

The Partition of Calcium and Protein in the Blood of Oviparous Vertebrates during Estrus

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ABSTRACT Six classes of vertebrate animals were injected with massive doses of estrogen for various periods of time necessary to produce a grossly recognizable response. Swelling of the liver, associated with hypercalcemia, hyperproteinemia, and lipemia, occurred in Teleostei, Amphibia, Reptilia, and Aves, but not in Elasmobranchii or Mammalia. Calcium that was added to the serum was bound as a calcium proteinate complex, synthesized in the liver, and liberated into the plasma. The concentration of calcium per gram was considerably higher than in other oviparous animals.

Teleostei, Amphibia, and Reptilia, produced one new component, protein- X_1 , having a sedimentation constant of approximately 17S, and many properties of a protein previously described in birds as X_2 . Calcium-binding capacity of teleostean and amphibian serum proteins was estimated at 25 to 35 mg. per gm. of the mixed proteins of the serum; reptilian serum proteins bound 55 mg. per gm.

Estrogen-treated birds clearly produced two, rather than one, new proteins, a phosphoprotein, X_1 , having a rate of 8.5S, and X_2 , a phospholipid-lipoglycoprotein, 17S. The calcium-binding capacity was approximately 50 mg. per gm. of the mixed proteins of the serums. Circumstantial evidence suggests that the plasma proteins of oviparity appeared simultaneously with the evolution of bone as a tissue and an ultimobranchial gland having parathyroid function.

INTRODUCTION

Estrus in oviparous and ovoviviparous animals is accompanied by striking alterations in the composition of the blood. Under the control of estrogenic hormones, the liver enlarges and liberates lipids, lipoproteins, and complex calcium phosphoproteinate molecules into the plasma to be transported to the ovary for deposition of egg yolk. During this period, some of the electrolytes, lipids, proteins, and carbohydrates normally present in the blood are increased in concentration; others are decreased. All these changes have been

induced under laboratory conditions as early as 24 to 48 hours after a single injection of a large dose of exogenous estrogen (Urist *et al.*, 1958; Schjeide and Urist, 1959). Viviparous species, whether as low as the selachians, or as high as the mammals, had no corresponding blood changes during estrus or estrogen treatment.

This report will review the comparative biochemistry of the blood and response of representatives of six different classes of vertebrates during artificial estrus. By means of correlated chemical, electrophoretic, and ultracentrifugal analyses, the individuality, concentration, and structure of the plasma proteins of reproduction will be described in detail. Special attention will be given to the structure of the calcium and phosphorus protein complexes, and the significance of these compounds in ontogeny and evolution.

EXPERIMENTAL

Methods

Two adult male sharks, one *Triakis semifasciatus* (ovoviviparous), and one *Heterodontus francisci* (oviparous, each egg enclosed in a horny case several inches long), were given intramuscular injections of 25 mg. of an aqueous suspension of estrone (Ayerst Laboratories, Inc.). An untreated male and female of the same species were used as controls.

Twenty male bass (*Paralabrax clathratus*) were each given an intramuscular injection of 10 mg. of estrone. Five females and five males of each species were untreated and used as controls. Five male frogs (*Rana catesbiana*) were similarly injected with 10 mg. of estrone; five were examined as controls.

Two male turtles (*Pseudemys scripta troostii*) were given an intramuscular injection of 50 mg. of estrone. Five females, with and without ripe ovaries, were used as controls. Twenty New Hampshire chicks, 5 days old, received an intraperitoneal injection of 5 mg. of estrone; twenty chicks were examined as controls. Fifty common white laboratory mice each received an intramuscular injection of 5 mg. per week of estrone for 4 weeks; fifty were examined as controls. All the animals were sacrificed 5 days after the treatment and the blood was examined as described below.

The yolks of the eggs of each of the oviparous species listed above were homogenized and dissolved in 10 per cent NaCl and analyzed for calcium and phosphorus. The proteins were resolved by ultracentrifugation.

The methods employed in this study were the same as those described in detail in previous publications (Urist *et al.*, 1958; Schjeide and Urist, 1959).

RESULTS

I. Serum Proteins

Tables I and II summarize the results of chemical analyses of the serums of various vertebrate animals before and after treatment with estrogen. Figs. 1

to 3 illustrate the results of electrophoretic and ultracentrifugal analyses of the same serums.

(A) ELASMOBRANCHII The high levels of 17.4 ± 2 mg. per cent calcium and low levels of 1.2 ± 0.5 gm. per cent protein in the blood were normal for the Elasmobranchii. A peculiar distribution of electrolytes and proteins and a concentration of 1 per cent urea in the plasma were special characteristics of this ancient class of fishes. The liver did not swell following an injection of estrogen. The composition of the blood was also unchanged with respect to the levels of Ca, total protein, protein-bound P, total P, phospholipid, total

TABLE I
RELATIONS BETWEEN CALCIUM, PROTEINS,
PHOSPHORUS, AND PHOSPHOLIPID IN THE PLASMA BEFORE
AND AFTER ESTROGEN TREATMENT

Class	Ca	Total protein	Protein-bound phosphorus, per cent of total protein	Total phosphorus	Phospholipid	Ca/gm. protein added to serum
	<i>mg. per cent</i>	<i>gm. per cent</i>		<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg.</i>
Elasmobranch	17.4 ± 2	1.2 ± 0.5	0.4 ± 0.21	6.8 ± 0.1	33.4 ± 2.8	—
Estrogen-treated elasmobranch	16.4 ± 3	1.6 ± 0.1	0.44 ± 0.29	8.6 ± 2.1	36.2 ± 1.1	—
Teleost	12.8 ± 2	5.5 ± 0.2	0.26 ± 0.1	20.0 ± 8.4	54.0 ± 1.9	—
Estrogen-treated teleost	108.2 ± 12	14.4 ± 0.3	0.78 ± 0.2	92.2 ± 16	2430	20.4
Amphibian	11.6 ± 1	1.8 ± 0.1	0.34 ± 0.1	15.0 ± 2.1	80.7 ± 1.0	—
Estrogen-treated amphibian	154.6 ± 14	10.6 ± 0.2	0.81 ± 0.18	22.8 ± 6.0	690 ± 7.0	16.1
Reptile	8.5 ± 0.5	2.9 ± 0.4	0.07 ± 0.02	14.0 ± 2.0	290 ± 10	—
Estrogen-treated reptile	36.8 ± 8	3.7 ± 0.1	0.58 ± 0.14	86.1 ± 11	660	47.0
Bird	10.2 ± 1	3.4 ± 0.2	$0.57 \pm .002$	16.0 ± 2.0	540 ± 1.4	—
Estrogen-treated bird	90.4 ± 8	7.4 ± 0.3	3.70 ± 1.0	184.9 ± 21	1170 ± 8	20.0
Mammal	9.9 ± 1.4	6.3 ± 0.1	—	15.2 ± 3	200 ± 10	—
Estrogen-treated mammal	11.0 ± 0.3	6.2 ± 0.4	—	10.0 ± 1.5	505 ± 36	—

lipid, sterol esters, triglycerides, or sterols. Paper electrophoresis showed various globulins but no albumin. Ultracentrifugal analyses confirmed these findings. The results were the same in both the viviparous and ovoviviparous species of sharks.

(B) TELEOSTEI The levels of calcium of 12.8 ± 2 mg. per cent and of protein of 5.5 ± 0.2 gm. per cent were normal for male bony fish and females with ripening ovaries. Following an injection of estrogen in kelp bass, the level

TABLE II
SERUM LIPIDS OF VARIOUS CLASSES OF ANIMALS BEFORE
AND AFTER TREATMENT WITH ESTROGEN

Class	Total lipid	Sterol ester	Triglycerides	Sterols	Phospholipids
	<i>mg. per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Elasmobranch	790	19.3 ± 2	33.4 ± 2	11.0 ± 2	36.2 ± 2
Estrogen-treated elasmobranch	940	21.4 ± 1	32.1 ± 2	13.2 ± 1	33.4 ± 3
Teleost	2210	23.0 ± 0	17.0 ± 1	6.0 ± 0	54.0 ± 1
Estrogen-treated teleost	4820	9.2 ± 1	34.4 ± 1	7.0 ± 1	49.4 ± 3
Amphibian	180	43.7 ± 0	6.3 ± 1	6.3 ± 0	43.7 ± 0
Estrogen-treated amphibian	940	23.0 ± 7	25.8 ± 6	17.0 ± 1	34.2 ± 2
Reptile	680	34.8 ± 4	9.4 ± 1	12.9 ± 4	42.9 ± 10
Estrogen-treated reptile	1870	12.3 ± 1	38.1 ± 4	14.5 ± 1	35.1 ± 4
Bird	1360	50.0 ± 2	2.5 ± 0	7.9 ± 2	39.6 ± 2
Estrogen-treated bird	3290	38.0 ± 1	20.2 ± 1	6.4 ± 1	35.4 ± 4
Mammal	565±	—	—	—	—
Estrogen-treated mammal	1080±	—	—	—	—

of calcium was elevated tenfold and the level of protein almost threefold. This was associated with corresponding elevations in the level of protein-bound P, total P, total lipid, triglycerides, and sterols. Paper electrophoresis showed various globulins and very little, if any, albumin. Ultracentrifugal analyses showed a large peak identifiable as teleost-X, having a sedimentation constant of approximately 17S, resembling the complex protein described previously as X₂. Phosphorus analyses, however, suggested that either a phosphoglycolipoprotein or a lipoglycoprotein, together with a separate phosphoprotein, having a similar sedimentation constant, was represented by this peak. In addition to these proteins, a new lipoprotein that reflected the beam negatively appeared in the plasma. An unusual component with a

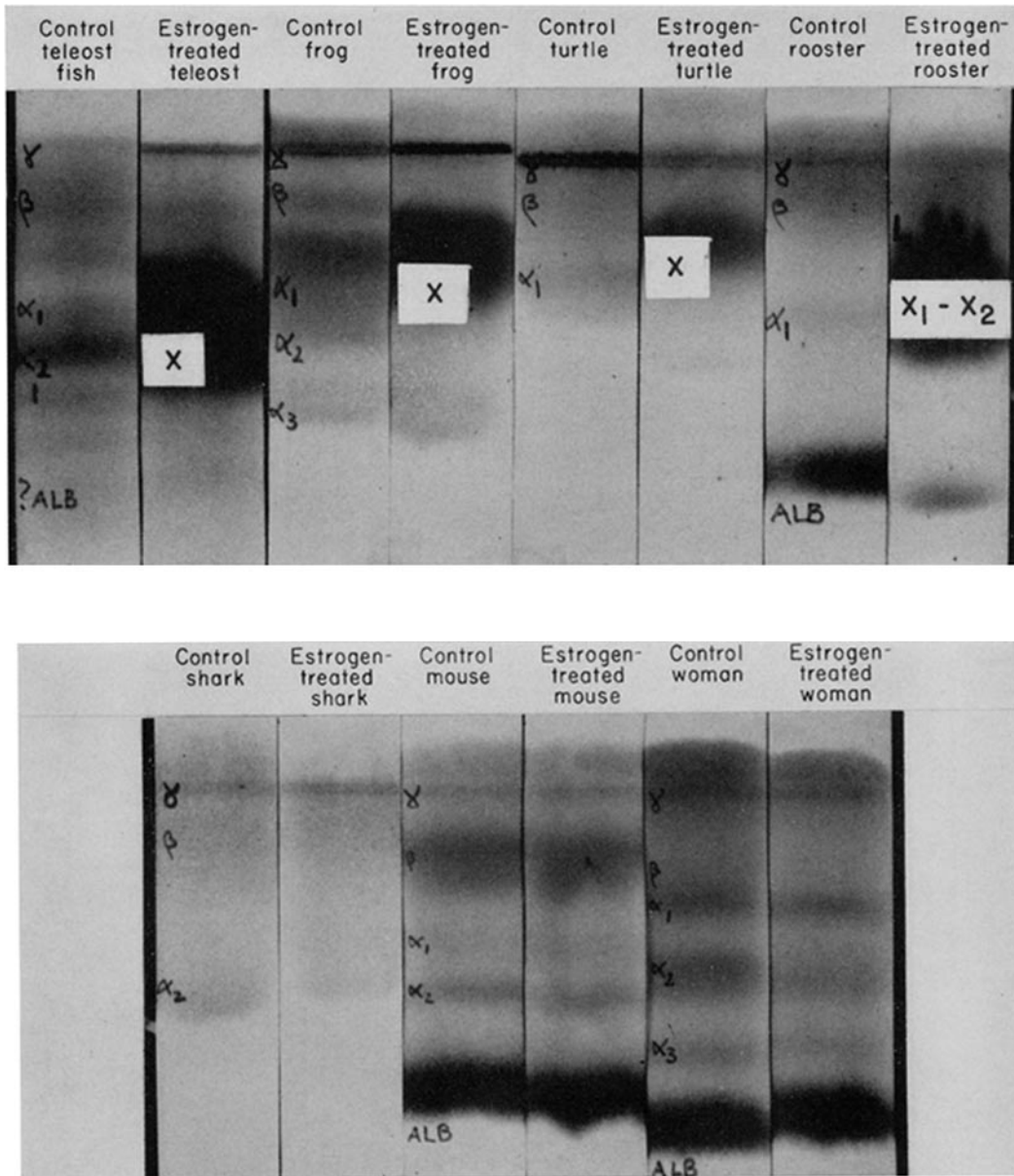


FIGURE 1. Filter paper electropherograms showing the proteins found in the serums of various classes of vertebrates before and after treatment with estrogen. Oviparous animals, as shown in the upper row of the paper strips, produced a new dense staining globulin, X or X₁-X₂, in response to estrogen. In viviparous animals, including a species as primitive as the leopard shark (having a pseudoplacenta), estrogen had no appreciable effect upon the serum protein. Ultracentrifugal and calcium analyses of these serums are shown in Figs. 2 and 3.

sedimentation constant of 1.0 was found in both control and estrogenized fish (Fig. 3). This moiety was not seen in the serums of any other class of animals.

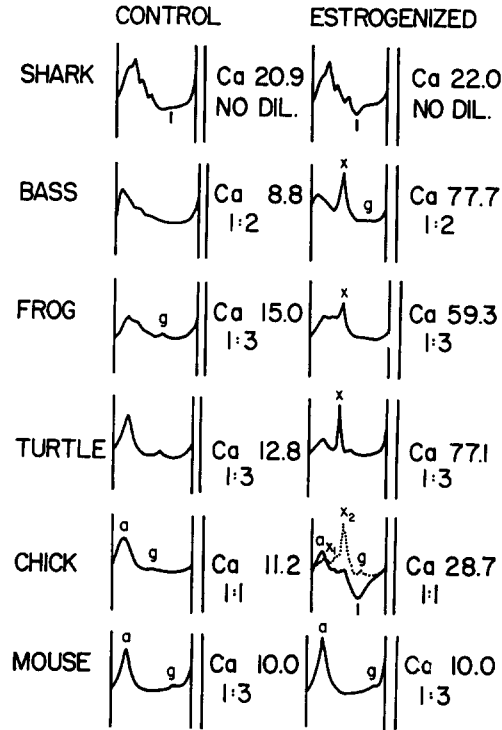


FIGURE 2. Ultracentrifuge patterns of serums from various species as resolved 48 minutes after reaching a speed of approximately 170,000 g . The peaks are sedimenting toward the right; g = globulin, X = phosphoprotein-phospholipid-lipoglycoprotein, X_2 = serum X_2 , phospholipid-glycolipoprotein, L = lipoprotein. The areas of peaks are directly proportional to concentration of those components. The elevations in the levels of calcium were always in proportion to the elevation in the level of protein in the serum. The levels of calcium in the serum and the dilutions used to resolve the proteinaceous peaks for each specimen are listed on the right side of each schlieren pattern. The corrected sedimentation rates of X and X_2 of various species were approximately the same (15–30S); X_1 , as a separate entity, was resolved only in the bird.

(C) AMPHIBIA Serum calcium levels of 11.6 ± 2 mg. per cent and serum protein levels of 1.8 ± 1 gm. per cent were within the normal range for male frogs and unripe females. Following an injection of estrogen in individuals of either sex, there was swelling of the liver; the level of calcium rose 14-fold and the level of protein rose sixfold. There was a concomitant elevation in the levels of protein-bound P, total P, total lipid, triglycerides, and sterols. The main elevation was in *frog-X* component, 17S, which appeared to be similar

to the *bass-X* protein described in bass. There was also a relatively small amount of lipoprotein reflecting the beam negatively. Paper electrophoresis revealed a mixture of globulins and a very small amount of albumin.

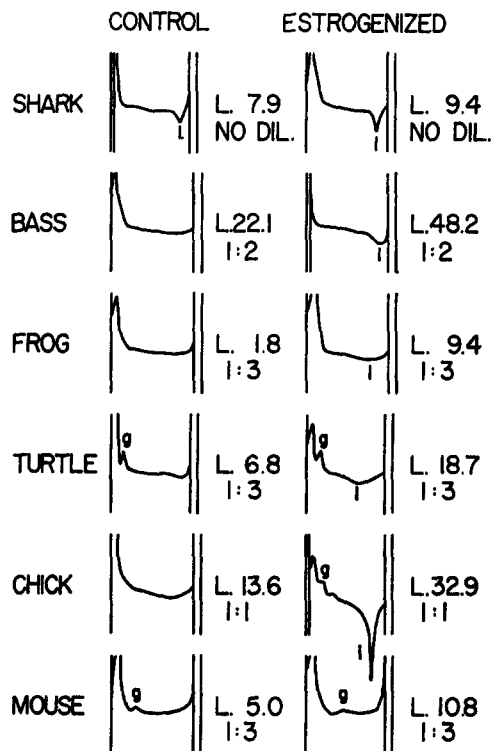


FIGURE 3. Ultracentrifuge patterns of serums from various classes of vertebrate animals as resolved at the time of reaching a speed of approximately 170,000 *g*. Negative peaks *L*. are lipoproteins, *g* = globulins. Areas of peaks are directly proportional to concentrations of those components. The concentrations of total serum lipids in milligrams per milliliter are listed along with the dilutions used to resolve the proteinaceous peaks which are shown on the right side of each schlieren pattern. In addition to the *X*, *X*₁, *X*₁-*X*₂ series of proteins shown in Fig. 2, estrogen produced more lipoprotein, *l*, (15-100S) than is normally present in the serum of viviparous vertebrates. The birds showed a larger increase in concentration of this component than any other species.

(D) REPTILIA The levels of calcium of 8.5 ± 0.5 mg. per cent and the levels of protein of 2.9 ± 0.4 gm. per cent were normal for the male turtle and for the female before or after ripening of the ova. Following an injection of a large dose of estrogen, the level of calcium rose fourfold and the protein, approximately 1.5-fold. However, there was an eightfold rise in the level of protein-bound P (compared with only threefold in teleost and amphibian species). There were variable elevations in the levels of triglycerides and sterols.

Paper electrophoresis showed various globulins and practically no albumin. Ultracentrifugal analyses revealed one large peak, having a sedimentation constant of approximately 17. *Turtle-X* contained 1.2 mg. P per gm. of protein, almost twice that found in the birds. The lipoprotein content, however, was relatively small, as seen by the slight amount of negative reflection of the beam in Fig. 3.

(E) AVES The level of calcium was 10.2 mg. per cent and for protein, 3.4 gm. per cent in immature and male individuals. In the laying hen, and immature or male birds treated with estrogen, the level of calcium rose ninefold and the protein twofold. This was associated with a rise in the level of protein-bound phosphorus of sixfold and in total P of 11-fold. The levels of phospholipid, total lipid, and triglycerides rose in proportion to the levels of total lipid.

Paper electrophoresis showed the appearance of high concentrations of at least three new proteinaceous components. Ultracentrifugal analyses revealed a small X_1 peak, 8.5S (a phosphoprotein), a large peak, 17S (a lipoglycoprotein), and an enormous lipoprotein peak that reflected the beam negatively (Urist *et al.*, 1958; Schjeide and Urist, 1959).

(F) MAMMALIA The levels of calcium of 9.9 ± 1.4 mg. per cent and 6.3 ± 0.1 gm. per cent of protein were normal for the mouse. After prolonged treatment with large doses of estrogen, there was no significant change in the levels of calcium, protein, total P, or phospholipid. Total lipid, however, was significantly elevated. Neither the electrophoretic nor ultracentrifugal analyses revealed any alteration in the composition of the plasma protein.

II. Calcium-Binding Capacity of the Mixed Proteins of the Serums of Various Classes of Vertebrate Animals

Table III presents estimates of the capacity factor of the proteins found in the serum of each class of animal, based on calculations in the literature (McLean and Hastings, 1935; Carr, 1953), on individual components, as well as mixtures of the components, before and after estrogen treatment, and identified by electrophoresis or ultracentrifugation (Urist, Schjeide, and McLean, 1958). The results suggest that because of the absence of albumin, and the low level of total protein in the serum, the Elasmobranchii had an extraordinarily small amount of protein-bound calcium, 1.6 mg. Ca/gm., in the blood. The Teleostei also had very little albumin in the serum but the mixed globulins, including *teleost-X*, had a calcium-binding capacity approximately 25 mg./gm. Serums having a similar mixture of proteins had a greater

capacity for calcium-binding as follows: Amphibia, 35 mg./gm.; Reptilia, 55 mg./gm.; Aves, 50 mg./gm.

III. *Lipemia*

Table II illustrates the point that all oviparous, or ovoviviparous classes, except Elasmobranchii, showed a characteristic shift toward elevation of the

TABLE III
CALCIUM-BINDING CHARACTERISTICS OF
MIXED PROTEINS OF VARIOUS CLASSES BEFORE
AND AFTER TREATMENT WITH ESTROGEN

Class	Capacity of mixed proteins of serum <i>mg. Ca/gm.</i>
Elasmobranchii	1.6
Estrogen-treated elasmobranchii	1.5
Teleostei	1.7
Estrogen-treated teleostei	25.0
Amphibia	1.7
Estrogen-treated amphibia	35.0
Reptilia	2.0
Estrogen-treated reptilia	55.0
Aves	3.0
Estrogen-treated aves	50.0
Mammalia	4.0
Estrogen-treated mammalia	4.0

level of the blood lipid following estrogen treatment. Viviparous animals produced no such response.

IV. *Egg Yolk*

Table IV summarizes an analysis of egg yolk for Ca, P, protein-bound P, and total protein in the yolk of the eggs of oviparous vertebrates. The concentration of total P was approximately the same in all four classes. However, the fraction of the total P bound to protein, and the capacity of the phosphoprotein for binding calcium, were higher in the reptile than in other vertebrates. This relationship was the same in egg yolk as in plasma.

Large yellow eggs were found in the oviduct of the female control shark, *T. semifasciatus*, but we have, thus far, not been able to extract protein from

them. For the most part, the yolk was insoluble in 10 per cent NaCl; the total Ca was 204 mg. per cent.

In the frog, the egg yolk is found in platelets, readily soluble in 10 per cent NaCl. Ultracentrifugal analyses reveal a large component with a sedimentation constant of 11S, and a much smaller component, 6S. The smaller component was found to be a phosphoprotein. Turtle egg yolk was also soluble in 10 per cent NaCl; ultracentrifugal analyses of the protein revealed two components, 5.3S and 11.0S, the latter being in relatively much larger concentration than the former.

Egg yolk of the reptile contained a twofold higher concentration of protein-bound phosphorus than was found in other oviparous classes. The 11S com-

TABLE IV
CALCIUM CONTENT OF THE EGG YOLK PROTEINS
OF VARIOUS OVIPAROUS VERTEBRATES

Class	No. of samples	Ca	P	Protein-bound P*	Total protein	Ca/gm. protein
		mg. per cent	mg. per cent	per cent	gm. per cent	mg.
Teleostei	3	160.6 ± 1	460 ± 21	0.79 ± 0.10	22.8	7.2
Amphibia	2	27.2 ± 1	472 ± 11	0.72 ± 0.12	41.7	0.6
Reptilia	6	212.0 ± 15	420 ± 20	1.27 ± 0.19	20.4	20.6
Aves	6	104.8 ± 8	460 ± 10	1.09 ± 0.11	16.	6.5

* Per gram of egg yolk protein.

ponent had some of the chemical properties of X_1 but yet showed the sedimentation properties of the X_2 of the bird, indicating that *turtle-X* is about twice the molecular weight of *fowl-X₁*.

Bird egg yolk was aggregated in granules. The protein was highly soluble in 10 per cent NaCl; ultracentrifugal analyses revealed a separate phosphoprotein, egg yolk- X_1 (4.75S), and a lipoglycoprotein, egg yolk- X_2 (10S); both of these components were apparently derived from the serum X_1 and X_2 and presumably split in half during transfer from serum to egg yolk (Schjeide and Urist, 1959).

DISCUSSION

Viviparity and Oviparity

The foregoing observations show that ovoviviparous elasmobranchs (species possessing a pseudoplacenta or communication between the maternal circulation and the yolk sac of the embryo), did not respond to estrogen treatment. It may be assumed either that the capacity of the liver to respond to estrogen

stimulation evolved only at a later stage of evolution than that represented by the elasmobranchs or that the evolution of viviparity precluded and eliminated the necessity for transport of egg yolk precursor substances in the blood. Possibly both assumptions are correct, but there remains the task of examining the blood of estrogen-treated cyclostomes and many more species of oviparous Elasmobranchii to determine the time of the evolution of the liver response to estrogen. The fact that the mice in the foregoing experiments did not produce hypercalcemia, hyperproteinemia, or gross lipemia in response to estrogen, suggests that the mammalian placenta, providing continuous communication between the fetal and the maternal circulation of blood, made it unnecessary to store calcium, phosphorus, or proteinaceous materials. In a general way, the phosphoprotein of egg yolk is not unlike the casein of milk, and it appears that during the preovulatory phase of the reproductive cycle, the chemistry of the swelling of the liver is analogous to the chemistry of the mammary glands during lactation in mammals.

Recent observations upon viviparous snakes in estrus (Dessauer and Fox, 1959) revealed enlargement of the liver, hyperproteinemia, and hypercalcemia, even during fasting. It was concluded that the calcium used in the formation of the calcium phosphoprotein complex must have come from bone. Whether ovoviviparous or oviparous, whether the calcium bound to protein is derived from the diet or the skeleton, the synthesis of calcium phosphoprotein complexes occurred in the liver, and (as in the experiments presented herewith) the correlation in the snakes was with deposition of egg yolk, the same as in all other vertebrates that reproduce without a structure having the function of a placenta. Complex relationships must exist between the character of the protein in blood and various factors, such as, structure and volume of egg yolk, type of enveloping embryonic membranes, mode of excretion of nitrogenous wastes, and water (Baldwin, 1949).

In vertebrates having bone tissue and an internal continuous supply of calcium and phosphorus, true oviparity can be determined by the presence of calcium phosphoprotein in the blood, either during reproduction, or following an injection of estrogen. Animals that lay eggs with shells are obviously oviparous, or ovoviviparous, but those that retain their eggs are sometimes difficult to classify. Ovoviviparity, or viviparity, terms often used arbitrarily and interchangeably, apply to several conditions and combinations of development of the yolk sac and chorioallantoic placenta (Fox, personal communication). For example, the viper snakes may have a yolk sac placenta like the pseudoplacenta of the shark at an earlier stage, and a chorioallantoic placenta like higher vertebrates at a later stage of development. However, during the preovulatory stage of the reproductive cycle, the plasma proteins of an ovoviviparous snake described by Dessauer and Fox resembled those we found in an oviparous turtle.

Hypercalcemia

As early as 1928 Hess *et al.* recognized the relationship of hypercalcemia to reproduction in the puffer codfish; there was no cognizance of the association with hyperproteinemia. Later, phosphoprotein was found in the serum of many egg-laying animals during the period of maturation of the ovarian follicles (Laskowski, 1936). Our experiments, as presented herewith, emphasize the relationship of both hypercalcemia and hyperproteinemia to calcium protein complexes and deposition of egg yolk, thus the processes of true oviparity. The hypercalcemia in oviparous species was proportional to the increase in the total protein, and not strictly related to alterations in the level of the total phosphorus. The elevation in the protein-bound phosphorus in the serum, however, was more pronounced in the reptile and avian species than in the teleost or amphibian. The schlieren patterns correlated with chemical data suggested that X_2 was evolved from the blood pattern of the bony fishes and the amphibians. At a later stage in evolution, X_1 , a phosphoprotein, was evolved in the birds. Apparently, in fish and amphibians, calcium is bound chiefly to a phospholipid-glycolipoprotein and other globulins, transported, and deposited with them in the yolk sac of the growing ova. The X_1 , the protein with the greatest capacity for binding calcium, probably did not appear in significant concentrations in the blood until vertebrates, such as reptiles and birds, came out of the water onto the land.

Relationship between Blood and Yolk Protein

Vitellin, whether derived from serum or egg yolk, consists of not one, but two, or more, proteins. *Phosvitin* is chemically altered phosphoprotein precipitated from egg yolk. Serum X_1 is the term we have employed to denote the unaltered, physiologically intact, phosphoprotein (Schjeide and Urist, 1959). The other components of the serum during estrus are uncharacterized, large molecules referred to as γ -*livetin*, *lipovitellin*, and several *lipoproteins*. Until more of the chemistry is known, the physiologic forms of the proteins of oviparity may be termed serum protein X_1 - X_2 and egg yolk X_1 - X_2 ; in birds, X_1 , the phosphoprotein, is a separate entity from X_2 , a phospholipid-lipoglycoprotein. In the process of the passage of these complex substances across the membranes between the blood and the yolk sac, their molecular weight is *decreased exactly in half* (Schjeide and Urist, 1959). The X_1 of serum has a sedimentation constant of 8.5 and a molecular weight of 144,000; egg yolk X_1 , 4.75S, has a molecular weight of 77,000. The X_2 of serum has an S of 17 and X_2 of egg yolk, 10S; the molecular weight of serum X_2 was estimated at 400,000, egg yolk X_2 , at 200,000. However, the exact structure

(and the calcium-binding capacity) of these proteins are an open controversy. One group of investigators (Common and Mok, 1959) holds that the protein consists of one large, complex, lipovitellin-phosphovitin-livetin (in the sense in which Joubert and Cook (1958) used the term *phosvitin* for phosphoprotein precipitated from a $MgSO_4$ solution of egg yolk). Another group (Abe *et al.*, 1958), using combined radioisotope and immunochemical studies, contends that phosphoprotein of serum is related to lipovitellin in egg yolk.

Calcium has been presumed to exist in blood during reproduction as colloidal calcium phosphate in fish (Bailey, 1957) and birds (Vanstone *et al.*, 1957). Our observations suggested that the calcium that was added to the serum was bound chiefly to protein and there was little, if any, that could be identified by the ultracentrifuge as colloidal calcium phosphate (Urist *et al.*, 1958).

Ultimobranchial glands with parathyroid function and regulation of calcium ions in the blood have not been described in Elasmobranchii (Camp, 1917), but seem present in Teleostei (Rasquin and Rosenbloom, 1954), the lowest class of animals we have found to respond to estrogen. It is possible that this process requires mobilization of calcium from bone in order to provide a constant minimum concentration of *calcium ions* in the extracellular and intracellular fluids of the liver cells (Urist *et al.*, 1960).

Dr. Kenneth Norris supplied the teleost fish and hospitality of his laboratory at Marineland of the Pacific, Palos Verdes, California. Dr. Eugenie Clark assisted in the collection of the blood at the Cape Haze Marine Laboratory, Placida, Florida. Mr. Loyal G. Goff and Mrs. Helen Hayes, and Dr. John Loeffler, Physiology and Biology Branches, Office of Naval Research, Department of the Navy, provided transportation and other valuable assistance. Dr. Albert H. Banner of the Coconut Island Laboratory, Hawaii, and Mr. Kenje Ego, Chief of the Bureau of Fisheries, Hawaii, gave valuable assistance and hospitality.

This work was aided by, in part, grants from the Easter Seal Research Foundation, Society for Crippled Children and Adults; Ayerst Research Laboratories, Inc.; Squibb Institute for Medical Research; the Josiah Macy, Jr. Foundation; and United States Public Health Service Grant-in-Aid No. A-3793(R1).

Received for publication, July 25, 1960.

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