The Effects of Estradiol Valerate upon the Serum and Bone of the Lizard *Sceloporus cyanogenys* (32608)

HOWARD K. SUZUKI AND ROBERT L. PROSSER, III
Department of Anatomy, University of Arkansas School of Medicine, Little Rock, Arkansas

Investigations which have been carried out on birds have indicated that estrogens inhibit bone resorption, cause an elevation in serum calcium, and the synthesis of new proteins not present in nonestrogenized birds(8,11). These manifestations are associated with the preovulatory buildup of reserve osseous tissues within the marrow cavities of long bones due to a high estrogen titer, followed by a lowering of the estrogen titer and the resorption of the bone. In this latter stage the calcium store is transferred from the bone into the serum and to the egg shell and yolk(9).

The hypercalcemic response produced by injection of estrogens is also evident in fishes, amphibians and reptiles. The target tissue is the liver, which produces a calcium–phosphoprotein–phospholipoprotein complex. When this protein is synthesized calcium is apparently mobilized from endogenous and exogenous sources and is stored with other nutrients in the yolk and is utilized by the growing embryo(3,9,11).

We have been interested in investigating the phylogenetic origin of estrogen-induced medullary bone. There is some question as to whether or not early reptiles possessed medullary bone since that group does not have heavily calcified eggshells as do the birds.

Edgren(4) observed that the carapace and plastron of the musk turtles has less density during the ovulatory period; a similar response was demonstrated microradiographically in the long bones of the slider turtles (10). Thus, at least in turtles, bone was apparently resorbed to take care of the ovulatory needs without a preovulatory buildup of medullary bone or calcium store.

Since studies of the effects of estrogens in reptiles have been limited to turtles, the ovoviviporous lizard *(Sceloporus cyanogenys)* was selected.

Materials and Methods. Adult *Sceloporus* were divided into two groups. One group was injected with 0.05 cc of sesame oil carrier and the experimental group was injected with 250 μg of estradiol valerate in the same volume of sesame oil. They were sacrificed 5, 11, 15, and 42 days after the injection.

Before sacrifice the lizards were injected with 100 IU of heparin, and blood was collected following decapitation. The right knee and half mandible were prepared for histological study, while the left knee and half mandible were fixed for microradiography by methods previously described(10).

The plasma levels of total protein and inorganic phosphorus were determined using the Technicon Auto Analyzer. Calcium was determined by the calcein method(1). Electrophoresis was run using the Beckman microzone cell with Beckman cellulose acetate membrane and barbital buffer, pH 8.6, with an ionic strength of 0.075. The voltage and time were 250 V at 20 min.

Results. The histological and microradiographic preparations did not manifest any significant differences between the experimental and control animals. The bones of the group treated with estrogen did not have any observable endosteal bone, nor were there appreciable numbers of resorption cavities on the endosteal surfaces.

Estrogen induced a significant elevation in the plasma levels of total calcium, inorganic phosphorus, and total protein (Table I). Both serum calcium and phosphorus increased rapidly, were significantly different from the controls within 5 days ($p<.001$), and appeared to reach a plateau in 2 weeks. Extremely high amounts of both serum calcium and phosphorus were found. Highest individual amounts recorded were 450 mg/100 ml for calcium and 117 mg/100 ml for inorganic phosphorus (Table I, Estrogen group treated for 15 days) as compared with 13 mg/100 ml for calcium and 7.4 mg/100 ml for phosphorus in the controls. The quantity of total proteins present in the serum did not increase proportionally to the amount of calcium and phosphorus, but they increased gradually in amount throughout the experi-

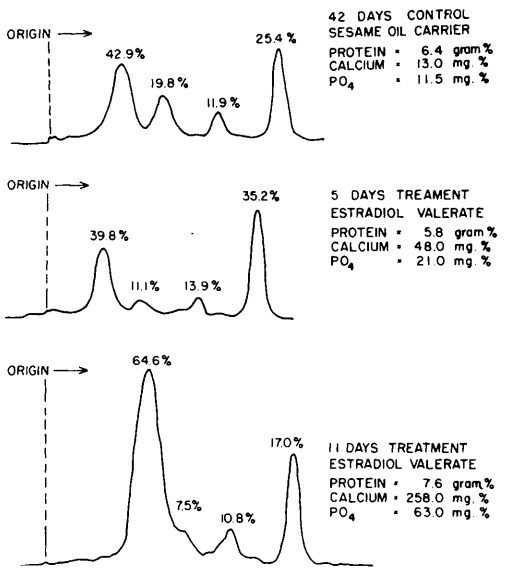

FIG. 1. Plasma electrophoretic pattern and plasma chemistry of 3 lizards. The upper one is that of a control lizard. Four fractions were separated and identified according to their mobilities. The fastest (fraction 1) comprising 25.4% of the proteins was named the "albumen." Fraction 2 (11.9%), fraction 3 (19.8%), and fraction 4 (42.9%) were named "alpha," "beta," and "gamma" globulins, respectively. Note that beta globulin is becoming confluent with gamma globulin in the 11-day estrogen-treated lizard.

mental period from 4.2 gm/100 ml in the controls to 10.3 gm/100 ml in the 6-week estrogen-treated group (Table I).

The electrophoretic pattern had four peaks in the controls, and were named according to their decreasing electrophoretic mobilities, i.e., "albumen" (fraction 1) and "alpha" (fraction 2), "beta" (fraction 3), and "gamma" (fraction 4) globulins (Fig. 1, control). There were no statistically significant differences between the electrophoretic patterns or total plasma proteins between the controls and the 5-day estrogen-treated group (Table II). The 11–42-day experimental animals manifested a steady increase not only in relative percentages but also in absolute quantities of gamma globulin (Table II). While the albumen appeared to decrease in relative percentages, it did not differ significantly in absolute amount between the controls and all of the experimental groups (Table II). The gamma globulin:albumen ratios were increased significantly the longer the treatment ($p<.001$). The alpha and beta globulin peaks were readily distinguishable in the controls but either disappeared or became confluent with the gamma globulin peak in the experimental groups (Figs. 1 and 2).

Discussion. Hypercalcemia, hyperphosphatemia, and hyperproteinemia have been produced by large doses of estrogen in turtles (11) and snakes (3). The estrogenic response in those reports were significantly lower than those reported in the present investigation. However, in agreement with their reports, our observations indicate that the response is directly related to time, the maximum being reached by 2 weeks.

Estrogen results in the synthesis of proteins within the liver which are released into the blood(11). In reptiles, these proteins have a high calcium binding capacity, approximately 55 mg of calcium per gram of protein(11). In the present study estrogen caused a relatively moderate rise from approximately 4 to 10 gm/100 ml of serum proteins, while inducing a large elevation from 13 to over 320 mg/100 ml of serum calcium. This corroborates the previous report of high calcium binding capacities of the proteins(11). Dessauer and Fox(3) observed hyperproteinemia, hypercalcemia, and enlargement of the livers in unfed snakes treated with estrogen and con-

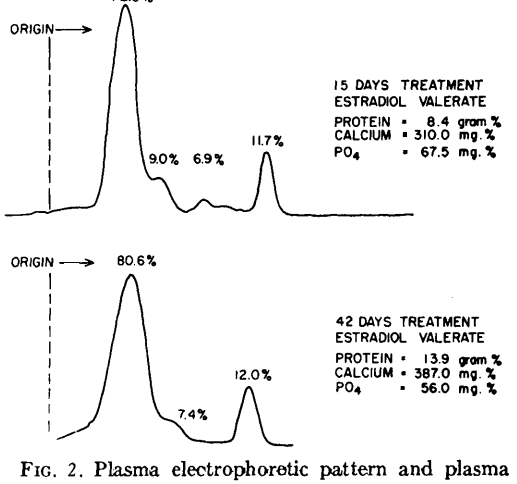

FIG. 2. Plasma electrophoretic pattern and plasma chemistry of two lizards treated with estradiol valerate. Note that the "alpha globulin" has nearly disappeared in the 15-day treated lizard, and is apparently gone in the 42-day treated lizard.

cluded that the calcium was released from the bone. Furthermore, Simkiss(9) suggested that calcium was necessary for the proteins to remain soluble in the serum, and thus explained why hypercalcemia took place even when no outside source of calcium was available.

Histologically, no apparent bone resorption was observed. If resorption of bone took place

TABLE I. Plasma Levels in *Sceloporus cyanogenys* Treated with a Single Injection of 250 μg of Estradiol Valerate.

Treatment		Length of treatment (days)	Total protein (gm)/100 ml	Calcium (mg)/100 ml	PO_4 (mg)/100 ml
Sesame oil control	(1)[a]	5	3.3	13.0	5.0
Estrogen	(5)	5	4.9 ± 0.75[b] (4.2–5.8)[c]	46.0 ± 14.2 (24–60)	24.8 ± 2.86 (21.0–29.0)
Sesame oil control	(1)	11	6.4	13.0	11.5
Estrogen	(4)	11	5.4 ± 1.70 (3.4–7.6)	224.0 ± 23.3 (210–258)	52.9 ± 6.9 (53–68)
Sesame oil control	(1)	15	3.9	13.9	9.5
Estrogen	(6)	15	8.7 ± 1.6 (6.7–11.4)	359.3 ± 62.1 (298–450)	88.1 ± 21.2 (60–117)
Sesame oil control	(15)	42	4.2 ± .92 (3.1–6.3)	13.0 ± 6.2 (8.5–30.0)	7.4 ± 1.3 (6.0–9.0)
Estrogen	(20)	42	10.3 ± 2.1 (7.0–13.9)	322.6 ± 89.6 (105–437)	55.3 ± 25.4 (21–105)

[a] Numbers in parentheses after treatment are numbers of animals in group.
[b] The values listed are ± one standard deviation.
[c] The numbers in parentheses under the values ± standard deviation are the total range.

TABLE II. Electrophoretic Analyses of Serum Proteins of *Sceloporus cyanogenys* Treated with a Single Injection of 250 μg of Estradiol Valerate.

Treatment	No. animals	Length of treatment (days)	Total plasma protein	Albumen	Gamma globulin	Gamma globulin/albumen
Sesame oil control	15	42	4.2 ± 0.9[c]	34.6 ± 7.2[a] (18.6–46.1)[b]	34.7 ± 8.7[a] (10.9–48.6)	1.09 ± 0.54[a] (0.30–2.61)
Estrogen	5	5	4.9 ± 0.75	36.0 ± 6.0 (27.3–43.1)	38.7 ± 5.7 (32.7–44.4)	1.11 ± 0.67 (0.81–1.62)
Estrogen	5	11	5.4 ± 1.7	22.4 ± 9.24 (16.6–38.6)	53.1 ± 17.3 (31.5–68.2)	2.8 ± 1.43 (.96–4.03)
Estrogen	6	15	8.7 ± 1.6	11.0 ± 3.0 (6.3–15.3)	72.9 ± 5.3 (71.8–82.3)	7.3 ± 3.02 (4.3–13.1)
Estrogen	20	42	10.3 ± 2.1	12.3 ± 3.9 (3.3–16.7)	78.6 ± 9.0 (54.5–95.2)	8.6 ± 5.68 (4.61–26.27)

[a] Albumen and gamma globulin given in percentages ± standard deviation.
[b] The numbers in parentheses under values are the total range.
[c] The total plasma proteins are given in gm/100 ml of plasma.

to obtain sufficient calcium to bind the proteins, it must have been generalized and took place in all of the bones. If we assume that *Sceloporus cyanogenys* has less than 2 ml of plasma, 4 mg of calcium could elevate the serum calcium levels to over 300 mg/100 ml. In the lizards used, the total skeleton weighed around 4 gm, and so the loss of only 0.1% of the skeletal weight could bring enough calcium into the circulating blood. Thus, the small amount of calcium removed from the lizard bones was difficult to observe histologically.

Previous investigators(3,9,11) interested in the role of estrogen in plasma protein synthesis have all considered that the liver was the site of origin. Our study has indicated that the "gamma globulin" component appears to be the main plasma protein that is increased in absolute quantity. Gamma globulin is synthesized in the reticuloendothelial system, particularly the plasma cells (2, 5, 6, 7), and estrogen causes not only an increased phagocytic index but also plasma cell hyperplasia and increased gamma globulin production(7). Therefore, it is suggested that in the lizard, estrogen induces an increased production of "gamma globulins" which may be produced by nonhepatic organs. Further studies will be conducted along these lines.

Summary. Adult *Sceloporus cyanogenys* were treated with 250 μg of estradiol valerate for 5, 11, 15 and 42 days. The bones were studied histologically and the plasma chemistry was determined. No morphological changes were observed in the bones. The plasma calcium and inorganic phosphorus were significantly elevated from the controls within 5 days after the injection. The proteins did not increase significantly until 11 days after injection, and the elevation occurred only in the "gamma globulin" component. It is suggested that estrogen induces increased "gamma globulin" synthesis whose origin is in part in the reticuloendothelial system.

1. Appleton, H. D., West, M., Mandel, M., and Sala, A. M., Clin. Chem. **5**, 36 (1959).

2. Askonas, B. A., and Humphrey, J. H., Biochem. J. **68**, 252 (1958).

3. Dessauer, H. C., and Fox, W., Am. J. Physiol. **197**, 360 (1959).

4. Edgren, R. A., Comp. Biochem. Physiol. **1**, 213 (1960).

5. Miller, L. L., Bly, C. G., and Bale, W. F., J. Exptl. Med. **99**, 133 (1954).

6. Miller, L. L., and Bale, W. F., J. Exptl. Med., **99**, 125 (1954).

7. Nicol, T., Bilbey, D. L. J., Charles, L. M., Cordingley, J. L., and Vernon Roberts, B., J. Endocrinol. **30**, 277 (1964).

8. Silberberg, M., Silberberg, R., in "The Biochemistry and Physiology of Bone," Bourne G. H., ed., Chap. 20. Academic Press, New York, 1956.

9. Simkiss, K., Biol. Rev. **36**, 321 (1961).

10. Suzuki, H. K., Ann. N. Y. Acad. Sci. **109**, 351 (1963).

11. Urist, M. R., and Schjeide, A. O., J. Gen Physiol, **44**, 743 (1961).

Received Oct. 26, 1967. P.S.E.B.M., 1968, Vol. 127.

Immunization of Mice to Sarcoma 180 and Ehrlich Carcinoma with Ultraviolet-Killed Tumor Vaccine* (32609)

M. L. LITTMAN, Y. C. KIM, AND D. SUK

Veterans Administration Hospital, Brooklyn, N. Y., New York University School of Medicine, New York, N. Y. and Catholic Medical College, Seoul, Korea

As early as 1906 Ehrlich (1) reported that animals bearing a progressively growing tumor would reject a second transplant of the same tumor. Since that time it has become known that once a transplanted animal tumor is actively established, it may either enlarge progressively and kill the host, or upon reaching a certain point in development, may undergo spontaneous regression and spare the host (2–4). Animals with spontaneously regressed tumors were found to be immune to sub-

* This investigation was supported by Public Health Service Research Grant No. CA-08861 of the National Cancer Institute.