3.3.2 Ammonium sulphate precipitation

Some research groups have used ammonium sulphate precipitation as a method of oestradiol receptor purification prior to affinity chromatography (165, 168, 172, 185, 189). The percentage recovery and purification reported for salt fractionation in these studies vary between 30% with an 8-fold purification (165) and 74,4% with a 2,4-fold purification (185). However, we obtained very low levels of purification (1,08-fold) and recovery (11,9%) of the HEBP in crude vervet monkey hepatic cytosol using 20% ammonium sulphate saturation (Table 3.2). The use of a saturated ammonium sulphate solution or a milder salt during the precipitation reaction, instead of the solid ammonium sulphate salt, may reduce the extreme loss of activity obtained here.

Although a concentrated receptor extract, that is basically free of contaminating proteins can occasionally be obtained with salt fractionation, this method can also result in a noteworthy receptor loss associated with a low increase in purity (177). Another disadvantage of ammonium sulphate precipitation is the formation of high levels of protein aggregates in the redissolved protein solution which contribute to a large amount of non-specific binding.

From the results obtained for ammonium sulphate precipitation (Table 3.2) it became apparent that a more suitable concentration method should be investigated.

3.3.3 Heparin-Sepharose chromatography

A summary of the results obtained for two heparin-Sepharose affinity chromatography experiments is given in Table 3.3. Figure 3.2 represents a heparin-Sepharose chromatogram in which the receptor was eluted with buffer containing potassium chloride (0,5 M). Results were much the same when the HEBP was eluted with buffer containg sodium thiocyanate (1 M).

TABLE 3.2: PARTIAL PURIFICATION OF THE HEBP BY AMMONIUM SULPHATE PRECIPITATION

Sample	Total binding activity [a] (dpm)	Total protein (mg)	Specific activity (dpm/mg)	Purification (-fold)	Recovery (%)
Cytosol	1 098 266	102,0	10 767	1,00	100,0
20% $(NH_4)_2SO_4$ ppt	130 412	11,2	11 644	1,08	11,9
30% $(NH_4)_2SO_4$ ppt	300 000	37,6	7 979	0,74	27,3
40% $(NH_4)_2SO_4$ ppt	185 951	35,6	5 223	0,48	16,9
50% $(NH_4)_2SO_4$ ppt	75 164	20,0	3 758	0,35	6,8

[a] Aliquots of redissolved precipitates were incubated with equal volumes of 10 nM ^{3}H-E_2 in the presence and absence of a 1 000-fold excess of DES at 4°C for 16 h.

TABLE 3.3: PARTIAL PURIFICATION OF THE HERP BY HEPARIN-SEPHAROSE AFFINITY CHROMATOGRAPHY

Sample	Total binding activity (dpm)	Total protein (mg)	Specific activity (dpm/mg)	Purification (-fold)	Recovery (%)
Cytosol	17 755	5,00	3,551	1,0	100,0
[a]Peak	12 885	0,27	47 722	13,4	72,6
Cytosol	26 182	7,1	3 688	1,0	100,0
[b]Peak	18 653	0,29	64 767	17,6	71,2

[a] Elution with 1 M NaSCN.

[b] Elution with 0,5 M KCℓ.

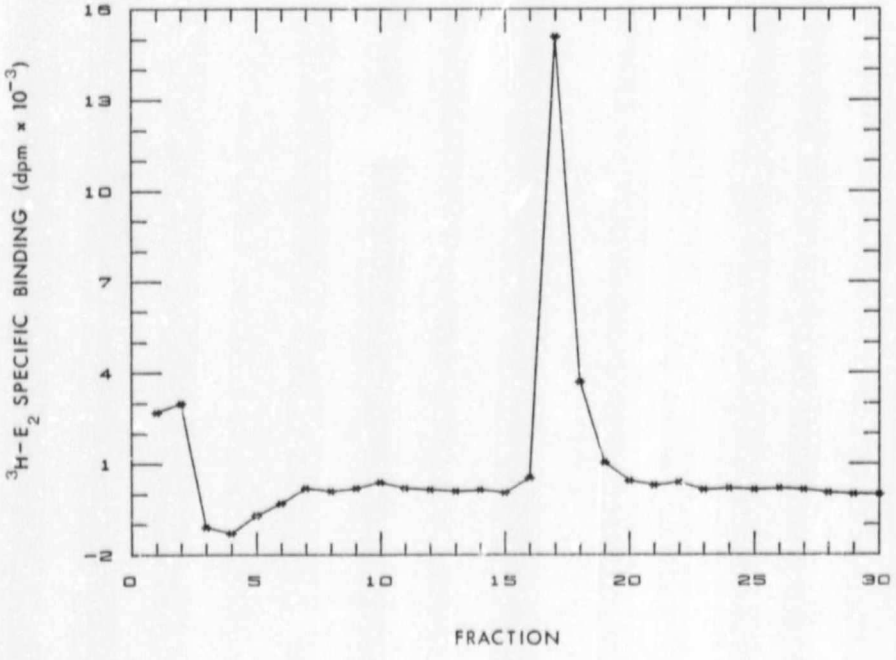


FIGURE 3.2

A representative heparin-Sepharose chromatography profile of the HEBP. The HEBP in cytosol was incubated with tritiated oestradiol in the presence or absence of a 1 000-fold excess of unlabelled DES. Heparin-Sepharose chromatography was executed on these samples as described in Section 3.2.8.b). The bound protein was eluted from the affinity adsorbent with buffer containing 0,5 M KCℓ. Specific binding was obtained by the subtraction of non-specific binding from the total binding.

Various cations like Mn^{2+}, Sr^{2+}, Fe^{2+} and Mg^{2+} stimulate binding of the receptor to heparin-Sepharose (176). Mn^{2+} is one of the most efficient cations in this stimulation and was therefore added to the vervet monkey hepatic cytosol before heparin-Sepharose chromatography. In addition, the recovery of adsorbed receptor protein is reportedly higher in the presence of this cation (177).

Heparin is a highly sulphated glycosaminoglycan. Covalent attachment of this polyanion to agarose provides an adsorbent that binds the oestradiol receptor with very high affinity. This property has been exploited by several research groups to achieve purification of the oestrogen receptor from target tissues (167, 169, 173, 176, 177). Reported values obtained during heparin-Sepharose chromatography range from 118-fold purification and 58% recovery (176) to 4,4-fold purification and 71% recovery (167). The hormone receptor complex exhibits a 10-fold higher affinity for heparin-agarose while the nuclear or uncomplexed oestrogen receptor shows very little affinity for this adsorbent (176). It was previously reported that heparin dissociates aggregates of oestradiol-receptor complexes to lower sedimenting forms (177, 169). However, the receptor form obtained from heparin-Sepharose chromatography eluted with heparin demonstrates a higher sedimentation coefficient than the salt-dissociated receptor molecule (169). A deleterious effect of salts like potassium chloride, sodium chloride and sodium bromide was previously observed on the oestradiol-free receptors, bound to heparin-agarose (190). This effect was, however, not observed on the heparin-agarose bound oestradiol-receptor complex. The receptor bound to heparin-agarose can be eluted either by increasing the ionic strength of the buffer (> 0,2 M sodium chloride, potassium chloride, potassium bromide or sodium thiocyanate) or with buffer containing heparin (1 - 5 mg/mℓ). Although elution of steroid receptors from the heparin-agarose column by high salt concentrations was reported to be much more efficient (52% against 7%) (177), it was apparently only employed for the

elution of oestradiol-receptor complexes (176). Heparin elution was primarily employed for oestradiol-free receptors (167, 169, 190). The advantages of an initial heparin-Sepharose purification step are: pre-purification of the oestradiol-binding proteins, a decrease in cytosol volume and the inhibition of protein aggregation. Heparin-Sepharose chromatography also provides a very efficient method to eliminate free oestradiol and salt used during the elution of hormone receptors from specific adsorbents (167).

A representative profile of the partially purified HEBP subjected to hypertonic (0,5 M KCl) sucrose density centrifugation is illustrated in Figure 3.3. It is evident that the majority of oestrogen binding activity is represented as the 4S form, most likely caused by salt dissociation as a result of the elution method employed.

From the results shown in Table 3.3 and illustrated in Figures 3.2 and 3.3 it became evident that heparin-Sepharose chromatography provides a convenient method for the pre-purification of the HEBP. This method holds the added facility to eliminate free oestradiol and salt used during elution of the HEBP from specific adsorbents.

3.3.4 DES affinity chromatography

a) Preparation of specific affinity adsorbents

Throughout the following section the term matrix will refer to the polymer, substituted with linker arm but not containing the steroid ligand. The affinity matrix on the other hand refer to the steroid-substituted matrix. Affinity matrices DES-Agarose and DES-Trisacryl were synthesized according to Van Oosbree et al. (173) and the LKB practical guide for affinity chromatography and related techniques (179). Minor modifications of these methods are described in Sections 3.2.7.a) and 3.2.7.b).

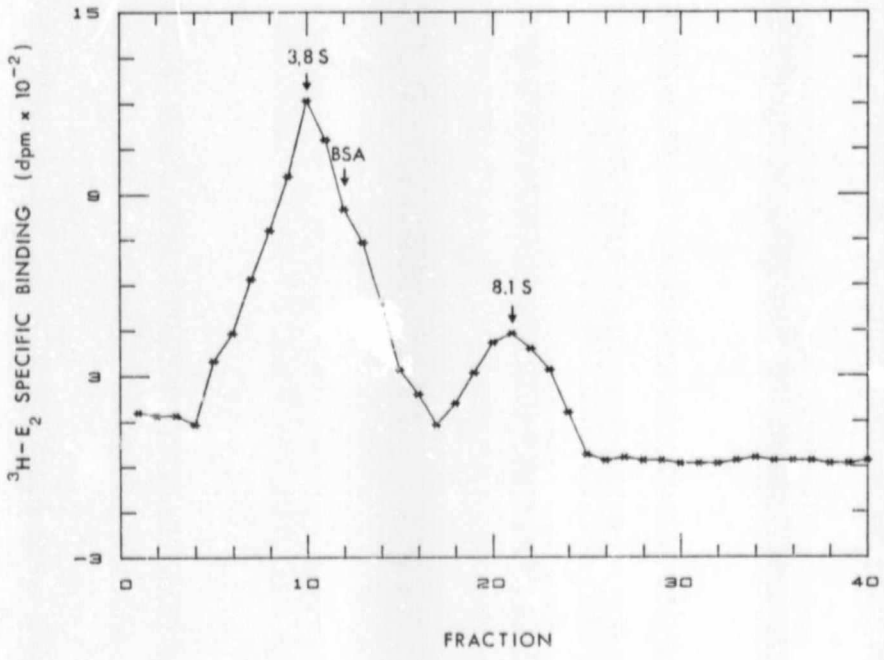


FIGURE 3.3

A representative sucrose density gradient (10 - 35%) profile of the HEBP, partially purified by heparin-Sepharose. The eluate from heparin-Sepharose chromatography (Section 3.2.8.b)) was subjected to hypertonic (0,5 M KCℓ) sucrose density gradient centrifugation. Fractions were collected manually and monitored for TB and NSB. A duplicate set of gradients with ^{14}C-methylated BSA was included with each run. The position of the ^{14}C-labelled marker protein (BSA, 4.6S) is indicated by an arrow.

The immobilization of DES onto matrices under various experimental conditions is summarized in Table 3.4. Because DES is more soluble in N,N-dimethylformamide (DMF) than dioxane, the DES used in the preparation of the affinity matrix, Des-Agarose, was dissolved in DMF rather than dioxane as previously described (173). Higher amounts (70%) of DES was bound to epoxy-activated Sepharose 6B when the coupling reaction was executed at a higher temperature. If any free steroid, used in the coupling reaction, is still present in the affinity matrix, during incubation with cytosol, it will compete with affinity matrix-bound steroid for receptor binding. Therefore affinity matrices were washed with large volumes of DMF and methanol. After the matrices were derivatized with DES, the remaining epoxy-activated sites on the affinity matrix were blocked by reacting it with ethyleneglycol or ethanolamine. If these sites were left underivatized it may lead to higher levels of non-specific binding. The amount of DES covalently bound to the matrices were determined by: i) including tritiated DES in the coupling reaction during the preparation of DES-Agarose, or by ii) calculation from the decrease in absorbance spectra (225 nm - 375 nm) of the ligand solution following reaction with the matrix. Radioactive labelling provides a far more reliable and sensitive method than difference spectra for the determination of the ligand concentration covalently bound onto the matrix.

b) Evaluation of procedures

In order to determine the optimal conditions for affinity chromatography, pilot experiments were set up. All reagents and buffers were pre-cooled to 4°C before use and the experiments were carried out at 4°C.

The interaction of HEBP with DES-Agarose containing 0,76 and 2,35 µmol DES/mℓ matrix and DES-Trisacryl containing

TABLE 3.4: THE IMMOBILIZATION OF DES ONTO MATRICES UNDER VARIOUS EXPERIMENTAL CONDITIONS.

Matrix	Activation of matrix	Coupling reaction	Blocking reaction	[a]Matrix steroid concentration (µmol/mℓ)
1. Epoxy-activated Sepharose 6B	-	12,3 mM DES 18h, 25°C	50% ethylene glycol; 18h, 25°C	0,83 ± 0,07 (4)
2. Epoxy-activated Sepharose 6B	-	12,3 mM DES 18h, 37°C	50% ethylene glycol; 18h, 37°C	2,76 ± 0,81 (6)
3. Trisacryl GF 2000	diepoxybutane; 18h, 25°C	28,0 mM DES 18h, 25°C	0,1M ethanolamine; 3h, 4°C	2,20 ± 1,9 (4)

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

2,0 µmol DES/mℓ is illustrated in Figure 3.4 and summarized in Table 3.5. Optimal conditions entailed the incubation of 1 mℓ cytosol (10 mg protein/mℓ) with 50 mg DES-Agarose (2,35 µmol DES/ mℓ matrix) or DES-Trisacryl (2,0 µmol DES/mℓ matrix). These incubation ratios were used in all further investigations. The effect of the matrix, homogenization buffer and method of incubation (column - and batchwise) on the interaction of cytosolic vervet monkey HEBP with DES covalently bound to the matrix is set out in Table 3.6. The results may be summarized as follows: (1) It became clear that DES-Agarose, with approximately the same bound steroid concentration as DES-Trisacryl, generally bound a higher percentage of the cytosolic HEBP. (2) When the incubation of HEBP with the affinity matrix is executed under high ionic strength conditions it appears that non-specific binding is depressed. (3) An overall lower percentage (3 - 5%) of cytosolic HEBP was bound to the affinity matrix in the presence of sodium molybdate. This depressed interaction of the vervet monkey HEBP with oestradiol in the presence of molybdate was also observed during Scatchard analyses (Chapter 2, Section 2.3.6). (4) Finally, when hepatic cytosol was incubated batchwise (18 h, 4°C), 15% more HEBP was bound to the affinity matrix as compared to column studies (5 mℓ/h, 4°C).

The elution of the HEBP from DES-Agarose by different concentrations of oestradiol in the presence or absence of sodium thiocyanate (0,5 M) is illustrated in Figure 3.5. From these results it was decided that 50 µM oestradiol (in the absence of sodium thiocyanate) would be an appropriate steroid concentration to elute bound HEBP from the affinity matrix. A lower percentage of bound HEBP is recovered when the HEBP was eluted in the presence of sodium thiocyanate. It is well known that this chaotropic salt disrupts intra- and intermolecular hydrophobic interactions and increases the turnover rate of the

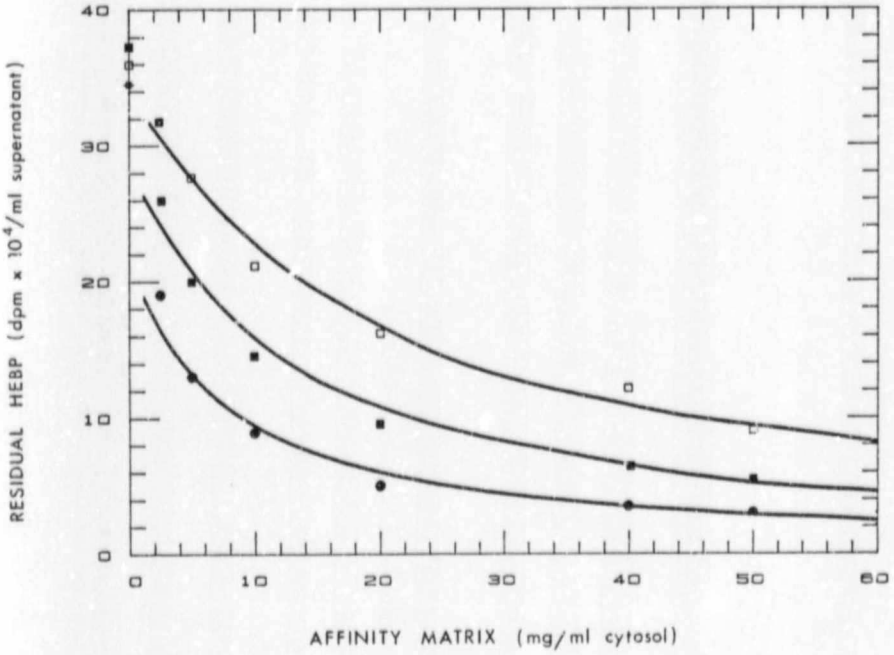


FIGURE 3.4

Interaction of HEBP with various affinity matrices. Increasing concentrations of DES-Agarose —□— (0,76 µmol DES/mℓ matrix), DES-Agarose —●— (2,35 µmol DES/mℓ matrix) and DES-Trisacryl —■— (2,0 µmol DES/mℓ matrix) were incubated with aliquots (0,5 mℓ) of cytosol (10 mg protein/mℓ) for 60 min. at 0°C. The residual HEBP in the supernatants (2 000 *g*, 15 min.) was subsequently measured as described in Section 3.2.9.a).

TABLE 3.5: THE INTERACTION OF HEBP WITH INCREASING CONCENTRATIONS OF DES AFFINITY MATRICES.

Affinity matrix concentration (mg/mℓ[a] cytosol)	Percentage cytosolic HEBP bound to affinity matrix		
	DES-Agarose: 0,76 µmol DES/ mℓ matrix	DES-Agarose: 2,35 µmol DES/ mℓ matrix	DES-Trisacryl: 2,0 µmol DES/ mℓ matrix
0,0	0	0	0
2,5	13	45	31
5,0	24	70	51
10,0	42	80	65
20,0	56	88	84
40,0	67	90	84
50,0	75	91	85

[a] Cytosol protein concentration = 10 mg/mℓ

TABLE 3.6: THE EFFECT OF THE MATRIX, HOMOGENIZATION BUFFER AND METHOD OF INCUBATION ON THE INTERACTION OF CYTOSOLIC VERVET MONKEY HEBP WITH DES, COVALENTLY BOUND TO THE MATRIX. THE SAME AMOUNT OF CYTOSOL WAS EMPLOYED IN EACH CASE.

Affinity matrix	Homogenization buffer	Percentage cytosolic HEBP bound to affinity matrix	
		Columnwise	Batchwise
DES-Agarose	TEDAG 0,5 M KCℓ	74,4	89,6
DES-Agarose	TEDAGM 0,5 M KCℓ	69,6	86,3
DES-Agarose	TEDAG	74,0	88,5
DES-Agarose	TEDAGM	69,0	83,0
DES-Trisacryl	TEDAG 0,5 M KCℓ	68,0	82,8
DES-Trisacryl	TEDAGM 0,5 M KCℓ	65,0	79,8
DES-Trisacryl	TEDAG	64,0	80,1
DES-Trisacryl	TEDAGM	60,3	75,4

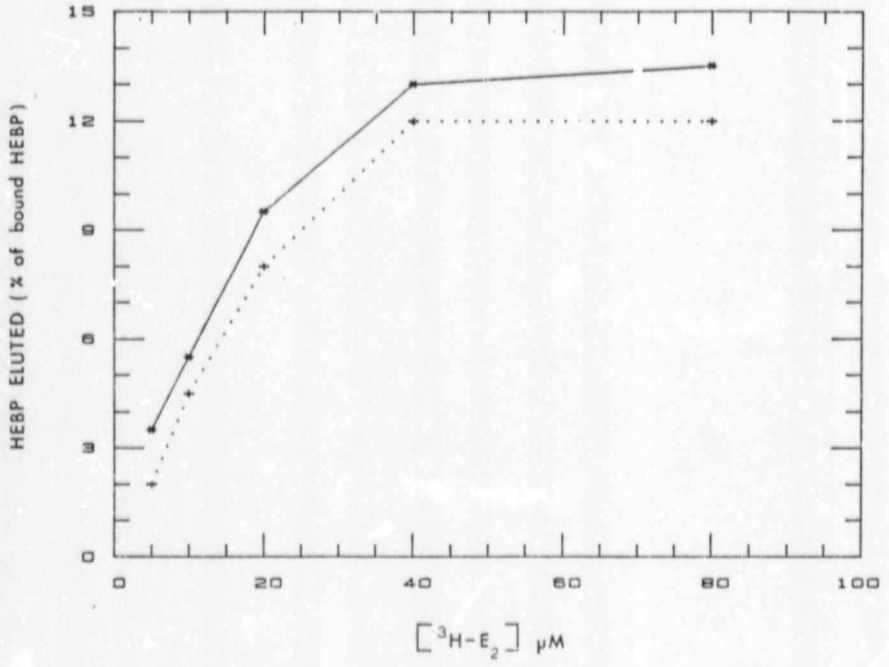


FIGURE 3.5

The elution of the HEBP from DES-Agarose as a function of increased hormone concentrations. Elution was carried out in TEDAG buffer containing ··+·· or not containing —×— sodium thiocyanate (0,5 M) at 20°C for 30 min. The eluates were subsequently treated with DCC (5 mℓ pellet) for 15 min. at 0°C and the resulting supernatant (2 000 *g*, 15 min.) assayed for oestradiol binding activity by the exchange assay described in Section 3.2.9.b).

the oestrogen receptor complex at 4°C (191, 192). Although hormone binding to the cytosolic androgen receptor is considerably inhibited by thiocyanate (0,5 M) this inhibition is reportedly absent with oestradiol-receptor interaction (193). However, the HEBP may be more susceptible to disruption of hydrophobic interactions by thiocyanate that may lead to denaturation of the protein. Despite the fact that a higher percentage of affinity matrix-bound HEBP is eluted in the absence of sodium thiocyanate the recovery of HEBP still appears to be extremely low (13,5%). This low recovery of HEBP during elution may be the result of one of two possible factors. Firstly the possibility exists that a large percentage of the HEBP is still bound onto the affinity matrix after elution. Thus, higher ligand concentrations and temperatures, longer elution periods or even the addition of a mild salt may possibly improve the recovery of affinity matrix-bound HEBP. Secondly there is the probability that, although a large percentage of the HEBP is eluted, there is a loss of binding activity. This loss of activity may primarily be caused by the relatively high temperature used during elution. In addition the relatively poor method (DCC treatment) employed to eliminate the excess oestradiol present in the eluates (Table 3.7) during this investigation, is probably the main reason for this 'loss' in activity. Oestradiol still present in the eluates after DCC treatment, competes with tritiated oestradiol binding during the assay for HEBP. This necessarily gives rise to the under-estimation of oestradiol-binding activity. Because of the relatively low solubility of oestradiol in an aqueous solution it is difficult to obtain an oestradiol solution at a concentration higher than 80 µM with an ethanol content which would not seriously affect the oestradiol receptor. The affinity matrix-bound HEBP was subsequently eluted employing shorter and multiple incubations with consequently higher recovery.

In Table 3.7 the different methods investigated for the elimination of excess oestradiol from eluates after specific

TABLE 3.7: ELIMINATION OF THE EXCESS OF OESTRADIOL PRESENT IN THE ELUATE AFTER THE SPECIFIC ELUTION OF HEBP FROM DES-AGAROSE.

Method	Recovery (%)	Purification (-fold)
Heparin-Sepharose	30,0	7 435
Sephadex G-25	19,8	4 911
DCC treatment	5,8	1 445

elution of the HEBP from DES-Agarose are summarized. From these results it became apparent that heparin-Sepharose is a more superior method by comparison with gel filtration and charcoal adsorption. Although the amount of protein, following gel filtration and charcoal adsorption was comparable to that after heparin Sepharose chromatography the specific oestrogen-binding activity was much less. This may be the result of an amount of oestradiol still present after gel filtration and charcoal adsorption that will compete with tritiated oestradiol for receptor binding during the assay for HEBP. Another disadvantage of gel filtration is the subsequent dilution of the partially purified HEBP.

Results obtained from the above-described pilot experiments assisted in defining the optimal conditions for affinity chromatography of HEBP on DES-Agarose as described in Section 3.2.8.c) (ii). The results achieved for the partial purification of the HEBP from the vervet monkey, by employing the optimized methods are summarized in Table 3.8. As a consequence of the high concentration of oestradiol in the eluate and the extreme dilution of protein, assessment of binding activity and protein concentration proved to be unreliable, following DES-Agarose affinity chromatography. The HEBP has been partially purified from the vervet monkey liver by affinity chromatography (DES-Agarose and heparin-Sepharose) to yield approximately 42 µg HEBP from 50 g liver with a recovery and purification of 30% and 7 435-fold respectively.

The purification of the oestrogen receptor to near homogeneity from 'classical' target tissue (uterus, mamma) cytosol achieved by using a combination of conventional methods and affinity chromatography has been reported frequently (164, 165, 167, 169, 173, 174, 189, 190) in contrast to the purification of cytosolic oestrogen receptor from liver tissue. Thus the efficiency of this method to purify HEBP from vervet monkey liver can only be determined in comparison with results

TABLE 3.8: THE PARTIAL PURIFICATION OF THE HEBP FROM VERVET MONKEY USING DES-AGAROSE AND HEPARIN-SEPHAROSE 6B. THE EXPERIMENT OF WHICH THE RESULTS ARE SUMMARIZED HERE WAS PERFORMED AS DESCRIBED IN SECTION

Purification step	Total volume (mℓ)	Total binding activity (dpm)	Total protein (mg)	Specific activity (dpm/mg)	Recovery (%)	Purification (-fold)
Cytosol	100	$5,4 \times 10^6$	1040	5 192	100	1
DES-Agarose	10	[a] n.m.	n.m	n.m	n.m	n.m.
Heparin-Sepharose	5	$1,6 \times 10^5$	0,042	$3,86 \times 10^7$	30	7 435

[a] n.m. = not measurable

obtained during purification of oestrogen receptors from 'classical' target tissue that contain far higher concentrations of the receptor than the liver. Van Oosbree et al. (173) reported a 792-fold purification of the oestrogen receptor from rabbit uterine cytosol with a 4,5% recovery by employing DES-Agarose and heparin-agarose chromatography. The oestrogen receptor has been purified sequentially by affinity chromatography (heparin-Sepharose and oestradiol-Sepharose) and gel filtration (Sephadex G-50) from the same tissue by Madhok and Leung (169). They obtained a significantly lower recovery when using fresh cytosol as the source of receptor and recommended higher temperatures (30°C) during elution in the presence of sodium thiocyanate and DMF. Sica and Bresciani (167) described the purification of the oestrogen receptor to homogeneity from uterus cytosol by affinity chromatography (heparin-Sepharose and oestradiol-Sepharose) in combination with gel filtration (Sephadex G-50). During this procedure a 15 585-fold purification and 53% recovery were obtained. Heparin-Sepharose chromatography was employed as a pre-purification step as well as a rapid and efficient manner to eliminate free oestradiol and sodium thiocyanate used during the elution of the receptor from the affinity matrix. A relatively high purification (25 700-fold) and recovery (39%) were reported by Puca et al. (190) for the 'native' oestrogen receptor from calf uterus. Heparin-agarose and oestradiol-agarose affinity chromatography in combination with Sephadex G-200 gel filtration were used during this investigation. The authors recommended the use of magnesium chloride during purification to decrease the aggregation phenomenon and employed a chaotropic salt in combination with a low oestradiol concentration for the elution of the oestrogen receptor. More recently Bond and Notides (194) described a rapid two-step procedure for the 900 - 1 700-fold purification of the calf uterine oestrogen receptor by using ammonium sulphate precipitation followed by affinity chromatography with Orange-Sepharose which rendered a 20,3% recovery. The advantage of this method is the purification of

the oestrogen receptor in the presence or absence of oestradiol under mild elution conditions. The selective purification obtained by Orange-Sepharose is a result of differential adsorption (ionic contacts) to the cation exchanger and differential elution with salt.

Other target tissues or cells like MCF - 7 human breast cancer cells (164, 165), mammary tumours (189) and myometrial tissue (177) have also been employed as sources from which the oestrogen receptor was purified. A purification factor of approximately 7 000-fold for the oestrogen receptor from mammary tumours was achieved by Al-Nuaimi et al. (189). This was obtained with the aid of salt fractionation, oestradiol-Sepharose chromatography and Sephadex G-25 gel filtration. During affinity chromatography they found it more beneficial to dilute the substituted gel three to five times with unsubstituted Sepharose. Heparin-Sepharose in conjunction with affinity chromatography were employed by Ratajczak and Hähnel (177) to obtain a 4 661-fold purification and 40% recovery of the oestrogen receptor from human myometrial cytosol. Greene and Press (164) reported a 6 424-fold purification of the oestrogen receptor from MCF - 7 human breast cancer cells with a 42% yield. These values were obtained by employing affinity chromatography and gel filtration.

As previously mentioned, very little has been published about the purification of the hepatic oestrogen receptor. The liver oestrogen receptor is generally purified from nuclear extracts rather than hepatic cytosol (168, 172). The nuclear oestrogen receptor has previously been purified (25 333-fold, 16% recovery) to homogeneity by using a combination of salt precipitation and affinity chromatography (172).

3.3.5 Isoelectric focussing

Isoelectric focussing of the vervet monkey HEBP following the partial purification described above (Section 3.2.8.c)(ii)) indicated the existence of two bands with oestradiol binding activity (Figure 3.6). One band focussed at a pH of 6,8 while a second band focussed at a pH of 6,9. The specific oestrogen binding component focussing at a pH value of 6,9 contained approximately 58% of the binding activity of the pI 6,8 entity. These results are illustrated photographically in Figure 3.7. In Figure 3.8 a representative calibration curve for the pI marker proteins is illustrated. In order to aid identification of the HEBP it was partially purified from cytosol pretreated with 5 µM oestradiol ('mock' purification) along with partial purification from cytosol without hormone pretreatment ('actual' purification). The results obtained during IEF of 'mock' and 'actual' purified HEBP is illustrated in Figure 3.9 and summarized in Table 3.9. From this IEF gel it appeared as if one protein band (pI 6,8) disappeared while another oestradiol band (pI 6,9) was slightly decreased in intensity following the oestradiol pretreatment of the cytosol from which the HEBP was partially purified. It is important to note that a one-step purification method (DES-Agarose) was used during this investigation compared to the two-step purification method (DES-Agarose and heparin-Sepharose) employed to obtain the results illustrated in Figures 3.6 and 3.7. Most of the other protein bands seen in the IEF gel (Figure 3.9) appear to be proteins non-specifically retained on DES-Agarose, since the binding was not influenced by oestradiol pretreatment.

It has been established that the steroid hormone receptors in target tissue cytosols appear to be heterogenous in IEF analysis (158, 195, 196). This microheterogeneity of oestrogen receptors has also been observed during other studies (197). Specific oestradiol binding components in lactating rat mammary gland reportedly exhibited pI values in the range

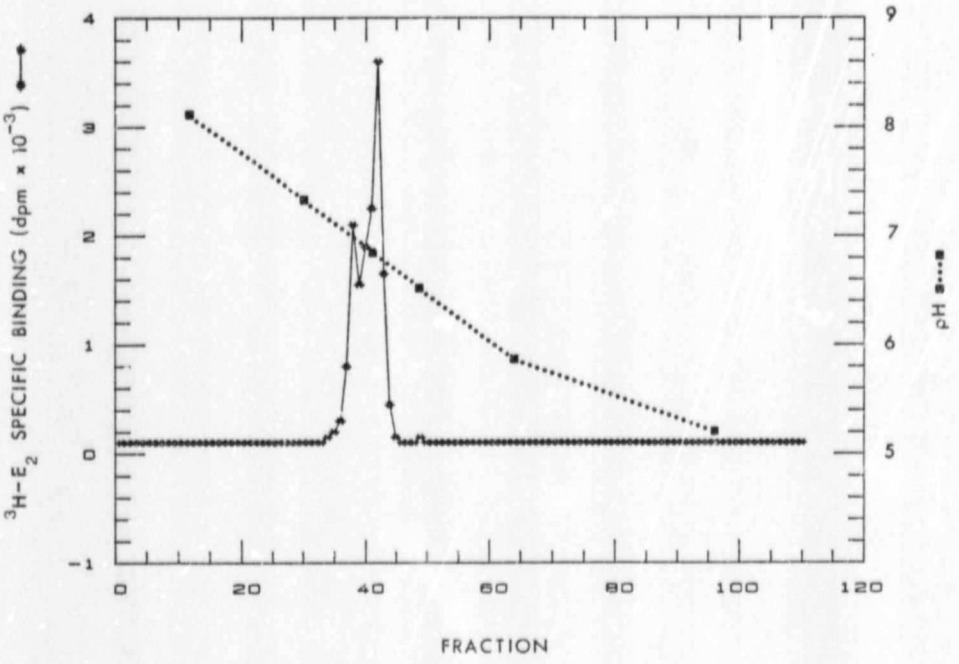


FIGURE 3.6

Isoelectric focussing on agarose gels of the partially purified vervet monkey HEBP. The ampholyte range employed was pH 5 - 8. The HEBP, partially purified as described in Section 3.2.8.c) (ii), was incubated with tritiated oestradiol (30 nM), with or without unlabelled DES (1 000-fold excess) at 20°C for 30 min. Reactions were terminated by DCC treatment and the 2 000 *g* supernatants applied to the prefocussed agarose gel. After focussing (Section 3.2.11.b)) the gel was cut into 1 mm slices and the radioactivity measured. Gels were calibrated by employing standard proteins: β-lactoglobulin A (pI 5,2), bovine carbonic anhydrase B (pI 5,85), human carbonic anhydrase B (pI 6,55), myoglobin-acidic band (pI 6,85), myoglobin basic band (pI 7,35), lentil lectin-acidic band (pI 8,15).

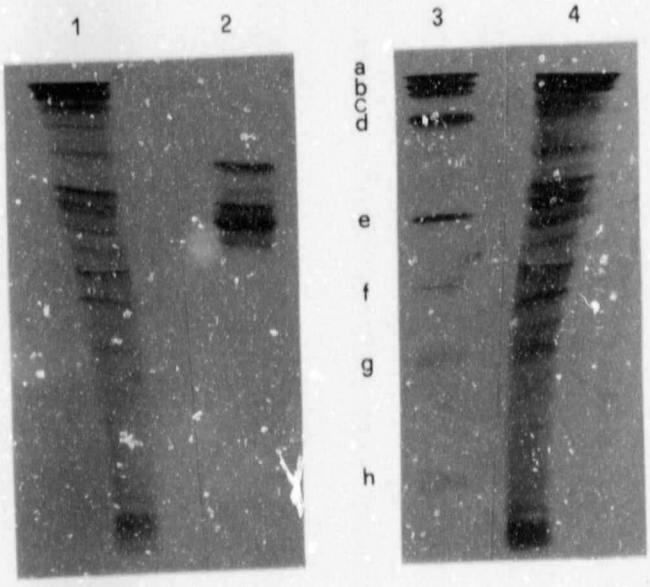


FIGURE 3.7

Isoelectric focussing on agarose gels of the vervet monkey HEBP partially purified by the optimum method of choice (Section 3.2.8.c) (ii)). The agarose IEF gel contained a pharmalyte range of pH 5 - 8. Isoelectric focussing was executed as outlined in Section 3.2.11. The samples focussed in the different lanes are 1) and 4) hepatic cytosol, 2) the partially purified HEBP and 3) the standard pI calibration proteins: a) lentil lectin - basic band (pI 8,65), b) lentil lectin - middle band (pI 8,45), c) lentil lectin - acidic band (pI 8,15), d) myoglobin - basic band (pI 7,35), e) myoglobin - acidic band (pI 6,85), f) human carbonic anhydrase B (pI 6,55), g) bovine carbonic anhydrase B (pI 5,85), h) β-lactoglobulin A ((pI 5,20). The cathode is represented by the top of the gel.

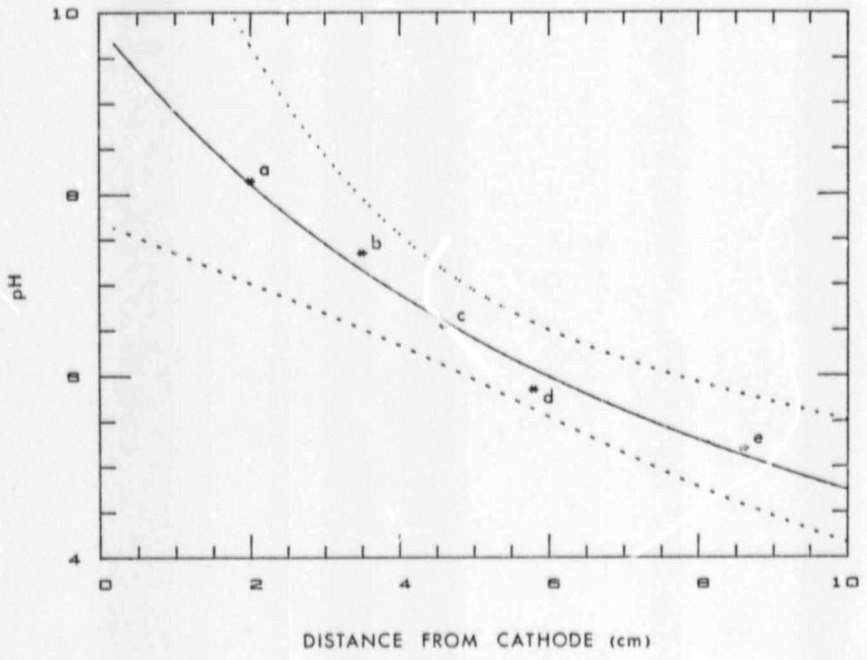


FIGURE 3.8

A representative pH profile for the IEF marker proteins were: a) lentil lectin - acidic band (pI 8,15), b) myoglobin basic band (pI 7,35), c) human carbonic anhydrase B (pI 6,55), d) bovine carbonic anhydrase B (pI 5,85) and e) β-lactoglobulin A (pI 5,2). IEF gels were focussed and stained as described in Section 3.2.11. The ampholyte range employed was pH 5 - 8.

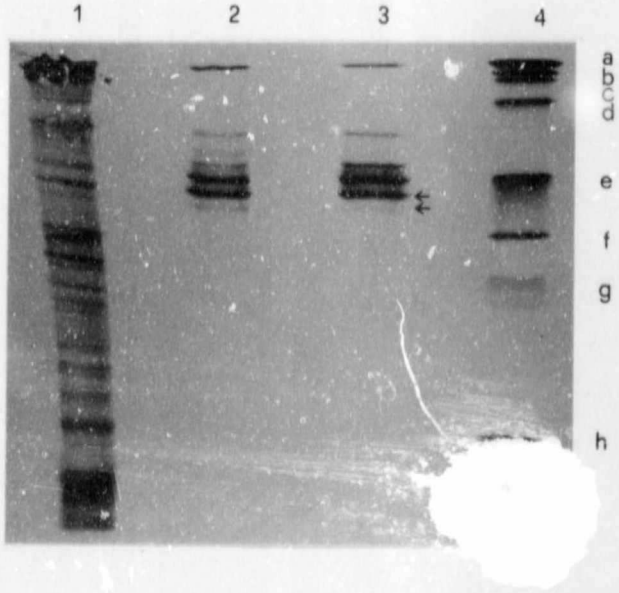


FIGURE 3.9

Isoelectric focussing of the vervet monkey HEBP partially purified (DES-Agarose chromatography) from hepatic cytosol pretreated with 5 μM oestradiol (lane 3) and from hepatic cytosol without hormone pretreatment (lane 2). The agarose IEF gel contained a pharmalyte range of pH 5 - 8. Isoelectric focussing was executed as outlined in Section 3.2.11. The samples focussed in the different lanes are 1) hepatic cytosol, 2) HEBP partially purified from cytosol without hormone pretreatment, 3) HEBP partially purified from oestradiol (5 μM) treated cytosol, 4) the standard pI calibration proteins: a) lentil lectin - basic band (pI 8,65), b) lentil lectin - middle band (pI 8,45), c) lentil lectin - acidic band (pI 8,15), d) myoglobin - basic band (pI 7,35), e) myoglobin - acidic band (pI 6,85), f) human carbonic anhydrase B (pI 6,55), g) bovine carbonic anhydrase B (pI 5,85) and h) β-lactoglobulin A (pI 5,20). The disappearance of the pI 6,8 component and the decrease in pI 6,9 component in lane 3 are indicated by arrows. The cathode is represented by the top of the gel.

TABLE 3.9: A SUMMARY OF THE RESULTS OBTAINED DURING IEF OF THE HEBP PARTIALLY PURIFIED BY DES-AGAROSE IN THE PRESENCE OR ABSENCE OF AN EXCESS UNLABELLED DES (5 μM).

Purification method:	DES-Agarose	DES-Agarose, 5 μM DES
pI values of protein bands:	8,15	8,15
	8,10	8,10
	7,95	7,95
	7,88	7,88
	7,60	7,60
	7,45	7,45
	7,29	7,29
	7,12	7,12
	7,05	7,05
	7,00	7,00
	6,90	(6,90)
	6,80	-

5,6 - 7,8 (195). The vervet monkey uterine cytosolic oestrogen receptor focussed in the pH range 5,9 - 6,8 (three peaks) in the absence and 5,0 - 6,8 (four peaks) in the presence of sodium molybdate (158). The cytosolic human uterine oestrogen receptor also appears to be very heterogenous as reported by Gibbons et al. (196). Al-Nuaimi et al. (189) obtained two peaks in IEF profiles for the cytosolic oestradiol binder in mammary tumour cells. Elimination of both peaks was obtained with an excess of DES. The pI value of their affinity purified oestrogen receptor was 6,4.

There is ample evidence that the apparent heterogeneity of the oestradiol receptor might be the result of aggregation of the receptor under non-denaturing conditions (150, 198). A problem experienced with aggregation is the inability of aggregates to enter the gel during IEF (198). IEF analyses in polyacrylamide gels executed by Wrange et al. (150) showed that the rat liver cytosolic oestrogen receptor focussed at a pH of 6,4. A sharper peak and improved recovery was observed after limited trypsin digestion of the tritiated receptor preparation before analysis. They also reported a similarity between IEF profiles of the liver and uterus cytosolic receptors after trypsin treatment. Monsma et al. (198) obtained a pI value of 5,7 with IEF performed in the presence of 8 M urea for the cytosolic and nuclear human breast cancer oestrogen receptor. The receptor proteins had been covalently labelled by tamoxifen aziridine during this investigation. IEF was performed in the presence of 8 M urea to overcome the aggregation and resultant heterodisperse appearance (2 - 3 peaks, pI 6,2 - 6,6) of the receptor under non-denaturing conditions.

3.3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The selective binding of proteins to DES-Agarose is illustrated in Figure 3.10. Proteins which were bound to DES-Agarose in the presence of oestradiol (5 µM) are illustrated in lane 3 and 7 and those bound in the absence of

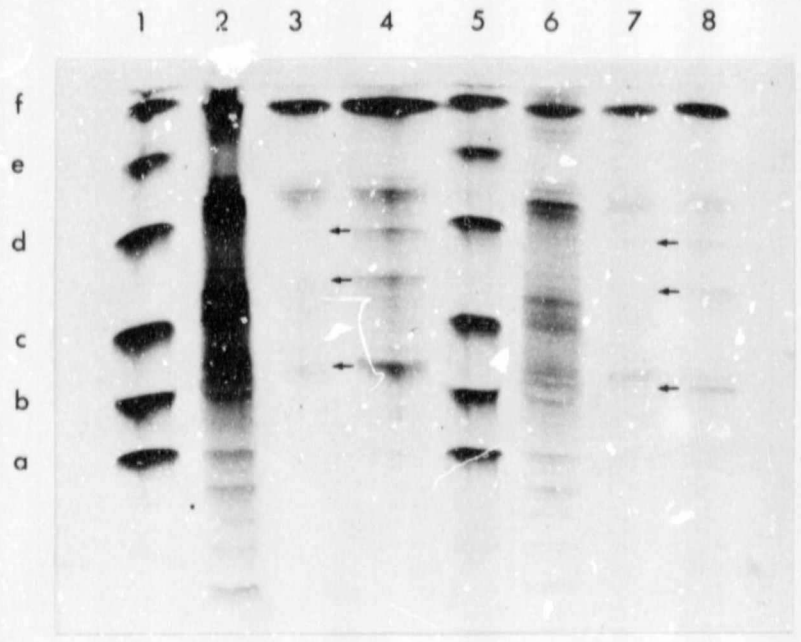


FIGURE 3.10

Selective binding of proteins to DES-Agarose as illustrated by SDS-PAGE. The HEBP was partially purified (DES-Agarose chromatography) from cytosol pretreated with 5 μM oestradiol (lane 3 and 7) and from cytosol without hormone pretreatment (lane 4 and 8). The samples were prepared and subjected to SDS-PAGE as described in Section 3.2.12.b). Following electrophoresis the gel was fixed and stained (Section 3.1.12.c) and d)). The samples in the different lane are 1) molecular mass marker proteins within brackets molecular masses in daltons: a) phosphorylase b (94 000), b) bovine serum albumin (67 000), c) ovalbumin (43 000), d) carbonic anhydrase (30 000), e) soyabean trypsin inhibitor (20 100) and f) x-lactalbumin (14 400), 2) hepatic cytosol, 3) HEBP partially purified from oestradiol (5 μM) treated cytosol, 4) HEBP partially purified from cytosol without hormone pretreatment, 5), 6), 7) and 8) corresponds to lane 1, 2, 3 and 4 except that samples in lane 6, 7 and 8 are diluted. The disappearance of 30 000, 38 000 and 68 000 dalton components in lane 3 and 7 are indicated by both sets of arrows. The bottom of the figure represents the point of sample application

steroid hormone in lane 4 and 8. It is evident that three protein bands (molecular masses: 5 000, 38 000 and 30 000 daltons) disappeared when hepatic cytosol was pretreated with oestradiol. The other protein bands seen in the acrylamide gel are bound non-specifically by DES-Agarose because steroid hormone treatment did not influence its binding. The molecular masses were calculated from the calibration curve (Figure 3.11) derived from the relative mobilities and molecular masses of the molecular mass marker proteins (lane 1 and 5).

Although evidence exists for a single hormone binding sub-unit with a molecular mass of 65 - 70 kilodaltons for the oestrogen receptor (ER) (163, 199, 200, 201), the molecular masses of the ER isolated from various sources differ. Reported molecular masses, as determined by SDS-PAGE, for the ER purified from the calf uterus ranged from 60 000 to 70 000 daltons (167, 171, 176). Van Oosbree et al. (173) and Olsen et al. (202) found clear evidence for two oestradiol-binding components (molecular masses: 50 000 and 65 000 daltons) in rabbit uterine cytosol purified by DES-Agarose. Both these bands disappeared when the cytosol had been pretreated with oestradiol (100 nM). The rat uterine ER appears as a major component (molecular mass: 60 000 - 66 000 daltons) together with a minor species (molecular mass 50 000 daltons) (34). The unfractionated cytosolic ER used during this SDS-PAGE was covalently labelled by tamoxifen aziridine. The nuclear ER extracted from MCF - 7 cell nuclei has a mobility consistent with a molecular mass of approximately 63 000 daltons, while the cytosolic ER showed a molecular mass of 62 000 daltons when subjected to SDS-PAGE (198). Rapid proteolysis of this ER was observed at room temperature with resultant smaller forms (molecular masses: 53 000 and 37 000 daltons). Inclusion of protease and phosphatase inhibitors, such as leupeptin and molybdate during the procedure reportedly prevents the conversion of the 63 000 daltons cytosolic receptor to lower molecular mass species. Smith and Schwartz (185) observed two

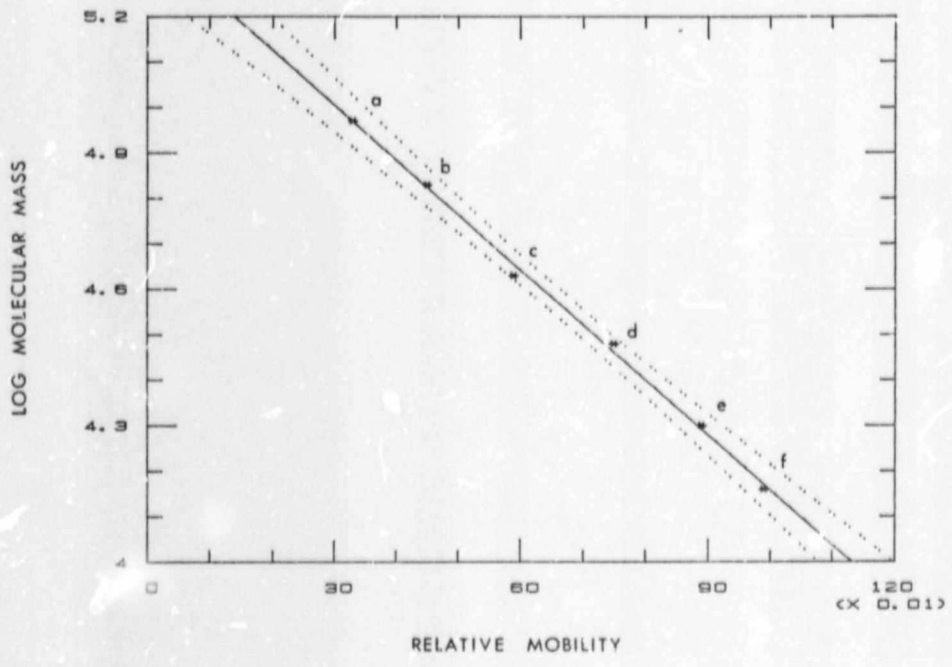


FIGURE 3.11

A typical calibration curve for the determination of the relative molecular mass of the partially purified HESP in the presence of SDS and 2-mercaptoethanol. The molecular mass marker proteins are a) phosphorylase b (94 000), b) bovine serum albumin (67 000), c) ovalbumin (43 000), d) carbonic anhydrase (30 000), e) soyabean trypsin inhibitor (20 100) and f) α-lactalbumin (14 400).

sub-units (molecular masses: 74 000 and 80 000 daltons) following SDS-PAGE of the ER purified from the nuclear fraction of hen oviduct, Gschwendt et al. (172) demonstrated that the nuclear ER purified from chick liver exists as a 55 000 dalton protein species (SDS-PAGE).

The molecular masses of the partially purified vervet monkey HEBP compare well with the molecular masses reported for the ER purified from other sources, as determined by SDS-PAGE under denaturing conditions. It is important to note that these analyses of the molecular mass of the HEBP have been performed employing denaturing conditions. These assays would therefore not reveal possible oligomeric forms of the HEBP.

3.4 SUMMARY

Extraction of the HEBP from the vervet monkey liver nuclei or the salt fractionation of hepatic cytosol proved to be insufficient for the prepurification of the HEBP. Heparin-Sepharose was advantageous in more than one way. Three oestrogen binding proteins (molecular masses: 68 000, 38 000 and 30 000 daltons) were partially purified from vervet monkey liver cytosol using an affinity chromatography procedure on diethylstilboestrol-Agarose. The overall purification achieved was 7 435-fold, by applying a combination of DES-Agarose and heparin-Sepharose affinity chromatography. The procedure yielded approximately 42 µg HEBP from 50 g vervet monkey liver, with a recovery of 30%. The partially purified HEBP migrated as three bands on a SDS-polyacrylamide ge with molecular masses of 68 000, 38 000 and 30 000 daltons. Isoelectric focussing of labelled, partially purified HEBP on agarose gels yielded two distinct oestradiol binding components with pI values of 6,8 and 6,9.

CHAPTER 4

THE ATTEMPTED MONOCLONAL ANTIBODY PRODUCTION AGAINST THE OESTROGEN-BINDING PROTEIN PARTIALLY PURIFIED FROM THE VERVET MONKEY LIVER

4.1 INTRODUCTION

The presence of other intracellular steroid-binding proteins and the low concentration of receptors in responsive tissues complicate the use of radiolabelled and unlabelled receptor ligands to detect and measure the ER in target tissues. Specific antibodies to the ER provide a means of detecting the receptor protein other than by binding to labelled hormone and therefore renders a new approach to the study of hormone receptor.

King and Greene (29) localized the ER in the nuclei of target cells with the aid of monoclonal antibodies to oestrophilin. Antibodies against the ER can be used for the preparation of immunoadsorbents. Similarities and differences between ER proteins present in different tissues of the same animal or between different animal species might be determined with ER antibodies. In fact, immunochemical similarities among ERs from several mammalian species as well as extranuclear and nuclear receptor forms have been demonstrated with antibodies against the ER (203-205).

Early assays for the ER such as the dextran coated charcoal (DCC) technique with Scatchard analysis, sucrose density gradient centrifugation and isoelectric focussing in polyacrylamide gels are based on the binding of radioactively labelled oestrogen (206). The disadvantage of relying upon ligand binding is that it is non-covalent and the ligand is subjected to displacement by dissociation. Moreover, heavy metal ions, enhanced temperatures, storage, manipulation of

the specimen and other oestrogen-binding proteins are factors influencing the hormone binding capacity (157, 207, 208). Considering these drawbacks it became clear that an immunoassay for detecting the ER may satisfy the demand for a rapid, simple and more stable method not based on the hormone binding capacity.

This chapter describes the attempted production of monoclonal antibodies against the vervet monkey HEBP, partially purified by affinity chromatography.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

17 -[^{3}H]-Oestradiol (130 Ci/mmol) was obtained from New England Nuclear. Radioligands were initially checked for radiochemical purity on reverse phase high performance liquid chromatography (HPLC). Diethylstilboestrol (DES) was supplied by Sigma Chemical Company. Freund's complete and incomplete adjuvants were purchased from Miles Laboratories. Chicken anti-mouse Ig (IgY) was from Bioclones (Pty.) Ltd. and Dulbecco's modified Eagle's medium from Flow Laboratories. All other reagents were obtained from Merck Chemicals.

4.2.2 Buffers and media

TEDAGM buffer was prepared as described in Chapter 2, Section 2.2.2.

TEDAGMT buffer: prepared as TEDAGM with the addition of Triton X - 100 (Merck Chemicals) to a final concentration of 0,1%.

Phosphate-buffered saline (PBS): Sodium chloride (150 mM), sodium phosphate (100 mM), EDTA (100 mM), pH 7,8.

Myeloma cells and hybrids were cultured in DMEM containing 2 mM L-glutamine and supplemented with sodium pyruvate (12 mM), penicillin (12 IU/mℓ), streptomycin (12 µg/mℓ), fungizone (1 µg/mℓ) and 12% heat inactivated horse serum (DMEM-HS). The Polyethylene glycol (PEG) solution used during cell fusion consisted of 2 g PEG in 2,8 mℓ DMEM. HAT- and HT-medium were prepared by the addition of 2 mℓ 50 x concentrated HAT (5 mM hypoxanthine, 20 µM aminopterin, 150 µM thymidine) or HT (5 mM hypoxanthine, 150µ M thymidine) to 100 mℓ DMEM-HS.

4.2.3 Preparation of the antigen

a) Preparation of DES-Trisacryl

DES-Trisacryl was prepared according to the method described in Chapter 3, Section 3.2.7.b) and Scheme 3.2

b) Partial purification of the antigen

One unit mass of vervet monkey liver was homogenized in 2 volumes of TEDAGM buffer. Following centrifugation Triton X - 100 was added to a final concentration of 0,1% to the resultant supernatant. It was then mixed with DES-Trisacryl (10 mℓ adsorbent per 100 mℓ cytosol) and gently stirred for 16 h at 4°C. The gel suspension was transferred to a sintered glass funnel and washed with four volumes of TEDAGMT buffer. The HEBP was eluted by incubating the adsorbent for 30 min. at 20°C with 5 µM oestradiol (0,3 volume). Excess oestradiol was removed from the eluate with DCC treatment. Eluates were concentrated by ultrafiltration (immersible CX - 10, Millipore) under reduced pressure. The antigen was stored in 50% glycerol at a protein concentration of 1 µg/µℓ (-4°C). The oestradiol binding capacity of various fractions was determined by adding an equal amount of tritiated oestradiol (25 nM) in the presence or absence of

a 1 000-fold excess of DES to aliquots of these fractions. Following an incubation period of 1 h at 4°C or 30 min. at 20°C, unbound ligand was removed with DCC treatment and the radioactivity measured. Protein concentrations were determined by employing the Bio-rad protein assay as described in Chapter 3, Section 3.2.10.

4.2.4 Immunization

The immunization program was performed on three six-week-old C 57 Black/6 mice of both sexes. For the primary immunization an emulsion of the antigen (50 µg protein/50 µℓ 50% glycerol) and Freund's complete adjuvant (one volume), prepared by sonification, was injected intradermally in the footpads. The first booster injection was given in incomplete adjuvant following a period of 14 days. Blood was collected seven days after the first booster injection. Following a rest period of three months the mice were injected intravenously and 24 h later intraperitoneally with 100 µg doses of antigen in saline. The animals were sacrificed two days later.

4.2.5 Radioactive hormone-receptor complexes

Tritiated oestradiol-HEBP complexes were prepared by the incubation of liver cytosol with an equal volume of tritiated oestradiol (25 nM) in the presence or absence of a 1 000-fold excess of DES. After an incubation period of 60 min. at 4°C unbound ligand was removed by means of DCC treatment. The radioactive hormone-receptor complex was used as labelled antigen during the immunoprecipitation assay.

4.2.6 Immunoprecipitation assay

In order to determine the amount of chicken anti-mouse Ig (IgY) sufficient to precipitate all the mouse Ig during the double antibody immunoprecipitation assay, a quantitative

precipitin curve was developed. A constant volume of normal mouse serum (11 µℓ) was added to a suitable range(5 - 35 µℓ) of accurately measured quantities of IgY. The total volumes were equalized with PBS. Each sample was mixed and incubated for 5 h at 4°C. Following centrifugation (2 000 *g* x 10 min.) in a refrigerator centrifuge the supernatants were removed and the drained precipitates washed with 0,5 mℓ volumes of PBS. The protein content of the fractions was determined with the Bio-rad protein assay described in Chapter 3, Section 3.2.10.

For immunoprecipitation, a solution of tritiated oestradiol-HEBP complex, normal mouse serum and immune serum in phosphate-buffered saline was incubated for 5 h at 4°C. Chicken anti-mouse Ig (IgY) was used for the precipitation of tritiated-E_2-HEBP-Ig complexes. Following 16 h the mixtures were centrifuged, washed with saline and dissolved in 0,2 M NaOH. The dissolved precipitates were neutralized with HCℓ containing scintillation cocktail and the radioactivity measured. Control assays were executed employing normal mouse serum tritiated-E_2-HEBP complex without immune serum.

4.2.7 Enzyme linked immuno sorbent assay

The partial purified HEBP protein was used in the preparation of antigen-coated microtitre plates. The antigen was dissolved in 0,05 M glycine, 0,1 M NaCℓ, pH 2,5 at a concentration of 1 - 10 µg protein per millilitre. After 5 - 10 min. the pH was adjusted to seven by the addition of solid Tris. Aliquots (100 µℓ) of the antigen solution were dispensed into the wells on a Linbro microtitre plate. The coated plate was incubated for 3 h at 37°C and freeze-dried. Remaining protein binding sites were blocked by incubation with 200 µℓ 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2 h at room temperature. After excess solution was removed the wells were washed with 1% BSA-PBS containing 0,05% Tween 20. Immune and control serum dilutions were prepared in 1% BSA-PBS

containing 0,05% Tween 20. Spent culture medium was used undiluted during screening stages as a result of the usually much lower concentration of antibody in the tissue culture supernatant. Aliquots (50 µℓ) were transferred to the antigen-coated wells. Following an incubation period of 30 min. The wells were washed with 1% BSA-PBS containing 0,05% Tween 20. A 1:300 dilution of a peroxidase conjugate (Horse radish peroxidase, goat anti-mouse IgG, Miles Yeda Ltd) was prepared in 1% BSA-PBS containing 0,05% Tween 20. Aliquots (50 µℓ) of this solution were transferred to the wells and incubated at room temperature for 30 min. The wells were then washed thrice with 1% BSA-PBS containing 0,05% Tween 20 and dried under vacuum. The substrate solution (5 mg O-phenylenediamine, 4 mg urea, hydrogen peroxide (H_2O_2), 10 mℓ 0,1 M citrate buffer pH 4,5) was then added to the wells (50 µℓ/well). Fifteen minutes later the enzyme reaction was monitored at 450 nm with a Titertek Multiskan MC. (Flow Laboratories, Helsinki, Finland).

4.2.8 Preparation of parental cells for fusion

Two mice were sacrificed by neck dislocation, dipped in 70% alcohol and placed on a dissection table in a sterile cabinet to remove the spleens under sterile conditions. The spleens were transferred to a petri dish containing DMEM. After cleaning the spleens they were transferred to a second DMEM-filled petri dish and washed gently. In a third DMEM filled petri dish the spleens were cut into pieces and gently torn apart. Afterwards the cells were transferred to a sterile plastic tube and placed on ice to allow sedimentation of larger tissue debris. The spleen cells were transferred to a 10 mℓ conical tube. The cells were spun down, resuspended in 10 mℓ DMEM and after a sample was taken for counting, spun down again.

A culture flask of $Sp^{2/0}$ (215) myeloma cells in logarithmic growth were pelleted by centrifugation after an aliquot was

taken for counting. Cell counting was performed on a hemacytometer employing the trypan blue exclusion method (222).

4.2.9 Cell fusion and selection of hybrids

Spleen cells and myeloma cells were mixed in a ratio of 10 : 1 in a total volume of 40 mℓ DMEM. Following centrifugation the supernatant was removed completely in order to avoid dilution of the PEG. The pellet was dispersed by gently flicking the cells in the tube. Pre-warmed (37°C) PEG solution (2 mℓ) was gradually added over a period of 1 min. while flicking the tube gently to suspend the cells. The mixture was then capped and incubated for 1 min. at 37°C. Pre-warmed DMEM, 5 mℓ, 10 mℓ and 25 mℓ quantities respectively were added to the cell suspension over intervals of 5 min. The cells were spun down, decanted and resuspended in 50 mℓ HAT-medium. A sample was taken for counting. The cells were diluted with HAT-medium to 500 000 cells per mℓ, applied to 5 microtiter tissue culture plates (474 wells) at approximately 100 µℓ per well and transferred to a CO_2 incubator at 37°C. After three days the wells were inspected for cell proliferation and 100 µℓ of HAT-medium added. Three days later clones of cells were seen growing in some of the wells. Half of the spent medium was removed from each well and more HAT-medium (100 µℓ) added. Following two more days of incubation the cultures were fed with HT-medium and the process repeated three days later. Fifteen days after the cell fusion, screening was done employing the ELISA technique described in Section 4.2.7.

4.2.10 Polyclonal cell cultures

The polyclonal cell cultures which showed a positive reaction against the immunized antigen were allowed to grow to confluence. These cell cultures were later transferred to individual wells on a multi dish and finally to culture flasks. The selected cultures were maintained for nearly two

months prior to cloning. During these two months common tissue culture contaminants (bacteria, yeast and fungi) were observed in some of the cell cultures and had to be controlled. Apart from the antibiotics routinely added to growth media, additional decontaminating measures were taken. Contaminated cell cultures were pelleted by centrifugation at 300 *g* for 5 min. (4°C). The supernatants were removed and the pellets resuspended in DMEM without heat-inactivated horse serum and with twice the amount of fungizone and penicillin-streptomycin usually added. Centrifugation was repeated and the pellet resuspended in DMEM-HS. The cell suspension was transferred to a new sterile growth flask. If necessary cell cultures were transferred back into a multidish.

4.2.11 Freezing in of cells

The freezing medium consisted of 35 mℓ DMEM (0,48 g DMEM powder with L-glutamine, 0,13 g sodium bicarbonate, 35 mℓ distilled de-ionised sterile water pH 7,2), 5 mℓ dimethyl sulphoxide, 10 mℓ heat inactivated horse serum. Cell cultures to be frozen were in full logarithmic growth phase. The number of cells pelleted by centrifugation (7 min., 400 *g*, 4°C) was in the range of 10^6 to 10^7. The cell pellet was resuspended in 0,5 mℓ freezing medium (4°C) following removal of the supernatant. This cell suspension was transferred to a freezing vial (Sterilin) which was placed in a polystyrene box (1 cm thick walls) in a -70°C freezer. A gradual temperature decrease was ensured by the insulating box at -70°C. The frozen cells were kept at -70°C.

4.2.12 Cloning

The following media solutions were used during cloning:

2 x DMEM: 0,819 g DMEM dry powder (Flow Laboratories), 88 mM $NaHCO_3$, 0,144 mℓ penicillin-streptomycin (5 000 units/mℓ, Flow Laboratories, 0,24 mℓ fungizone (250 µg/mℓ Flow laboratories),

0,72 mℓ sodium pyruvate (100 mM, Flow Laboratories) in 30 mℓ double distilled, deionised sterile water (pH 7,2).

DMEM: 9 mℓ 2 x DMEM and 9 mℓ distilled de-ionised sterile water.

DMEM - HS consisted of 15 mℓ 2 x DMEM, 18 mℓ DMEM and 12 mℓ heat inactivated donor horse serum (Flow Laboratories). This was filtered through a 0,22 micron filter and kept at 45°C.

2% Agar: 1 g Agar (Bacto Agar) in 50 mℓ double distilled deionised sterile water. This was autoclaved for 15 - 20 min. and kept at 45°C.

A 0,5% agar solution (15 mℓ) was poured into each petri dish (9 cm diameter, tissue culture grade, Sterilin) and allowed to set at room temperature. Several cell suspensions (5×10^3, $2,5 \times 10^3$, 1×10^3 cells/mℓ) were prepared. A volume (1,2 mℓ) of 0,5% Agar (at 45°C) was added to 0,8 mℓ of each cell suspension (at room temperature). Each tube was mixed and poured immediately onto the solidified agar base. The agar was allowed to set at room temperature and incubated at 37°C in a CO_2 incubator.

Clones were picked following 5-6 days. The picked clones were transferred to individual cups on a Linbro plate and allowed to grow to confluence. The spent medium of each clone was assayed for antibody activity using an ELISA.

4.3 RESULTS AND DISCUSSION

4.3.1 Partial purification of the antigen

Two strategies are generally used in monoclonal antibody (McAb) studies: (a) purification of the desired antigen, or (b) employing relatively impure tissue extracts to immunize the spleen-donor animal (209). The concept behind the second

strategy is that the amount of labour put into the purification of the antigen will be decreased or even substituted by the selective screening of antibody producing hybridomas and by cloning. However, in practice this idea is complicated by immunogenicity of antigenic determinants on other macromolecules present in the preparation. The purity of the immunogen becomes important if it gives weak specific responses compared to immunodominant contaminants or if the screening assay employed following fusion and cloning can not distinguish between antibodies to the desired specific components and antibodies to unwanted impurities (210).

The partial purification of the vervet monkey HEBP used as antigen during this investigation was obtained by DES-Trisacryl GF 2000 affinity chromatography. A 6 091-fold purification together with a recovery of 13,5% was achieved (Table 4.1). In order to obtain the 'native' (8S) HEBP to avoid the risk of immunising against additional exposed sites, 0,1% Triton X - 100 was added to buffers during the purification procedure rather than salts that favour the 4S receptor entity. Several groups reported the production of polyclonal (PcAb) (203-205) and monoclonal antibodies (McAb) (175, 211, 212) to the oestrogen receptor by the immunization with purified preparations of the oestrogen receptor. Purification procedures employed, range from affinity chromatography (175) and nuclear extraction (203) to salt precipitation, gel filtration and PAGE (205).

4.3.2 Immunization

Special attention should be given to the immunization program since the success of the cell fusion depends on the immune state of the spleen-donor animal. Various immunization protocols have been suggested for different antigens (210). Because of the possibility of different individual reactions to the immunogen following immunization, three mice were immunized with the partially purified HEBP. A primary subcutaneous injection of 50 µg HEBP in Freund's complete

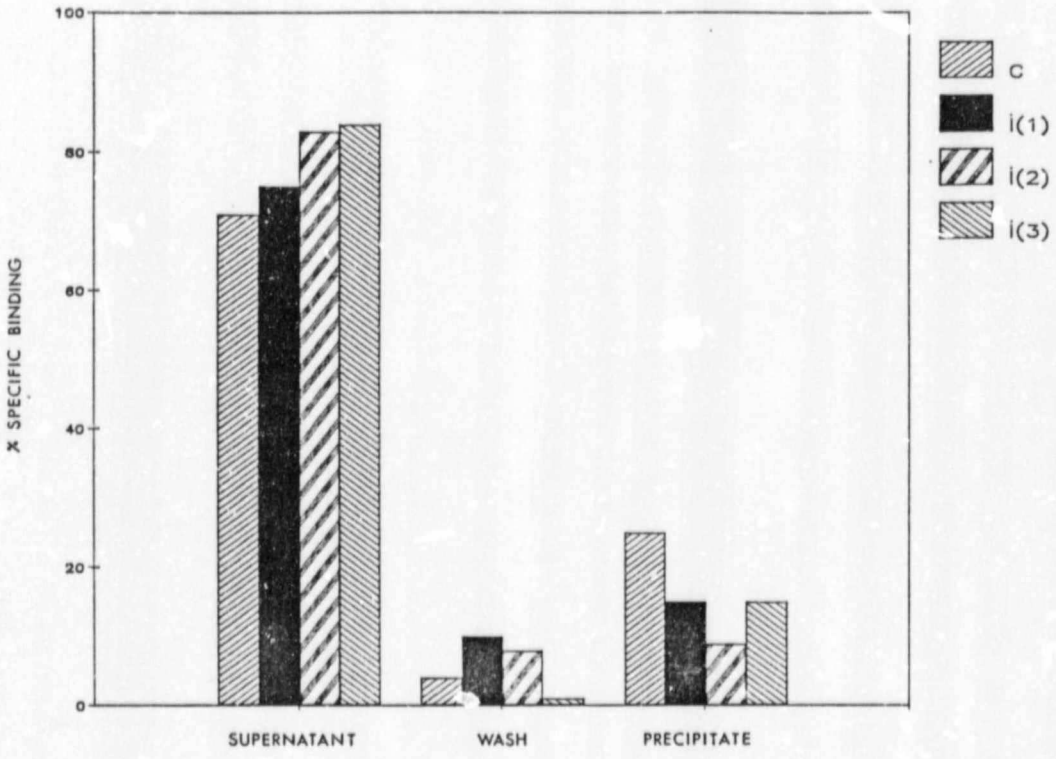
TABLE 4.1: THE PARTIAL PURIFICATION OF THE VERVET MONKEY HEBP USING DES-TRISACRYL GF 2000 AFFINITY CHROMATOGRAPHY. THE EXPERIMENT SUMMARIZED HERE WAS PERFORMED AS DESCRIBED IN SECTION 4.2.3.b).

Purification step	Total volume (mℓ)	Total binding activity (dpm)	Total protein (mg)	Specific activity (dpm/mg)	Recovery (%)	Purification (-fold)
Cytosol	100	$1,1 \times 10^7$	2 279	4 939	100	1
Eluate (Following DCC treatment)	30	$1,5 \times 10^6$	0,050	$3,0 \times 10^7$	13,5	6 091

adjuvant was followed by a booster injection in Freund's incomplete adjuvant two weeks later. Blood was collected seven days after the booster injection to determine the quality and quantity of antibody generated. Results obtained by immunoprecipitation and ELISA on control and immune sera are illustrated in Figures 4.1 and 4.2. The data illustrated in Figure 4.1 is summarized in Table 4.2. No positive reaction could be observed when employing immunoprecipitation as the screening assay, probably because of the extremely low levels of antibody. Nevertheless, immune sera 1 and 2 demonstrated antibody activity when screened by ELISA. However, the ELISA does n distinguish between specific and non-specific binding, thus the demonstrated antibody activity could be non-specific. Following a rest period of three months and an intravenous antigen injection the animals were boosted intraperitoneally (24 h later) and the fusion executed 24 h later. The best time for fusion appeared to be 1 - 4 days following the booster since antigen-induced proliferation is strong during this period (213).

Small amounts of ER, together with immunodominant contaminants in the partially purified ER preparate as well as a weak immunogenicity of the antigen could possibly be responsible for the observed inconclusive immuno response. Different approaches have been suggested by several authors to overcome this problem: (a) conjugation of the antigen to a highly immunogenic carrier (213), (b) adsorption of the antigen to nitrocellulose, (c) direct injection of the antigen into the spleen (213) or, (d) specific adsorption of an immunodominant contaminant on a monoclonal antibody affinity matrix prior to immunization (209).

The production of McAb to a receptor protein by the immunization of a structurally constrained agonist of the specific receptor, conjugated to BSA, has previously been reported (214). This is based on the hypothesis that macro= molecules of the same specificity will show structural homologies in their binding sites. Thus, antibodies to a



FIGURES 4.1

A representative histogram of results obtained for the immunoprecipitation reaction between ^{3}H-E_2 HEBP-antibody complexes and chicken anti-mouse immunoglobulin (IgY). The immunoprecipitation reaction was executed on immune (i(1-3)) and control serum (c) as described in Section 4.2.6. Antigen-antibody complexes were precipitated by the addition of IgY to a concentration that gives maximum precipitation.

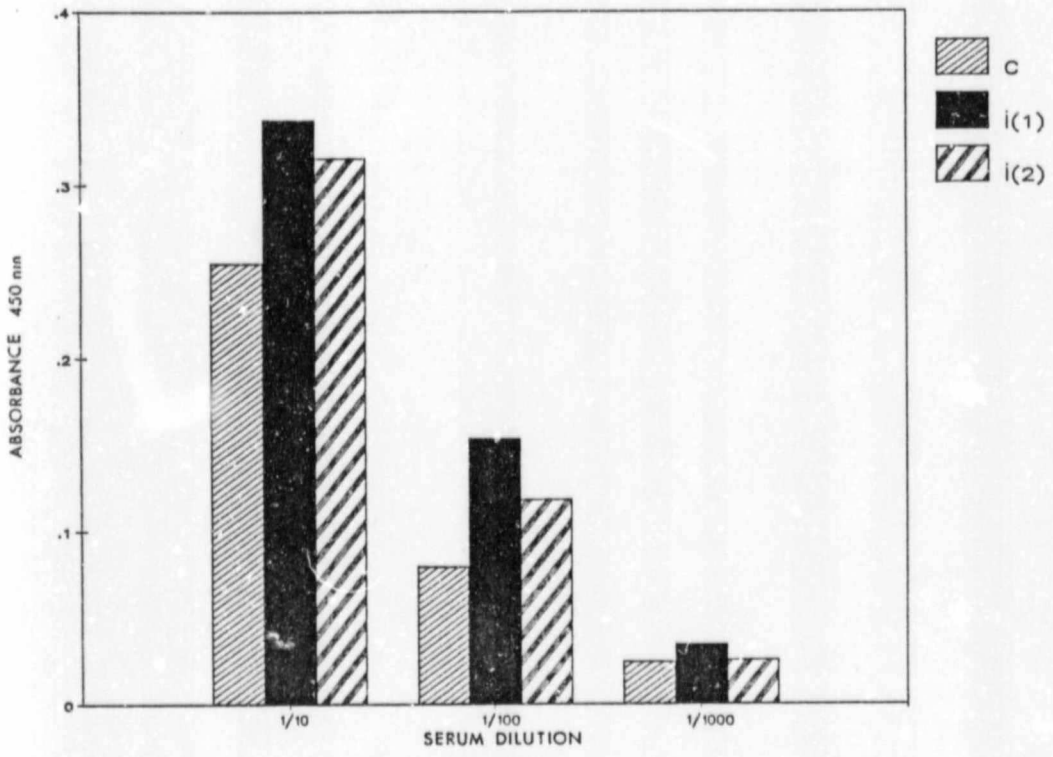


FIGURE 4.2

Results obtained on ELISAs executed on control (c) and immune sera (i(1), i(2)) to detect any reaction against the partially purified antigen (HEBP) bound to a solid support. The ELISA was performed as described in Section 4.2.7 on three different serum dilutions (0,1, 0,01 and 0,001).

TABLE 4.2: RESULTS OBTAINED FOR THE IMMUNOPRECIPITATION REACTION BETWEEN $^{3}H\text{-}E_2$ HEBP-ANTIBODY COMPLEXES AND CHICKEN ANTI-MOUSE IMMUNOGLOBULIN (IgY).

Serum	PBS	$^{3}H\text{-}E_2$ HEBP[a] (TB/NSB)	IgY	$^{3}H\text{-}E_2$ HEBP Content (% specific binding)		
(11 µℓ)	(µℓ)	(µℓ)	(µℓ)	Supernatant	Saline wash	Precipitate
Control	875	100/100	25	71	4	25
Immune 1	875	100/100	25	75	10	15
Immune 2	875	100/100	25	83	8	9
Immune 3	875	100/100	25	84	1	15

[a] $^{3}H\text{-}E_2$ HEBP = radioactive hormone-receptor complex prepared as described in Section 4.2.5