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ESTRADIOL RECEPTOR FUNCTIONS OF SOLUBLE PROTEINS FROM TARGET-SPECIFIC LYSOSOMES

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SUMMARY

Recent work from these laboratories has implicated lysosomal function in the abrupt and preferential uptake of isotopically labeled steroid hormones in specific target cells *in vivo*. The present investigations were designed to analyze this interaction in detail by means of a rigorously controlled Dextran–coated charcoal method. Lysosome-enriched fractions were prepared from preputial glands of ovariectomized rats by the non-destructive methods previously described. The membrane-bounded organelles were then subjected to osmotic shock in the hypotonic media such as are in general use for the extraction of "soluble" steroid-binding proteins from hormone-sensitive tissues. The resultant particle- and membrane-free ("lysosol") extracts contained macromolecules which possessed the high-affinity, low-capacity characteristics requisite to authentic hormonal receptors, as analyzed by the Scatchard plot. Binding was suppressed by proteolytic enzymes and also in the presence of unlabeled estradiol-17β. The 17α-congener was ineffective in competition, except at extremely high molar ratios. Interaction of [^{3}H]-estradiol-17β was target selective, as indicated by relative inertness of "lysosol" from liver. Direct comparison of binding capacities of both high- and moderate-affinity sites revealed that while the affinity constants were similar, lysosomal proteins were substantially more effective than those of corresponding whole cytoplasm of preputial gland within the same experiment. Prolonged extraction of these preparations in the cold promoted an increase in concentration of specific binding proteins in "cytosol" at the expense of structurally latent macromolecules of related function in the lysosomal fraction. These data thus support the possibility that lysosomal proteins extruded from these fragile organelles during vigorous homogenization in hyposmotic media may serve as the source of the "cytosol" receptor molecules for steroid hormones.

INTRODUCTION

It is now well established that estradiol interaction with "soluble" cytoplasmic ("cytosol") proteins is a primary step in the two-stage transfer of the hormone into the nucleus of the specific target cell [1, 2]. What is not at all clear, however, is the precise subcellular source of such proteins, either for the estrogens or for other steroidal ligands. This gap in our understanding is the more glaring in view of the predictably disruptive effects of vigorous mechanical homogenization in the hypotonic buffers generally employed for extraction of "receptor" proteins from steroidal targets [3], were such macromolecules found to occur within membrane-bounded organelles.

Evidence linking organ-selective lysosomal function to the action of the steroid hormones has been presented [4–8]. In the course of these investigations, it had been noted that intense and rapid accumulation *in vitro* of [^{3}H]-estradiol-17β [1,3,5(10)-estratriene-3,17β-diol] occurred in macromolecular fractions extracted by hypotonic saline from highly purified lysosomes following isolation of these organelles in structurally intact state from preputial glands of ovariectomized rats [5]. These sex accessory structures are target organs for gonadal hormones of both sexes [9] and are highly enriched in lysosomes [6]. These results, which had their counterpart *in vivo* [8], were consonant with the characteristics of hormone uptake by reproductive targets in the intact organism [1, 2].

We were prompted by these observations to examine in greater detail certain features of the association of [^{3}H]-estradiol-17β with macromolecules which are extracted with hypotonic buffers from purified lysosome preparations of preputial gland. The present investigations reveal that such macromolecules possess the typical properties of steroid hormonal receptors, including target selectivity, stereospecificity, high affinity and ready saturability. Binding activity was sensitive to proteolysis. Comparison of these binding properties of "lysosol" proteins to those of the corresponding "cytosol" preparations demonstrated close similarity of association constants. Moreover, the relatively higher specific radioactivities of lysosomal proteins than those of the "cytosol", coupled with the progressive increase in "cytosol" binding at the expense of

structurally latent lysosomal protein on prolonged extraction, are compatible with the possible lysosomal origin of at least a portion of the "cytosol" binding function for estradiol in the preputial gland.

MATERIALS AND METHODS

Female rats of an inbred Sprague–Dawley strain were ovariectomized at 6 weeks of age and approximately 160 g body wt. The animals were then kept for 3 weeks in a low-steroid environment under controlled conditions of light and temperature [8]. On the day of experiment, preputial glands were excised under light Nembutal (sodium pentobarbital; 5 mg/100 g body wt.) anesthesia, rapidly chilled, weighed, and minced in pools of 15–20 paired glands. Comparable samples of uteri and liver were also prepared in some experiments. Unless otherwise indicated, no prior treatment of the experimental animals was undertaken. Subsequent preparative procedures were conducted at 0–4°C. Cellular disruption was carried out in an Emanuel and Chaikoff press [10]; (Microchemical Specialties Co., Inc., Berkeley, California), as described elsewhere [6]. With isosmotic media, use of this instrument instead of homogenization in glass provided particulate preparations of superior integrity [6].

Lysosome preparations

The post-nuclear supernatant from the organ homogenate which had been prepared in 0·25 M sucrose or in the homogenization medium described elsewhere [11], was processed for isolation of a lysosome-enriched fraction as previously reported [6, 7, 11]. The resultant washed pellet was then resuspended in 0·054 M sodium phosphate buffer, pH 7·7, generally in a concentration of 7·5 ml/15 paired preputial glands or 5 ml/20 uteri, respectively. These preparations were then extracted in the hypotonic buffer with stirring in the cold, in most cases for 1 h. They were then centrifuged for 1 h at 105,000 ***g*** to yield a particle-free supernatant, or "lysosol", which was subsequently analyzed for steroid-binding activity.

In some early experiments, solubilization of constituents of the isolated lysosomes was carried out by extraction in the above buffer with gentle shaking for 2 h at 37°C, followed by rapid chilling and centrifugation at 105,000 ***g*** for 1 h.

Preparation of "cytosol"

Parallel samples of tissue were suspended in the hypotonic phosphate buffer, 15% (w/v), and subjected to cellular disruption in this medium directly, by means of the special press. Unless otherwise indicated, the samples were then stirred for 1 h in the cold, followed by centrifugation for 1 h at 105,000 ***g*** to yield a particle-free supernatant ("cytosol") fraction.

Steroids

[6,7-$^3H_2$]-Estradiol-17β (48 Ci/mmol), was purchased from New England Nuclear Corp. Its homogeneity and identity with authentic standard was greater than 98%, as verified by thin-layer chromatography (t.l.c.) on Silica Gel G with 2 solvent systems* [12].

Stock solutions of unlabeled estradiol-17β (Schering)† and estradiol-17β (1,3,5(10)-estratriene-3,17α-diol; Mann Research Laboratories, New York, N.Y.) were prepared in 95% ethanol and refrigerated until just before use. Their molar extinction coefficients were within 96% of the theoretical values.

Dextran–coated charcoal

Stock solutions of 0·5% (w/v) Dextran T 70 (Pharmacia, Uppsala) and 5% (w/v) suspensions of charcoal (Norit A, American Norit Co., Inc., Jacksonville, Fla.), were prepared in deionized, glass-distilled water. Unless otherwise specified, equal volumes were mixed and used without further dilution. In some early experiments, the 1:1 mixture was further diluted with 3 parts of 0·054 M phosphate buffer just before use.

Protein-binding assay

From a working solution of [^{3}H]-estradiol-17β, diluted just before use, duplicate or triplicate aliquots of suitable concentration were delivered into 12 × 75 mm polypropylene tubes (Falcon Plastics Co., Oxnard, Calif.), using Eppendorf automatic pipettes with disposable plastic tips. The samples were placed in a water bath at 32°C and evaporated to just dryness under dry, purified nitrogen. For competition experiments, the unlabeled steroid was added to the tube and mixed well with tritiated steroid prior to evaporation.

The assay was conducted by the general procedure of Shutt [13] as modified by Soloff *et al.* [14] and Liang-Tang and Soloff [15], with further alterations as indicated. To the sample tubes in an ice bath, 0·3 ml of the lysosomal or corresponding cytoplasmic extract were added, to yield concentrations of tritiated hormone which ranged from 10^{-11} to 10^{-9} M. "Blank" tubes, to which 0·054 M phosphate buffer was added instead of tissue extract, were carried in triplicate in each assay to confirm the efficiency of removal of unbound steroid by the Dextran–coated charcoal. The final values for radioactivity in such control tubes were

* Methylene chloride–acetone, 4:1 and chloroform–glacial acetic acid, 85:15.

† Kindly donated by Dr. Preston L. Perlman of the Schering Corp., Bloomfield, New Jersey.

consistently those of background. Recovery data from additional control tubes without charcoal verified the complete solubilization of the steroid in the buffer, in the presence or absence of tissue extract.

The assay tubes were then capped and transferred to a Dubnoff metabolic incubator and equilibrated with gentle shaking for 15 min at 32°C unless otherwise indicated. The tubes were then transferred to ice. With stopwatch timing, 0·5 ml of the Dextran–charcoal mixture, which was kept in uniform suspension by continuous stirring at ice temperature, was rapidly added to the assay tube with a Chaney-type Hamilton syringe. The sample was agitated with a Vortex mixer, returned to ice, stirred once more at 55 s, and, at exactly 1 min, decanted onto a 25 mm, 0·45 μm Millipore filter which had been pre-wetted with buffer. The tube was washed twice with 0·5 ml each of chilled phosphate buffer delivered rapidly from a spring-loaded syringe, and the filtrate and washings collected in a Kimble graduated polystyrene tube. The final volume was made to 2·0 ml at ice temperature, prior to thorough mixing and removal of a 1 ml aliquot to a counting vial containing 10 ml of Aquasol® (New England Nuclear Co., Boston) for determination of bound radioactivity. In practise, the assay was carried out by processing staggered sets of six samples, beginning with addition of the 0·3 ml tissue extract, in order to maintain conditions of timing uniform throughout the series for all stages of the procedure.

Counting was carried out in a Packard-TriCarb liquid scintillation spectrometer, Model 3310, at an efficiency of approx. 53%. The values were corrected for quenching (approx. 18%) by addition of internal standard.

Protein was determined on the tissue extracts by the method of Lowry *et al.* [16].

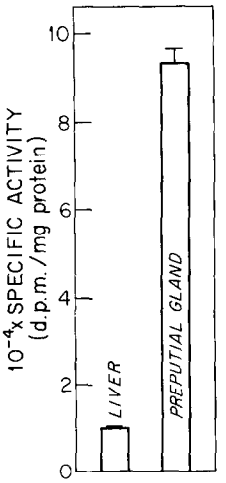

Fig. 1. Target selectivity of binding of estradiol-17β to lysosomal macromolecules. Lysosome-enriched fractions were prepared from liver and preputial gland of ovariectomized rats as described in the text. Unless otherwise indicated, purified lysosome preparations were isolated from cells which had been disrupted by the special press (see text). In the case of liver, a 15 s-treatment with a Teflon pestle and unfrosted glass homogenizer was utilized for preparation of the initial homogenate. The resultant lysosomal preparations were subjected to extraction for 2 h in 0·054 M sodium phosphate buffer, pH 7·7, at 37°C, followed by rapid chilling and centrifugation at 105,000 ***g*** for 1 h. The particle-free supernatant fractions, in which the protein concentrations were 760 and 238 μg/ml for liver and preputial gland, respectively, were than analyzed for binding of [^{3}H]-estradiol-17β at a concentration of $1{\cdot}43 \times 10^{-9}$ M, which constituted a region of linearity of binding for both preparations, using a Dextran–coated charcoal method, modified as described in the text. The means and the positive segments of the S.E.M. of triplicate determinations are depicted.

RESULTS

Target selectivity of binding of [^{3}H]-estradiol-17β by lysosomal macromolecules

Lysosome-enriched fractions of liver and preputial gland were prepared from ovariectomized rats as described above. These preparations were extracted with hypotonic (0·054 M) sodium phosphate buffer as indicated, followed by centrifugation at 105,000 ***g*** for 1 h. The particle-free supernatants were analyzed for binding of [^{3}H]-estradiol-17β by the modified Dextran–coated charcoal procedure described. Under these conditions, the "lysosol" from the preputial gland was far more effective in binding of the tritiated steroid than was the corresponding preparation from liver (Fig. 1).

Steroid specificity of the binding of lysosol constituents

Binding to soluble macromolecular fractions of preputial gland possessed the steroid specificity requisite to the hormonal target. Thus, displacement of tritiated hormone from macromolecular association was achieved by unlabeled estradiol-β (Fig. 2). As indicated in the Figure, the degree of inhibition of binding was proportional to the relative molar concentration of antagonist. The 17α-epimer, on the other hand, was ineffective at moderate concentrations (Fig. 2). When the molar ratios of potential antagonist:agonist were increased to 10 and 100 times, however, evidence of concentration-dependent competitive interaction was obtained for the 17α-cogener, with corresponding reduction in binding of [^{3}H]-estradiol-17β by 36 and 63%, respectively [17]. These results were in accord

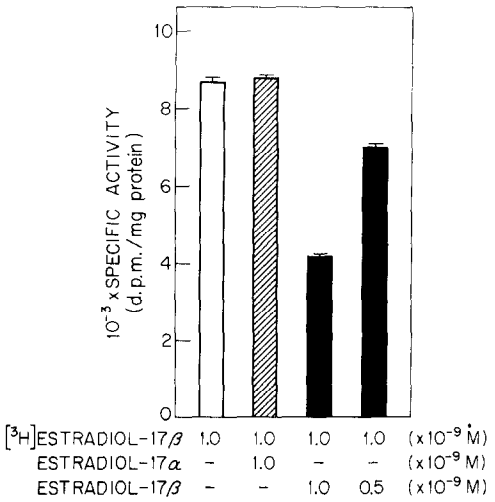

Fig. 2. Stereospecificity of steroid structure in binding of estradiol by macromolecular fractions of preputial-gland lysosomes. Preparations and procedures were as in Fig. 1, including cellular disruption by the special press; however, extraction of the lysosome-enriched fraction was carried out by stirring for 2 h at 4°C in the presence of 0·054 M sodium phosphate buffer, pH 7·7, followed by centrifugation at 105,000 *g* at 0°C. Shown are the means and the positive segments of the S.E.M. of quadruplicate determinations at the steroid concentrations indicated.

with the relative hormonal effectiveness of the unnatural epimer in the literature [18, 19]. Steroidal specificity of the binding was further indicated by the observation that occupation of a portion of the binding sites as a result of estradiol-17β administration *in vivo* immediately prior to the isolation of lysosomal constituents reduced the amount of binding subsequently determined *in vitro* [17].

Protein nature of the binding components

As anticipated from previous data with preputial gland [5] and from the many observations of others on receptor molecules for the steroid hormones in their various target cells [1, 20], the interaction of [^{3}H]-estradiol with lysosomal macromolecules was sharply inhibited by prior exposure to proteolytic enzymes. As Table 1 demonstrates, brief pre-incubation of lysosomal extracts with pronase, trypsin or chymotrypsin, even at very low concentrations of these enzymes, was highly effective in curtailing subsequent binding of [^{3}H]-estradiol-17β. It will be noted, however, that while binding activity was unaffected by similarly low concentrations of lysozyme and ribonuclease, a moderate reduction in binding activity was induced by relatively large excesses of these latter enzymes [21]. At least in the case of ribonuclease, proteolytic contamination was excluded. These results suggest potential participation of carbohydrate and nucleic acid functions on the protein molecules primarily responsible for the steroid binding [22].

Influence of temperature on binding kinetics

Analysis of the time-course of interaction of [^{3}H]-estradiol-17β with "lysosol" of preputial gland was carried out at 0° and 32°C (Fig. 3). As previously observed

Table 1. Protein nature of the [^{3}H]-estradiol-binding components of "lysosol" from rat preputial gland*

Enzymic treatment			Specific radioactivity			
Enzyme	Concentration (μg/ml)		d.p.m./mg protein		Per cent of control	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Control†	None	None	8913	14,709	100	100
Pronase	5		6626		74·3	
	10		5339		59·9	
		50		3692		25·1
		100		2000		13·6
Trypsin	5		8205		92·1	
	10		5240		58·8	
		50		2418		16·4
Chymotrypsin	5		7433		83·4	
		50		3194		21·7
Lysozyme	5		8702		97·6	
	10		8825		99·0	
		50		5154		35·0
Ribonuclease‡	5		8632		96·9	
	10		8614		96·7	
		50		5517		37·5

* Lysosomal extracts, as in Fig. 2, incubated with enzymes for 20 min at 23°C, then rapidly cooled to 0°C. Equilibration with isotopic steroid, 1×10^{-9} M, 30 min at 0°C. Means of duplicate or triplicate samples are shown. "Lysosol" from Exp. 1 stored 1 month at −88°C before analysis.

† Preincubated without enzyme under identical conditions. Incubation alone resulted in 10·9% decline in binding activity.

‡ Hirs component A, "protease free".

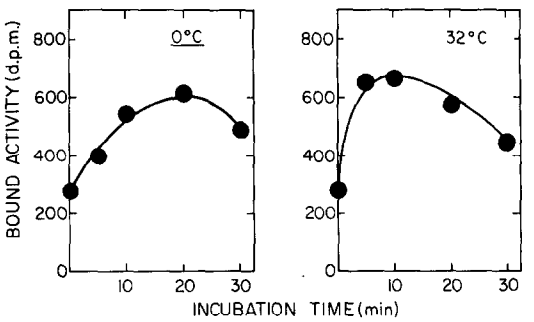

Fig. 3. Influence of temperature on the binding kinetics of [^{3}H]-estradiol-17β by "lysosol" proteins of preputial gland. Preparations and procedures for obtaining the "lysosol" extract were as in Fig. 2. The subsequent assay was conducted at the variable times and at the two temperatures specified in the presence of $7{\cdot}15 \times 10^{-10}$ M tritiated hormone. Results are the means of duplicate analyses.

using Sephadex column chromatography (Fig. 2 in ref. 8), there was virtually instantaneous binding of a substantial proportion of radioactivity, even at 0°C, with an apparent maximum at 20 min of equilibration at the latter temperature. Although the maxima were essentially equivalent at either temperature, these levels were achieved sooner at 32°C. With continued incubation under either set of conditions, there was a decline in the amount of isotope bound, suggesting denaturation or destruction of the binding sites.

Binding parameters

Preliminary analysis of the interaction of [^{3}H]-estradiol-17β with macromolecules of preputial-gland lysosomes was carried out at a series of estrogen concentrations. Figure 4 reveals that this system was approaching saturation at relatively low levels of hormone, as required by general receptor theory. Hence, more detailed analyses of binding characteristics were undertaken to permit resolution of high- and low-affinity binding sites.

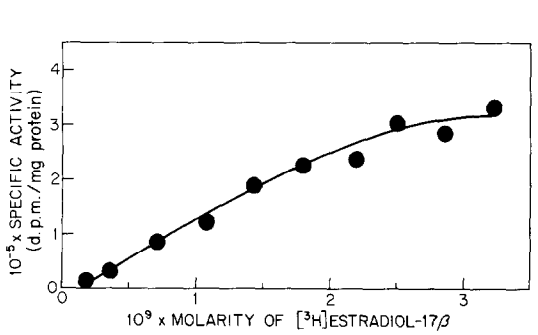

Fig. 4. Concentration dependence and saturability of binding of [^{3}H]-estradiol-17β by lysosomal proteins of preputial gland. Preparations and procedures as in Fig. 1.

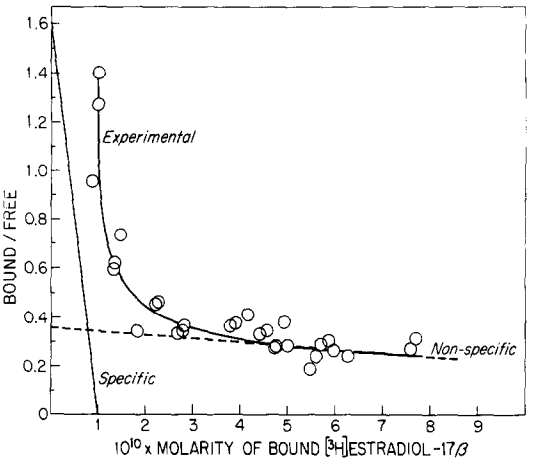

Fig. 5. Binding of [^{3}H]-estradiol-17β to preputial-gland "lysosol" at multiple concentrations of steroid, expressed as a Scatchard [23] plot. "Specific" binding is the resultant of application of the Rosenthal [38] correction. The "lysosol" preparation was obtained from preputial gland as indicated in Fig. 1 and text.

Figure 5 demonstrates the coexistence of both high- and low-affinity binding sites in soluble macromolecular constituents of preputial-gland lysosomes, as revealed by the classical Scatchard [23] plot. The mean association constant and its standard error for the high-affinity binding from a series of five similar experiments was $1{\cdot}60 \pm 0{\cdot}75 \times 10^{10}$ l/mol (uncorrected). The magnitude of the K_a was in close agreement with the mean value obtained under identical conditions for comparable lysosomal preparations for uteri of ovariectomized rats, namely $2{\cdot}87 \times 10^{10}$ l/mol (uncorrected). The order of magnitude of binding affinity, in turn, is in general accord with the values reported for cytoplasmic receptor of rat uterus by other investigators, using a variety of techniques [1, 2, 24, 25]. The similarity of magnitude is striking despite the differences in methodology, thus demonstrating that with rigorous control of time and temperature, accurate measurements are obtained with the charcoal method [15]. Saturability of the high-affinity binding at approximately 1×10^{-10} M ligand (corrected), as revealed in Fig. 5, was further evidence for the existence of a limited number of specific receptors in protein fractions extractable in hypotonic buffer from target-selective lysosomes which are readily disrupted by anisosmotic media.

These considerations prompted us to carry out simultaneous analyses of the characteristics of the binding of [^{3}H]-estradiol-17β by proteins extracted by the

Table 2. Binding parameters of [^{3}H]-estradiol-17β in proteins extracted from isolated lysosomes and unfractionated homogenates of target organs of ovariectomized rats*

	"Lysosol"					"Cytosol"				
Affinities	K_a ($\times 10^{10}$ l/mol)	Binding sites† ($\times 10^{-10}$ mol/l)	Binding index‡	Binding capacity§ ($10^{13} \times$ mol/mg protein)	Affinities	K_a ($\times 10^{10}$ l/mol)	Binding sites† ($\times 10^{-10}$ mol/l)	Binding index‡	Binding capacity§ ($10^{13} \times$ mol/mg protein)	Binding capacity ratio (L/C)
Preputial gland										
H	1·10	2·32	2·55	17·98	H					
L	0·02	18·00	0·36	139·53	L					
H					H					
L	0·02	11·75	0·24	45·90	L	0·05	32·70	1·64	11·49	3·99
H	0·65	3·80	2·47	16·31	H					
L	0·01	48·30	0·48	207·30	L					8·42
H	0·80	0·49	0·39	1·01	H	1·10	1·16	1·28	0·12	
L					L					
H	0·80	1·32	1·06	0·99	H					
L	0·04	12·40	0·50	93·23	L					
H	4·67	0·26	1·21	0·85	H	4·67	0·85	3·97	0·09	9·44
L	0·06	2·69	0·16	8·76	L	0·08	10·60	0·81	1·14	7·68
Uterus										
H	1·25	0·43	0·54	8·90	H	0·30	1·85	0·56	0·72	12·36
L	0·01	3·55	0·04	73·46	L	0·01	19·50	0·20	7·63	9·63
H	4·50	0·04	0·18	0·80	H	3·13	0·33	1·03	0·13	6·15
L	0·07	0·15	0·01	3·00	L	0·04	5·40	0·22	2·14	1·40

* Within same experiments by procedures described in the text.

† Intercept on abscissa in Scatchard plot, without application of the Rosenthal [38] correction.

‡ $K_a \times$ molar binding sites [25].

§ Molar binding sites normalized to 1 mg ml protein in assay tube. In the case of preputial gland, the actual concentrations of protein ranged from 0·13 to 0·70 mg ml for "lysosol" and 2·85 to 9·40 mg ml for "cytosol"; for uterus the corresponding values were 0·05 and 0·05 mg ml for "lysosol" and 2·56 and 2·53 mg ml for "cytosol".

present procedures from whole homogenates of preputial gland and from the corresponding purified lysosomes isolated from this source within the same experiment. There was a distinct parallel in the respective association constants for the high-affinity binding (Table 2). Moreover, the association constants for non-specific binding by lysosomal and "cytosol" proteins were likewise similar, averaging $3{\cdot}0 \times 10^8$ l/mol for "lysosol" and $6{\cdot}0 \times 10^8$ l/mol for "cytosol". The latter figures are of an order somewhat higher than certain of the values reported for uterine "cytosol" [25]; they correspond to the data for the medium-affinity binding seen with the β-globulins in the plasma [25–27]. A large part of the low-affinity (rapidly dissociated) non-specific binding is removed by the charcoal method [21, 28]. This may account for the lack of truly low-affinity binding sites in our experiments.

The "binding index", a product of the association constant and the molar binding sites [25], gives an indication of the relative affinities of the two classes of binding sites. The high-affinity sites in "lysosol" as well as in "cytosol" accounted for substantially greater binding at physiological concentrations [25]. However, when the molar binding sites were normalized to correspond to the equivalent of 1 mg of protein/ml in the assay tube to yield a figure for binding capacity, the low-affinity sites took precedence, by definition, indicating their relatively non-specific nature. Thus, the data in both lysosomal and "cytosol" fractions of preputial gland demonstrate the high-affinity, low-capacity character of one class of binding sites in this organ which is in accord with the observations in the literature for other steroidal targets [cf. also, 29].

Table 2 permits additional comparisons between the

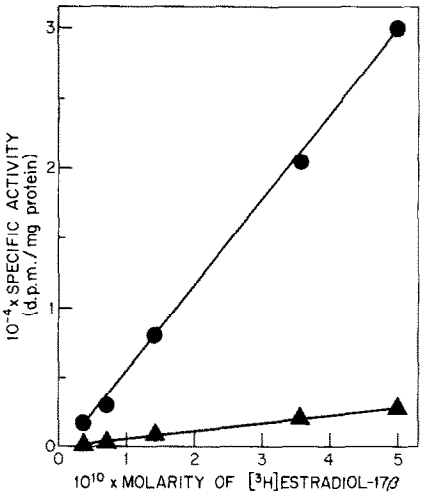

Fig. 6. Relative specific activities of "lysosol" and "cytosol" proteins from the preputial gland at varying concentrations of [^{3}H]-estradiol-17β. Preparations and procedures as in Fig. 1, with the exception that extracts of lysosome-enriched fractions (●) or whole homogenate (▲) were obtained as indicated in Fig. 2.

binding parameters for soluble protein components of lysosomes and of corresponding unfractionated "cytosol" within the same experiment. It will be noted that binding capacities of both the high- and low-affinity sites were consistently higher in the "lysosol" preparations than in the whole homogenates of origin, as indicated by the ratio, L/C. This finding also extended to comparable analyses of the uterine preparations (Table 2).

These observations prompted a direct comparison of the specific radioactivity of "cytosol" and "lysosol"

Table 3. Influence of prolonged extraction in hypotonic buffer on the binding parameters of "lysosol" and "cytosol" from preputial gland

Criterion	"Lysosol" High affinity binding	"Lysosol" Low affinity binding	"Cytosol" High affinity binding	"Cytosol" Low affinity binding
*Extraction for 1 h**				
K_a (l/mol)	$0{\cdot}80 \times 10^{10}$		$1{\cdot}10 \times 10^{10}$	
Binding sites (mol/l)†	$0{\cdot}49 \times 10^{-10}$		$1{\cdot}16 \times 10^{-10}$	
Binding index‡	0·39		1·28	
Binding capacity§	1·01		0·12	
*Extraction for 5 days**				
K_a (l/mol)	$2{\cdot}50 \times 10^{10}$	$0{\cdot}10 \times 10^{10}$	$2{\cdot}20 \times 10^{10}$	$0{\cdot}13 \times 10^{10}$
Binding sites (mol/l)†	$0{\cdot}36 \times 10^{-10}$	$3{\cdot}10 \times 10^{-10}$	$0{\cdot}71 \times 10^{-10}$	$6{\cdot}30 \times 10^{-10}$
Binding index‡	0·90	0·31	1·57	0·82
Binding capacity§	0·52	4·45	0·25	2·21

* With stirring at 0–4°C; otherwise as described in text.

† ‡ As in Table 2.

§ As in Table 2. The actual concentrations of protein in the "lysosol" samples were 0·49 mg/ml and 0·70 mg/ml for the 1 h and 5-day extracts, respectively. The corresponding values for "cytosol" were 9·40 and 2·85; the latter value was the result of dilution.

proteins of preputial gland following incubation of the soluble proteins with tritiated estradiol. Figure 6 demonstrates in a typical experiment that the binding capacity of "lysosol", although readily saturable (Figs. 4, 5; Table 2), exceeded that of the unfractionated "cytosol" several fold at low concentration of steroid.

These combined data appeared to be compatible with possible lysosomal origin of at least a portion of the "cytosol" binding function for tritiated estradiol in preputial gland. This suggestion, which is in keeping with the property of structural latency of lysosomal proteins [30], is supported by data presented in Table 3 which demonstrate that the binding capacity of the high-affinity "cytosol" proteins doubled, while that of lysosomal cryptoproteins declined to one-half, on prolonged extraction in the cold of purified lysosome fractions and corresponding whole homogenates from preputial gland.

In very recent experiments (Horton *et al.* unpublished work), attempts have been made to fractionate soluble lipoprotein components of rat prepuital gland by differential flotation ultracentrifugation, according to the methods of Goldstone *et al.* [13]. Binding activities of the resultant low- and high-density lipoproteins for [^{3}H]-estradiol-17β were intense, with specific activity of the latter greater than 50 times that of the crude "lysosol" [17]. These proteins migrated rapidly toward the anode in polyacrylamide gel electrophoresis at pH 8·5 and possessed additional properties which reflected their acidic nature. The significance of these features of the lysosomal matricular constituents are discussed elsewhere [8, Horton and Szego, in preparation].

DISCUSSION

The present investigations extend preliminary observations from these laboratories which indicated preferential uptake of [^{3}H]-estradiol-17β by target-specific lysosomes [5, 8]. It has now been demonstrated that macromolecular constituents of highly purified lysosomes that were isolated from preputial gland of untreated ovariectomized rats possess binding properties for tritiated hormone which conform to the stringent requirements of classical receptor theory. Thus, proteins extracted from such lysosome preparations by hyposmolar buffer possessed the high-affinity, low-capacity properties for interaction with [^{3}H]-estradiol which are characteristic of the binding proteins described in the "cytosol" of a series of steroid-sensitive cells [1–3, 20]. The order of magnitude of the association constants observed in the present investigation by the use of a carefully controlled Dextran–charcoal procedure was in close agreement with the values reported for cytoplasmic receptor of rat uterus by other investigators, using a variety of techniques (*vs.*). Moreover, the organ-selectivity characteristic of the binding function of soluble lysosomal ("lysosol") constituents was likewise in accord with properties requisite to receptor molecules. This was illustrated by the limited steroid binding to macromolecules solubilized from a lysosome-enriched fraction of liver, in contrast to the avidity of proteins extracted from corresponding preparations of preputial gland and uterus. Evidence of stereospecificity of receptor function was obtained by competition from unlabeled estradiol-17β and lack of significant suppression of binding by the 17α-epimer, except at overwhelming concentrations of the latter. Stereospecific binding to "lysosol" proteins was characterized by extremely rapid onset, even at 0°C.

Analysis of binding parameters by the Scatchard plot [23] revealed a striking parallelism of the slopes and thus, of association constants, of ligand interaction with "lysosol" and "cytosol" proteins which were extracted from lysosome-enriched preparations and corresponding unfractionated cytoplasm, respectively. Moreover, the product of the K_a and the molar binding-sites for "lysosol" vs "cytosol", each adjusted to 1 mg of protein/ml assay mixture, was consistently higher for both high- and low-affinity binding in the case of proteins solubilized by hypotonic buffer from lysosomal origin, compared to those extracted from the whole homogenate under similar conditions. This preferential binding capacity of "lysosol" proteins was 4 to 9 times greater than that of the corresponding "cytosol" preparations for preputial gland, and up to 12 times in the case of the comparable relationship in uterus. These combined observations suggested that such properties might be attributable to macromolecules of overlapping source in the intact cell. On prolonged extraction of the unfractionated homogenates with cold hypotonic buffer, it was evident that the binding capacity of the "lysosol" declined with time in contrast to the increase in this function for "cytosol". Evidence from the present investigations, therefore, supports the possibility that proteins extruded from lysosomes during preparative procedures may serve as the source of the "cytosol" receptor molecules.

It has appeared implicit in the work of previous investigators [1–4] that proteins which are present in soluble form in the customary "cytosol" preparation may well exist in membrane-bounded state in the intact cell [32]. For reasons related to the striking and abrupt uptake of steroid hormones by lysosomes [5, 8, 17], the extraordinary hormone-induced nucleotropic migration of these organelles from their generally peripheral distribution within the resting cell [7, 8, 33], and from the potential function of lysosomal

peptidases in the transformation of receptor–protein to the subunit or fragment of lower mass [4, 8], it was postulated that lysosomal protein might serve as the source of the cytoplasmic "receptor" [8]. This function was considered the more likely on the basis of the extreme fragility of the bounding membrane of the lysosome to damage during vigorous homogenization and to the ionic imbalance to which it is exposed in the hyposmolar buffers universally used to obtain the soluble receptor. It has already been noted that the special press [10] used in these laboratories for cellular disruption is more conservative of membrane-bounded particulate components than are the glass homogenization methods in more general use [6]. Recognition of this factor is essential for analysis of the potential origin of structurally latent proteins solubilized by preparative procedures from fragile subcellular organelles, such as lysosomes, as reviewed elsewhere [4, 8]. The structural labilization of these organelles by exposure to hormone, with consequent loss of latent proteins into the extraction medium, such as would occur to an exaggerated extent after *in vivo* injection of the hormone [6, 7], was even more highly suggestive of the postulated relationship. Indeed, the present results are in harmony with the indications that selective hormone accumulation in lysosomes [8] and in "mitochondria" [34, 35] precedes nuclear uptake. Lysosomes have long been recognized as contaminants of mitochondrial preparations [36, 37].

The extraordinary avidity of lysosomal proteins isolated from selective targets for preferential binding of tritiated estradiol-17β and testosterone as well as the extremely rapid time-course of this association, as reported in the present work and elsewhere [5, 8, 17], are compatible with the available literature for kinetics of hormone reception at the target cell [2]. The observations are likewise in harmony with the time delay involved in delivery of the agonist to the nucleus bound to constituents of hormone-activated lysosomes, a phenomenon for which both morphological and biochemical evidence has now become available [8]. These combined data bear significantly upon the hypothesis that lysosomal migration serves as a potential means of translocation of the hormone from cell membrane, through the cytoplasm, and into the nucleus.

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REFERENCES

1. Gorski J., Toft D., Shyamala G., Smith D. and Notides A.: *Recent Prog. Horm. Res.* **24** (1968) 45–80.
2. Jensen E. V. and DeSombre E. R.: *Ann. Rev. Biochem.* **41** (1972) 203–230.
3. Steroid–Hormone "Receptors"; *Advances in the Biosciences* (Edited by G. Raspé). Pergamon Press, New York, Vol. 7 (1971) 420 p.
4. Szego C. M.: In *The Sex Steroids: Molecular Mechanisms* (Edited by K. W. McKerns). Appleton-Century-Crofts, New York, (1971) pp. 1–51.
5. Szego C. M.: *Proc. 3rd. Int. Congr. Hormonal Steroids. Excerpta Medica Int. Cong. Ser.* **219** (1971) 642–650.
6. Szego C. M., Seeler B. J., Steadman R. A., Hill D. F., Kimura A. K. and Roberts J. A.: *Biochem. J.* **123** (1971) 523–538.
7. Szego C. M. and Seeler B. J.: *J. Endocr.* **56** (1973) 347–360.
8. Szego C. M.: *Recent Prog. Horm. Res.* **30** (1974) 171–233.
9. Freinkel R. K.: In *The Sebaceous Glands; Advances in Biology of Skin* (Edited by W. Montagna, R. A. Ellis and A. F. Silver). Pergamon Press, New York, Vol. 4 (1963) pp. 125–134.
10. Emanuel C. F. and Chaikoff I. L.: *Biochim. biophys. Acta* **24** (1957) 254–261.
11. Szego C. M., Steadman R. A. and Seeler B. J.: (1974) *Eur. J. Biochem.* In press.
12. Waldi D.: In *Thin Layer Chromatography* (Edited by E. Stahl), Academic Press, New York, (1965) pp. 249–278.
13. Shutt D. A.: *Steroids* **13** (1969) 69–82.
14. Soloff M. S., Creange J. E. and Potts G. O.: *Endocrinology* **88** (1971) 427–432.
15. Liang-Tang L. K. and Soloff M. S.: *Biochim. biophys. Acta* **263** (1972) 753–763.
16. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–275.
17. Hirsch P. C.: Thesis, University of California, Los Angeles (1974).
18. Whitman B., Wintersteiner O. and Schwenk E.: *J. biol. Chem.* **118** (1937) 789–795.
19. Toft D. and Gorski J.: *Proc. natn Acad. Sci. U.S.A.* **55** (1966) 1574–1581.
20. Kellie A. E.: *Ann. Rev. Pharmacol.* **11** (1971) 97–112.
21. Alberga A., Massol N., Raynaud J.-P. and Baulieu E.-E.: *Biochemistry* **10** (1971) 3835–3843.
22. Soloff M. S.: Doctoral dissertation, University of California, Los Angeles (1968) p. 46.
23. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
24. Toft D., Shyamala G. and Gorski J.: *Proc. natn Acad. Sci. U.S.A.* **57** (1967) 1740–1743.
25. Ellis D. J. and Ringold H. J.: In *The Sex Steroids: Molecular Mechanisms* (Edited by K. W. McKerns). Appleton-Century-Crofts, New York, (1971) pp. 73–106.
26. Szego C. M. and Roberts S.: *Endocrinology* **41** (1947) 322–324.
27. Lea O. A.: *Biochim. biophys. Acta* **317** (1973) 351–363.
28. Méšter J., Robertson D. M., Feherty P. and Kellie A. E.: *Biochem. J.* **120** (1970) 831–836.
29. Bullock L. P. and Bardin C. W.: *J. clin. Endocr. Metab.* **35** (1972) 935–937.
30. de Duve C.: In *Lysosomes in Biology and Pathology* (Edited by J. T. Dingle and H. B. Fell). North-Holland Publishing Co., Amsterdam, Vol. 1 (1969) pp. 3–40.
31. Goldstone A., Szabo E. and Koenig H.: *Life Sci.* **9**(II) (1970) 607–616.

32. Pain V. M. and Clemens M. J.: *FEBS Lett.* **32** (1973) 205–212.
33. Szego C. M. and Smith R. E.: *Ann. Int. Med.* **78** (1973) 725–738.
34. Williams M. A. and Baba W. I.: *J. Endocr.* **39** (1967) 543–554.
35. Fraser R.: In *The Biochemistry of Steroid Hormone Action, Biochemical Society Symposium no.* 32 (Edited by R. M. S. Smellie). Academic Press. London. (1971) pp. 101–127.
36. de Duve C. and Wattiaux R.: *Ann. Rev. Physiol.* **28** (1966) 435–492.
37. Vignais P. M. and Nachbaur J.: *Biochem. biophys. Res. Commun.* **33** (1968) 307–314.
38. Rosenthal H. E.: *Analyt. Biochem.* **20** (1967) 525–532.