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Estradiol receptor in the lizard liver (*Podarcis s. sicula*). Seasonal changes and estradiol and growth hormone dependence

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Summary

This study shows that in the liver of the oviparous lizard, *Podarcis s. sicula*, the estrogen receptor (ER) level increases during the reproductive period (spring) when vitellogenesis occurs. This phenomenon interested both unfilled and filled ER present in the cytosolic and nuclear fractions. The increase in unfilled cytosolic and filled nuclear receptor was positively correlated to the plasma level of vitellogenin.

The level of liver ER approximated that of mammalian liver ER and, therefore, it is higher than that reported for the liver of several nonmammalian species.

At electrofocusing, liver ER distributes in two pH ranges (pH 6.5–7.5 and 8.0–8.8, respectively). The first form predominated in nuclei of reproductive females or of spayed estrogenized females and could represent the activated form of receptor.

Ovariectomy was followed by a decrease in liver ER which can be induced in spayed females by estradiol administration. Pituitary growth hormone (GH) seemed to exert a synergic effect on estradiol liver estrogen receptor regulation. In lizards treated both with estradiol and GH, in fact, there was a significant increase in nuclear filled ER rather than an increase in the level of total nuclear ER.

Introduction

In the oviparous lizard, *Podarcis s. sicula*, like in other nonmammalian vertebrates (Wallace, 1985; Ho, 1987), vitellogenin synthesis takes place in the liver and is primarily an estrogen-dependent process. In fact, estradiol, administered to nonreproductive females, increases liver vitellogenin production. In these animals many oocytes in the ovary take up blood vitellogenin and accumulate yolk (Botte, personal communication).

Estradiol binding to specific receptor is a prerequisite for hepatocyte stimulation. The resulting estradiol-receptor complex acts on nuclear constituents and induces gene transcription (cfr. Callard and Callard, 1987). Liver estrogen receptors have been found in the cyclostome *Eptatretus stouti* (Turner et al., 1981), the Atlantic salmon (Lazier et al., 1985), the winter flounder *Pseudopleuronectes americanus* (Sloop et al., 1984), the amphibia *Xenopus laevis* (Westley and Knowland, 1978; Wright et al., 1983), *Discoglossus pictus* (Ozon and Bellé, 1973), *Rana esculenta* (Paolucci and Botte, 1988) and *Ambystoma mexicanum* (May et al., 1981), the reptiles *Chrysemys picta* (Riley et al., 1987; Ho et al., 1988; Riley and Callard, 1988; Yu and Ho, 1989) and *Podarcis s. sicula* (Paolucci

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and Botte, 1987), the chicken (Mester and Baulieu, 1972; Schneider and Gschwendt, 1977; Lazier and Haggarty, 1979), the sea trout *Cynoscion nebulosus* (Smith and Thomas, 1989) and mammals (Eisenfeld et al., 1976; Powell-Jones et al., 1976; Marr et al., 1980).

In several nonmammalian species, moreover, there is a positive correlation between the levels of estrogen receptor in hepatocytes and plasma vitellogenin. This is additional evidence that these receptors are directly involved in the mechanism of induction of vitellogenin synthesis in hepatocytes (Westley and Knowland, 1978; Westley, 1979; Paolucci and Botte, 1988; Riley and Callard, 1988).

Other hormones, besides estrogens, can influence liver vitellogenin production (cfr. Ho, 1987). For example, in some teleostean species, several nonestrogen hormones induce liver vitellogenin synthesis (Hori et al., 1979; Le Menn, 1979; Le Menn et al., 1980). In other vertebrates, pituitary hormones seem to be necessary for the estrogen action to be effective. In the hypophysectomized female frog, *Rana esculenta*, the plasma vitellogenin level is higher if estradiol administration is coupled to an homologous pituitary extract (Gobbetti et al., 1975). In the female turtle, *Chrysemys picta*, hypophysectomy depresses estrogen-induced liver vitellogenin synthesis; this effect is partially counteracted by growth hormone administration (Ho et al., 1982a,b, 1985). Hypophysectomy markedly reduced cytosolic and nuclear hepatic estrogen receptor content in the female (Ho et al., 1989) while replacement with growth hormone causes an increase in nuclear estradiol receptor level (Riley and Callard, 1988). In the female lizard, *Dipsosaurus dorsalis*, the estradiol-dependent lipophosphoprotein synthesis (an index of vitellogenesis) is abolished by hypophysectomy (Callard et al., 1972; Gerstle and Callard, 1972).

These observations suggested that in reptiles, pituitary hormones, and in particular growth hormone, play a prime role in the regulation of liver vitellogenin production by acting on estradiol receptor level. To help clarify this regulatory mechanism we studied the dynamics of the level of liver estradiol receptor in relation to blood estradiol and vitellogenin levels in the female lizard, *Podarcis s. sicula*, during the reproductive period. Moreover, we investigated whether estradiol and growth hormone administration can influence liver estradiol receptor levels.

Material and methods

Animals

Adult females, *Podarcis s. sicula*, were captured in the outskirts of Naples during April–November 1988. These lizards emerge from winter semihibernation in March and reproduce in May–June when ovulation and egg deposition take place (Filosa, 1973; Angelini et al., 1982). At the end of June the genital apparatus regresses and remains quiescent until the following April (Licht, 1969).

The lizards were killed after ether anaesthesia. Blood was withdrawn through a heparinized capillary inserted in the heart. Plasma, generated after brief centrifugation, was kept at $-20\,^{\circ}C$ until use. The liver was exposed and perfused with 0.7% cold saline to eliminate all traces of blood. Thereafter it was excised and kept in liquid nitrogen until use. The sexual stage of gonads was established by direct inspection of ovaries at autopsy (evaluation of number and diameter of vitellogenetic follicles; presence of eggs in the oviduct).

November nonreproductive lizards were used for experimental administration of estradiol. These animals were reared in terraria at a temperature of $26\,^{\circ}C$, with a photoperiod of dark : light = 16 : 8. Meal worms and fresh vegetables were furnished ad libitum. Except for a few animals, all the females were ovariectomized. A week later they were divided into four groups of ten animals each. The animals of group I received 0.1 μg of 17β-estradiol (Sigma, U.S.A.) dissolved in 0.1 ml of 0.7% saline, intraperitoneally every 2 days. Those of group II were given 0.04 IU of growth hormone (GH, Serono Umano Italy, GRORMA-HGH) dissolved in 0.1 ml 0.7% saline, intraperitoneally every 2 days. Group III animals were administered both estradiol and GH. Group IV animals received only the solvent. After 8 days of treatment, the lizards were killed and processed as described above.

Preparation of liver subcellular fractions

All the procedures were conducted at $0–4\,^{\circ}C$. Analytical-grade chemicals were used. The method

reported by Wright et al. (1983), slightly modified as previously described (Paolucci and Botte, 1987), was used to prepare cytosol and nuclear extracts. Briefly, each liver was chopped and homogenized in 4 volumes (wet weight/ml) of 5 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.4, containing 0.33 M sucrose, 5 mM MgCl, 1 mM 2-mercaptoethanol, 150 μg/ml phenylmethylsulfonyl fluoride (homogenization buffer). The suspension was centrifuged at $800 \times g$ for 10 min. The supernatant was centrifuged at $189000 \times g$ for 1 h and constituted the cytosol. The $800 \times g$ pellet was suspended in 4 volumes of homogenization buffer containing 0.25 M sucrose and washed twice with the same buffer by subsequent centrifugations at $800 \times g$ for 10 min. A smear of final pellet was examined under phase-contrast microscope to check the nucleus integrity. The final pellet was suspended in 4 volumes of Hepes buffer, pH 7.4, containing 0.5 M KCl, 1 mM EDTA, 1 mM mercaptoethanol, 10% glycerol, and 150 μg/ml phenylmethylsulfonyl fluoride (extraction buffer). The suspension was frozen and thawed and left on ice for 1 h, and then centrifuged at $189000 \times g$ for 1 h. The supernatant constituted the nuclear extract.

Measurement of labeled estradiol binding

[2,4,6,7-^{3}H]Estradiol (90–110 Ci/mM) was obtained from Amersham Radiochemical Centre (Amersham, Bucks, U.K.); unlabeled estradiol and diethylstilbesterol (DES) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aliquots (0.2 ml) of samples (cytosol or nuclear extract) were incubated with 5 mM labeled estradiol with and without a 100-fold excess of DES for 2 h at 4°C or at 20°C to reveal, respectively, free and total binding sites. After incubation, the mixtures were supplemented with 0.6 ml of a carbon-dextran suspension (0.5 and 0.05 w/v) at 4°C and incubated for 10 min. The suspensions were then centrifuged at $800 \times g$ for 10 min. 5 ml Maxifluor (Packard, Milan, Italy) were added to the supernatants and radioactivity was counted (efficiency approximately 40%).

Isoelectrofocusing

The method described by Matsumada and Goldman (1974) was used for these experiments. A glass column (31×0.5 cm) was filled with a mixture of 12.5% sucrose in water containing 0.01% Triton X-100 and 3% Ampholine (Pharmacia, Sweden) pH 3.5–10. After a prerun of 1 h at 4°C, 200 V, 0.2 ml of sample (cytosol or nuclear extract preincubated with 5 nM labeled estradiol for 1 h at 20°C) were layered on top of the gradient. Electrofocusing was carried out for 18 h at 4°C, 200 V. At the end of the run, 0.4 ml aliquots were removed from the bottom of the column and used for the evaluation of pH and labeled estradiol binding, as described above.

Measurement of plasma estradiol

A radioimmunoassay method was used to measure estradiol (Ciarcia et al., 1986). Sensitivity was 3 pg (intraassay variability, 7%; interassay variability, 13%).

Measurement of plasma vitellogenin

Plasma vitellogenin was determined with an enzyme-linked immunosorbent assay (ELISA) method (Giorgi et al., 1982). The specific antibody was a gift from Dr. O. Carnevale of the University of Camerino (Italy).

Statistical methods

Numerical data were analyzed by a two-way analysis of variance (ANOVA) method, followed by the Duncan test, and by correlation methods.

Results

Table 1 shows the changes in the level of estradiol receptor in the hepatocytes of the female lizard, *Podarcis s. sicula*, during the reproductive cycle. The plasma titres of 17β-estradiol and vitellogenin are also reported. In reproductive females, in which vitellogenetic processes are very active, there was an increase in estradiol binding sites. This phenomenon was statistically significant for both filled and unfilled cytosol ($P < 0.01$) and nuclear ($P < 0.05$) binding sites. Plasma vitellogenin levels were higher during the reproductive period ($P < 0.05$), whereas the 17β-estradiol titre remained unchanged. Table 2 shows the correlation coefficients between the parameters reported in Table 1. The levels of cytosol unfilled estradiol receptor and of nuclear filled estradiol receptor

TABLE 1

CHANGES IN THE LEVEL OF LIVER ESTRADIOL-BINDING MOLECULES AND PLASMA ESTRADIOL AND VITELLOGENIN IN THE LIZARD, *PODARCIS S. SICULA*, DURING THE REPRODUCTIVE PERIOD

Six animals were examined for each period.

	Stages of the sexual cycle		
	Prerepro-ductive	Repro-ductive	Postrepro-ductive
1. *Liver specific estrogen receptor* (fmol/g wet tissue)			
Cytosol			
unfilled	1186±112	3262±354 **	1512±147
filled	44±19	760±143 **	147±56
Nuclear extract			
unfilled	358±43	1284±190 *	380±100
filled	107±23	530±75 *	145±29
2. *Plasma estradiol* (ng/ml)	1.83±0.38	1.19±0.40	1.15±0.35
3. *Plasma vitellogenin* (mg/ml)	140±63	580±24 *	368±93

The increase during the reproductive period was statistically significant: * $P < 0.05$; ** $P < 0.01$.

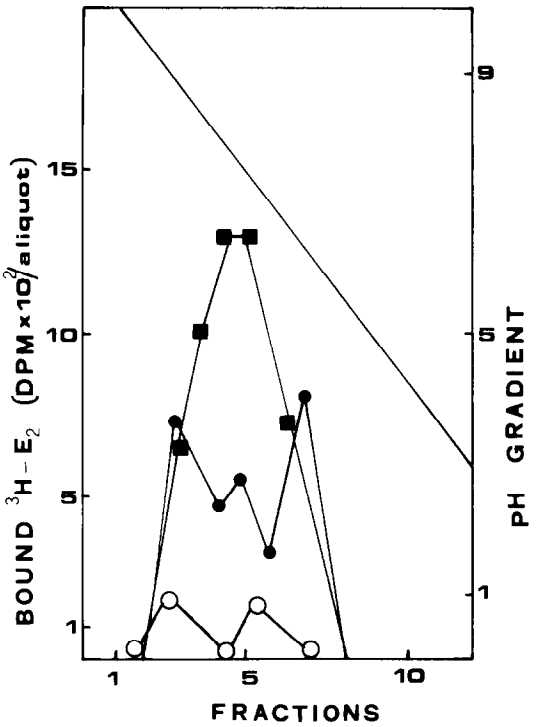

Fig. 1. Isoelectrofocusing of cytosolic estradiol receptor from the liver of prereproductive (○———○), reproductive (●———●) and estradiol-treated (□———□) female lizard, *Podarcis s. sicula*. The experiment is representative of three different separations. Oblique line on the graph represents the pH gradient. Only specific estradiol binding is shown. For other conditions see Material and Methods section.

were significantly and positively correlated to plasma vitellogenin titres.

Figs. 1 and 2 illustrate a typical distribution along the pH gradient, obtained with electrofocusing of, respectively, cytosol and nuclear liver estradiol receptor. The estradiol receptor of prereproductive females was distributed in two pH ranges: pH 6.5–7.5 and pH 8.0–8.5. In reproductive lizards the pH 6.5–7.5 binding molecules predominated and their level was higher in the nuclear compartment. In nonreproductive spayed and estradiol-treated females, the distribution of estradiol receptor was similar to that observed in normal reproductive females, whereas the estradiol receptor p*I* was slightly higher (pH 7.5–7.8).

Figs. 3 and 4 illustrate the effects of ovariectomy and 17β-estradiol and/or GH adminis-

TABLE 2

CORRELATION COEFFICIENTS OF DATA REPORTED IN TABLE 1

	cRU *	cRF	nRU	nRF	E	Vt
cRU	1	0.400	0.309	0.401	−0.222	0.647 **
cRF		1	0.236	0.450	−0.004	0.407
nRU			1	0.872 **	0.340	0.204
nRF				1	0.386	0.570 **
E					1	−0.122
Vt						1

* cRU = cytosolic unfilled receptor; cRF = cytosolic filled receptor; nRU = nuclear unfilled receptor; nRF = nuclear filled receptor; E = estradiol; Vt = vitellogenin. $n = 18$.

** $r = 0.468$ ($P < 0.05$) and 0.590 ($P < 0.01$) (FD = 16).

tration on, respectively, cytosol and nuclear liver estradiol receptor levels. Ovariectomy caused a significant depletion of estradiol receptor. In spayed animals, estradiol injection was followed by a significant increase in estradiol receptor, both unfilled ($P < 0.05$) and filled ($P < 0.01$), in the nuclear compartment. Some increase in unfilled estradiol receptor was observed in cytosol, but it was not significant. Growth hormone, when administered with 17β-estradiol, caused an even greater increase in nuclear filled estradiol receptor ($P < 0.01$).

Discussion

We have previously shown that hepatocytes of the female lizard, *Podarcis s. sicula*, contain an estrogen receptor ($K_d = 5.3 \times 10^{-10}$ M for cytosol and $K_d = 2.5 \times 10^{-10}$ M for nuclear extract) whose level is higher during the reproductive period, i.e., when vitellogenetic processes take place in the ovary (Paolucci and Botte, 1987). There is a similar correlation in the female turtle, *Chrysemys picta* (Riley and Callard, 1988). The data reported in the present paper confirm and extend these observations. In *Podarcis s. sicula* both unfilled and filled estradiol receptor was found in cytosol and nuclear extract of hepatocytes. Its level increased significantly during the breeding season. Moreover, unfilled cytosol and nuclear filled binding sites were positively correlated to plasma levels of vitellogenin. A positive correlation between liver nuclear filled binding sites and plasma vitellogenin titre has also been reported in the vitellogenetic female green frog, *Rana esculenta* (Paolucci and Botte, 1988). This indicates that nuclear filled estradiol receptor is probably involved in specific gene transcription (see Callard and Callard, 1987).

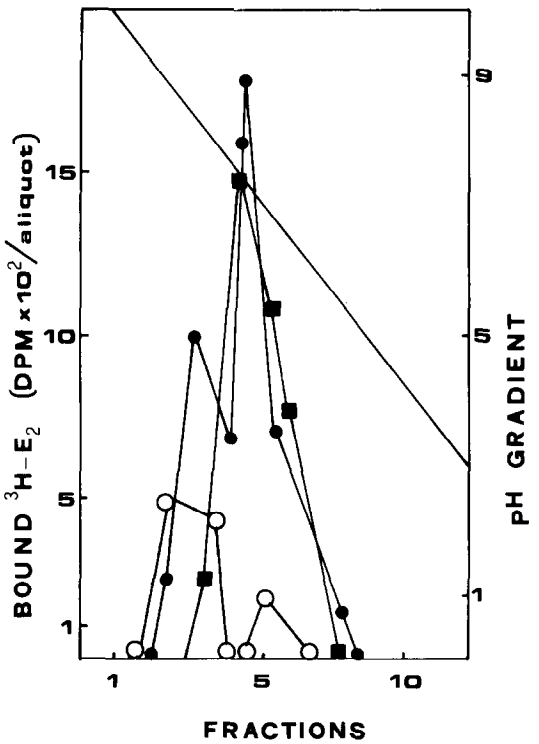

Fig. 2. Isoelectrofocusing of nuclear estradiol receptor from the liver of prereproductive (○———○), reproductive (●———●) and estradiol-treated (□———□) female lizard, *Podarcis s. sicula*. Other indications as in Fig. 1.

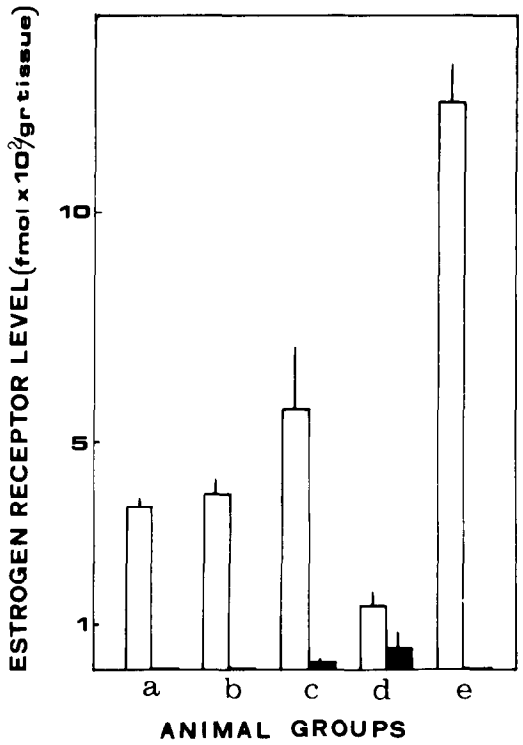

Fig. 3. Effects of ovariectomy and of estradiol and/or growth hormone treatment on liver unfilled (□) and filled (■) cytosolic estradiol receptor level in nonreproductive (November) female lizard, *Podarcis s. sicula*. (a) ovariectomized treated with solvent (saline); (b) ovariectomized treated with growth hormone; (c) ovariectomized treated with estradiol; (d) ovariectomized treated with estradiol and growth hormone; (e) intact lizards.

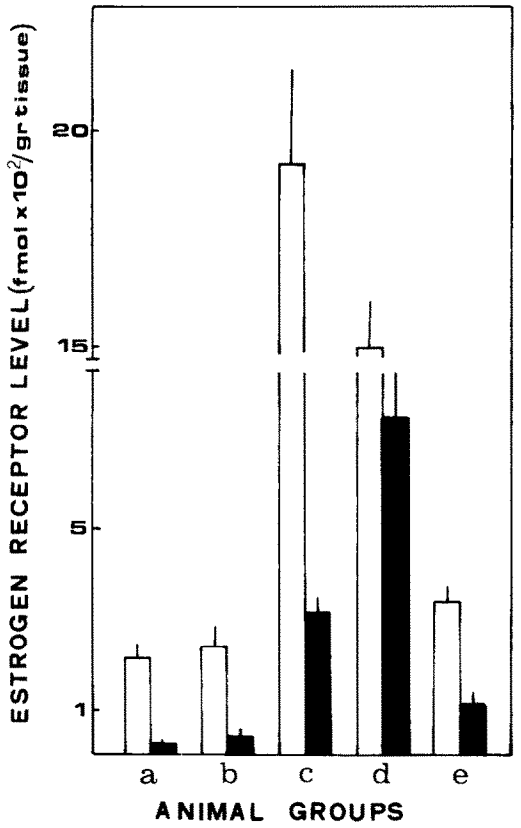

Fig. 4. Effects of ovariectomy and of estradiol and/or growth hormone treatment on liver unfilled (□) and filled (■) nuclear estradiol receptor level in nonreproductive (November) female lizard, *Podarcis s. sicula*. For abbreviations see legend to Fig. 3.

The levels of liver estradiol receptor in *Podarcis s. sicula* (almost 4000 fmol/g wet liver in the cytosol and 1800 fmol/g wet liver in nuclear extract, in reproductive females) approximate those reported for mammals (Rochman et al., 1985), but they are higher than that found in several other nonmammalian vertebrates (Lazier and Haggarty, 1979; Lazier et al., 1985; Paolucci and Botte, 1988) including the turtle, *Chrysemys picta* (Ho et al., 1988). The liver receptor level, therefore, is not coupled to low levels of circulating estradiol, as would be expected from the hypothesis of Ho et al. (1988): low hepatic estradiol receptor content in egg-laying vertebrates is related to high levels of estrogen present in their circulation. In fact, in female *Podarcis s. sicula*, blood estradiol titre reaches a relatively high value (1.8–1.15 ng/ml of plasma) during reproduction.

In *Podarcis s. sicula*, we have shown that liver estradiol receptor synthesis appears to be an estradiol-dependent process since estradiol, given in physiological doses to spayed females, induces a significant increase of unfilled and filled nuclear binding sites. However, changes in the level of liver estradiol receptor during the reproductive cycle were not correlated to hormone plasma titres. This discrepancy could be due to the rapid rise in plasma estradiol levels that precedes vitellogenin synthesis (Ciarcia et al., 1986). This early increase in estradiol seems to be better correlated to oviduct spring growth, a process that is estradiol-dependent (Botte, 1974).

The presence of steroid receptor molecules in the cytosol of target cells has been considered an artifact resulting from preparation procedures (King and Greene, 1984; Welshons et al., 1984). In fact, during tissue homogenization, unfilled estradiol receptor is easily extracted from nuclei and accumulates in cytosolic fraction. Filled estradiol receptor (ER), on the contrary, tends to adhere tenaciously to nuclear components (Callard and Callard, 1987). The last statement, however, disagrees with the presence of unfilled ER in liver nuclei of *Podarcis s. sicula* and with some observations on the androgen receptor (AR) behaviour in goldfish brain. In this teleost, the percent of occupancy of nuclear brain AR, in fact, showed no changes throughout the annual cycle while receptor level fluctuates with circulating steroids and brain aromatase activity (Psmanik and Callard, 1988). The cytosol versus nuclear shift of unfilled and filled estradiol receptor in liver of *Rana esculenta* (Paolucci and Botte, 1988) and of *Chrysemys picta* (Riley and Callard, 1988) following estrogen treatment is in accordance with a mainly nuclear localization of sex hormone receptors. The extent of estradiol receptor extraction from cell nuclei during tissue preparations, however, differs in various target organs and in different vertebrate species. It is not known which factors govern these differences. In dogfish liver, as an example, the level of estradiol receptor extracted from nuclei seems to depend on body fluid osmolarity (Callard and Mak, 1985).

In *Podarcis s. sicula* hepatocytes, cytosol un-

filled and filled estradiol receptor steadily predominates and, like nuclear estradiol receptor, it increases during the breeding cycle. In *Rana esculenta* liver, on the contrary, cytosol filled estradiol receptor is very scarce and does not show any significant change during the sexual cycle (Paolucci and Botte, 1988). An explanation of this discrepancy could be that in reproductive female *Podarcis s. sicula* more liver filled estradiol receptor is present than the chromatin binding sites can accommodate. Therefore, filled estradiol receptor can spill, together with unfilled estradiol receptor, into the cytosol fraction during tissue preparation.

Liver estradiol receptor of *Podarcis s. sicula*, subjected to electrofocusing, is distributed in two pH ranges, respectively pH 6.5–7.5 and 8.0–8.8. Since the first form of estradiol receptor prevails in nuclear extracts of both vitellogenetic and spayed estrogenized females, it could represent activated estradiol receptor. p*I* changes in rat prostate androgen receptor have been observed following activation (Mulder and Brinkman, 1985). No information is yet available on the p*I* of liver estradiol receptor in other reptilian species. Electrofocusing of liver estradiol receptor extracted from other vertebrates shows that it focuses in a narrow pH range (pH 7.6–7.7 in the female green frog, *Rana esculenta* (Paolucci and Botte, 1988) and pH 6.4 in the rat (Wrange et al., 1980)). It cannot be ruled out that the different pH range found in liver estradiol receptor is due to the presence of various estradiol receptor forms known to exist in several sex steroid target tissues. In hen oviduct two progesterone receptor forms focus respectively at pH 6.8 and 7.3 (Kon et al., 1980); in the calf uterus two progesterone receptor forms focus at pH 6.6 and 6.8 (Molinari et al., 1977; Puca et al., 1977). Should this interpretation be correct, the next step would be to study the specific function of the various estradiol receptor forms in relation to the lizard breeding period.

It seems unlikely that all unfilled estradiol receptor serves to bind estrogens in vivo (Clark et al., 1982). In several target organs a subpopulation of these molecules is normally utilized by lower affinity ligands, such as catechol estrogens (Martucci and Fishman, 1976; Merriam et al., 1980) or lipoidal estrogens (Mellom-Nussbaum et al., 1980). High concentrations of these ligands are present in target organs after estradiol administration, and rapidly dissociate from their receptor during tissue preparation (Clark et al., 1982). Therefore, it is possible that in *Podarcis s. sicula* liver unfilled estradiol receptor that binds to estrogen is overestimated. The meaning of the above-mentioned ligands, if present, in the physiology of lizard liver is unknown.

As reported above, liver estradiol receptor in female *Podarcis s. sicula* appears to be mainly under ovarian control. Its amount significantly decreases in spayed females and increases after estradiol administration. Moreover, pituitary growth hormone seems to exert a synergic effect on estrogen liver estradiol receptor regulation, because growth hormone injected in spayed females improves the estradiol-induced increase in nuclear filled estradiol receptor. These results agree with a study carried out on the liver estradiol receptor of the chelonian *Chrysemys picta* (Riley and Callard, 1988; Ho et al., 1989), in which, however, growth hormone seems to exert a more important function than in *Podarcis s. sicula*. It is feasible that, in vivo, pituitary hormones contribute to vitellogenin synthesis regulation by affecting filled estradiol receptor levels. This effect, however, could have different consequences in different reptilian species.

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