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ESTROGEN BINDING PROTEINS IN RAT PANCREAS

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SUMMARY

Estrogen binding proteins (EBP) of pancreatic acinar cells of a number of species have been reported by us previously. This work examines the organ specificity of EBP. Microsomal fractions of pancreas, liver, parotid gland, kidney, lung, abdominal muscle and submaxillary gland were incubated with estradiol (E_2). After the microsomal pellets of each tissue had adsorbed E_2, the pellets were washed twice by resuspension and recentrifugation and the last supernatant obtained was tested for E_2 binding by sucrose density gradient. Over 80% of the E_2 was found in the bound form in the supernatant of the pancreatic microsomal pellet, whereas no bound E_2 was found in the supernatants of the microsomal pellets of any of the other tissues tested. Organ specificity became also apparent by immunodiffusion tests of extracts of different tissue against antibodies to EBP. One strong and some weaker precipitin lines are produced by pancreatic extracts. Weaker precipitin lines were observed by extracts of submaxillary gland and liver. Precipitin lines were also visible from extracts of epididymis, prostate, testis, muscle, parotid gland, lymph nodes, and, maybe, a weak reaction with thyroid. No precipitin lines were observed with hypothalamus, uterus or ovaries. Organ specificity was made noticeable by Ouchterlony immuno-diffusion plates which showed that the *strong* precipitin line of pancreatic extracts is not shared with those of extracts from other tissues.

INTRODUCTION

The role that specific cytoplasmic receptors for reproductive steroid hormones play in the processes of transcription in target organs has been under investigation for some time [1]. A now-classical picture has emerged whereby the steroid–receptor complex translocates to the nucleus and stimulates RNA synthesis followed in time by protein synthesis. More recent reports include binding to the microsomal fraction of reproductive organs and its relation to cytoplasmic receptors [2, 3]. Specific binding for reproductive steroids have also been reported in tissues that are considered to be non-reproductive, e.g., E_2 and DHT* in the kidney [4, 5], E_2 and E_3 in the pancreas [6], and androgens in the pancreas [7]. High capacity binding with association constants lower than $10^8\ M^{-1}$ has in general been ignored in the past in favor of investigations of receptors, which are present at very low concentrations but with high association constants. The long residence period of estrogens in the pancreas after *in vivo* injections [8] of these steroids led us to look not only for receptors in the pancreas but also for high capacity binders. In this paper we are reporting on experiments designed to examine the organ specificity of high capacity cytosolic and microsomal estrogen binders in the pancreas.

* DHT = 17β-hydroxy-5α-androstan-3-one. E_2 = estradiol-17β. E_3 = estriol. EBP = esrtrogen binding protein.

EXPERIMENTAL

Estradiol-17β[2,4,6,7-^{3}H(N)] and Estriol[2,4,6,7-^{3}H(N)] with specific activities of 105 and 109 μCi/nmol, respectively, were purchased from the New England Nuclear Corporation and checked for purity by chromatography on Sephadex G50. The following buffers were used: TK(0.01 M Tris–HCl, 0.15 M KCl, 0.02% NaN_3; pH 7.4), TE(0.01 M Tris–HCl, 0.0015 M Na_2EDTA, 0.02% NaN_3; pH 7.4), TEK (0.01 M Tris–HCl, 0.0015 M Na_2EDTA, 0.4 M KCl, 0.02% NaN_3; pH 7.4), and TKMgCa (0.01 M Tris–HCl, 0.15 M KCl, 0.002 M $MgCl_2$, 0.002 M $CaCl_2$, 0.02% NaN_3; pH 7.4).

Distribution of E_2 and E_3 over cellular fractions

Mature, male Sprague–Dawley rats of average weight of 450 g were anesthetized with ether and killed by cervical dislocation. Surgically removed pancreases were rinsed with ice cold saline. The tissue was minced, and incubated at 35°C for 20 min in RPMI 1640 tissue culture medium (1:1 by vol.) containing 5 nM or 1 μM of ^{3}H labeled E_2 or E_3. All procedures thereafter were carried out at 0–4°C. RPMI 1640 (obtainable from Grand Island Biological Co., NY 14072, under Cat. No. H-18) contains inorganic salts, amino acids and vitamins, but no calf serum. The mince was centrifuged at 600 *g* for 10 min and the pellet washed twice with two vol of TK and once with TE by resuspension and recentrifugation at 600 *g*. The pellet was then homogenized with an

equal volume of TE with a motor-driven Potter–Elvehjem Teflon–glass homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min, the pellet resuspended 3 times in two vol. of TE and once in two vol. of TEK and recentrifuged at 1000 *g* for 10 min after each suspension. The supernates of the TE suspensions were considered the "rinse" of the crude nuclear pellet and those from the TEK the "extract". The supernatant from the first 1000 *g* centrifugation was recentrifuged at 10,000 *g* for 20 min and the crude mitochondrial pellet rinsed and extracted in the same manner as the nuclear pellet. The supernatant of the first 10,000 *g* centrifugation was recentrifuged at 105,000 *g* for 75 min and the crude microsomal pellet was rinsed and extracted with TE and TEK as above. The supernate of the first 105,000 *g* centrifugation (the cytosol), the various rinses, extracts, and remaining pellets of the tissue were measured for radioactivity.

Partial purification of estrogen-binding proteins

A microsomal extract, which was prepared in the above manner from a mince of 25 rat pancreases incubated with 0.57 μM labeled E_2, was dialyzed against a twenty-fold volume of TE and chromatographed on Sephadex G100 in a 2.5 × 100 cm column from Pharmacia. Eluting buffer was TE in an upward flow of about 12 ml/h. This chromatography was followed by a DEAE-cellulose chromatography in 0.01 M Tris–HCl, pH 7.6 with a linear gradient of 0 to 0.3 M KCl from two 250 ml reservoirs. The column dimension was 0.9 × 60 cm. The peak E_2-binding areas were rechromatographed on Sephadex G100 in columns of 0.9 × 60 cm. The partially purified EBPs were tested by SDS- polyacrylamide gel electrophoresis according to Laemmli[9].

Uptake of E_2 by microsomal pellets of different tissues

Protocol I. Minced tissues were washed twice in two volumes of TKMgCa and homogenized in TKMgCa. The homogenate was centrifuged at 10,000 *g* followed by a centrifugation of the supernatant at 105,000 *g*. The microsomal pellets were washed twice in two vol. TKMgCa and incubated at 35° for 20 min with two vol. of cytosol or buffer preincubated with 10 nM of ^{3}H-labeled E_2. The microsomal incubates were centrifuged and the supernatants counted and tested by sucrose density gradient. The pellets were washed twice with three vol. TKMgCa and counted for radioactivity. The second washing was tested by a 5–20% sucrose density gradient, centrifuged in a Beckman Ultracentrifuge (model L2-65B) with a SW41 rotor at 41,000 rev/min. (205,000 $g_{av.}$) for 20 h. The approximately 40 fractions of the sucrose gradient were counted for radioactivity. Per cent steroid bound was calculated from the amount of free steroid found at the top of the gradient and the amount of bound steroid that traveled into the gradient.

Protocol II. Microsomal pellets were prepared as in Protocol I. The pellets were suspended in 50 vol. TE and incubated with ^{3}H-labeled E_2. The suspensions were centrifuged at 105,000 *g* the pellets counted, and the supernatant tested by sucrose density gradient.

Immunodiffusion

Rabbit antiserum to partially purified microsomal E_2 binder(s) was obtained by intradermal injections of about 1 mg protein in Freund's complete adjuvant at multiple sites. Booster injections of about 0.5 mg each were given after three and five weeks. The rabbits were bled once a week. Bleedings of the third through the eighth week were pooled and concentrated by precipitation with $(NH_4)_2SO_4$. To that end, 3 vol. of 4 M $(NH_4)_2SO_4$ were added under stirring at room temperature to 2 vol. rabbit serum, which resulted in a precipitation at 40% $(NH_4)_2SO_4$ saturation. The precipitate was left overnight at 4°C, centrifuged at 10,000 *g* for 20 min, separated from the supernatant, washed twice with $(NH_4)_2SO_4$ of 40% saturation, dissolved in saline-borate buffer, pH 7.9, at about 14% the vol. of the original rabbit serum, and extensively dialyzed against the same buffer.

To obtain tissue extracts for the immunodiffusion experiments, liver, spleen, kidney, testes, epididymis, lung, muscle, and pancreas were minced and homogenized in 0.25 M sucrose–TKMgCa. The homogenate was centrifuged at 10,000 *g*. The pellet was sonicated in TEK, let sit at 4°C overnight, centrifuged at 10,000 *g*, the supernatant recentrifuged at 105,000 *g* and the supernatant after this centrifugation tested for precipitin reaction. The supernatant of the first 10,000 *g* centrifugation mentioned above was recentrifuged at 105,000 *g*. This supernatant, i.e., the cytosol, was also tested. The microsomal pellet was sonicated in TE, let sit overnight, recentrifuged at 105,000 *g* and the supernatant tested. The smaller tissues, prostate, adrenal, parotid, lymph, thyroid, pituitary and hypothalamus were sonicated in TE with or without prior mincing and centrifuged at 105,000 *g*. The supernatants were tested. The pellets were sonicated in TEK, let sit overnight, and recentrifuged at 105,000 *g*. The last supernatants were also tested. Sonifications were done at 50 W in 10–20 s bursts with a Branson sonifier.

Immunodiffusion was carried out on microscope slides covered with 1% agarose in 0.025 M veronal buffer of pH 8.6.

Autoradiography of immunodiffusion plates

After the development of precipitin lines in agarose on microscopic slides, the slides were thoroughly rinsed free of unprecipitated protein. The washed slides were submerged in water containing 1 μCi of [^{14}C]-E_2 per 100 ml and left at 4°C with gentle stirring. The slides were removed after 2 days, drained free of excess [^{14}C]-E_2-containing water and air-dried under filter paper. The dried slides were exposed to X-ray film, Kodak XR-1, for 2–4 weeks before development of the film. After the conclusion of the auto-

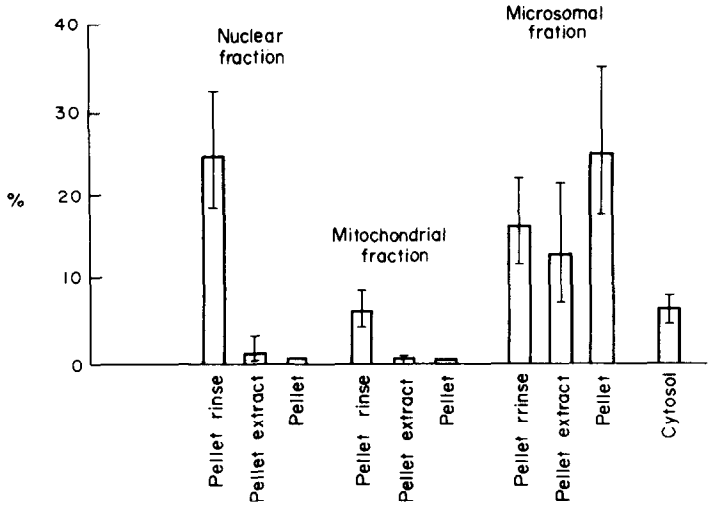

Fig. 1. Minced pancreas was incubated with ^{3}H-labeled E_2, homogenized and fractionated. About 76% of the radioactivity was taken up by tissue mince. The bar diagram shows the distribution in per cent of the radioactivity recovered from the minced tissue.

radiography, the slides were stained with naphtol blue-black for better visualization of the precipitin lines.

RESULTS

The distribution of E_2 or E_3 in cellular fractions of pancreatic tissue after *in vitro* incubations of minced tissue with radioactive estrogens is shown in Figs 1 and 2. Most of the estrogens were recovered in two cellullar fractions, i.e., the cytosol and microsomal pellet. The distribution was the same whether incubations of mince were carried out in 5 nM or 1 μM concentrations of estrogen. Per cent binding was also unaffected by the increase in estrogen concentration. About 91% of the E_2 was bound in cytosol or microsomal extract when measured by sucrose density gradient, and about 67% of E_3. Relatively large amounts of estrogen were recovered in the TE rinses of the pellets. This observation prompted us to continue the TE rinses of a microsomal pellet. Each subsequent rinse released about as much E_2 as the previous ones for at least 3 more rinses, with % binding remaining as high as in the TEK microsomal extracts. The pancreatic microsomal E_2 binder(s) are precipitable by $(NH_4)_2SO_4$ at 55–60% saturation and are denatured by heating to 50°C.

The uptake of E_2 from a buffer solution by the microsomal pellet of pancreas was compared with the uptake by microsomal pellets of liver or abdominal muscle tissue according to protocol I (Table 1, 3rd column). The uptake was high in all three pellets, i.e.,

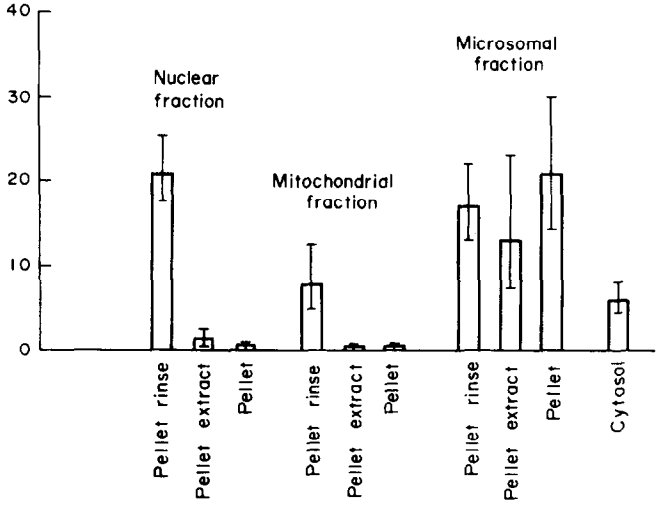

Fig. 2. Minced pancrease was incubated with ^{3}H-labeled E_3, homogenized and fractionated. About 72% of the radioactivity was taken bup by tissue mince. The bar diagram shows the distribution in per cent of the radioactivity recovered from the minced tissue.

Table 1. Incubation of microsomal pellet with E_2-containing buffer or cytosol

Buffer or cytosol	Microsomal pellet	% E_2 in pellet	% Bound E_2 in supernatant of washed pellet
Buffer	Pancreas	93	81
Buffer	Liver	91	0
Buffer	Muscle	88	0
Pancreas	Pancreas	84	74
Pancreas	Liver	49	~10
Pancreas	Muscle	19	42
Liver	Liver	80	0
Muscle	Muscle	79	0

93% in pancreas, 91% in liver, and 88% in muscle. Only a small per cent of E_2 was recovered in the washings of the pellets, i.e., 3–4% in the second washing. There was, however, a marked difference between pancreas and liver or muscle concerning the state of the E_2 found in this second washing of the pellet; E_2 was over 80% bound in the supernate of the pancreatic pellet, but totally free in the supernates of the liver or muscle microsomal pellets (Table 1, 4th column). A smaller amount of bound E_2 could be found in the second washing of a microsomal pellet of muscle (<45%) when the microsomal pellet was incubated with E_2-containing pancreatic cytosol instead of buffer. Incubations of the microsomal pellets of liver or muscle with their respective cytosol resulted only in free E_2 in the second washing of the pellets. These results (of 2 experiments) are summarized in Table 1.

In another set of experiments, the uptake of E_2 from a buffer solution by pancreatic microsomal pellet was compared with the uptake by microsomal pellets of kidney, submaxillary gland, lung, parotid, and liver according to protocol II. Although the microsomal pellets of each tissue took up E_2, per cent bound was high (~84%) only in the supernates of the pancreatic pellets (2 experiments) as shown in Table 2.

Partial purification of the microsomal E_2 binder(s) on a large Sephadex G100 column followed by a DEAE-cellulose column resulted in two peaks of bound E_2. Peak I eluted at about 0.13 M KCl and peak II at about 0.16 M KCl. Each peak was rechromatographed on a small Sephadex G100 column. Thus, two different materials were obtained by the partial purification of one microsomal extract. Samples, marked I and II respectively, were analyzed by SDS-acrylamide gel electrophoresis (Fig. 3). Two strong bands with mol. wt. of 27,000 and 45,000 daltons and a number of minor bands are noted. Rabbit antiserum was obtained to the material that had fewer minor bands. A concentrated γ-globulin fraction was used for most of the precipitin tests against extracts of various tissues. Ouchterlony immunodiffusion plates showed that the strong precipitin lines of pancreatic extracts are not shared with precipitin lines of extracts from other tissues. Precipitin lines of pancreas are compared with those of spleen, liver, kidney, lung, or adrenal in Fig. 4. Only some weaker precipitin lines of pancreatic extract are shared. Similar results were obtained for most of the other tissue extracts tested, i.e., epididymis, prostate, testis, muscle, parotid gland, submaxillary gland, lymph nodes, and, maybe, a weak reaction with thyroid. The strongest reactions with non-pancreatic tissues were obtained by extracts of submaxillary gland and liver. Autoradiography of slides exposed to [^{14}C]-E_2 showed that the strong pancreas-specific precipitin line as well as the weaker, non-organ-specific lines contain E_2 binders. No precipitin lines were observed with hypothalamus, uterus, or ovaries. Also, no reactions were obtained with rat serum or serum albumin. Figure 5 shows lines of identity for pancreas cytosol and microsomal extract.

Table 2. Incubation of microsomal pellet in 50× vol. of E_2-containing buffer

Tissue	% E_2 in pellet	% Bound in supernatant
Pancreas	17	84
Liver	74	*
Lung	36	*
Submaxillary	43	*
Parotid	46	*
Kidney	48	*

* Between 0 and 10, too low to measure in this assay.

DISCUSSION

In vitro incubations of pancreatic minces show that binding protein(s) of high capacity and substantial affinity for estrogens are present primarily in the cytoplasm and in association with the microsomal fraction. A unique organ specificity of the microsomal EPB(s) is demonstrated by incubations of microsomal fractions from various tissues with E_2. Only extracts of pancreatic microsomal pellets contained bound E_2. Another demonstration of organ specificity are the

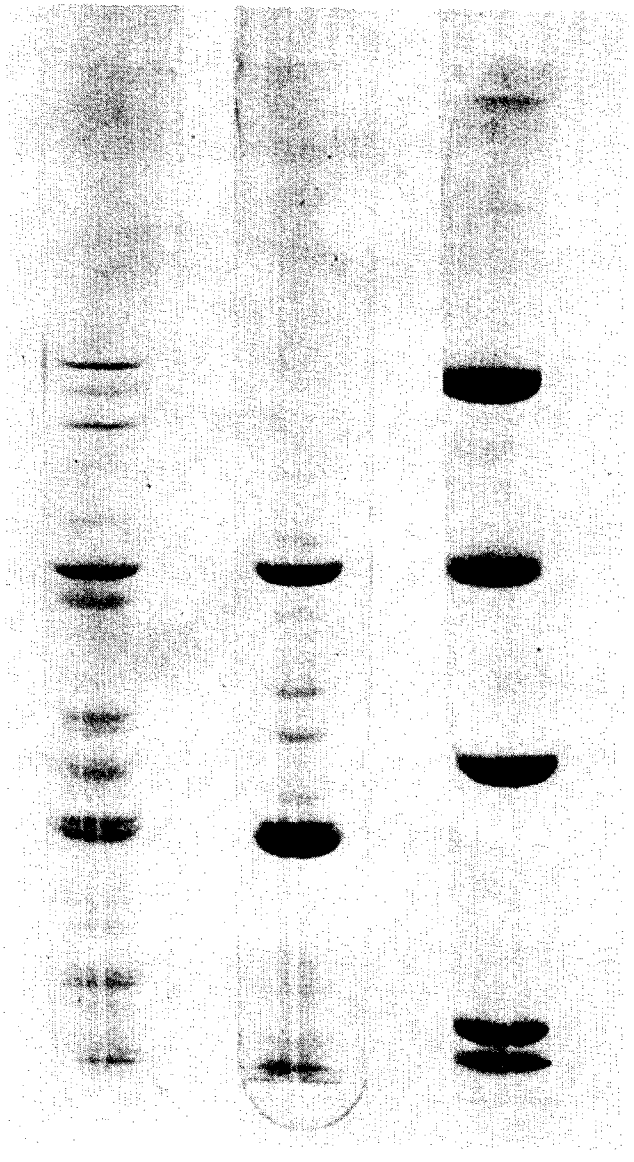

Fig. 3. SDS acylamide gel electrophoresis. From left to right: Sample II, Sample I, and standards. The standards applied where BSA, ovalbumin, carbonic anhydrase, β-lactoglobulin, and cytochrome *C* with 68,000, 43,000, 32,000, 18,000 and 12,000 mol. wt., respectively. To obtain samples I and II, microsomal estrogen binders were partially purified on Sephadex G100 followed by DEAE cellulose chromatography, which yielded 2 EBP peaks, peaks I and II. These were rechromatographed, separately, on Sephadex G100, thereby providing for Samples I and II, respectively.

Ouchterlony immunodiffusion plates that were prepared with antiserum to partially purified pancreatic EBP(s). A strong precipitin line obtained with pancreatic extracts is not shared with lines obtained from extracts of other tissues. The precipitin lines of other tissues are much weaker and are shared with the weaker line(s) of pancreatic extract. By a technique involving autoradiography we could show that the strong as well as the weaker precipitin lines contain E_2 binders. Precipitin lines of tissues other than pancreas were strongest for liver and submaxillary gland. It is interesting to note that the submaxillary gland and pancreas are embryologically related.

The importance of steroid receptors for transcription has been well documented for many organs [10]. Less is known about the effects of estrogens on the microsomal level. Blyth *et al.*[11] showed that estrogens together with androgens affect the attachment of ribosomes to liver endoplasmic reticulum. A tissue and hormone specific E_2-binding component of low affinity ($K_D = 5 \times 10^{-6}$ M) in chicken liver has been reported by Geschwendt[12]. The pancreatic estrogen binder(s) may be related to the sex steroid binding macromolecules detected in liver endoplasmic reticulum. An androgen receptor in the rat pancreas was demonstrated by Pousette[7]. The author specu-

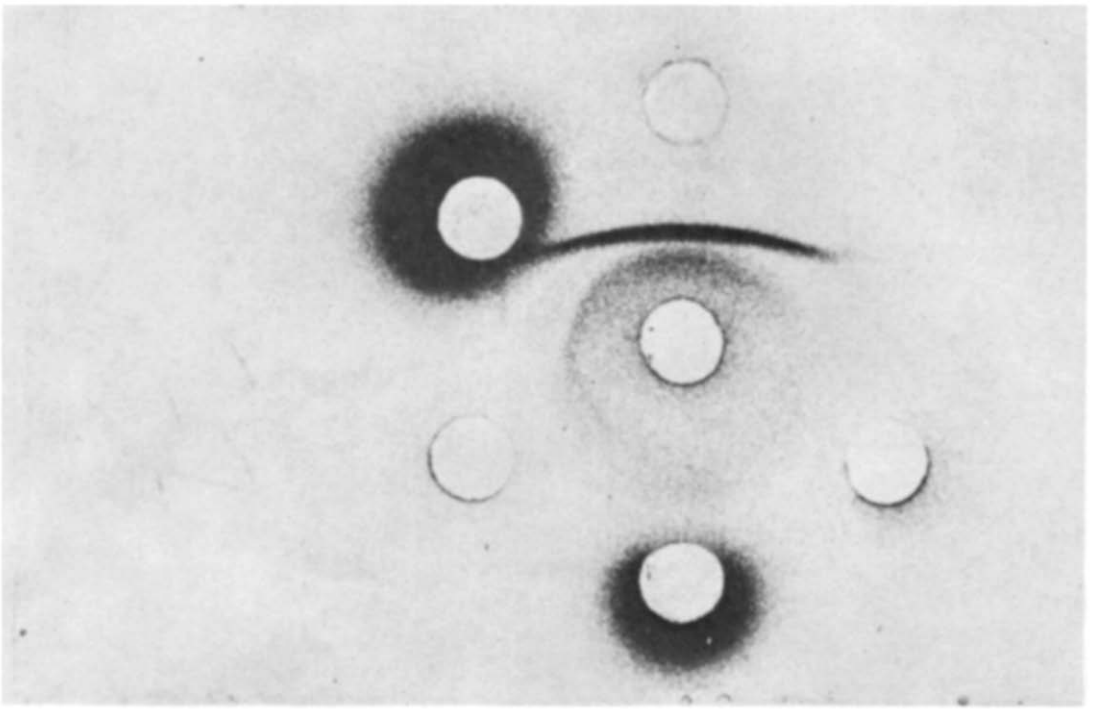

Fig. 4. Ouchterlony immunodiffusion plate with antibodies to Sample I in center well. Clockwise from top: pancreas, adrenal, spleen, kidney, liver, lung.

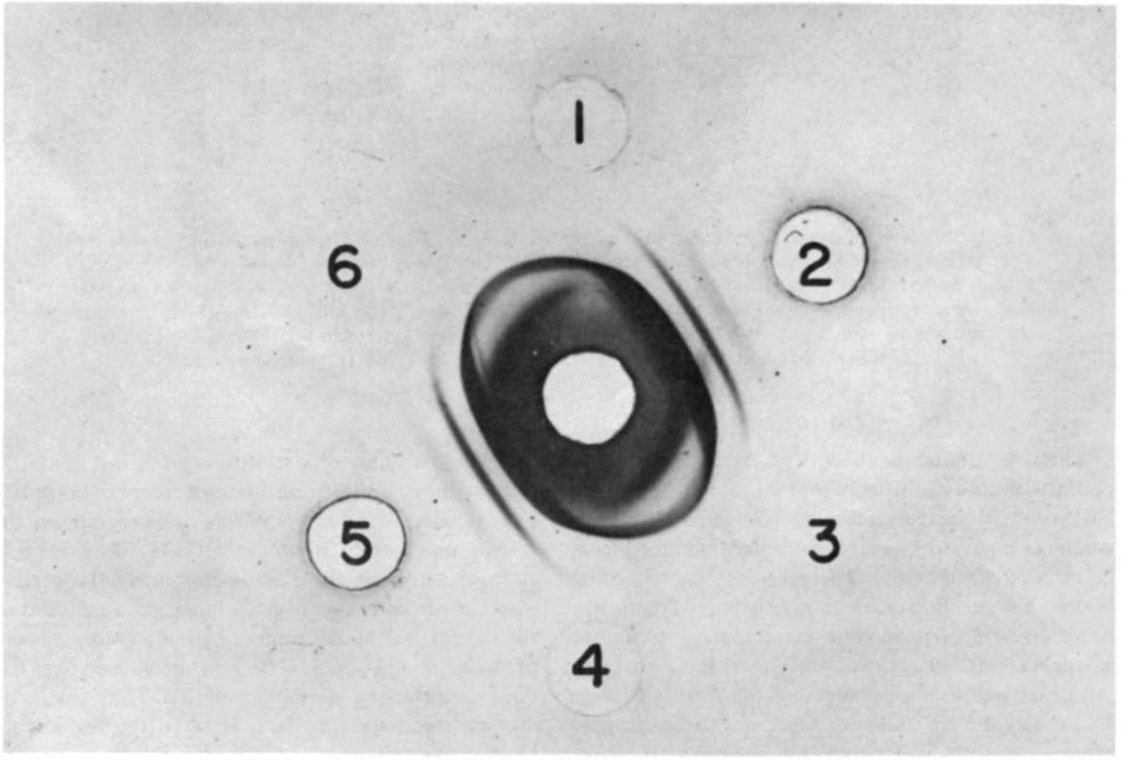

Fig. 5. Ouchterlony immunodiffusion plate with antibodies in center well. In outer wells are; 1 and 4, microsomal extract; 2 and 5, cytosol; 3 and 6, purified E_2 binder.

lated that the pancreatic androgen receptor may play a role in sex steroid regulation of pancreatic function. The presence of this receptor, as well as the estrogen binders described by us [6 and this paper], raise the possibility that an equilibrium in pancreatic function is related to the ratio of androgen and estrogen in the acinar cells.

Our findings may be of potential importance in the field of pancreatic physiology and pathology. Current investigations in our laboratory indicate that estrogen administration to dogs leads to decreased levels of bicarbonate, and increased levels of amylase and lipase. Electrolyte movement within the kidney has been reported by DeVries *et al.*[4] to be under the influence of estrogens. Estrogens are probably involved in the function of excretory glands. In this connection, it is interesting to note that pancreatitis and other changes related to pancreatic function have been described in patients who have been receiving either estrogens or contraceptive pills containing estrogens [13–16]. The recent increase reported in the incidence of cancer of the pancreas is taking place primarily in males, so that this increase is sex dependent.

We think that non-specific binders as well as specific binders are of biological importance. The pancreas offers a unique model to investigate the possible biological function of a high capacity binding system for estrogens.

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