP are discussed.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Some of the reagents employed for this part of the investigation were identical to those described in Chapter 2, Section 2.2.1. Additional reagents used were obtained from the following sources.

[Monoethyl-^{3}H] diethylstilboestrol, with specific activity (S.A.) of 89,5 Ci/mmol, was purchased from Amersham, England. The purity of radioactive steroids was monitored for radiochemical purity by reverse phase high performance liquid chromatography (HPLC). Heparin-Sepharose, Epoxy-activated Sepharose and Sephadex G-25 were supplied by Pharmacia Fine Chemicals. Heparin used for elution of receptor adsorbed on heparin-Sepharose was a product of Sigma Chemical Company (grade I, S.A. 170 USP units/mg). Trisacryl GF 2000 and DL-1,2: 3,4 diepoxybutane were obtained from LKB and Merck Chemicals respectively. Coomassie brilliant blue G were bought from Sigma Chemical Company. Molecular mass marker proteins, IEF marker proteins (high pI calibration kit), Pharmalyte carrier ampholytes (pH 5 - 8) and Agarose (IEF grade) were purchased from Pharmacia Fine Chemicals. All other chemicals for polyacrylamide gels, buffers, fixation and staining were supplied by Merck Chemicals.

3.2.2 Buffers

TEDAG and TEDAGM buffers were prepared as described in Chapter 2, Section 2.2.2.

TEM buffer: Tris-HCl (10 mM), EDTA (1,5 mM), sodium molybdate (10 mM), pH 7,4 (4°C).

TEDMg buffer: Tris-HCl (10 mM), EDTA (1,5 mM), dithiothreitol (1 mM), magnesium chloride (5 mM), pH 7,4 (4°C).

TEDMgM buffer: Prepared as TEDMg buffer with the addition of sodium molybdate (10 mM).

3.2.3 Dextran coated charcoal

This suspension was prepared as described in Chapter 2, Section 2.2.3.

3.2.4 Liver tissue

Liver tissue was obtained and treated as reported in Chapter 2, Section 2.2.5.

3.2.5 Preparation of cytosol

Cytosol was prepared as explained in Chapter 2, Section 2.2.6 with the exception that one unit mass of liver tissue was homogenized in two volumes of pre-cooled buffer.

3.2.6 Preparation of nuclear extract

Vervet monkey liver was homogenized with a TP 10 Ultra Turrax (Janke and Kunkel) in five volumes of TEDAG buffer containing oestradiol (30 nM) and phenylmethylsulfonylfluoride (1 mM) (Sigma Chemical Company). The homogenate was centrifuged, directly or after an incubation period (15 min., 30°C) at 800 *g* for 10 min. at 4°C. The supernatant was removed and the crude nuclear preparation centrifuged at 105 000 *g* for 30 min. at 4°C. The crude nuclear pellet was washed three times with TEDAG buffer by resuspension, vortexing and centrifugation at 800 *g* for 10 min. at 4°C. The crude nuclear extract was prepared through extraction of the nuclear pellet by way of TEDAG buffer containing potassium chloride (KCl, 0,4M) and potassium thiocyanate (KSCN, 0,1M). Alternatively, the nuclear

pellet was extracted with the KCl-KSCN solution, containing deoxyribonuclease I (DNase I; 50 µg/350 µg DNA). DNA content was assayed according to the method of Burton (178). The supernatant and resulting extractions were assayed for oestrogen-binding activity. Results appear in Section 3.3.1. A control experiment was executed by homogenizing the liver tissue in the absence of oestradiol.

3.2.7 Diethylstilboestrol affinity adsorbents

a) Preparation of DES-Agarose

Affinity adsorbent DES-Agarose was synthesized as described by Van Oosbree et al. (173) with the following modifications. The DES (50 mg), used in the preparation of the affinity adsorbent, was dissolved in N,N-dimethylformamide (DMF, 5 mℓ). The pH of a 5 mℓ volume of distilled water was adjusted to 10 with KOH. The aqueous alkaline solvent was added to the DES solution and the pH re-adjusted to 10 at room temperature. The resultant solution was then added to a suspension of epoxy-activated Sepharose in 5 mℓ. In order to obtain high levels of binding of DES to the Agarose, coupling reactions were performed at 37°C for 18 h with gentle agitation. The Agarose was subsequently filtered off and washed with methanol (200 mℓ) and distilled water (200 mℓ). A 50% aqueous ethyleneglycol solution (pH 10) was employed to block the remaining activated sites on the agarose (18 h, 37°C). Finally the DES-Agarose was filtered off and washed with distilled water (100 mℓ), DMF (200 mℓ), 80% methanol (2 ℓ) and finally again with distilled water (1 ℓ) prior to storing it in TEDAG buffer at 4°C. A flow diagram of the preparation of the affinity matrix is depicted in Scheme 3.1.

SCHEME 3.1: SYNTHESIS OF DES-SEPHAROSE 6B

Epoxy-activated Sepharose 6B (5 mℓ)

↓

DES immobilization

DMF (5 mℓ), d.H_2O (5 mℓ), pH 10

DES (50 mg)

↓ 37°C, 18h

Wash: methanol (200 mℓ)

d.H_2O (200 mℓ)

Blocking

50% aqueous ethyleneglycol, pH 10

↓ 37°C, 18h

Wash: d.H_2O (100 mℓ)

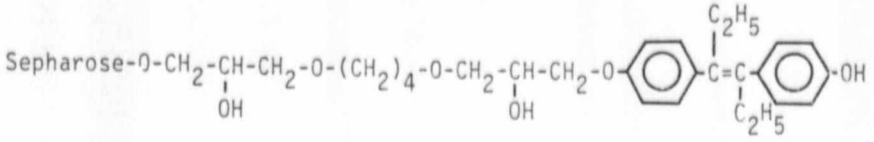
DMF (200 mℓ)

80% methanol (2 ℓ)

d.H_2O (1 ℓ)

↓

DES-Sepharose 6B



b) Preparation of DES-Trisacryl

DES-Trisacryl was prepared according to the LKB practical guide (179). Trisacryl GF 2000 (20 mℓ of the aqueous suspension of 300 mℓ gel supplied by LKB) was washed with distilled water (70 mℓ), suspended in 12 mℓ distilled water and agitated. DL-1,2: 3,4-diepoxybutane (2 mℓ) and 6 mℓ carbonate buffer (0,5 M, pH 10) containing sodium borohydride were added to the suspension. The reaction mixture was agitated for 18 h at room temperature (20 - 25°C) and washed with distilled water (500 mℓ). Following the resuspension of the gel in 10 mℓ 50% dioxane: 50% distilled water (pH 8,5 - 11) containing DES (0,3 g) it was agitated for 18 h at room temperature. The gel was then filtered off and washed with 50% aqueous dioxane (500 mℓ) until the excess ligand was eliminated. Remaining oxirane groups were blocked by treatment of the gel with 18 mℓ carbonate buffer (pH 7,8 - 8,5) containing 0,1 M ethanolamine for 3 h at 4°C. The affinity gel was finally washed and stored in TEDAG buffer at 4°C. A flow diagram of the synthesis of the affinity matrix is depicted in Scheme 3.2.

c) Determination of DES immobilized onto Agarose/Trisacryl

[Monoethyl-^{3}H] diethylstilboestrol (S.A. 89,5 Ci/mmol) was used to determine the quantity of DES covalently coupled to Agarose. The synthesis of DES-Agarose was performed as outlined in Section 3.2.7. a) except that 100 µCi of tritiated DES was added to the 50 mg of unlabelled DES. The quantity of tritiated DES immobilized onto the Agarose was calculated by monitoring the radioactivity in an aliquot (5 µℓ) of finally prepared affinity gel. In a standard preparation 2,76 µmol of DES was covalently bound per millilitre of agarose. The amount of DES bound to activated Trisacryl was calculated from the decrease in absorbance spectra (225 nm - 375 nm) of the ligand solution after reaction with the gel. From these spectra

SCHEME 3.2: SYNTHESIS OF DES-TRISACRYL GF 2000

Trisacryl GF 2000 (20 mℓ)

↓

Gel activation

d.H_2O (12 mℓ)
DL-1,2:3,4 diepoxybutane (2 mℓ)
Carbonate buffer, pH 10 (6 mℓ)
Sodium borohydride (40 mg)

↓ 20-25°C, 18h
Wash: d.H_2O (500 mℓ)

DES immobilization

50% aqueous dioxane (1 vol)
DES (0,3 g)

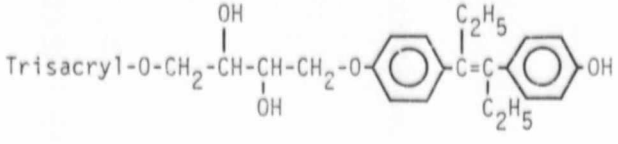
↓ 20-25°C, 18h
Wash: aqueous dioxane (500 mℓ)

Blocking

Carbonate buffer, pH 8
Ethanolamine (0,1 M)

↓ 4°C, 3h
Wash: d.H_2O (100 mℓ)
DMF (200 mℓ)
80% methanol (2 ℓ)
d.H_2O (1 ℓ)

DES-Trisacryl GF 2000



it could be calculated that 2,20 µmol of DES was bound per millilitre of activated Trisacryl.

d) Interaction of HEBP with DES affinity adsorbents

Hepatic cytosol prepared as described in Section 3.2.5 was incubated with increasing concentrations of DES affinity matrices for 60 min. at 4°C. After the suspension was centrifuged (2 000 *g*, 15 min.) the residual oestradiol binding activity in the supernatants was determined as outlined in Section 3.2.9.

e) Regeneration of DES affinity adsorbents

Following an affinity chromatography experiment, any remaining proteins on the affinity adsorbent were removed by washing the beads successively with SDS (2%), methanol (80%), distilled water and TEDAG buffer. The affinity matrix appeared to be relatively stable, with no loss in HEBP binding capacity after four successive experiments.

3.2.8 Partial purification of the HEBP

a) Ammonium sulphate precipitation

Finely powdered ammonium sulphate was slowly added to four cytosol preparations over a period of one hour during continuous magnetic stirring to obtain final saturation levels of 20%, 30%, 40% and 50% respectively. The suspensions were then centrifuged at 10 000 *g* for 20 min., the supernatant decanted and the precipitates redissolved in TEDAG buffer to one-half of the original volume. The resulting ammonium sulphate fractions were assayed for protein concentration and specific oestradiol binding activity as described in Sections 3.2.10 and 3.2.9.

b) Heparin-Sepharose chromatography

Cytosol prepared in TEM buffer was incubated (60 min., 4°C) with an equal volume of tritiated oestradiol (5 nM) in the presence and absence of a 1 000-fold excess of unlabelled DES. The unbound ligand was removed by the addition of DCC. The suspension was vortexed, incubated for 15 min. and centrifuged at 2 000 *g* for 15 min. Magnesium chloride and dithiothreitol were added to the 2 000 *g* supernatant to a final concentration of 5 mM and 1 mM respectively. Heparin-Sepharose was added to the resultant solution (0,75: 1, v/v) and incubated (1 h, 0°C) with gentle agitation. The suspension was packed into a Econo-column (Bio-rad Laboratories) and washed with 10 column volumes of TEDMgM buffer. Adsorbed protein was eluted with 10 column volumes of TEDMgM buffer containing either NaSCN (1 M) or KCℓ (0,5 M). Radioactivity of the collected fractions (0,3 mℓ) were determined with a liquid scintillation counter. The partially purified oestrogen-binding protein was subjected to hypertonic (0,5 M KCℓ) sucrose density gradient centrifugation (Section 2.2.14).

c) DES affinity chromatography

(i) Evaluation of procedures

Two types of HEBP preparations were subjected to affinity chromatography: (i) cytosol prepared in TEDAG/TEDAGM buffer in the presence or absence of 0,5 M KCℓ or (ii) the eluate from heparin-Sepharose chromatography, employed as a prepurification step prior to chromatography on the affinity matrix. The HEBP preparations were loaded onto the affinity gel (DES-Agarose or DES-Trisacryl) in a column (5 mℓ/h, 4°C) or batchwise (18 h, 4°C). The adsorbent was subsequently transferred to a sintered glass funnel and washed with 100 gel volumes of TEDAG/TEDAGM

buffer containing 0,5 M KCℓ and 50 gel volumes of TEDAG/TEDAGM buffer until the absorbance (280 nm) reached a constantly low level. To determine the amount of HEBP bound to the affinity matrix, an aliquot of cytosol before and after incubation with the affinity matrix was assayed for protein and oestradiol binding activity. Finally the adsorbent was incubated with an equal volume of eluting buffer containing unlabelled oestradiol (5 µm - 80 µM) in the presence or absence of NaSCN (0,5M) at 20°C for 30 min. Additional procedures were examined following affinity chromatography in order to eliminate the large excess of oestradiol and NaSCN employed in the specific elution of HEBP in the previous isolation step and to concentrate the partial purified HEBP. Procedures investigated were: heparin-Sepharose chromatography, gel filtration (PD - 10 Pharmacia column prepacked with Sephadex G - 25M), DCC treatment and sample concentration by using the Minicon-B sample concentrator (Amicon Corporation).

(ii) Optimum method of choice

The following procedure was selected to render optimum levels of HEBP: Liver tissue was pulverized and homogenized in TEDAG buffer containing 0,5 M KCℓ (two volumes of buffer per unit mass of tissue). The suspension was homogenized (TP 10 Ultra Turrax, Janke and Kunkel), followed by centrifugation at 105 000 *g* for 30 min. The 105 000 *g* supernatant fraction was added to the affinity resin (DES-Agarose) (maximum volume cytosol per gram affinity matrix = 20 mℓ). In general each millilitre of the adsorbent contained 2,76 µmol of DES and was able to bind at least 80% of the receptor present in the preparation. The reaction suspension was gently

agitated for 16 h at 4°C. It was then transferred to a sintered glass funnel and filtered. The amount of receptor bound to the affinity gel was determined by recording the quantity of receptor in the cytosol before and after incubation with the affinity matrix. The adsorbent was filterd and washed with 100 mℓ TEDAG (0,5 M) and 50 mℓ TEDAG buffer per gram adsorbent over a period of 30 min. The affinity adsorbent was gently stirred with a thin glass rod during the washing procedure. Following the removal of the affinity matrix residue from the sintered glass funnel it was suspended in TEDAG buffer, containing oestradiol (50 µM, S.A. 15 Ci/mol). The oestrogen-binding protein was then eluted twice from the affinity matrix by batch incubation (15 min) in the presence of a large excess of oestradiol (50 µM) at 25°C with intermittant stirring. One mℓ of elutant was used per gram resin. The affinity matrix was washed with 2 mℓ TEDAG buffer (25°C) per gram resin and the eluate passed over a heparin-Sepharose column at a flow rate of 4 mℓ/h. The heparin-Sepharose was rinsed with TEDAG until the absorbance (280 nm) or the radioactivity reached a constantly low level. Finally the matrix was removed from the column and suspended in TEDAG buffer containing heparin (4 mg/mℓ) and KCℓ (0,5 M). The HEBP was eluted from the heparin-Sepharose column material by batch incubation for 30 min. at 4°C. In order to aid identification of the HEBP, affinity isolations were performed in the presence of 5 µM unlabelled oestradiol added to the cytosol prior to incubation with the affinity matrix.

3.2.9 Assay of specific oestradiol-binding activity

a) Direct assay

The direct determination of available binding sites were performed following the incubation of uncomplexed HEBP preparations with an equal volume of TED/ containing tritiated oestradiol (30 nM) for 16 h at 4°C. Non-specific binding was measured by competitive incubation of similar samples in the presence of a 1 000-fold excess of unlabelled DES. Bound and free oestradiol was separated with DCC, Chapter 2, Section 2.2.8.

b) Exchange assay

Purification of the HEBP by affinity chromatography implied dilution in the presence of high excess levels of unlabelled oestradiol which saturates the binding sites on the high affinity binding proteins. In order to measure the binding sites, it was essential to remove unbound ligand by means of heparin-Sepharose chromatography, gel filtration or DCC treatment. The preparation was then incubated with saturating quantities of tritiated oestradiol (30 nM) (30 min., 20°C) to exchange the bound hormone for radioactive hormone. The assay was completed as described in Chapter 2, Section 2.2.8.

3.2.10 Protein determination

The protein concentration of cytosol was determined as described in Chapter 2, Section 2.2.7. Protein concentrations of samples containing microgram quantities were analysed by means of the Bio-rad microassay procedure using bovine serum albumin as standard. Samples and standards were analysed in

duplicate at the same time. A standard curve for the microassay procedure is depicted in Figure 3.1.

3.2.11 Isoelectric focussing

a) Preparation of agarose gels

Agarose (IEF grade) and sorbitol were dissolved by boiling in distilled water to give a final concentration of 1% and 12% (w/v) respectively. The solution was cooled down to 75°C and the Pharmalyte carrier ampholyte (pH 5 - 8) added to a final dilution of 1 in 16. The gel solution (33,7 mℓ) was poured onto GelBond film (Pharmacia Fine Chemicals) into a gel casting frame (114 x 225 mm) resting on a horizontally leveled plate. Gels used were 1 mm thick throughout. After casting, gels were stored in a humid chamber for 16 h at 4°C.

b) Application of proteins and focussing

The anode and cathode electrodes were soaked in 0,05 M H_2SO_4 and 1 M NaOH, respectively. A multiphore electrophoresis apparatus was employed and thermoelectric cooling was set at 10°C. To obtain optimal results gels were prefocussed under the following conditions: 1 500 V, 50 mA and 4 W for 30 min. After prefocussing the samples and IEF marker proteins were applied 1,5 - 2 cm from the cathode into slots of a polyester sample application template. IEF was conducted under prefocussing conditions for two hours. The power was increased gradually to a maximum setting of 17 W during the third hour.

Gels that had to be analysed for radioactivity were cut into slices (1 mm) immediately after focussing. The slices were dissolved in 2 mℓ of scintillation cocktail and radioactivity was measured after 16 h in a scintillation counter (Beckman LS 5800).

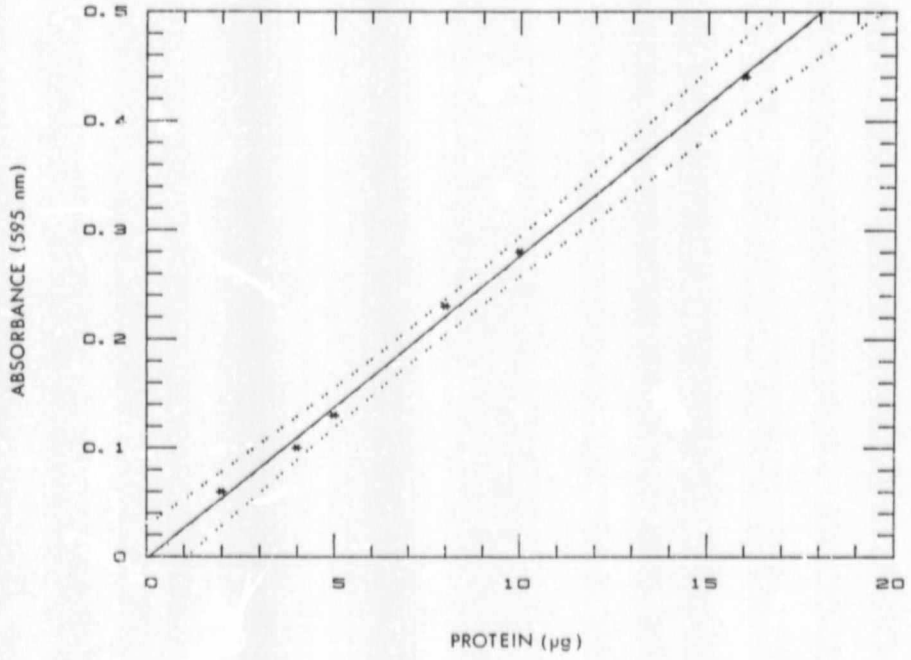


FIGURE 3.1

A typical standard curve for the determination of microgram quantities of protein. The Bio-rad microassay procedure, according to the method of Bradford (152), was employed. Bovine serum albumin (BSA) was used as protein standard. The 99% confidence limits are indicated by the dotted lines.

c) Fixing of proteins

Once the gel was focussed it was fixed for 30 min. in a fixing solution containing 10% trichloroacetic acid (w/v), 5% sulfosalicylic acid (w/v) and distilled water. The gel was then soaked in an aqueous solution of 35% ethanol (v/v) and 10% acetic acid (v/v) for 30 min., with one change of fresh solution after 15 min. The gel was dried by covering it with three layers of filter paper, a glass plate and a weight of about 1 kg. After 10 min. the gel was dried completely with a hairdryer.

d) Staining of proteins with Coomassie blue

An aqueous solution of 0,2% (w/v) Coomassie brilliant blue G was prepared in 35% ethanol (v/v) and 10% acetic acid (v/v). The dried gel was stained for 1 - 2 h in this solution. Destaining was done in the same solution excluding the Coomassie brilliant blue G until the background became clear.

e) Staining of proteins with silver

Silver staining was executed according to Budowle (180). The dried gel was washed once in a 4% glutaraldehyde (v/v) solution and twice in distilled water for 5 min. respectively. Two solutions were used for the silver staining. Solution A contained 25 g of anhydrous sodium carbonate dissolved in 500 mℓ distilled water. Solution B contained 1 g ammonium nitrate, 1 g silver nitrate, 5 g tungstosilicic acid and 7 mℓ 37% formaldehyde in 500 mℓ distilled water. Solution B was added to solution A in a ratio of 68 to 32 by volume just before use. The washed gel was put into this mixture and stained for 15 min. The staining was stopped by transferring the gel to a 10% solution of acetic acid.

3.2.12 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

a) PhastGel gradient media

SDS-PAGE was executed on a PhastSystem employing a PhastGel Gradient 10 - 15 and PhastGel SDS Buffer Strips supplied by Pharmacia Fine Chemicals (181, 182). The PhastGel Gradient 10 - 15 had a 13 mm stacking gel zone and a 32 mm continuous 10 - 15% gradient gel zone with 2% crosslinking. The gel was approximately 0,45 mm thick. Buffer systems in the gel and PhastGel SDS Buffer Strips were 0,112 M acetate, 0,112 M Tris, pH 6,4 and 0,2 M tricine, 0,2 M Tris, 10% SDS, pH 7,5 respectively. The buffer strips were made of 2% Agarose (IEF Grade).

b) Preparation of samples and developing conditions

Samples were prepared in Tris-HCℓ buffer (10 mM), pH 8 at a protein concentration of 1 µg/µℓ. SDS (2,5%) and β-mercaptoethanol (5%) were added. Following the incubation at 100°C for 5 min., bromophenol blue was added to approximately 0,01%. Insoluble material was removed by centrifugation. Molecular mass marker proteins were treated in the same way. Following sample application electrophoresis was carried out (250 V x 3,0 W x 15°C, 60 Vh) using a Pharmacia PhastSystem.

c) Fixing of proteins

Gels were fixed in a 30% ethanol (v/v), 10% acetic acid (v/v) for 5 min.

d) Staining of proteins with Coomassie blue

The following solutions were used during the Coomassie staining method (183). Stain: 0,1% PhastGel Blue R solution (Pharmacia Fine Chemicals) in 30% methanol and

10% acetic acid in d.H_2O. Stock solution: 1 tablet PhastGel Blue R (Pharmacia Fine Chemicals) in 80 mℓ d.H_2O and 120 mℓ methanol. Final solution: 1 part filtered stock solution and 1 part 20% acetic acid in d.H_2O. Destain: 30% methanol and 10% acetic acid in d.H_2O (3 : 1 : 6). Preserving solution: 5% glycerol and 10% acetic acid in d.H_2O. Following fixing the gel was stained in the final solution for 8 min., destained for three periods of 5, 8 and 10 min. and preserved for 5 min. All steps were carried out at 5°C.

3.3 RESULTS AND DISCUSSION

3.3.1 Extraction of the nuclear HEBP

Liver tissue appears to contain only minute amounts of the oestrogen binding proteins. The concentration (pmol/g) of the oestrogen receptor in uterine tissue of the vervet monkey is approximately 2 - 5 times higher than the concentration present in the liver (168). It was assumed that the extraction of nuclear HEBP, under conditions where all the HEBP were translocated to liver nuclei, might render a higher yield of these proteins. An initial attempt was launched to obtain a nuclear extract of the HEBP from the vervet monkey liver. Table 3.1 summarizes the results obtained during the extraction of vervet monkey HEBP from liver nuclei and compares this with the cytosol. Exchange assays revealed that cytosols prepared with TEDAG buffer in the presence or absence of unlabelled oestradiol (30 nM) contain approximately the same binding capacities (B_{max}: 52,8 and 48,1 fmol/mg cytosolic protein respectively). Pre-incubation of extracts at 15 min. for 30°C was thought to be sufficient for the translocation of activated HEBP to liver nuclei. However, there was still not much difference between the B_{max} values of cytosols prepared with or without oestradiol. These values were, however, slightly lower than the B_{max} values obtained for cytosols without any incubation. No significant difference could be observed between the binding capacities of nuclear extracts

TABLE 3.1: A SUMMARY OF THE RESULTS OBTAINED FOR THE EXTRACTION OF VERVET MONKEY HEBP FROM LIVER NUCLEI.

Homogenization buffer	Activation	[a]Binding capacity (B_{max}) ([b]fmol/mg [c]protein		
		Cytosol	Nuclear extract KCℓ, KSCN	Nuclear extract KCℓ, KSCN, DNase
TEDAG (30 nM E_2)	15 min., 30°C	38,5	2,0	1,4
TEDAG	15 min., 30°C	40,3	3,3	1,9
TEDAG (30 nM E_2)	-	52,8	2,1	3,2
TEDAG	-	48,1	1,3	1,4

[a] Mean values (n = 3)

[b] fm/mg = femtomol per mg

[c] The protein concentration of cytosol was determined by the method of Bradford (152).

obtained by the different methods of extraction described in Section 3.2.6. Binding capacities in the extracts were generally 4 - 6% of the corresponding cytosolic B_{max} values.

Steroid hormones act on their target cells by the formation of high-affinity complexes with receptor proteins present in the cytoplasm (22, 23). These hormone-receptor complexes are subsequently translocated to the nucleus (184). A major difference between the liver and other target organs for oestrogen is the extremely low concentrations of the oestrogen receptor in the liver relative to the levels of other tissue proteins. In order to obtain a preparation of HEBP with a receptor protein concentration comparable with 'classical' target organ cytosol, the receptor can be extracted from liver nuclei after receptor translocation (168, 172, 185). Translocation of the cytoplasmic oestrogen receptor to the nucleus is necessary in order to ensure a good recovery with extraction. This can be done *in vivo* by injecting the animal to be used with oestradiol before sacrificing (103, 168, 172), or *in vitro* by incubating the tissue homogenate with oestradiol prior to centrifugation (103). A nuclear oestrogen receptor was previously purified from the nuclear fraction of the hen oviduct (185) and chick liver (172) (90 000 and 25 000-fold purification respectively) by using a combination of cell fractionation, ammonium sulphate fractionation and affinity chromatography.

It was not possible to treat the vervet monkeys with oestrogen prior to sacrificing them, therefore an alternative method (*in vitro*) for translocation was employed. The results obtained for the extraction of vervet monkey HEBP from liver nuclei are summarized in Table 3.1. It is apparent that most of the HEBP is located within the cytosol. Failure of pro-translocation conditions to render higher yields of HEBP may be the result of several factors. The liver is known to be the major organ responsible for metabolism of steroid hormones, including oestrogens (103). Metabolism of oestradiol

to less active binding metabolites in hepatic cytosol may limit oestrogen-receptor interaction and the subsequent translocation to the nucleus. Eisenfeld and Aten (103) reported that the five-fold higher oestrogen concentration requirement for receptor binding in liver compared to uterine tissue is the result of oestrogen metabolism. Thus, it should be kept in mind that a large amount of the oestradiol incubated with liver cytosol will be metabolized. Limited oestrogen-receptor interaction and translocation resulting from steroid metabolism may have been a reason for the poor recovery of HEBP in nuclei as depicted in Table 3.1.

Steroid hormone receptors can be hydrolyzed by a formidable array of proteolytic enzymes present in target tissues (66). It is therefore necessary to protect steroid hormone receptors by effective protease inhibitors (66). Phenylmethylsulfonyl-fluoride may have been insufficient for the inhibition of the protease activity known to be present in liver cytosol. Hazato and Murayama (66) indicated that the oestrogen receptor can be specifically protected by relatively low concentrations (0,5 mM) of leupeptin or antipain; inhibitors of serine and thiol proteases respectively. Activation of cytosolic hormone receptors and subsequent translocation to the nucleus can be achieved by incubation with steroid hormone, exposure to high salt concentrations or by exposure to elevated temperatures (186). It is possible that the method employed to translocate vervet monkey HEBP to liver nuclei (15 min., 30°C, 30 nM oestradiol) may have been insufficient.

Finally, the suggested phosphorylation-dephosphorylation, associated with the binding of the steroid hormones to the receptor and the subsequent translocation should not be neglected (92, 187, 188). A definite relationship between ATP and receptor binding capacity has been observed (92). Thus without sufficient ATP, binding capacity will decrease and as a result subsequent translocation of the hormone receptor to the nucleus.