ANDROGEN, ESTROGEN AND PROGESTERONE RECEPTORS IN VULVAR DYSTROPHY

A. ONNIS, G. B. NARDELLI, L. BECAGLI, T. MAGGINO, M. MARCHETTI

Institute of Gynecology and Obstetrics
University of Padua (Italy)

SUMMARY

Assays have been developed for the quantitation of androgen, estrogen and progesterone receptors in the cytosol of 45 vulvar dystrophic tissues. In 27 patients we assayed only cytoplasmatic receptors, while in 18 patients both cytoplasmatic and nuclear receptors.

The workers verified the possibility to assay these carriers, finding significant correlations neither with patient's age nor between atrophic and hypertrophic dystrophies. The research concerning the cytoplasmatic depletion and nuclear accumulation allowed us to understand the action mechanism of the androgen in vulvar tissues in patients in menopause.

INTRODUCTION

The existence of interdependence between androgen levels and E_2R and PgR has been confirmed by various workers. Garcia [1] demonstrated that in immature rat uteri, the injection of 100 μg (10^{-8} M = physiological dose) induces the maximum occupation of the androgenic receptorial sites but provokes no translocation of E_2R. On the other hand, at doses $\geq$ 3 mg (10^{-6} M = pharmacological dose), the DHT induces nuclear translocation (NT) of E_2R. The Author concludes that the trophic action of the androgens in vivo and vitro is correlated to the grade of NT of E_2R and not to the AR which are present in very small quantities. Ruh [2], on the immature rat uteri came to the same conclusions, noting estrogens and androgens nuclear accumulations of E_2R with the same mechanism. The Author also observed that the antiandrogens block the bond of the androgens with their receptors, but don't interfere with the androgen-induced NT of E_2R while the antiestrogens inhibit the androgen-induced NT of E_2R. Zava and McGuire [3, 4] on the MCF-7 cell line, demonstrated that the DHT interacts directly with E_2R with an affinity 1000 fold lower than E_2. This explains the necessity of pharmacological doses. With such doses (10^{-6} M) the androgens mimic the action of the estrogens in stimulating the NT of E_2R. What's more, since the synthesis of PgR is a product of the estrogenic action, the Authors have demonstrated that while physiological doses of DHT (10^{-8} M) don't provoke an increase of PgR, pharmacological doses of DHT (10^{-6} M) exercise a stimulation of the synthesis of PgR equal to physiological doses (10^{-8} M) of estradiol. The Authors also specify the androgens do not cause NT of PgR.

Rochefort [5] confirms that the trophic effect of the androgens is mediated by estrogenic receptors and not through the AR system. In regards to vulvar tissue in par-

ticular, Johansson ([6]) demonstrated, in three cases of vulvar kraurosis and four cases of apparently normal vulva, that the estrogen receptors are present in these tissues even though in very low concentrations. These receptors were also found in four neoplastic vulvar tissues in contrast with Ford's ([7]) experience in which, in five neoplastic vulvar tissue, neither estrogen nor progesterone receptors were found.

MATERIAL AND METHODS

The sample of 45 cases included two groups of patients. In 27 patients with vulvar dystrophy, only cytoplasmatic receptors were assayed; in another 17 patients with vulvar dystrophy and in one normal vulvar tissue, both cytoplasmatic and nuclear receptors were assayed.

The first sample was used to verify the real possibility of assaying hormonal receptors in vulvar tissues, to investigate a possible correlation with the patient's age, and to study atrophic and hyperthophic dystrophy in relation to the concentration of the receptors. The second sample was used to study the action mechanism of androgen, estrogen and progesterone in dystrophic vulvar tissues. To this aim, these 18 cases were subdivided according to a Clinical Grading of the Dystrophy:

— Light Dystrophy: All cases which have presented symptoms for less than two years and lesion which partially interests the vulvar region.

— Moderate Dystrophy: All cases which have presented subjective symptoms for two to five years and lesion limited to the vulvar region.

— Severe Dystrophy: All cases which have presented intense subjective symptoms more than 10 years with macroscopically visible lesion diffused in the entire vulva, which may, occasionally, interest the perineum, perianal and groincrural regions as well.

Tissue biopsies: the vulvar biopsies were carried out under local anaesthesia with Marcain 0.50%-Pierrel by Luer needle 25 × 5/8″-0.50-16 mm (B. Braun Melsungen AG-W. Germany), and Stainless Surgical Blade n. 11 (Paragon Sheffield-England).

The seat of the extirpation was sutured with 35 mm needle-Chromic Catgut 3-75 cm (W 463-Ethicon, Italy).

Tissue preparation: the removed tissue were divided into two parts: one part was used for the histologic examination and was conserved in formalin; the other part, used for the assaying of hormonal receptors, was frozen in liquid nitrogen and conserved in a freezer at −70 °C until the moment of processing.

The dry weight of the tissues was from 0.1 to 0.3 gms; the proteic concentration for the cytoplasmatic assay was standardized at 2.3 mg/ml for AR, at 1.8 mg/ml for PgR, at 0.8 mg/ml for E_2R using the estimate of the nucleic acids which guided the dilutions. The proteic concentrations of nucleic assays were standardized at 0.7 mg/ml for AR, PgR and E_2R ([15]).

Buffers: P-Buffer pH 7.4 at 4°C: KH_2PO_4/K_2HPO_4 10 mM (Merck-Schuchardt, Bracco, Milan, Italy); DTT 0.5 mM (Calbiochem-Behring Co.-La Jolla-Ca.); Glycerol 10% (MCB-Manufacturing Chemical Co., St. Louis-Mo.).

This buffer was used in the first 27 dystrophic vulvar tissues. In the second part of the experiment (18 cases) the pulverized tissues were suspended in C-Buffer pH 7.4 at 4°C: TRIS-Ultra Pure 50 mM (Schwarz/Mann - Spring Valley, N.Y.); EDTA 1.5 mM (Fisher Scientific Co. Fair Lawn - N.J.); Glycerol 10% (MCB-Manufacturing Ch. Inc. - Cincinnati - Ohio); Molybdic Acid 10 mM; Mersalyl Acid (Sigma Ch. Co. - St. Louis - Mo.).

The Molybdate and the mercurial reagent Mersalyl Ac. are used to promote dissociation of more than 90% receptor bound steroid. The reaction is reversible by addition of Dithiothreitol.

The pellets were resuspended in KCl-Buffer pH 7.4 at 4°C: TRIS 10 mM, EDTA 1 mM, Glycerol 10%, KCl 0.6 M (Merck-Schuchardt, Bracco, Milan, Italy) and homogenized in polycarbonate tubes for ultracentrifuge.

The radioactive and radioinert steroids were diluted in H-Buffer pH 7.4 at 4°C: TRIS 50 mM, EDTA 1.5 mM, Glycerol 10%, DTT 25 mM.

Steroids: Androgen Receptor: Methyltrienolone-^{3}H-R-1881 (NEN-NET 590), with S.A. 87 Ci/mmol; Cold-R-1881 (NEN-NLP 005); Triamcinolone Acetonide (T.A.) (Sigma Ch. Co. - St. Louis, Mo.).

Progesterone Receptor: Promegestone-^{3}H-R-5020 (NEN-NET 555) with S.A. 85 Ci/mmol; Cold-R-5020 (NEN-NLP 004); DHT (Sigma Ch. Co. - St. Louis, Mo.); Hydrocortisone Acetate (Calbiochem, Behring Co., La Jolla, Ca.).

Estrogen Receptor: 2,4,6,7 - ^{3}H - Oestradiol (Amersham-TRK 322) with S.A. 114 Ci/mmol; DES (Sigma Ch. Co., St. Louis, Mo.).

Chemicals: Dextran Grade C 0.025 gms/l (BDH Ch. Ltd., Poole, England); Charcoal Activated 2.5 gms/l, Albumine Bovine-Fraction V 1% (Sigma Ch. Co., St. Louis, Mo.); Toluol, PPO

(2.5-diphenyloxazone-scintillation grade), POPOP (2,2'-p-phenylene-bis(4-methyl-5-phenyloxazole) scintillation grade) (Merk-Schuchardt, Bracco, Milan, Italy).

Cytoplasmic Receptor. The frozen samples had a dry weight of 0.1-0.3 gms. Each piece was pulverized in microdismembrator-Braun or in Mortar-AISI 304, accurately frozen beforehand.

The powder was then transferred into prechilled glass tubes of 20 × 34 mm and transferred to the cold-room at 4°C. All the steps of the processing were carried out in the cold-room to assure a constant reaction temperature.

The powder was suspended in P-Buffer (27 cases) and in C-Buffer (18 cases) in a W/V ratio and homogenized with Polytron-Brinkman-PT 10 ST by three 10 sec. bursts at the very lowest setting. The homogenized material was carefully transferred with a spatula into polycarbonate ultracentrifuge tubes with a screw cap (Kontron 13.5 ml, 5/8" × 3", 16 × 76 mm). The homogenate has been centrifuged at 105,000 g/60 min with Kontron Rotor TFT 65.13 in Kontron TGA 65.

The cytosol was sucked with cold Pasteur and, after having carried out the ([15]) estimate of the nucleic acids, was suitably diluted. 200 λ of sample, in triplicate, was incubated in the cold-room with 1 hour intervals with the respective competitors, then for 16 hours with radiolabeled steroids. DCC 1%-BSA was added to each tube and, after 15 minutes of vigorous shaking, the tubes were put into the centrifuge for 15 minutes at 3000 rpm in ALC-965 R ([16]).

A 500 λ part was read in a Kontron Betamatic beta-counter with an efficiency of 57%. The sparkling liquid was prepared with Toluol-PPO-POPOP.

The proteins were assayed according to the Lowry method on the LKB-Ultrospec-4050 Spectrophotometer, and the results were expressed in fmol/mg ([11]).

Nuclear Receptor — The pellets of 18 pieces, previously homogenized in C-Buffer, were resuspended in KCl-Buffer and homogenized with a teflon pestel directly in the polycarbonate tubes. They were then shaken gently for 30 min in the cold-room on Varvel-FVF 20, and, afterwards, they were centrifuged at 105,000 g/30 min.

After the standardization of the proteic concentration with the estimate of the nucleic acids, the cytosol was suitably diluted; the samples, in triplicate, were incubated with the modality of the cytoplasmic receptors.

Saturation Analysis — A Saturation Analysis and sucessive correction of the Scatchard-Plots biphasic according to Chamness-McGuire ([8]) were carried out on four abundant dystrophic vulvar tissues, derived from a simple vulvectomy. Assays were performed in six points in duplicate with graded concentrations of ^{3}H-R-1881 6×10^{-10} M, Cold-R-5020 10 fold excess, DHT 1000 fold excess, F 1000 fold excess, ^{3}H-E_2 8×10^{-11} M, DES 100 fold excess ([9, 10]).

The DPM-Data are transferred on-line to a PSI-80 Microcomputer Kontron.

The following Kd were found: AR = Kd 0.6 $\pm$ 0.2 $\times 10^{-9}$ M; PgR = Kd 0.4 $\pm$ 0.1 $\times 10^{-9}$ M; E_2R = Kd 0.4 $\pm$ 0.2 $\times 10^{-9}$ M.

Single Saturation Analysis (S.S.A.) — Since the quantity of removable tissue from a biopsy done on an out-patient basis is always small, and since a part of the taken sample must be used for the histological examination, the assay method of hormonal receptors is important.

In a six-point method 0.1-0.3 gms of tissue must be taken to a volume of 2.5-2.6 ml for each hormone; this causes an excessive proteic dilution under the standard minimum, with a consequent underestimate of the receptorial concentration.

For this reason, we established the optimal doses of radiolabeled and radioinert steroids for only one point of the saturation; what's more, the object of our research was a cognitive analysis of the receptorial situation of vulvar tissues.

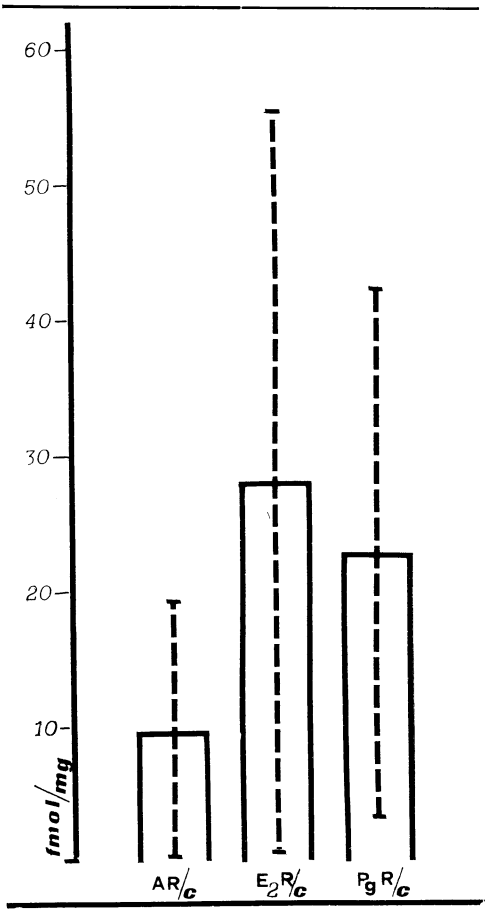
This method offers credible results if the steps of elaboration, the purity of the steroids and reagents and the working temperature are rigorously respected.

In SSA the following concentrations were used: ^{3}H-R-1881 3×10^{-9} M, Cold-R-1881 3×10^{-7} M, TA 18×10^{-7} M; ^{3}H-R-5020 2.5×10^{-9} M, Cold-R-5020 2.5×10^{-7} M, DHT 1×10^{-6} M, F 1×10^{-6} M; ^{3}H-E_2 8×10^{-11} M, DES 8×10^{-9} M. Protein concentration was quantitated by the Method of Lowry ([11]) and the results were represented in fmol/mg.

RESULTS

In analysing the results of the AR/c, E_2R/c and PgR/c assays we found that the concentration of receptors is stably inferior as compared with the receptorial levels found in endometrial and breast tissues. The only notable element concerns the PgR/c which, when present, resulted significantly higher than the AR/c and E_2R/c levels. What's more, in this group of patients between the ages of 42 and 82 years, and in any case, in menopause, we found no significant modification related to age. Unfortunately data concerning

Table 1. — *The results of the AR/c, E_2R/c and PgR/c assays show that the concentrations of receptors is stably inferior as compared with the receptorial levels found in endometrial and breast tissues.*



women in the fertile age are not yet available (tab. 1).

As regards the histologic correlations, in 14 cases of atrophic dystrophy, no correlation was found between the levels of E_2R/c and AR/c, AR/c and PgR/c; between PgR/c and E_2R/c the index of correlation was very low: 0.69.

The tab. 2 shows the range of variability which is discretly large. Even so, the comprehensive level of the three receptors was shown to be positive, that is to say biologically significant in 8 cases out of 14 (>10 fmol/mg).

In the 13 cases of hypertrophic dystrophy examined, except for a weak correlation (index 0.67) between E_2R/c and AR/c, no particular correlation between the three receptors could be seen. From the biological point of view, only 2 cases out of 13 presented a positive level of the three receptors (>10 fmol/mg) (table 2).

Furthermore, the hypertrophic forms showed to be characterized by a stably higher level of PgR/c in 10 out of 13 cases.

Finally, in these 27 cases of vulvar dystrophy, the presence of AR/c, E_2R/c and PgR/c was noted in 30% of the hypertrophic forms, 42% of the atrophic forms and in 37% of all dystrophies.

In the second group of 18 patients, both cytoplasmatic and nuclear receptors were assayed to study the cytoplasmatic depletion mechanism and the consequent nuclear accumulation. We found that the percentage of assayable receptors in the nucleus is significantly graduated, starting from 82% of AR/n, going to 76% of E_2R/n and to 70% of PgR/n. In the cytoplasm the percentage of assayed receptors was found to be more stable for AR/c and E_2R/c, while the percentage of progesterone receptors was clearly different: 76% (table 3).

Table 2. — *In the cases of atrophic dystrophy there was a low correlation (index 0.69) between PgR/c and E_2R/c, but there was no evident correlation between E_2R/c and AR/c, and between AR/c and PgR/c. In the cases of hypertrophic dystrophy there was no particular correlation among the three receptors; there was a weak correlation only between E_2R/c and AR/c with an index of 0.67.*

	Vulvar dystrophy	
	atrophic range (fmol/mg)	hypertrophic range (fmol/mg)
AR/c	1.3 ↔ 19.2	0.74 ↔ 9.7
E_2R/c	0.56 ↔ 55.2	2.2 ↔ 24.7
PgR/c	3.7 ↔ 34.0	3.4 ↔ 42.6

Table 3. — *We found that the percentage of assyable receptors in the nucleus is significantly graduated. In the cytoplasm the % of assayed receptors was found to be more stable for AR and E_2R.*

% of receptors assayed in the Cytoplasm	Nucleus
100%	82%
100%	76%
76%	70%

Furthermore, the NT mechanism was conserved for all three hormones in 33% of the cases; in 67% of the cases NT was absent for one or two hormones. In particular the NT of PgR was observed in 57% of the cases if the NT of E_2R was already present, in 53% of the cases if the NT of AR was present and in 54% of the cases if the NT of AR + E_2R was present (table 4).

In clear contrapposition, the NT of E_2R was observed in 76% of the cases only if the NT of AR was already present and in 75% of the cases only if the NT of AR and PgR was already present.

Finally, we carried out an analysis of the C/N ratio of E_2R, AR and PgR. If we don't consider the absolute values in fmol/mg, but only the presence or absence of a phenomenon, we can observe from table 5 that we more frequently found inherent cinetic defects in PgR. Therefore, this parameter could be considered as the most sensitive index of the break of the A-E_2-Pg balance hand by hand with the dystrophy to get worse: 8 cases out of 12 in the moderate-severe dystrophy against 4 cases out of 12 of E_2R and against 3 cases out of 12 of AR.

DISCUSSION

From the first case sample of 27 patients, we can draw some important informations: that the hormonal receptors are present and assayable even in vulvar tissues. The current status of hormonal knowledge for this organ does not allow us to apply, as with the breast and endometrium, the quantitative parameters of positivity (>10 fmol/mg), of borderline (3-10 fmol/mg), and negativity (< 3 fmol/mg), also because we haven't yet any sure information concerning normal vulvar tissues. So, we were not surprised by the lack of correlation between receptorial levels and the patient's age, since, in any case, all of the patients were in menopause and therefore underwent the same hypergonadotrophinic situation.

The awaited correlation between hypertrophic and atrophic forms and hormonal receptors was not observed. This observation confirms once again, that histological grading is not surely correlated to the level of hormonal receptors.

On the other hand, we found the results of the percentage of the presence of all three receptors contemporaneously (AR + E_2R + PgR) assayed with different reagents, interesting: in the first case sample 37% of the cases, in the second case sample 33% of the cases. This affinity of results assures us about the efficiency of the assays carried out in SSA. From the second sample of 18 cases, the assay of nuclear receptors besides cytoplasmatic, allowed us to establish a sequential order, in the form of steps, in the interpretation of the results of AR, E_2R, PgR which we initially evaluated singularly or in couples.

It is not, in fact, by chance, that the kinetic cellular defects are more accentuated for progesterone receptors (70% of

Table 4. — *Interdependence in nuclear translocation.*

If there was NT of		There was NT of	Of all dysrophy
AR	→→	E_2R	too → in 76%
E_2R	→→	PgR	too → in 57%
AR	→→	PgR	too → in 53%
AR + E_2R	→→	PgR	too → in 54%
AR + PgR	→→	E_2R	too → in 75%

Table 5. — *Analysis of the $^c/_n$ ratio of AR, E_2R, PgR.*

AR c	AR n	E_2R c	E_2R n	PgR c	PgR n	
N		N			–	
N			–		–	
N			–		–	
N		N			–	
	–	N		N		
	–	N		N		moderate-severe dystrophy
N			–	N		
N			–	N		
N		N		–	–	
N		N		–		
	–	N		–		
N		N		–		
3/12		4/12		8/12		
N		N		N		
N		N		N		
N		N		N		
N		N		↓N		light dystrophy
N		N		↓N		
N		N		N		normal tissue

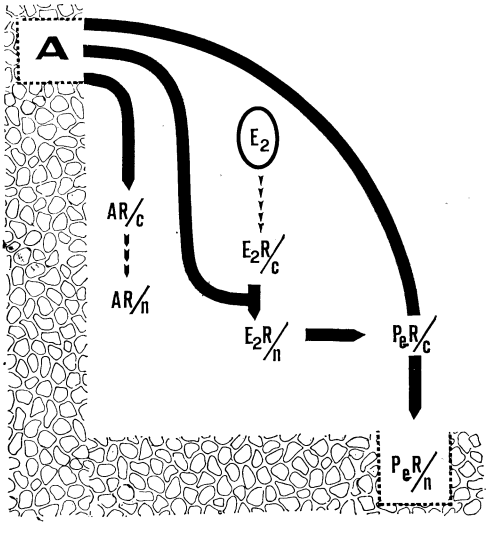
N = normal $^c/_n$ ratio; c = cytoplasm; n = nucleus; – left = cytoplasmatic defects; – right = nuclear defects; ↓ = lower cytop. levels.

presences in the nucleus), more subdued for E_2R (76% of presences in the nucleus) and lighter for AR (82% of presences in the nucleus). In fact, 33% of the cases in which NT was conserved for all three hormones included 5 cases of light dystrophy and one case of normal tissue, as if the dystrophies, when still light, had an integral hormonal regulator mechanism in each step as in normal tissue.

Moreover, from the table 5 we can deduce that the dystrophy picture is correlated to the loss of hormonal regulator mechanism since the integrity of the mechanism of the first (AR) and the second E_2R) steps is not always conserved. Therefore the probability of having the NT of PgR is shown to be equal to the presence of NT of only one of the two receptors. On the contrary, when the first and last steps (AR+PgR) are integral, the NT of E_2R is present in the same percentage of cases in which the NT of AR is integral (76%).

Consequently, our hypothesis concerning the regulator mechanism of vulvar trophism in menopause, is, even in light of other experience published, that androgens play a supporting role in answer to steroids and that this support is verified as a mechanism which we've called FALL-SYSTEM. Such a mechanism would explain how the nuclear concentrations of E_2R and PgR can be androgen-induced and how PgR/n is the final aim of this mechanism the action of which, well known, is to balance the action of estrogens (table 6).

Table 6. — *Fall-system.* A. Androgens stimulate the synthesis of their own receptors and the passage in to the nucleus. B. Androgens stimulate only the NT of E_2R but not their synthesis. C. This is a compulsory step in both the estrogenic and androgenic paths. D. The synthesis of progesterone receptors may come about by way of both androgens and estrogens. E. Even in menopause, the estrogenic path acts on the synthesis of E_2R and PgR. F. PgR/n is the final product of this fall-system.



CONCLUSION

From this experience of ours we can hypothesize that in menopause the hypergonadotrophinic situation and/or the androgenic ovary may be responsible for a mechanism capable of regulating the type of response of the vulvar tissues to steroids. In fact, the estro-progestinic balance in premenopause is supported by low levels of gonadotropins and an active ovary, while in menopause androgens, which don't carry out a direct trophic function, support the estro-progestinic balance at the cellular level even though with a reduced modality and even though being responsible for vulvar trophism ([12]).

To support this information, we recall two of our clinical observations:

1) We found that the vulvar dystrophies are more frequent in patients without androgenic habitus, but not the contrary ([13]).

2) We find that if the patient with vulvar dystrophy shows a clear improvement after 2 or 3 months of topic treatment with testosterone, the benefit is strictly bound to the continuity of long-term treatment. On the contrary, if the patient doesn't show clear improvement after 2 or 3 months, therapy with topic testosterone can be abandoned ([14]).

Finally, it has been demonstrated that vulvar dystrophic tissue transplanted in a healthy area of the thigh lost its dystrophic characteristic, and viceversa, a transplant of healthy skin in a dystrophic area, also became dystrophic. This demonstrates that a pathology which regards the organ itself and not tissue does exist.

BIBLIOGRAPHY

1) Garcia M., Rochefort H.: *Steroids, 29*, 111, 1977.
2) Ruh T. S., Wassilak S. G., Ruh M. F.: *Steroids, 25*, 257, 1975.
3) Zava D., McGuire W.: *Endocrinology, 103*, 624, 1978.
4) Zava D., McGuire W.: *Science, 199*, 787, 1978.
5) Rochefort H., Garcia M.: *Effect of androgens mediated by the estrogen receptor in uterus.* Research on Steroids Vol. VIII - A. Klopper, L. Lerner, H. J. Van der Molen, F. Sciarra Ed. - Academy Press - N.Y., 1979, p. 323-325.
6) Johansson R., Gronroos M., Kouvonen I., Lovgren T.: *Acta Obst. Gyn. Scand., 58*, 213, 1979.
7) Ford L. C., Berek J. S., Lagasse L. D., Hacker N. F., Heins Y. L., De Lange R. J.: *Gyn. Oncol., 15*, 27, 1983.
8) Chamness G. C., McGuire W.: *Steroids, 26*, 538, 1975.
9) Nardelli G. B., Tambuscio G., Marchesoni D., Gangemi M.: *Tumori, Suppl., 66*, 92, 1980.
10) Scatchard G.: *Ann. N. Y. Acad. Sci., 51*, 660, 1979.
11) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J.: *J. Biochem., 193*, 265, 1951.
12) Ambrosini A., Becagli L., Scrimin F., De Salvia D., Resta P., Gambato M.: *Topical testosterone propionate in the therapy of vulvar dystrophies. Clin. Exp. Obst. Gyn.,* 1983 (in press).
13) Ambrosini A., Becagli L., Resta P., Nardelli G. B., D'Antona N.: *Clin. Exp. Obst. Gyn., 7*, 1, 1980.
14) Becagli L., Scrimin F., De Bastiani B., De Salvia D., Nardelli G. B., Ambrosini A.: *Eur. J. Gyn. Oncol., 4*, 229, 1983.
15) Layne E.: *Spectrophotometric and turbidimetric methods for measuring protein. Methods in Enzimology*, Vol. III, 1975.
16) Powell B. L., De La Garza M., Clark G. M., Mc Guire W. L.: *Breast Cancer and Treatment, 1*, 33, 1981.