Acta Chemica Scandinavica B 38 (1984) 407–414

Purification and Characterization of an Estradiol-17 β Binding Macromolecule in Rat Pancreas

ÅKE POUSETTE,[a] LARS-ERIK APPELGREN,[d] KJELL CARLSTRÖM,[b] HOLGER SKÖLDEFORS,[c] NILS WILKING[c] and NILS OLOF THEVE[c]

[a] Department of Clinical Chemistry, Karolinska Institutet, Huddinge University Hospital, S-141 86 Huddinge, Sweden, [b] Hormone Laboratory, Department of Obstetrics and Gynecology, Huddinge University Hospital, S-141 86 Huddinge, Sweden, [c] Department of Surgery, Sabbatsbergs Hospital, Box 6401, S-113 82 Stockholm, Sweden and [d] Department of Pharmacology, The Swedish University of Agricultural Sciences, BMC, Box 573, S-751 23 Uppsala, Sweden

A high capacity estradiol-17β binding protein has been purified from rat pancreatic cytosol. The protein constitutes about 2 % of the protein content in the cytosol. Using estradiol-17β as ligand the K_d *in vitro* has been calculated to 2.0×10^{-7} M. Estrone and estriol also show affinity to the protein but testosterone, progesterone and dexamethasone do not. The protein has been purified to homogeneity by chromatography on Concanavalin-A and hydroxylapatite followed by preparative polyacrylamide gel electrophoresis. The purified protein formed a single protein band when analyzed in different systems.

The partially purified [^{3}H]estradiol-macromolecule complex formed a 2.6 S complex using sucrose gradient analysis, a Stokes radius of 35 Å when analyzed using gel chromatography and focused at pI 4.6. The complex did not bind to phosphocellulose or DNA-cellulose and showed no similarities to [^{3}H]estradiol-receptor complexes.

The biological function of this protein is unknown, but may include regulation of estrogen fluxes in pancreatic cells. The purified protein from rat pancreas is similar but not identical to the earlier purified estrogen binding protein from human pancreas. The presence and purification of the protein from rat pancreas will make it possible to use rat pancreas as a model system to study the biological function of a high capacity intracellular steroid binding protein.

Estrogens have long since been known to affect several tissues and the general mechanism of action has been elucidated during the last decade.[1,2] The estrogen molecule binds to a cytoplasmic receptor protein and the complex formed is translocated into the nucleus where transcription of specific mRNAs is initiated. In this way estrogens direct the protein synthesis in estrogen sensitive cells. Specific estrogen receptor proteins are recognized by a high ligand affinity (K_d 10^{-9} M) but a low binding capacity (usually less than 1 000 fmol/mg of cytosolic protein).*

Although not considered as classical estrogen sensitive tissue, the pancreas has been shown in

* The abbreviations and trivial names used are:

estradiol	estradiol-17β
estriol	1,3,5(10)-estratriene-3,16α,17β-triol
estrone	3-hydroxy-1,3,5(10)-estratriene-17-one
testosterone	17β-hydroxy-4-androstene-3-one
dexamethasone	9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione
estramustine	1,3,5(10)-estratriene-3,17β-diol, 3-*N*-bis(2-chloroethyl)carbamate (Leo 275)
EBP	Estradiol Binding Protein in rat pancreas

0302-4369/84 $2.50

several investigations to respond to sex hormone stimulation, and estrogen treatment has been shown to cause changes in the patient's pancreatic function.[3-5] In the rat, estrogen treatment causes marked changes in the morphology of the pancreas as well as in the biochemical composition of the pancreatic juice.[6-7] Although these findings indicate estrogen sensitivity of the pancreas, it is doubtful whether classical estrogen receptors are present in pancreatic tissue. Some groups have reported the detection of estrogen receptor proteins[8-9] while others including ourselves have failed to demonstrate the presence of classical receptor proteins.[10]

After administration of [^{3}H]estradiol to rats, the highest degree of accumulation was found in the pancreas.[11,12] We have recently demonstrated the presence of a high capacity–low affinity estrogen binding protein in human pancreatic cytosol[13] and this high retention of radioactivity is probably due to the binding of [^{3}H]estradiol to a similar protein in the rat pancreas. Sandberg and co-workers,[6,8] have studied the retention of estrogens in rat pancreas and have demonstrated that binding to a specific protein is responsible for the estrogen uptake. More recently Boctor and Grossman[14] have further characterized this protein and have shown that a small molecule (accessory factor), being probably a peptide, is necessary for maximal binding of the ligand.

In order to further elucidate the use of rat pancreas as a model for studying the biological role of a high capacity–low affinity intracellular estrogen binding protein, we found it necessary to purify this protein in order to achieve a homogenous system and to be able to raise antibodies against it. The present communication describes the purification to homogeneity and the partial characterization of the main estradiol-protein complex present in rat pancreatic cytosol.

MATERIALS AND METHODS

Steroids. [2,4,6,7-^{3}H]estradiol-17β (3400 GBq/mmol) was purchased from New England Nuclear Chemicals, GmbH, Dreieichenhain, West Germany. Unlabeled steroids were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Preparation of cytosol. Pancreas from 8 week old male rats of the Sprague-Dawley strain were used in these experiments. After decapitation pancreatic tissue was removed and homogenized in 3 volumes of ice cold buffer A (Tris 50 mM, EDTA 1 mM, NaCl 50 mM, pH 7.4) using an Ultra-Turrax homogenizer. The homogenate was centrifuged for 60 min at 105 000×g. After removal of the floating lipid layer, the cytosol was decanted and immediately frozen (−20 °C). Pools of cytosol were prepared from 20 rats and stored in 10 ml aliquots. Aliquots to be used were thawed on ice before being further processed.

Equilibrium dialysis. 0.5 ml of cytosol diluted 1:10 was added to a well washed dialysis tube, and dialyzed against 10 ml of buffer A containing 500 000 dpm ^{3}H-estradiol diluted with increasing concentrations of unlabeled estradiol. The tubes were continuously rotated for 12–16 h before aliquots were taken inside and outside the bag for measurements of radioactivity. Aliquots taken outside the bag were assumed to represent the free protein-unbound fraction. The protein-bound fraction was calculated as total radioactivity per ml inside the bag minus total radioactivity per ml outside the bag. Calculation of K_d and total binding was performed according to Scatchard.[15] Protein determination was performed according to Lowry *et al*[16] using bovine serum albumin as standard.

Competition experiment. In order to study the steroid specificity of the macromolecule, competition experiments were performed using the same techniques as above. 500 000 dpm ^{3}H-estradiol was diluted with 5 μg of unlabeled estradiol, which saturates the estradiol-binding protein. Increasing amounts of unlabeled steroids were added to the buffer and dialysis was performed as described above.

Metabolism of ^{3}H-estradiol. Dialyses were performed using 5×10^6 dpm ^{3}H-estradiol diluted with 0.1 and 5 μg of unlabeled estradiol-17β. After dialysis, aliquots (0.5 ml) were taken inside and outside the bag and extracted 3 times using 3 ml of ethyl acetate. More than 99 % of the radioactivity was recovered in the ethyl acetate phase. The pooled extracts were evaporated to dryness under nitrogen, redissolved in a minimal amount of acetone and subjected to TLC on precoated silica gel plates (250 μ, Merck AG, Darmstadt, West Germany) using toluene–ethanol, 87:13 (v/v) as solvent. After drying, the TLC plates were scanned for radioactivity using a Berthold thin-layer scanner, Model II (Berthold, Wildbad, West Germany).

Purification of the estradiol-binding protein. Pools of 200 ml of cytosol were prepared and frozen in aliquots containing 10 ml. Forty ml were thawed on ice and divided into two 20 ml portions. In order to follow the purification, the cytosol was incubated with 50 000 dpm ^{3}H-

estradiol-17β/ml for 1 h at 0 °C. Each portion was chromatographed on a 40 ml Concanavalin-A column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in buffer A. After application of the sample, the column was washed with buffer A until no further protein was eluted (measured at 280–310 nm). The washing was repeated using buffer A, 1 M KCl, followed by elution of the estradiol-17β-binding protein with buffer A, 1 M KCl, 0.1 M α-D-mannose-glycosidic acid.

The eluate was collected in 2 ml fractions. Fractions containing radioactivity were pooled from two columns and the pooled eluate was applied to a 20 ml hydroxyl-apatite column. The column was washed using buffer A, 1 M KCl until no further radioactivity or protein was eluted. The estradiol-binding protein was then eluted using buffer A, 1 M KCl 1 M K_2PO_4. The sharp radioactivity peak (about 5 ml) was collected and dialyzed against buffer A for 12–16 h. After concentration to about 0.5 ml using a Sartorius membrane (Sartorius GmbH, 34 Göttingen, West Germany), the concentrated eluate was incubated using 500 000 dpm ^{3}H-estradiol-17β/ml for 16 h. The sample was then subjected to electrophoresis on a 10 % cross linked polyacrylamide gel.[13] The area containing the estradiol binding protein was sliced in 1.2 mm thick slices. The individual slices were transfered to test tubes and crushed before 0.5 ml buffer A was added. After continuous stirring for 12–16 h, 2×10 μl was taken for determination of radioactivity. Aliquots from tubes containing high radioactivity were analyzed for purity using the gel system above or using electrode buffer pH 7.4. For testing purity gels were cut in 2 parts along the gel using one part for calculation of radioactivity and one for protein staining.

High Performance Liquid Chromatography (HPLC). A Varian HPLC 5010 with an SW 3000 column (27 cm) was used for analysis of eluates from preparative polyacrylamide gel electrophoreses. This column separates proteins according to molecular weights. The column was equilibrated in buffer A and 100 μl samples were analyzed. The chromatography was peformed at a flow of 1.5 ml/min and the eluate was collected in 1 ml fractions. Aliquots were analyzed for protein concentration and radioactivity.

Other procedures. A partly purified preparation was used for further characterization of the 17β-estradiol-protein complex. Cytosol was prepared as described above and chromatographed on Concanavalin-A and hydroxylapatite. The eluate was dialyzed, concentrated and reincubated before further analyses were carried out.

Sucrose gradient analysis was essentially carried out as earlier described.[13] The eluate from the hydroxylapatite chromatography was concentrated 5 times and reincubated with 50 000 dpm [^{3}H]estradiol/0.1 ml for 1 h. The incubate was passed over a Sephadex G-25 (medium) column before an aliquot (0.2 ml) of the protein bound fraction was taken for analysis. Bovine serum albumin (Sigma) and cytochrome C (Sigma) were used as markers.

Ultrogel ACA-44, DEAE-Sephadex, DNA-cellulose and Phosphocellulose chromatography were performed as described previously.[17,18,19]

Preparation of serum. Blood was collected from normal male rats and allowed to clot before centrifugation at 3,000×g for 10 min. The supernatants were pooled before the serum samples were treated in the same way as cytosol.

Isoelectric focusing. Analytical isoelectric focusing was performed as earlier described.[19]

Table 1. Relative binding affinity (RBA) of various ligands for the estradiol binding protein in rat pancreatic cytosol. Binding was analyzed using the equilibrium dialysis technique. For compounds giving curves not parallel to that of estradiol, RBA was not calculated.

$$\text{RBA} = \frac{\text{Excess of 17}\beta\text{-estradiol required for 50 \% inhibition}}{\text{Excess of competitor required for 50 \% inhibition}}$$

Ligand	RBA
Estradiol	1
Estrone	0.73
Estriol	0.56
Testosterone	No competition
Progesterone	No competition
Dexamethasone	No competition

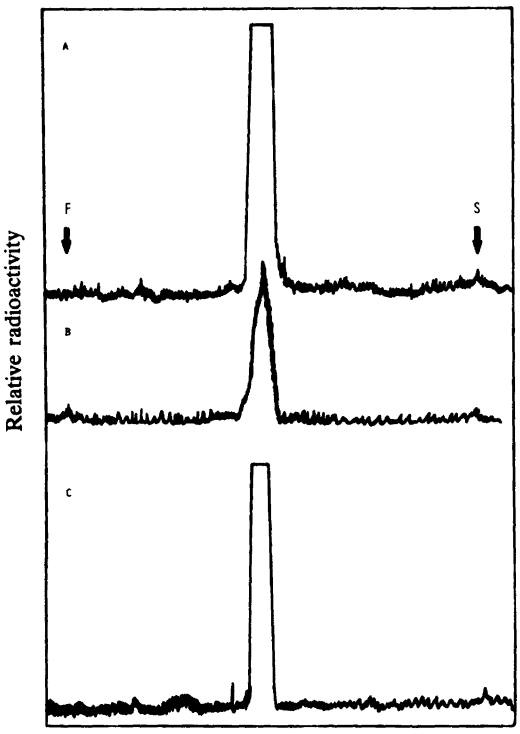

Fig. 1. Radioactivity scanner chromatograms of thin-layer plates with extracts from incubations of [^{3}H]estradiol (A) and [^{3}H]estradiol diluted with 5 μg estradiol (C) with cytosol. Following incubations and extractions the incubates were analyzed by thin-layer chromatography. The plates were developed using the solvent system, ethanol–toluene, 13:87. ^{3}H-estradiol was used as standard (B). Increasing sensitivity was used but no metabolism could be detected. S=start of chromatography. F=front.

RESULTS

Using cytosol prepared from male rat pancreas and estradiol as ligand a K_d of 1.7×10^{-7} M and B_{max} of 80 nmol/mg of cytosolic protein was calculated. Assuming an estradiol macromolecule binding ratio 1:1 and a MW around 50,000 it was calculated that the estradiol binding protein (EBP) constituted about 2 % of the proteins in the cytosol. Competition data summarized in Table 1 indicate high ligand specificity, since only estrogens showed any affinity for the protein. Rat serum showed nonspecific nonsaturable binding of ^{3}H-estradiol when binding was analyzed using equilibrium dialyses. However, no binding could be detected when serum had been passed over a Concanavalin-A column and no ^{3}H-estradiol-macromolecule complex was retained when serum was labeled with ^{3}H-estradiol prior to chromatography on Concanavalin-A. These results strongly indicate that EBP is formed in the pancreas. No metabolism of estradiol-17β could be detected using the incubation conditions described above (Fig. 1).

Purification of EBP. The purification of EBP is summarized in Table 2. Because of the dissociation of the estradiol-macromolecule complex no reliable calculation of the purification in every single step can be made (Figs. 2 and 3). The macromolecule-[^{3}H]-estradiol complex extracted following polyacrylamide gel electrophoresis appeared as a single protein band comigrating with the radioactivity when the eluate was ana-

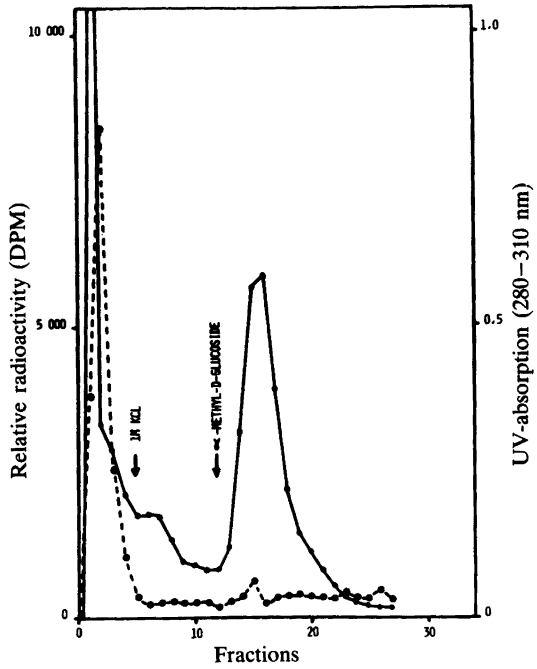

Fig. 2. Chromatography of ^{3}H-estradiol labeled cytosol on Concanavalin-A. Labeled cytosol was added to the column that was washed with buffer A, Al–1 M KCl before the flucoproteins were eluted using α-D-*manno*-glycosidic acid 0.1 M in Al, 1 M KCl buffer, ○ radioactivity. --- UV absorption (280–310 nm). (See material and methods.)

Table 2. Purification of the ^{3}H-estradiol-17β binding protein in rat pancreatic cytosol.

	Total protein (mg)	Purity	Total EBP	
Cytosol	400	2 %	8 mg	Purity calculted from equilibrium dialyses
Eluate from Concanavalin-A	32	nd[a]	nd	—
Eluate from OH-apatite dialyses and concentration	4	40 %	nd	Purity calculated from analytical polyacrylamide gel electrophoreses
Polyacrylamide gel electrophoresis	0.4	>95 %	400 μg	Purity calculated from different analytical polyacrylamide gel electrophoreses

[a] nd=not determined.

lyzed using the same gel system with electrode buffers of pH 7.4 or 8.4.

HPLC analysis revealed one single protein band that was comigrating with the radioactivity (Fig. 3). These results indicate a very high purity of the preparations. The overall recovery was 5–10 % in different preparations. Higher recovery could be obtained when less starting material was used, but the total recovery calculated in g was then lower. It should be stressed that an accessory factor is needed for maximal binding, and calculation of recovery in every single step is impossible.

Phosphocellulose and DNA-cellulose chromatography. The ^{3}H-estradiol-macromolecule complex either in crude cytosol or in cytosol partially purified using Concanavalin-A and hydroxylapatite chromatography did not bind to phosphocellulose or DNA-cellulose.

Ultrogel ACA-44 gel chromatography and density gradient centrifugation. Chromatography of the ^{3}H-estradiol-macromolecule complex on an ACA-44 gel column showed a Stokes radius of 35 Å, and sucrose gradient analysis a sedimentation constant of 2.6 S (Fig. 4).

Isoelectric focusing. The [^{3}H]estradiol-Macromolecule complex in crude cytosol or in cytosol partially purified by Concanavalin-A and hydroxyl-apatite chromatography focused at pH 4.6.

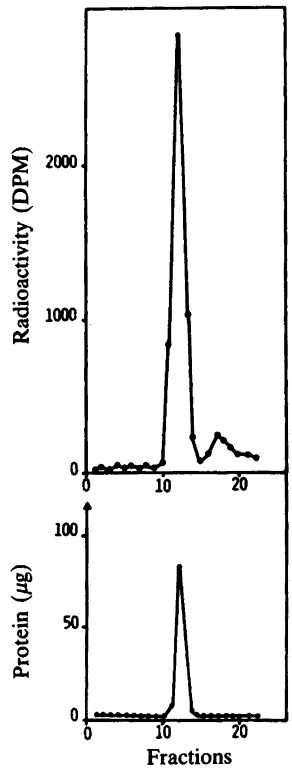

Fig. 3. Analyses of eluate from polyacrylamide gel electrophoresis using high pressure liquid chromatography. After chromatography 1 ml fractions were collected and aliquots were taken for analyses of protein and radioactivity.

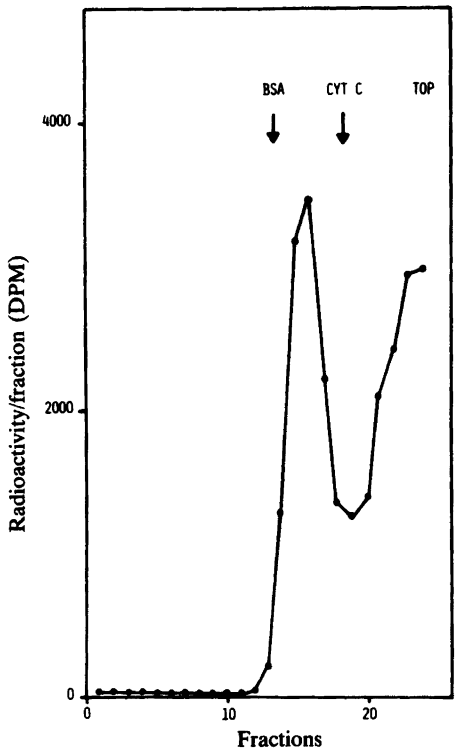

Fig. 4. Sucrose density gradient centrifugation of partly purified ^{3}H-estradiol protein complex. Centrifugation was performed at 40 000 rpm for 15 h at 2 °C in an SW 50.1 rotor. Standards: Bovine serum albumin (BSA) and Cytochrome C (Cyt C) were run in parallel tubes. The radioactive peak corresponds to a protein with a sedimentation coefficient of about 2.6 S.

DISCUSSION

Based upon the reports on effects of estrogens on pancreatic function, morphology and biochemistry,[3-7] and our recent finding of an estrogen binding protein, similar to EBP, in human pancreas,[13] we are now evaluating antiestrogens for the treatment of pancreatic carcinoma.[20] However, animal model systems will be necessary for further studies on estrogen-pancreatic interaction and on the role of these high capacity–medium affinity estrogen binding proteins. The rat was chosen as model animal, based on our and other groups' previous work with this animal.[9,14,18] The purification protocol used in the present study does not compensate for variations in estrogen binding due to loss of the accessory factor necessary for maximal binding.[14] Since the recovery of protein in some cases was calculated as the recovery of the [^{3}H]-labeled ligand, this might be influenced by the concentration of the accessory factor.

Estrogen action in most tissues is undoubtedly mediated via cytoplasmatic receptor proteins. However, besides these high affinity binding proteins, steroid binding proteins with medium affinity ($K_d \cdot 10^{-7}$ M) are also present in many tissues. The biological role of the latter proteins is unknown. Concerning the pancreas, several studies have shown the exocrine part to be sensitive to estrogens.[6-7] The results of estrogen receptor determinations in this organ are, however, inconclusive.[8-10] Estrogen receptor analysis in pancreatic samples are hampered by two technical problems: The presence of high levels of the medium affinity estrogen binding protein EBP and high protease activity. Binding to EBP has

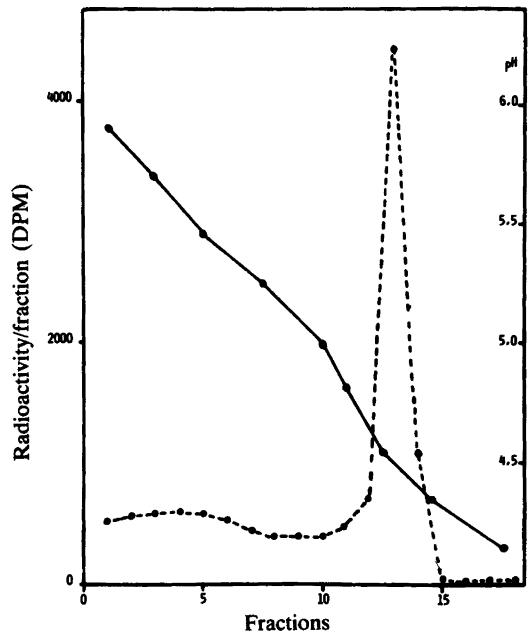

Fig. 5. Isoelectric focusing of ^{3}H-estradiol-protein-complex performed on polyacrylamide gels. Pancreatic cytosol was incubated with ^{3}H-estradiol (10 μg/ml) overnight and aliquots were analyzed by isoelectric focusing. pH was measured before the gel was sliced and radioactivity was calculated.

Table 3. Comparison between the estradiol binding protein from pancreas cytosol, EBP, described in this paper and the prostatic secretion protein, PSP.[21a]

	PSP	EBP	Comment
Isoelectric point	5	5 (4.6)	
S-value	3	2.6	
Stokes radius (Å)	35	35	Calculated on ACA-44
Binding to DNA-cellulose	No	No	
Binding to phosphocellulose	No	No	
Binding to heparin-sepharose	No	No	(data not shown)
Binding to Concanavalin-A	Yes	Yes	
Binding to octyl-Sepharose	Yes	Yes	(data not shown)
Ligand specificity	Different		

[a] Partial characterization shows two similar proteins differing only in ligand specificity. K_d for the ^{3}H-estradiol-EBP complex is calculated to 2×10^{-7} M, but estradiol does not compete with estramustine for binding to PSP.[12]

undoubtedly in some reports been described as receptor binding. On the other hand, it is quite possible that negative findings are due to destruction of the estrogen receptor protein by proteases.

If estrogen receptors are present in pancreatic tissue, their concentration is probably rather low. Therefore it is tempting to speculate that EBP might be involved in the action of estrogens in the pancreas. The high retention of [^{3}H]estradiol in the pancreas, shown in the rat, suggests concentration of estrogen in the tissue as a possible function of EBP. High local estrogen concentrations would lead to saturation of the estrogen receptor protein. A direct regulatory function of the EBP-estrogen complex is another possibility. If so, the absence of binding of the complex to DNA or phosphocellulose and the fact that the complex is not taken up by nuclei, indicate an extranuclear effect.

The high ligand specificity of EBP makes an involvement in estrogen action mechanism very likely. In this respect a comparison with the rat prostatic secretion protein (PSP) is of interest.[21] PSP is a secretory steroid binding protein with properties similar to EBP, with exception of the ligand specificity (Table 3). PSP has been shown to modulate the nuclear androgen uptake *in vitro*.[17] It would therefore be of interest to study the effect of EBP on the nuclear uptake of steroid hormones including androgens. Androgen receptors have previously been demonstrated in pancreatic tissue.[18]

The present protein was purified from the 105,000×g supernatant, but nothing is actually known about its intracellular localization. This as well as the tissue distribution of the protein can be studied by immunological techniques. Work is now in progress to raise antibodies against this protein in order to study the function of this estradiol binding protein using the pancreas as a unique model system.

Acknowledgements. This work was supported by grants from the Swedish Medical Research Council, LEO Research Foundation, *Alex och Eva Wallströms* and *Hagströms Stiftelse.*

REFERENCES

1. King, R. and Mainwaring, W. *Steroid Cell Interactions,* Butterworths, London 1974.
2. Jensen, E., Mohla, S., Gorell, S. and Taud de Sombre, E. *Vitam. Hormon. 32* (1974) 89.
3. Davidoff, F., Tishler, S. and Rosoff, C. *N. Engl. J. Med. 289* (1973) 552.
4. Gluech, C., Scheel, D., Fishbach, J. and Stener, P. *Metabolism 21* (1972) 657.
5. Greenberger, N., Hatch, F., Drummey, G. and Isselbacher, K. *Medicine (Baltimore) 45* (1966) 161.
6. Sandberg, A. and Rosenthal, J. *J. Steroid Biochem. 11* (1979) 293.
7. Grossman, A., Boctor, A. M. and Lane, B. *Endocrinology 85* (1969) 956.
8. Sandberg, A. and Rosenthal, H. *J. Steroid Biochem. 5* (1974) 969.
9. Boctor, A. M. and Grossman, A. *Fed. Proc. 40* (1981) 1875.
10. Greenway, B., Iqbal, M. and Johnson, P. *Br. Med. J. 283* (1981) 751.

11. Forsgren, B., Gustafsson, J.-Å., Pousette, Å. and Högberg, B. *Cancer Res. 39* (1979) 5155.
12. Ullberg, S. and Bengtsson, G. *Acta Endocrinol. (Copenhagen)* (1963) 75.
13. Pousette, Å., Carlström, K., Sköldefors, H., Wilking, N. and Theve, N. O. *Cancer Res. 42* (1982) 633.
14. Boctor, A. M. and Grossman, A. *Proc. Natl. Acad. Sci. U.S.A. 78* (1981) 5648.
15. Scatchard, G. *Ann. N. Y. Acad. Sci. 51* (1949) 660.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randell, R. *J. Biol. Chem. 193* (1951) 265.
17. Pousette, P., Björk, P., Carlström, K., Forsgren, B., Högberg, B. and Gustafsson, J.-Å. *The Prostate 2* (1981) 23.
18. Pousette, Å. *Biochem. J. 157* (1976) 229.
19. Pousette, Å., Snochowski, M., Bression, D., Högberg, B. and Gustafsson, J.-Å. *Biochim. Biophys. Acta 582* (1979) 358.
20. Theve, N. O., Pousette, Å. and Carlström, K. *Clin. Oncol. 9* (1983) 193.
21. Forsgren, B., Björk, P., Carlström, K., Gustafsson, J.-Å., Pousette, Å. and Högberg, B. *Proc. Natl. Acad. Sci. U.S.A. 76* (1979) 3149.

Received August 12, 1983.