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TRANSFER OF MATERNAL SERUM PROTEINS INTO THE EGG OF *RANA PIPIENS* AND THEIR ROLE IN DEVELOPMENT

by

Priyambada Mohanty Hejmadi

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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ABSTRACT

TRANSFER OF MATERNAL SERUM PROTEINS INTO
THE EGG OF RANA PIPIENS AND THEIR ROLE IN DEVELOPMENT

by

Priyambada Mohanty Hejmadi

Chairman: George W. Nace

Although serum-like proteins have been reported in the amphibian oocyte and embryo, little is known about their origin, nature, and ontogeny. To approach this problem, specific antisera were prepared against Frog Serum Albumin (A.FSA) and Frog Serum γ-Globulin (A.FSG) fractions and were used to examine the possibility that these proteins were transferred from the maternal serum into their oocytes as well as to follow their ontogeny. Immunochemical, immunohistological, chromatographic, and electrophoretic methods were used.

Albumin and γ-globulin antigens were detected in the saline soluble fraction and yolk platelets of oocytes throughout oogenesis. The distribution of these antigens in the female and her oocytes suggested that these macromolecules are transferred into the oocytes. Further investigations indicated that concurrent with vitellogenesis, the level of two other serum proteins, α-Female Serum Protein (α-FSP) and β-Female Serum Protein (β-FSP), increased. The levels of these two proteins decreased with the maturation of the

oocytes, suggesting their transfer into the oocytes. Ovariectomy led to an increase of circulating serum albumin, α-FSP, β-FSP, and γ-globulins, suggesting that removal of the ovary had deprived the animal of the depot in which these proteins were deposited and, thus, suggesting that these serum proteins are normally accumulated by the oocytes. Direct support for the transfer of maternal serum proteins into oocytes came from observations that parenterally administered heterologous bovine serum albumin, rabbit γ-globulin, and human serum were transferred antigenically intact, into the oocytes.

Two albumin and one γ-globulin antigens were utilized between blastula and gastrula stages. As yolk platelets remain intact until the tailbud stage, it is proposed that these albumin and γ-globulin antigens were degraded to provide the amino acids and polypeptides needed for autosynthesis until yolk is utilized. Two other albumin and one other γ-globulin antigens persisted until the tailbud stage. There was an increase in the pre-existing albumin and γ-globulin antigens and the appearance of one more albumin and one more γ-globulin antigens between the tailbud and gill circulation stages. This increase in albumin and γ-globulin antigens occurs at the time embryonic circulation is established and the organs associated with serum protein synthesis are still rudimentary and therefore probably still non-functional. This suggests that these late appearing constituents were of maternal serum origin, that prior to this stage they were sequestered in the yolk and that they function as embryonic serum proteins. It is also

proposed that some of the γ-globulin antigens are immunoglobulins which provide the embryo with immunological protection.

When eggs containing heterologous serum were fertilized development was blocked prior to gastrulation. This indicated that the heterologous proteins affect development drastically and adversely. The implication of these observations to the control of differentiation was discussed.

ACKNOWLEDGEMENTS

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INTRODUCTION

Fundamental to the development of the individual is the organization of the egg and the programming which controls the progressive formation of a complex patterned system from a relatively simple one. This organization and programming must arise from either or both of two sources: endogeneously from within the egg, or exogenously by transmission to the egg from elsewhere within the maternal body. With the development of knowledge of genetics, the origin of programming or specificity has been generally attributed to endogenous sources, whereas exogenous sources have been credited as supplying raw materials to be used at the discretion of the specifying systems of the egg. This latter view has been challenged by Brambell, Hemming, and Henderson (1951), Schechtman (1955), and others, but the description and experimental evidence remains incomplete. The relative significance of the two possible sources of developmental control has not been clearly evaluated.

Since formative centers and axes are already organized within mature oocytes, they are unsuitable as starting material. Therefore, such studies must be initiated at the level of the relatively simple primordial germ cell (Nace and Lavin, 1963; and Twitty, 1966, p 51).

Oogenesis, the growth of the primordial germ cell into the mature oocyte, has been a classical subject of studies by developmental biologists, and much descriptive

information is available (Raven, 1961; Guraya, 1965; and Norrévang, 1968). However, the study of the nature, origin, and distribution of the macromolecules which are involved in oogenesis has been initiated only very recently (Needham, 1942). The information available is not yet sufficient to reconstruct the events of oogenesis in sufficient detail to understand the relative contributions of endogenous and exogenous sources, or to interpret them with respect to the subsequent execution of the program of development (Nace, Suyama, and Smith, 1961).

During oogenesis, the growing oocyte synthesizes some of its macromolecules. These endogenous materials have been referred to as "autosynthetic" constituents. The exogenous materials which the oocyte receives from sites of synthesis elsewhere in the maternal body have been referred to as "heterosynthetic" constituents (Schechtman, 1955). Depending on the species, the nature of the autosynthetic as well as the heterosynthetic macromolecules can be either nucleic acids or proteins (Schechtman, 1955; Nace and Lavin, 1963; Schjeide et al., 1963; Telfer, 1965; Korfsmeier, 1966; Davidson, 1968; Glass, 1970; and Nace, 1970). Each of these contributes unique characteristics and potential to the egg and must be considered separately as well as together.

The most extensively studied "heterosynthetic" materials are serum proteins. This does not deny the significance of other constituents; but the physiological functions of serum proteins are well known, they can be isolated, and

their antigenic nature permits the use of immunochemical tools to trace their site of synthesis, to examine their transport to the oocyte, and in some cases to examine their fate and function during development. All these aspects have been reviewed and discussed in several recent articles (Solomon, 1965; Brambell, 1966; Glass, 1970; and Nace, 1970). A summary of the work on various groups of animals is presented in the Appendix.

Examination of these studies reveals that the consideration of function has been limited to the later stages of development, and possible important roles for these materials at stages prior to the development of circulation have not been adequately investigated. Various studies indicate that, regardless of the species, the transfer of serum proteins into the growing oocyte involves two overlapping events, one concerned with maternal physiology, and the other with morphological conditions in the ovary.

The maternal physiological changes involve alterations in the composition of the blood, particularly the accumulation of lipids, lipoproteins, and complex phosphoprotein molecules (Urist and Schjeide, 1961). Such changes are related to the endocrinological cycles associated with reproduction and have been demonstrated in insects (Telfer, 1965), fishes (Booke, 1964), amphibians (Follett and Redshaw, 1968; and Wallace and Jared, 1969), reptiles (Dessauer and Fox, 1959), and birds (Vanstone, Maw, and Common, 1955; and Schjeide et al., 1963). The study of Urist and Schjeide (1961) is representative of an extensive body of work which

has demonstrated that, by administration of estrogen, oviparous vertebrates can be induced to synthesize the reproduction-related serum proteins; the viviparous vertebrates do not seem to respond to such stimuli. However, in the rat a reproduction-related serum protein not controlled by estrogen has been reported by Heim (1962).

Morphological changes in the ovarian tissues consist of the appearance of interfollicular spaces (Hope, Humphries, and Bourne, 1963; Wischnitzer, 1966; Kessel and Panje, 1968; Wallace and Dumont, 1968; and Norrévang, 1968), and the development of macrovilli from follicle cells which interdigitate with the oocyte microvilli and make contact with the oocyte cytoplasm (Kemp, 1956; Hope et al., 1963; Press, 1964; Wischnitzer, 1966; Schjeide et al., 1966; Kemp and Istock, 1967; Kessel and Panje, 1968; Wallace and Dumont, 1968; and Glass, 1968). Following the vitellogenic stage when oocytes deposit yolk and grow rapidly, the interfollicular spaces disappear and the macro- and microvilli are withdrawn. Heterosynthetic materials are transferred during the vitellogenic but not during the post-vitellogenic stage (Telfer and Anderson, 1968; and Glass, 1970).

The identity, fate, and function of the heterosynthetic serum proteins in the embryo after the establishment of the circulation have been studied. This is especially true for albumin and immunoglobulin in birds (Schechtman, 1947; Nace and Schechtman, 1948; Nace, 1953; Knight and Schechtman, 1954; Brierly and Hemming, 1956; Solomon, 1965; and Nace, 1970) and in mammals (Brambell et al., 1951; Solomon, 1965;

Brambell, 1966; and Nace, 1970), but similar information on amphibians is incomplete.

Serum-like molecules have been reported at all stages of amphibian development from the immature oocyte to the larval stages (Cooper, 1948, 1950; Flickinger and Nace, 1952; Flickinger and Rounds, 1956; and Chalumeau-Le Foulgoc, 1967). By use of the fluorescent antibody technique, Glass (1959) demonstrated and localized adult serum-like molecules in the oocytes of Rana pipiens at all stages of oogenesis. Furthermore, Cooper (1948, 1950), Flickinger and Rounds (1956), Glass (1959), and Nace and Lavin (1963) reiterated the suggestion that serum proteins are transferred from mother to oocyte in R. pipiens as in other vertebrates (Appendix). But, because their data was descriptive, they could not firmly establish this suggestion. Evidence from other amphibians also points to the transfer of macromolecules. Thus, on the basis of studies on antibody synthesis in larvae of R. catesbeiana, Cooper, Pinkerton, and Hildemann (1964) speculated that maternal immunoglobulins probably protect the amphibian embryo before it becomes immunocompetent. Also, Chalumeau-Le Foulgoc (1967) reported adult serum albumin and globulin-like molecules in the eggs and embryos of Pleurodeles waltlii.

In an attempt to obtain direct evidence for macromolecular transfer in R. pipiens, Kaster and Schechtman (1957) examined oocytes for human serum albumin administered parenterally to females. Since they could not detect this heterologous protein in the oocytes, they concluded that it was

too foreign a protein for transmission across the membranes of the R. pipiens ovary.

Other studies, however, have directly demonstrated that macromolecules can be transferred across the ovarian membrane systems in amphibians. Wartenberg (1964) demonstrated the transfer of iron-containing dextran (myofer) into the vitellogenic oocytes of Triton alpestris. In a series of studies, Wallace and his coworkers (Wallace and Jared, 1969), using Xenopus, demonstrated the transfer of a serum lipophosphoprotein (SLPP) which eventually contributes to the formation of the crystalline body of the larger yolk platelets.

Background of the Present Study

Using both the chicken and the amphibian, this laboratory has long been concerned with the question of transfer of macromolecules between females and their eggs (Nace and Schechtman, 1948; Flickinger and Nace, 1952; Nace, 1953; Glass, 1958, 1959; Nace and Lavin, 1963; and Nace, 1970). However, unanswered questions abound.

1) Although serum-like proteins have been reported in various stages of oogenesis and embryogenesis, the identity of these proteins in terms of adult serum proteins remains obscure both in R. pipiens and in other species.

2) Wartenberg (1964), in demonstrating the transfer of foreign macromolecules into the oocytes of T. alpestris, emphasized the importance of using vitellogenic females. Kaster and Schechtman (1957) could not detect parenterally

administered human serum albumin in the oocytes of R. pipiens, but did not specify the condition of the experimental females. This left open the possibility that foreign serum proteins could be transferred into vitellogenic oocytes of this species.

3) Although serum-like proteins have been identified in R. pipiens embryos, their function and fate are not known. Indeed, the ontogenetic changes among the serum proteins has not been studied in amphibians. After the establishment of embryonic circulation in birds and mammals, maternal serum proteins, especially albumin and γ-globulin, make important contributions to the osmotic pressure, help regulate ionic concentration, and provide immunological defense for the embryo until it synthesizes its own serum proteins (Solomon, 1965; and Nace, 1970). Such transfer of albumins and γ-globulins may also take place in amphibians, and during embryogenesis these serum proteins could serve functions similar to those known or suggested for other species.

4) Their role prior to the establishment of circulation remains completely unknown in this or other species.

These questions concerning the heterosynthetic endowment of the developing organism which remain enigmatic for all species can be appropriately studied in R. pipiens. This species was chosen for study because of its efficacy for such studies, and because it is available from biologically controlled conditions in the Amphibian Facility of The University of Michigan. The present study was designed: first, to obtain descriptive information on

specified serum proteins, specifically albumin and γ-globulin, in the eggs and embryos of R. pipiens; second, by using heterologous proteins, to demonstrate unequivocally that serum proteins can be transferred from the mother to the egg; and third, to obtain information which might suggest the functions of these proteins in the egg, early embryo, and larva. In the process of attaining these objectives it was found necessary to describe the serum proteins in the maternal organism and to determine whether or not the mechanisms controlling the synthesis of these proteins during vitellogenesis were comparable to those operating in other species. To accomplish these objectives, immunochemical, fluorescent antibody, chromotographic, and electrophoretic methods were used.

MATERIALS AND METHODS

Animals and Their Care

Leopard frogs, Rana pipiens, obtained from C.H. Mumley, Alburg, Vermont, were maintained either in hibernation at 4 C or at room temperature, 21 to 26 C, in conditions of optimal nutrition, in accordance with methods currently utilized at the Amphibian Facility of The University of Michigan (Nace, 1968). Females maintained at room temperature were all post-ovulatory.

Hibernating frogs were bled and Adult Frog Serum (AFS) was pooled for fractionation and other tests. Females maintained at room temperature in the Amphibian Facility or within one week of arrival following collection in the field were used to follow changes in the electrophoretic pattern of the serum during vitellogenesis. Tissues were collected from both hibernating and room temperature animals for histological study and antigen preparation.

Vitellogenic females obtained in July and August were injected with foreign serum proteins within a week of their arrival in the laboratory. It was necessary to maintain these animals under conditions of optimal nutrition since starved females kept either at 18 C or room temperature for as short a period as four days sometimes absorbed their oocytes, making them unsuitable for experimentation.

Embryos were obtained by induced ovulation and artificial insemination following standard methods (Nace and

Lavin, 1963; DiBerardino, 1967). For comparative studies full sibling embryos were used.

Adults and juvenile males and females raised in and provided by the Amphibian Facility were used to determine the effects of hormones on serum proteins.

Frogs were anaesthetized by intraperitoneal injection of 1% Tricaine Methanesulfonate (MS 222)[1], 0.1 ml per 10 g body weight, for in situ examination of the ovary or ovariectomy. Oocyte developmental stages were classified according to the system described by Kemp (1953).

Preparation of Antigens

Adult Frog Serum (AFS)

To collect Adult Frog Serum (AFS), frogs were spinal pithed, and the sternum reflected to expose the heart which was slipped through a hole in the pericardium and wiped free of pericardial fluid. Each frog was placed on an aluminum foil holder mounted on a tripod in such a manner that the heart protruded through a perforation in the foil and hung over a suitably placed centrifuge tube. The tip of the ventricle was lanced; the position of the frog permitted cardiac action to assist the free flow of blood into the tube. In this manner 2 to 5 ml of blood from each 50 to 70 g frog was collected separately. For pooled samples, blood from three to five frogs of the same sex and reproductive maturity was collected in each tube. The

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[1]Finquel, Ayerst Laboratories, Inc.

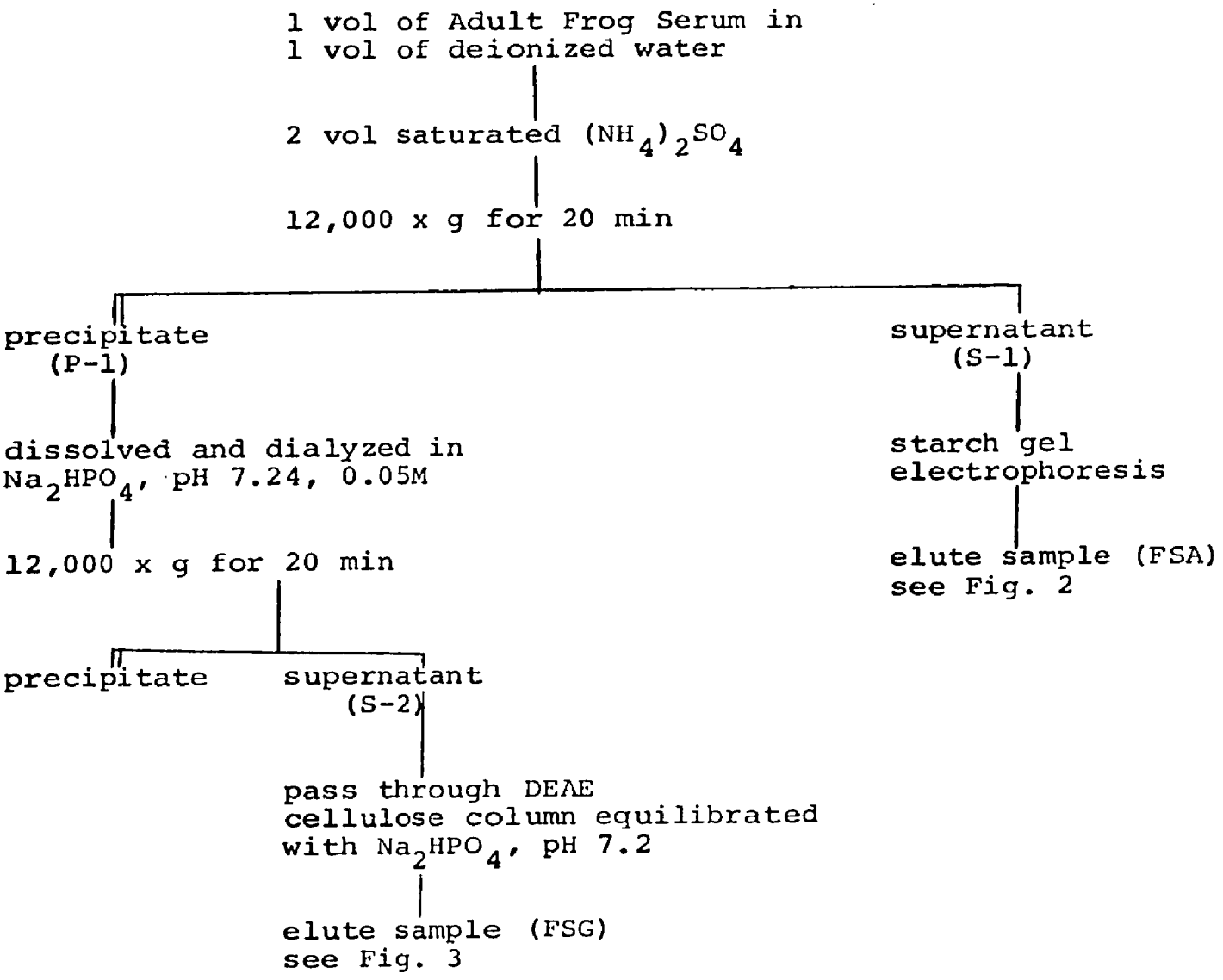
blood was clotted at room temperature for 1 to 2 hr and overnight at 4 C. Serum was separated from the clot by centrifugation and each sample was divided into several aliquots, one for lyophilization and storage at -20 C, the others for storage at 4 C. As a preservative, fluoro-azide at a concentration of 1:50 was added to the serum before storage. Fresh or stored sera were dialyzed to equilibrium against saline before fractionation. Both reconstituted lyophilized and fresh sera were used for immunochemical tests.

For serial collection of the serum of live frogs, blood was obtained by free flow from the musculo-cutaneous vein after cleaning the skin with 70% alcohol and lancing with a sharp needle. As much as 100 µl of blood per bleeding were aspirated into a heparinized tube, transferred into a vial, and the serum separated by centrifugation before storage at -20 C.

Fractionation of AFS

A method was developed for fractionation of AFS to obtain Frog Serum Albumin (FSA) and Frog Serum γ-Globulin (FSG). Procedures developed for birds and mammals, such as repeated precipitation with ammonium sulfate (Kabat and Mayers, 1961), and column chromatography (Peterson and Sober, 1962) were modified as a result of a series of preliminary experiments. This resulted in the protocol outlined in the diagram (Fig. 1). In the development of this protocol, each product was evaluated by disc electrophoresis.

Fig. 1 Fractionation of Adult Frog Serum*

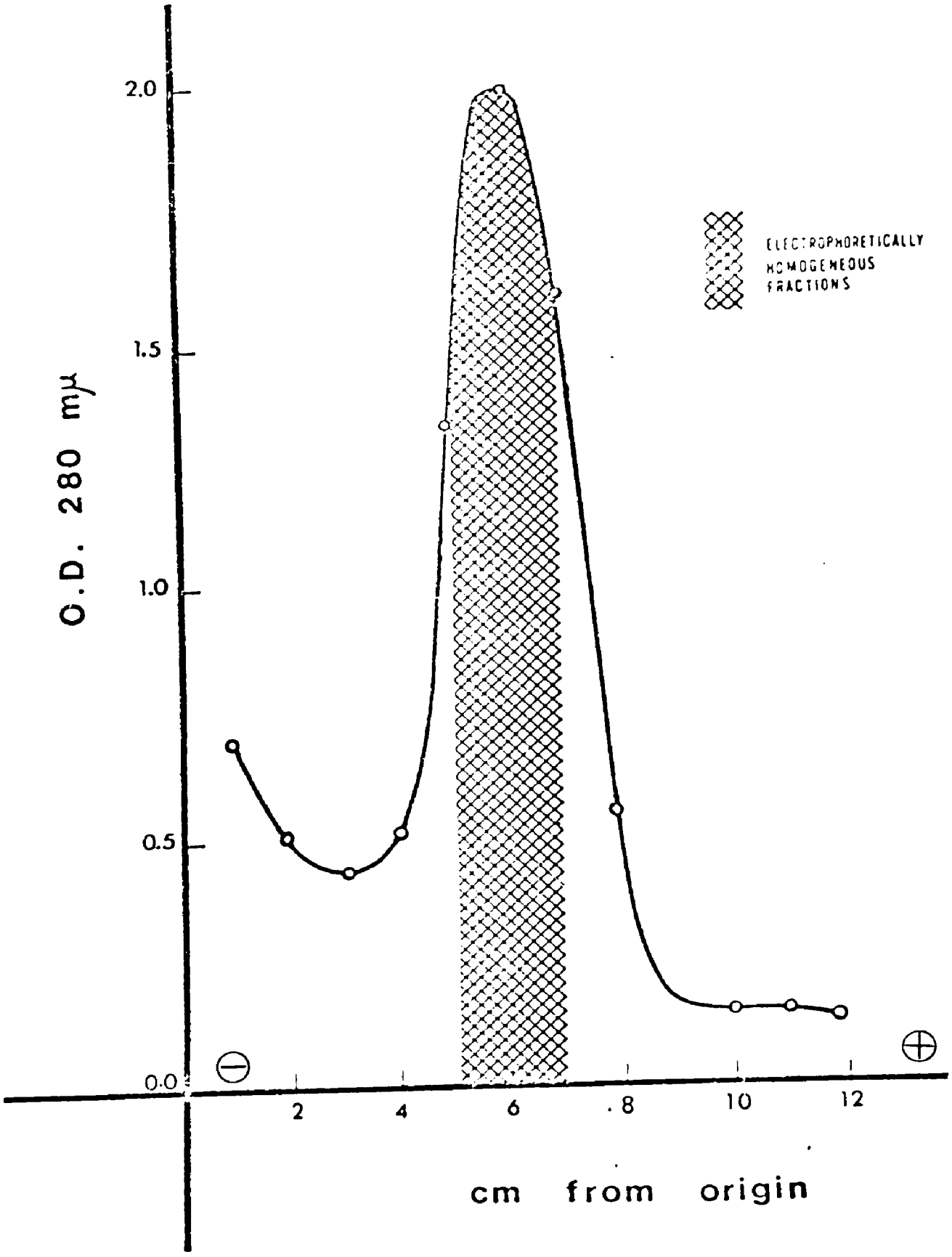


*Critical steps carried out at 0-4 C.

The most homogeneous FSA was obtained by ammonium sulfate fractionation followed by preparative starch gel electrophoresis. The best preparations of FSG were obtained by ammonium sulfate fractionation followed by chromatography.

Preparation of Frog Serum Albumin (FSA). To prepare FSA (Fig. 1), AFS was diluted with an equal volume of deionized water and brought to 50% saturation by the slow addition of saturated ammonium sulfate. The supernatant, S-1, separated by centrifugation at 12,000 X g for 20 min, was dialyzed against repeated changes of 0.05 M phosphate buffer, pH 7.2, until ammonia could not be detected in the buffer using Nessler's reagent (Koch and McMeekin, 1924). After concentrating the S-1 fraction by dialysis against 1 vol of 20 M carbowax in 3 vol of deionized water, it was subjected to starch gel electrophoresis. Electrophoresis was done at 4 C, with 15 mA, 100-140 v for 24 hr, in 0.05 M phosphate buffer. Using an Amido black stained blotting of the plate as a guide, the starch gel was sectioned into pieces and the protein was eluted with the phosphate buffer. The optical density (OD) of the fractions at 280 μ was plotted and a single peak was obtained (Fig. 2). The homogeneity of each fraction from 4-9, the region which comprised the symetrical peak, was tested by disc electrophoresis. Fractions 5-7, the most homogeneous fractions, were pooled, dialyzed to equilibrium with an effective volume of phosphate buffer, lyophilized, weighed, and stored at -20 C. A total of 70 mg FSA was obtained from 5 fractionations

Fig. 2 Homogeneity of albumin fraction (S-1) on starch gel electrophoresis.



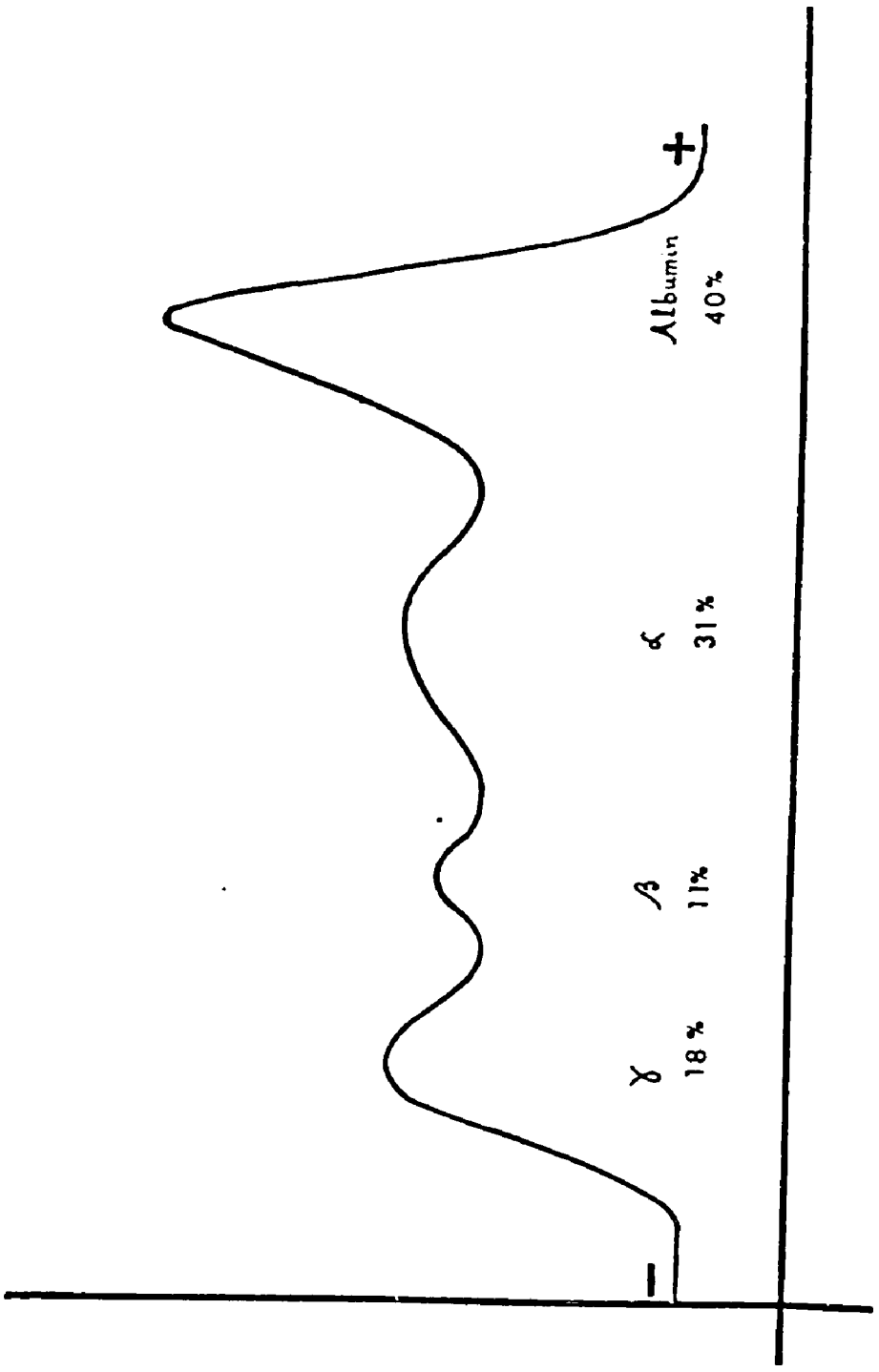


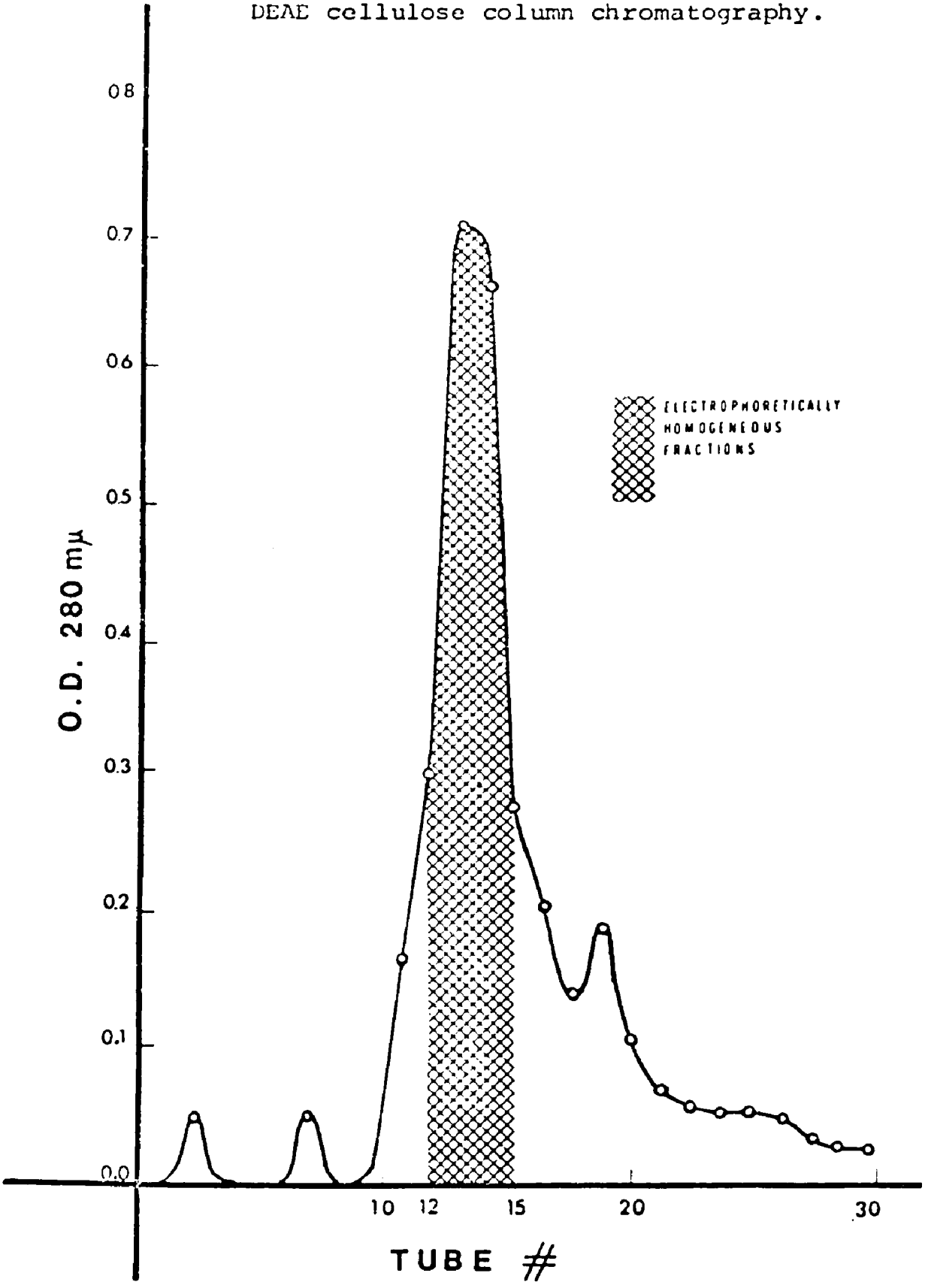
Fig. 3 Densitometric tracing of adult frog serum electrophorogram.

using 10 ml of AFS each, a yield of about 80% since the AFS contained 209 mg protein per ml of which 40% was FSA (Fig. 3).

Preparation of Frog Serum γ-Globulin (FSG). The precipitate, P-1, obtained at 50% saturation with ammonium sulfate (Fig. 1), was dissolved in 0.05 M phosphate buffer, pH 7.2, at 1/4 vol of the original sample, freed of ammonia by dialysis against an effective volume of the buffer, and eluted through a basic form DEAE-cellulose column equilibrated with the same buffer. The OD of the fractions at 280 μ was determined. A major peak was obtained (Fig. 4), and the fractions 10-18 comprising this peak were tested by disc electrophoresis. Fractions 12-15, the most homogeneous, were pooled, dialyzed to equilibrium with an effective volume of buffer, lyophilized, weighed, and stored at -20 C. A total of 20 mg FSG was obtained from 5 fractionations, each using 10 ml of AFS. The yield was 52%, since AFS had 209 mg protein per ml of which 18% was FSG (Fig. 3).

Preparation of α- and β-FSP. In the course of analyses to be discussed below, two constituents of female serum were identified as α- and β-FSP. These were isolated from the sera of appropriate females for use as test antigens in agar diffusion studies. To accomplish this, the sera were resolved electrophoretically on acrylamide gels, and the proteins were eluted from these gels which were sectioned in accordance with the mobilities of the

Fig. 4 Homogeneity of γ-globulin fraction on DEAE cellulose column chromatography.



desired proteins. These protein preparations were used immediately after elution.

Oocyte and Embryo Supernatants, and Yolk Platelets

Mature ovaries, post-ovulatory ovaries with immature oocytes, and intermediate stage ovaries with vitellogenic oocytes, all from adults perfused with saline, and whole embryos of Shumway stages 7 (32-cell), 11 (mid-gastrula), 14 (neural fold), 17 (tailbud), and 20 (gill circulation), mechanically removed from their jelly coats, were homogenized in equal volumes of standard Holtfreter's solution in a Teflon glass homogenizer at 0 to 4 C, and centrifuged at 20,000 X g for 20 min. The precipitate was discarded. The aqueous supernatant was collected, lyophilized, and stored at -20 C. When needed, lyophilized material was reconstituted with deionized water.

Yolk platelets were isolated by the method of Ringle and Gross (1962) as modified by Wallace (1965). This product contains very little pigment or nuclear contamination (Wallace and Karasaki, 1963). Yolk platelets were brought into solution by the use of 10% NaCl (Flickinger and Nace, 1952) or 0.25, 0.5 and 1.0 % Triton-X.

Preparation of Antisera

Antisera against Adult Frog Serum (AFS), Frog Serum Albumin (FSA), and Frog Serum γ-Globulin (FSG) were produced in 3 to 5 kg New Zealand White rabbits by injecting 5 to 20 mg of antigen in saline with Freund's Complete

Adjuvant[2] by a combination of subscapular, intramuscular, and subcutaneous routes. Antisera were collected at regular intervals for four months, starting ten days after the single injection. Fluoro-azide was added to the antisera as a preservative at a ratio of 1:50.

Rabbit Anti-Bovine Serum Albumin (A.BSA), sheep Anti-Rabbit γ-Globulin (A.Rγ), rabbit Anti-Human Serum (A.HS), and rabbit Anti-Large Oocyte Supernatant (A.LOS) were available from the stocks in the laboratory which were prepared according to the method described in Nace, Suyama, and Smith (1961). These antisera were designated by standard notations (Nace et al., 1961), and those under consideration here will be dealt with in the text.

Gamma globulin fractions at 5 mg/ml protein, prepared by a cold ethanol procedure (Deutsch, 1952), were conjugated with either fluorescein isothiocyanate[3] (Riggs et al., 1958), or with rhodamine[4] (Nairn, 1964). To remove unreacted dyes, conjugated fractions were adsorbed with Sephadex (G-25) and DEAE-cellulose (Nairn, 1964). In some cases, whole serum at 60 mg/ml protein was conjugated with rhodamine and adsorbed with activated charcoal (Nairn, 1964). Protein determination was made by the method of Lowry et al. (1951).

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[2]Difco Laboratory.

[3]Baltimore Biological Laboratory.

[4]ICI, England.

Electrophoretic and Immunochemical Analyses

Frog serum proteins were studied by vertical electrophoresis (Davis, 1964; and Ornstein, 1964) in 6% acrylamide gel using 5 µl of serum, with 0.01 M Tris and 0.08 M glycine buffer, pH 8.3. The current was kept at 3 mA per sample, and the voltage varied according to the number of samples. The migration of individual samples was usually stopped as soon as the marker dye, brom phenol blue, moved 65 mm from the origin.

Immunodiffusion was carried out using the micro-method standardized in the laboratory (Nace, In Press). Immunoelectrophoresis was carried out in 2% agarose (Hegenauer and Nace, 1965), using 0.05 M Tris and 0.01 M maleic acid buffer at pH 8.3.

After electrophoresis or immunodiffusion, the gels were stained with 1% Amido Black, destained in 7% acetic acid, and representative patterns were photographed (Nace and Alley, 1961).

To determine whether the surface of the yolk platelets possessed antigens with FSA and FSG specificities, a constant volume of total yolk platelets was mixed with 1, 2, or 4 volumes of antisera, incubated at 37 C for one hr, and at 4 C overnight, and centrifuged. The supernatants, from which antibodies to these antigens should have been removed if the antigens were present on the yolk platelets, were tested for these antibodies with FSA and FSG by immunodiffusion.

Fluorescent Antibody Localizations

On the basis of histological integrity and fluorescence consistency, Carnoy fixation was chosen in preference to 4 other fixatives which were tested. Sections of ovary fixed at room temperature in Carnoy, Smith's solution, 10% formalin, and frozen sections fixed in acetone confirmed the observations (Glass, 1959; and Lavin, 1963) that Carnoy-fixed material showed minimum autofluorescence, and that frozen sections, though free of autofluorescence, were morphologically distorted (Nace and Clarke, 1958).

Fixed tissues were dehydrated in ethanol, cleared in xylene, and embedded in 48 C paraffin or tissuemat. Paraffin sections cut at 5 μ were mounted on coverslips coated with chicken egg white albumin, hydrated, flooded with unconjugated Normal Rabbit Serum (NRS), incubated at 37 C for 10 min, and at 4 C for 10 min in a humid chamber. After this pretreatment with NRS, the sections were washed with buffered saline, pH 7.1, at 4 C for 10 min on a rocking device, and flooded with conjugated test antisera. Controls were flooded with saline or NRS, either conjugated or unconjugated. Both control and test sections were incubated at 37 C for 15 min, and at 4 C for 15 min in a humidified chamber. These sections were washed at 4 C for 15 min each in two changes of buffered saline, rinsed in deionized water, and then mounted in Elvanol 51-05 (Rodriguez and Dunhardt, 1960).

For the indirect technique, the same procedure was used except that the sections were first treated with unconjugated test sera and then treated with conjugated Anti-Rabbit γ-Globulin (A.Rγ).

Observations were made on a Leitz Ortholux microscope following standard practices (Nace and Clarke, 1958). Photographs were taken with a Leitz 35 mm camera using Kodak daylight highspeed Ektachrome, ASA 160. The exposure time varied from 15 to 45 sec, depending on the intensity of fluorescence. After observation and photography, the Elvanol was washed from the sections with phosphate buffer, and the sections were dehydrated and stained with either hematoxylin and eosin, or with Heidenhain's triple stain (Lillie, 1965). Selected stained sections were later photographed.

Steroid Stimulation of Female Serum Proteins

Estrogens have been implicated in the synthesis of vitellogenic serum proteins in Xenopus laevis (Wallace and Dumont, 1968; and Follett and Redshaw, 1968) and Rana catesbeiana (Urist and Schjeide, 1961). Knowledge of the possible occurrence of this phenomenon in R. pipiens seemed essential to an understanding of the relationship between maternal serum proteins and antigens with serum protein specificity in the egg and embryo. To answer this question, estrogen was administered to juveniles, mature males and females, and ovariectomized females. Since 17-B estradiol is more potent than other estrogens (Redshaw, Follett, and

Nicholls, 1969), it was administered into the dorsal lymph sac in the following dosages which were selected on the basis of the dosages used by Wallace and Jared (1968): 1.0, 10.0, 100.0, 250.0, 1,000.0, and 5,000.0 µg in 0.1 ml saline per animal.

To distinguish between the action of estrogen and the possible actions of other steroids, mature males and females received 500.0, 1,000.0, and 5,000.0 µg in 0.1 ml saline of N-testosterone or deoxycorticosterone. Controls were injected with 0.1 ml saline. After a single injection of the hormone, the changes in the serum proteins of control and test animals were followed for three months by vertical disc electrophoresis.

Administration of Foreign Serum Proteins

To test for the transfer of heterologous serum proteins from mother to egg, vitellogenic frogs were injected with Normal Rabbit Serum (NRS) from the colony in this laboratory, Human Serum (HS) drawn from the investigator, and Bovine Serum Albumin[5] (BSA). Protein concentrations of the NRS and HS were determined by the method of Lowry et al. (1951) using BSA standards, and the NRS and HS solutions were then diluted with standard Holtfreter's solution to 60 mg protein per ml, the serum protein value typical for frogs. The crystalline BSA was made up at 60 mg protein per ml in standard Holtfreter's solution.

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[5]Armour Pharmaceutical Company.

Frogs weighing 50 to 60 g were given single injections via the dorsal lymph sac with 0.5 ml of serum containing 30 mg of protein. Based on preliminary tests using other procedures, this route and dosage were selected for optimal transfer of foreign serum protein into the oocytes. The serum of the recipient females did not react with test antisera prior to the injection of foreign serum proteins.

Each of these heterologous serum proteins was injected into four frogs. Two from each group were maintained at room temperature under optimal nutritional conditions until their eggs matured and could be fertilized. The other two from each group were spinal pithed, one two hours and the other three hours after receiving its injection. These frogs were perfused with saline and an ovary removed and fixed for immunohistology. The other ovary was used for the preparation of Vitellogenic Oocyte Supernatant (VOS). This VOS was tested for the presence of foreign serum proteins by double diffusion against antiserum homologous to the foreign protein.

To minimize the possibility that blood remaining in the ovarian tissue might produce positive reaction when VOS and the antisera were tested, the effectiveness of perfusion was followed under a binocular microscope, and the tissues were manipulated to clean all visible blood from the vessels. In addition, oocytes were dissected from their follicles and were divided into 2 groups: white oocytes in Y_1 and Y_2 stages, and gray oocytes in Y_3 stage (Kemp, 1953),

were then punctured and their internal contents used to prepare VOS.

Similar experiments were conducted using 0.5 ml of 60 mg protein per ml BSA conjugated with 1% Evan's blue or rhodamine (Nairn, 1964). One ovary from each frog was treated in a manner similar to that used for immunochemical tests, while the other ovary was fixed in Carnoy solution for histological examination of the oocytes.

EVALUATION OF REAGENTS AND PROCEDURES

Antigens and Antisera

The antigenic homogeneity of the injection antigens and the specificity of the antisera were determined by the use of appropriate immunodiffusion and immunoelectrophoretic analyses. In these tests, Adult Frog Serum (AFS) and Anti-Adult Frog Serum (A.AFS) were used as broad spectrum reagents to detect contaminants.

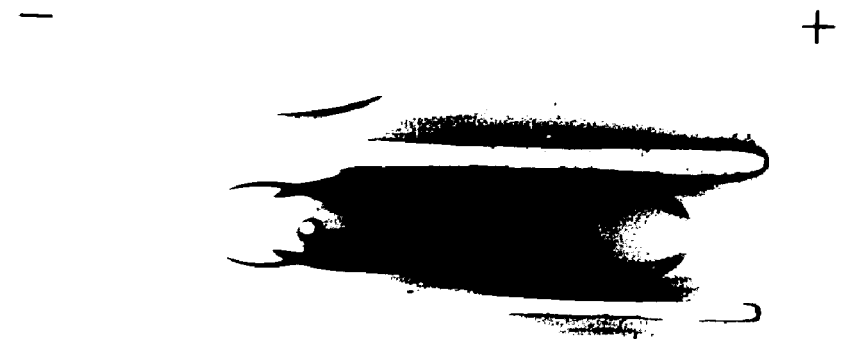
Specificity of FSA and A.FSA

Using the injection antigen, Frog Serum Albumin (FSA), which was characterized as homogeneous by the physical procedures described in Materials and Methods, three concentric anodic components were detected (Fig. 5a) in immunoelectrophoretic analyses with Anti-Adult Frog Serum (A.AFS) selected because of its broad spectrum of specificity. Thus, the injection antigens were not immunochemically homogeneous, and the antisera resulting from their use were also expected to be heterogeneous. When the antisera were tested, they did show heterogeneity. Therefore, the antisera were tested individually prior to pooling. The intense precipitation produced by the antisera collected between 25 to 30 days following immunization indicated that the highest antibody levels were reached at that time. Antisera collected during this period (D6 and D7) from each rabbit (H7 and H23) were tested by radial immunoduffusion

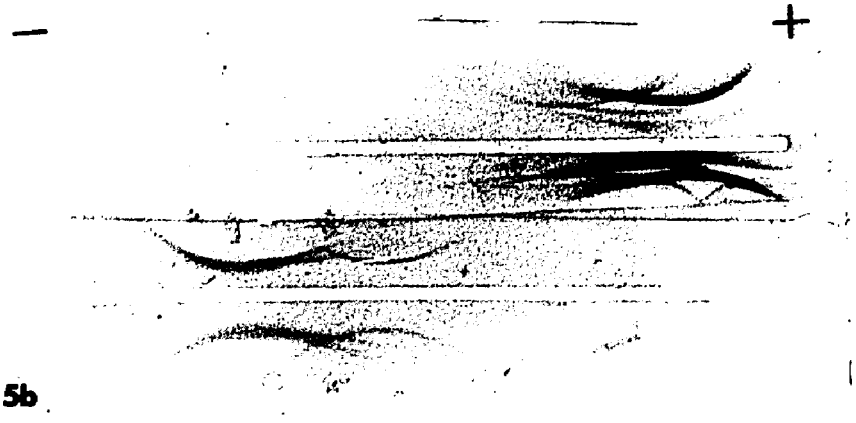
Fig. 5 Immunoelectrophoretic tests of FSA and FSG and of the specificity of A.FSA and A.FSG.

a. A.AFS, H21, D7 (top and bottom troughs).
FSG, 5 mg/ml (top well).
AFS, 10 mg/ml (center well).
FSA, 5 mg/ml (bottom well).

b. A.FSA, pooled D6 and D7 from H7 and H23 (top trough).
A.FSG, pooled D6 and D7 from H9 and H29 (bottom trough).
AFS, 10 mg/ml (all four wells)



5a



5b

analyses with Adult Frog Serum (AFS) and FSA, and by immunoelectropnoresis. Since they gave identical results, they were considered qualitatively identical, and were then pooled.

In immunoelectrophoresis, the pooled A.FSA formed three lines with FSA and three intense and two faint lines with AFS (Fig. 5b), all of which were in the electrophoretic mobility range of albumin which, in the frog, is a complex family of molecules (Nace, unpublished results). This showed that five components of FSA evoked antibody production, but only three in the antigenic preparation were capable of giving a visible reaction. This could have resulted from differences in concentration or antigenicity among the series of components under consideration and concentration seems the most plausible explanation because of variations in the frequency of the several components in the population of Vermont frogs from which serum was pooled.

A.FSA failed to react with FSG in immunodiffusion tests (Fig. 6a).

Thus it may be concluded that A.FSA was specific for determinants on the class of antigens showing the highly anodic, but overlapping mobilities characteristic of albumin.

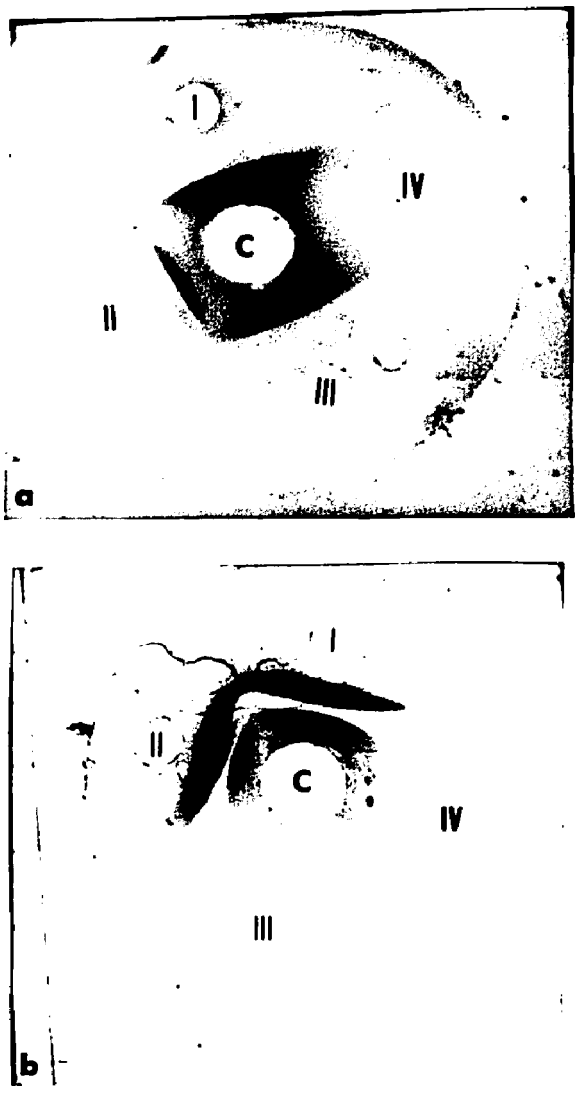


Fig. 6 Radial immunodiffusion tests of the specificity of A.FSA and A.FSG.

a. Center well: A.FSA, same as in Fig. 5b.
Well I: FSA, 10 mg/ml.
Well II: FSA, 2.5 mg/ml.
Well III: FSA, 5 mg/ml.
Well IV: FSG 5 mg/ml.

b. Center well: A.FSG, same as in Fig. 5b.
Wells I and II: FSG, 5 mg/ml.
Well III: FSA, 5 mg/ml.
Well IV: FSA, 10 mg/ml.

Specificity of FSG and A.FSG

Using the injection antigen, Frog Serum γ-Globulin (FSG), which was characterized as homogeneous by the physical procedures described in Materials and Methods, three overlapping cathodic components were detected (Fig. 5a) in immunoelectrophoretic analyses with Anti-Adult Frog Serum (A.AFS). Thus, the injection antigens were not immunochemically homogeneous, and the antisera resulting from their use were also expected to be heterogeneous. When the antisera were tested, they did show heterogeneity. Therefore, the antisera were tested individually prior to pooling. The intense precipitation produced by the antisera collected between 25 to 30 days following immunization indicated that the highest antibody levels were reached at that time. Antisera collected during this period (D6 and D7) from each rabbit (H9 and H29) were tested by radial immunodiffusion analyses with Adult Frog Serum (AFS) and FSG, and by immunoelectrophoresis. Since they gave identical results, they were considered qualitatively identical and were then pooled.

When the pooled A.FSG was tested with AFS (Fig. 5b) and FSG (not illustrated here) in immunoelectrophoresis, the same three overlapping lines which developed when A.AFS was tested with FSG (Fig. 5a), were observed. Thus, A.FSG didnot reveal any additional components.

A series of immunodiffusion (Fig. 6b) and immunoelectrophoretic tests failed to show any reactions between A.FSG and FSA.

Thus, A.FSG was specific for antigens in the cathodic region characteristic of the γ-globulins.

Summary

The data presented here demonstrate that the antisera, A.FSA and A.FSG used as test reagents in this study, did not show cross reactions. Each antiserum reacted with antigens characteristic of the class, either albumins or γ-globulins. Each had the advantage of not being selective for a particular molecular form among the group of materials designated either albumin or γ-globulins.

Immunohistological Procedures

Sections of Carnoy-fixed and paraffin embedded ovary, oviduct, and liver treated with saline or unconjugated Normal Rabbit Serum (NRS) showed low intensity orange-yellow autofluorescence under the conditions for immunohistological microscopy. Control sections for nonspecific reactions of the tissue with rabbit serum were treated either with conjugated NRS, or with unconjugated NRS followed by either conjugated NRS or conjugated sheep Anti-Rabbit -globulin (A.R). These control sections produced low intensity fluorescence, the color being red for rhodamine and orange-green for fluorescein. However, the fluorescence was diffuse and showed no tendency to localize on any part of a section, a finding consistent with other observations (Nace and Clarke, 1958; Glass, 1959; and Lavin, 1963).

The direct and indirect methods of antigen localization gave identical results with regard to intensity and site of fluorescence with both A.FSA and A.FSG. Rhodamine and fluorescein-labeled reagents gave identical localization, but differed in that the red color of the rhodamine label produced a better contrast between the autofluorescent and stained areas.

Sections pre-treated with homologous, unconjugated antiserum followed by conjugated antiserum gave localized low intensity fluorescence, demonstrating the replacement of some of the unconjugated antibody molecules by the conjugated ones.

In testing the immunohistological specificity of the reagents, sections treated with either unconjugated A.FSG or unconjugated NRS followed by conjugated A.FSA showed identical fluorescence. This demonstrated that neither globulin nor "unspecific" reaction sites interfered with the detection of albumin sites. Similarly, appropriate tests also demonstrated that neither the albumin nor the "unspecific" reaction sites interfered with the detection of the globulin sites.

These tests and controls demonstrate that, when applied to tissue sections, these reagents were capable of detecting only the albumins and globulins for which they were specific.

RESULTS AND CONCLUSIONS

Serum Proteins During the Reproductive Cycle

Definition of Serum Proteins of Frogs During Hibernation

Electrophoretic patterns of the sera of hibernating gravid females collected from nature ("nature" frogs) are shown in Fig. 7 and are summarized in a composite diagram (Fig. 8). Bands showing mobilities in the range of 0.60 to 0.80 were designated as albumin on the basis of mobility characteristics meeting criteria suggested by Engle and Wood (1960) and Petrakis and Brown (1970) - the retention of brom phenol blue stain and high protein concentration (Fig. 3). These criteria were also used to identify the albumin fraction considered earlier (p. 13).

Bands showing mobilities in the range of 0.40 to 0.56, 0.10 to 0.39, and less than 0.10 were classified as α, β, and γ-globulins, respectively (Fig. 8). This classification was based on the mobilities of similarly designated globulins in mammalian serum.

The female serum protein patterns (Fig. 7) showed qualitative and quantitative differences among individuals. Though nine bands were common to all frogs analyzed, as many as 12 bands were found in the serum of one animal. In the serum pattern of each individual there were bands in the mobility ranges of the albumin α, β, and γ-globulins. Seventy seven of 80 females had an albumin band with a

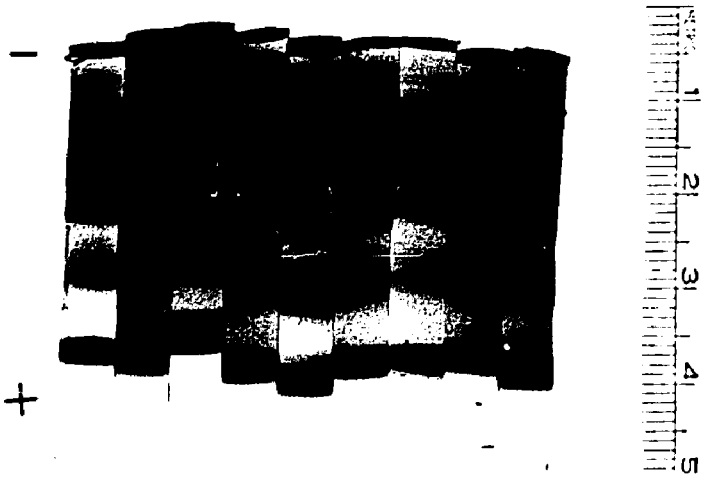
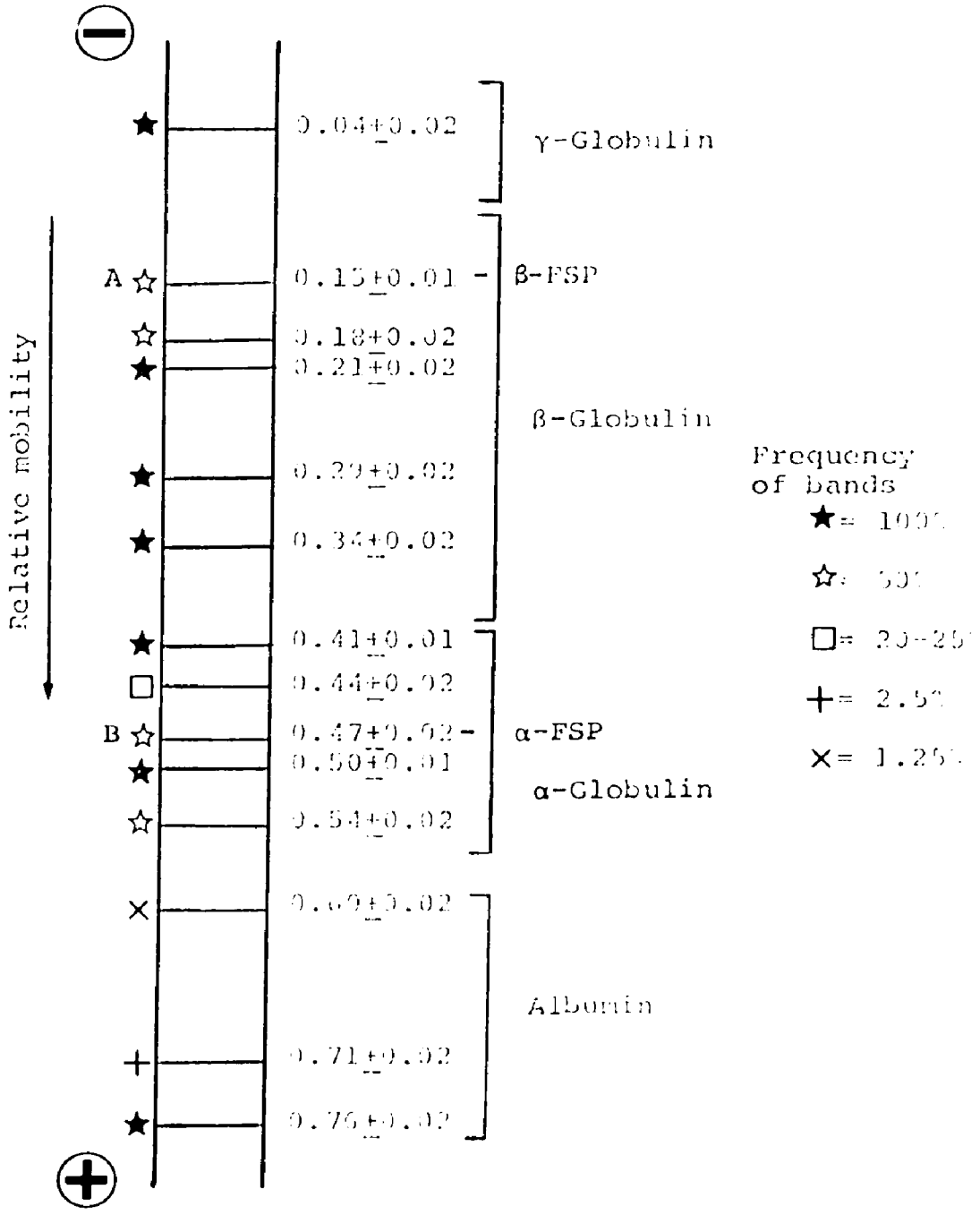


Fig. 7 Serum Patterns of individual hibernating females. All were analyzed under comparable conditions with time, rather than marker migration, held constant. Although major features are constant, note the variations in the number and intensity of bands among individuals.

Fig. 8 A composite diagram of serum protein bands based on the analysis of patterns from 80, female frogs. The average mobility is indicated next to each band, and a suggested identification system is described in the text. Mobilities calculated relative to brom phenol blue as a marker.



mobility of 0.76 ± 0.02. Each of two females had double albumin bands with mobilities of 0.76 ± 0.02 and 0.71 ± 0.02. Another female had double albumin bands with mobilities of 0.76 ± 0.02 and 0.60 ± 0.02. There were considerable variations in the concentrations of comparable serum bands among individuals as judged by staining intensity and band width. These variations are reflected in the data on the frequencies of the several bands which are summarized in Fig. 8.

The serum protein bands of males (Fig. 9a) were similar to those of females, although albumin polymorphism was not observed among the 24 males analyzed.

Serum Proteins of Lab-Raised Juvenile Frogs

Nine to 12 bands were found in the sera of each of 24 18-month-old post-fertilization frogs which were judged to be juveniles on the basis of their gonad development (Fig. 9b). The serum pattern of each individual included bands in the mobility ranges of albumins, and of α, β, and γ-globulins. Serum bands of juveniles had a weaker staining intensity than those of adults, indicating a lower protein concentration.

Serum Proteins During Vitellogenesis

Under normal conditions the ovary of a gravid female if filled with mature (Y_5) oocytes, but also contains well advanced immature or pre-vitellogenic (Y_o) oocytes which will mature for the next generation of eggs. These are shown and identified in Fig. 10. After ovulation, however,



Fig. 9 Representative serum patterns of:
a. Hibernating males and
b. Juveniles. Conditions as described for Fig. 7.



Fig. 10 An "asynchronous" ovary of a laboratory maintained female showing immature to mature oocytes. 2X. The transparent and translucent immature or pre-vitellogenic (Y_{1-2}^{o}) oocytes go through opaque white (Y_{1-2}^{o}), gray (Y_3), and small black and white (Y_4) stages to become larger black and white mature (Y_5) oocytes. The irregular black specs and masses represent degenerating oocytes.

the ovary of the female contains only pre-vitellogenic (Y_o) oocytes. These oocytes increase in diameter, accumulate yolk, and become opaque white (Y_{1-2}). The opaque white oocytes turn gray (Y_3) following initial pigment deposition. In time the additional synthesis and migration of pigment to discrete areas of gray oocytes results in the formation of black and white oocytes (Y_4). These black and white oocytes increase in diameter to become the mature (Y_5) oocytes.

In laboratory frogs which were stripped of eggs and maintained at room temperature with ad lib feeding (Table 1), Y_o oocytes reached Y_{1-2}, Y_3, Y_4, and Y_5 stages in roughly 40, 60, 70, and 100 days respectively. This was compared with frogs in nature in which Y_o oocytes advanced to the Y_5 stage in 120 days.

During the interval between ovulation and the maturation of the next batch of oocytes, the following sequential changes occurred in the serum patterns of "nature" and laboratory frogs.

Post-Ovulation Serum Pattern. The serum patterns of females immediately after ovulation, i.e. with pre-vitellogenic oocytes, and in the hibernation (Fig. 7) or pre-ovulation condition, i.e. with mature (Y_5) oocytes (e.g. Frog 650, Table 1), were similar.

Vitellogenesis Serum Pattern. The density and width of all the serum protein bands increased in females with only translucent (Y_o) oocytes (Fig. 11). This was especially

TABLE 1. Correlation between vitellogenesis and the presence of α and β-FSP in frogs at room temperature.

Frog #	Days from ovulation	Stage of vitellogenesis[2]	β-FSP[3]	α-FSP[4]
704	40	Y_o & few absorbed eggs	L	0
669[1]	30	only Y_o oocytes	H	0
670	60	Y_o through Y_3 oocytes	H	0
672[1]	50	Y_o through Y_3 oocytes	H	+
703	40	Y_o through Y_4 oocytes	H	+
664	60	Y_o through Y_4 oocytes	H	+
700[1]	60	Y_o, Y_4, and Y_5 oocytes	H	+
653	70	Y_o, Y_4, and Y_5 oocytes	H	+
651	90	Y_o, Y_4, and Y_5 oocytes	H	+
668	80	Y_o, Y_4, and Y_5 oocytes	L	+
650	100	Y_o and Y_5 oocytes	L	0

[1]Ovaries and serum patterns of these frogs are shown in Figs. 11-14. Note that the time course for ovarian development varies among individuals.

[2]Oocytes classified after Kemp, 1953.

[3]H = high level, L = low level.

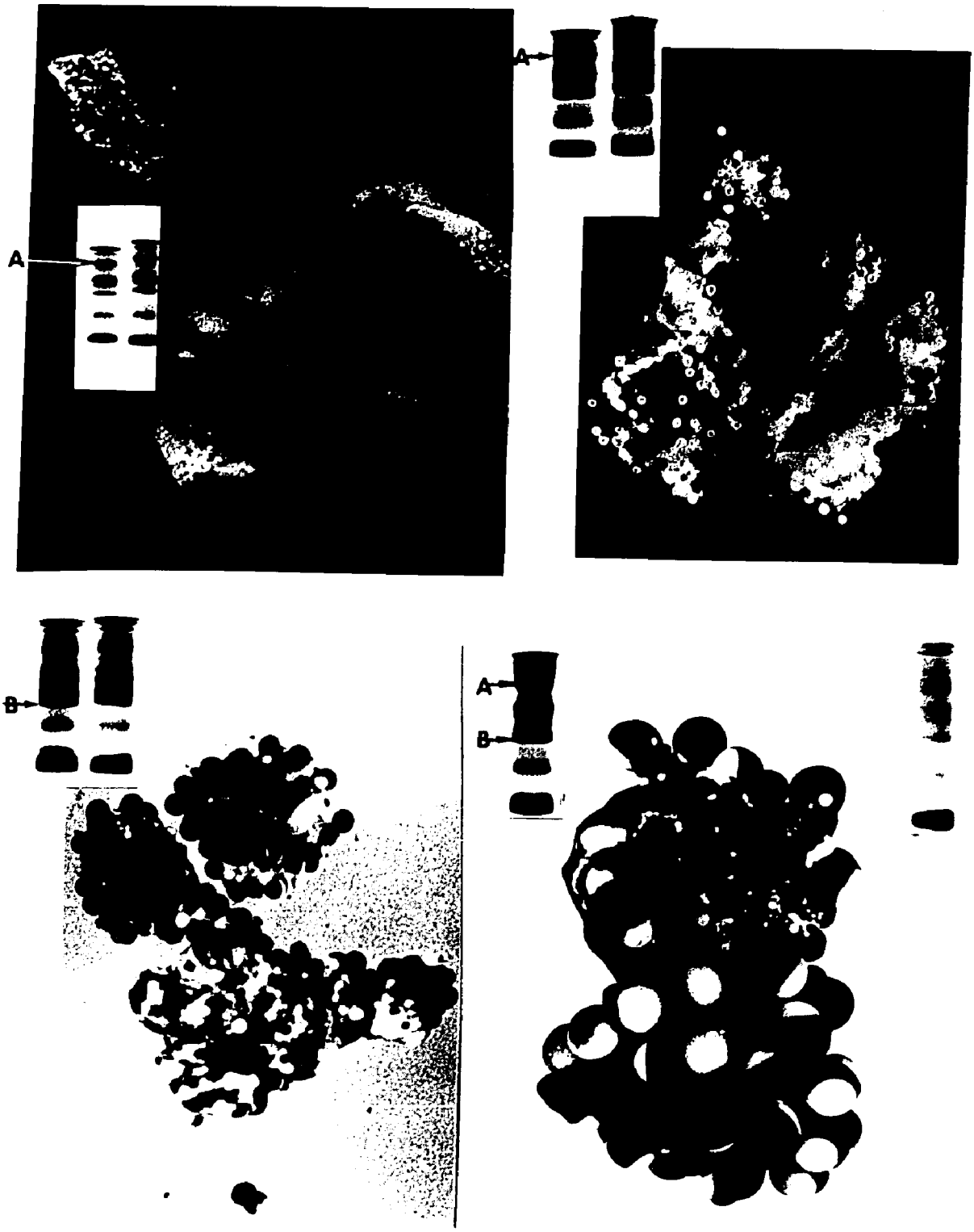
[4]+ = present, 0 = absent.

Fig. 11 Ovulated ovary. 3X. Note the empty follicles (white, elliptical), Y_0 oocytes (white, round), and a few mature unovulated oocytes being absorbed (black). The insert shows the corresponding serum pattern (in duplicate). Note that band A (see Fig. 12) is weak and band B (see Fig. 13) is absent.

Fig. 12 Ovary of a female (Frog 669, Table 1) 30 days after ovulation. 5X. Note pre-vitellogenic oocytes (white, round). The insert shows the corresponding serum pattern (in duplicate). Note the increase in concentration of band A in comparison to that of Fig. 11.

Fig. 13 Ovary of a female (Frog 672, Table 1) 50 days after ovulation. 5X. Note the vitellogenic oocytes at Y_{1-2} (white, round), and Y_3 (gray, round) stages. The insert represents the serum pattern of the female (in duplicate). Note the strong band B which was absent in the serum patterns represented in Figs. 11 and 12. Band A is still present.

Fig. 14 Ovary of a female (Frog 700, Table 1) 60 days after ovulation. 5X. Note the post-vitellogenic Y_4 (black and white, smaller), and mature Y_5 (black and white, larger) oocytes. The insert on the left hand side represents the serum pattern of this female. The insert on the right hand side is the serum pattern of a female with mature ovary in which the intensity of band A has decreased and band B has disappeared.


A
A
B
A
B

noticeable in a band (A) with a mobility characteristic of β-globulin (Fig. 11). A new band (B) with a mobility in the α-globulin range appeared in the serum of females (e.g. Frogs 670 and 700, Table 1) which had vitellogenic (Y_{1-4}) oocytes (Figs. 12-14). There was a decrease in the intensity of most bands, and a disappearance of the B band in 90% of the gravid females from nature. Further decrease in the intensities of the bands was noted in the sera of mature females kept in hibernation for two months (right hand serum pattern, Fig. 14).

Female Serum Proteins. Bands A and B were present in trace amounts in the sera of about 25% of juveniles and adult males. Since the increase of bands A and B coincided with vitellogenesis, and since these bands were not found in most of the juveniles and adult males, the serum proteins represented by these bands are hereafter designated β-Female Serum Protein (β-FSP) for Band A, and α-Female Serum Protein (α-FSP) for Band B (Table 1 and Figs. 11-16). β-FSP was in low concentration and α-FSP was not detectable in 90% of gravid females from nature.

Serum Proteins in Ovariectomized Females

Both the decrease in all serum proteins and the disappearance of α-FSP with maturation of oocytes suggested that serum proteins are removed by the maturing oocytes. If this was true, ovariectomy should remove the depot and lead to an increase in concentration of the serum proteins. To

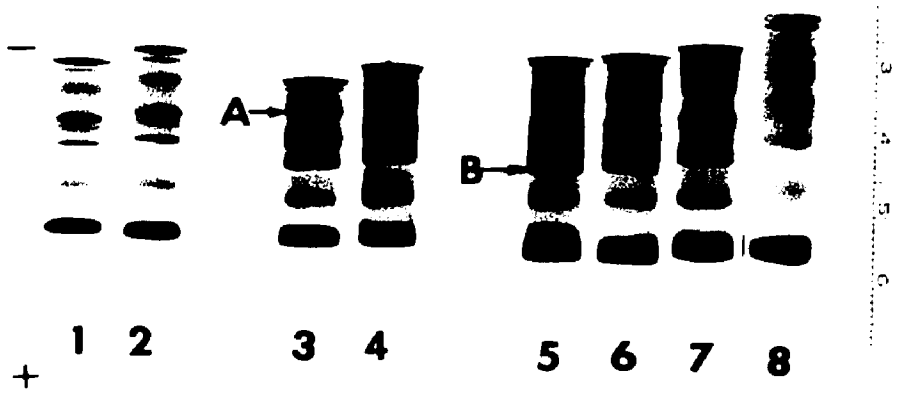
Fig. 15 Changes in serum patterns during vitellogenesis.

1 and 2: post-ovulatory females.
3 and 4: pre-vitellogenic females.
5 and 6: vitellogenic females.
7: female with almost mature ovary.
8: female with mature ovary.

A and B: β- and α-FSP, respectively.

Fig. 16 Changes in the serum pattern during vitellogenesis of a frog bled at 10-day intervals from 20 to 80 days after ovulation. This female gave full ovulation 10 days after the last bleeding, i.e. 90 days after the first ovulation.

A and B: as in Fig. 15.



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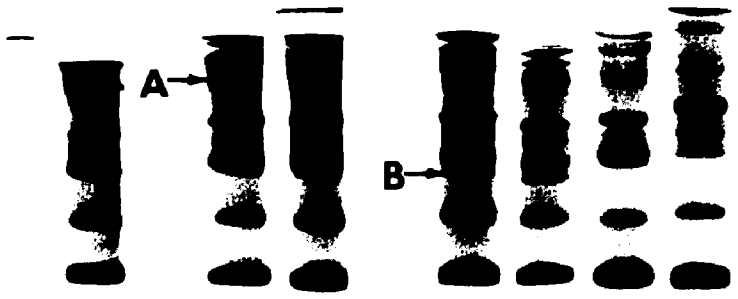

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test this hypothesis, vitellogenic females with high levels of serum proteins were ovariectomized.

When the serum protein bands of ovariectomized females were compared with controls 20 days after ovariectomy, an increase in the intensity of all protein bands was observed (Fig. 17). This provided suggestive confirmation for the hypothesis that maturing oocytes remove serum proteins, although, of course, other mechanisms, such as disruption of serum protein degradation systems, could also explain the result.

Observations on Hormone-Treated Animals

The serum patterns of adult males, normal females and ovariectomized females receiving 1.0 to 100.0 µg of estrogen were examined to determine whether this hormone influenced the electrophoretic patterns of the serum proteins. Estrogen doses in the range of 1.0 to 10.0 µg led to a concentration increase of β-FSP (Band A) in all test groups within 7 days. Higher doses of estrogen, 100.0 to 5000.0 µg, led to an increase in the concentration of one or two additional cathodal bands in the same time interval (Fig. 18, small arrows). Figs. 19a and b show the progressive changes in the serum patterns of adult males which received the vehicle and 100.0 µg of estrogen over 50-day periods. These changes were characterized by the gradual loss of anodal and increase of cathodal components.

There was an elevation of β-FSP and other protein bands in the β and γ-globulin regions (illustrated in Fig. 18,

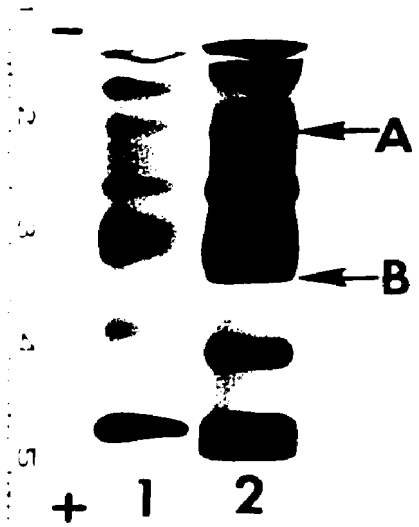


Fig. 17 Serum pattern of a control (1) and ovariectomized adult female (2) 20 days after ovariectomy. Note the remarkable increase in all serum components in contrast to the control.

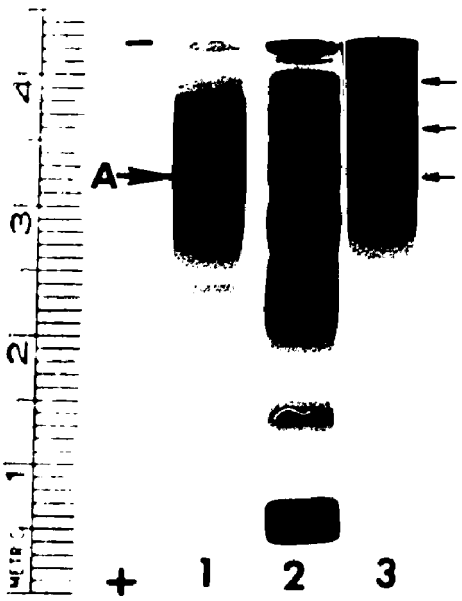


Fig. 18 Serum patterns of estrogen-treated adult frogs 40 days post-injection.

1: female which received 100 µg of estrogen.
2: control.
3: Male which received 100 µg of estrogen.

small arrows) in juveniles that received 10.0 to 100.0 µg and adults that received 100.0 to 5000.0 µg of estrogen. The more negatively charged serum proteins, including α-globulin and albumin, gradually disappeared (Fig. 19b). All these effects were more pronounced in females than in males.

In addition to serum protein patterns, hematocrit and clotting times were also affected by high doses of estrogen. An average control hematocrit value of 27 ± 7% was obtained from 12 males and 12 females. This value dropped to as low as 10 and 15 ± 5% in females and males respectively after hormone treatment. Clotting time varying from 10 to 30 sec in control animals extended up to 75 sec in the experimental groups. Estrogen treatment also resulted in a change in serum color from straw to yellow and a reduction in spleen size.

Although the oviducts of estrogen treated animals were hypertrophied irrespective of sex and maturity, the oocytes of post-ovulatory females did not develop beyond the translucent (Y_o) stage, and females receiving more than 100.0 µg of estrogen during vitellogenesis absorbed the vitellogenic oocytes. This suggested that, rather than influencing protein synthesis, per se, these pharmacological doses of estrogen may have influenced the transport of serum proteins into the oocytes as well as producing marked changes in the protein clearance mechanisms. If the change in clearance mechanism involved increased retention of the cathodal proteins,

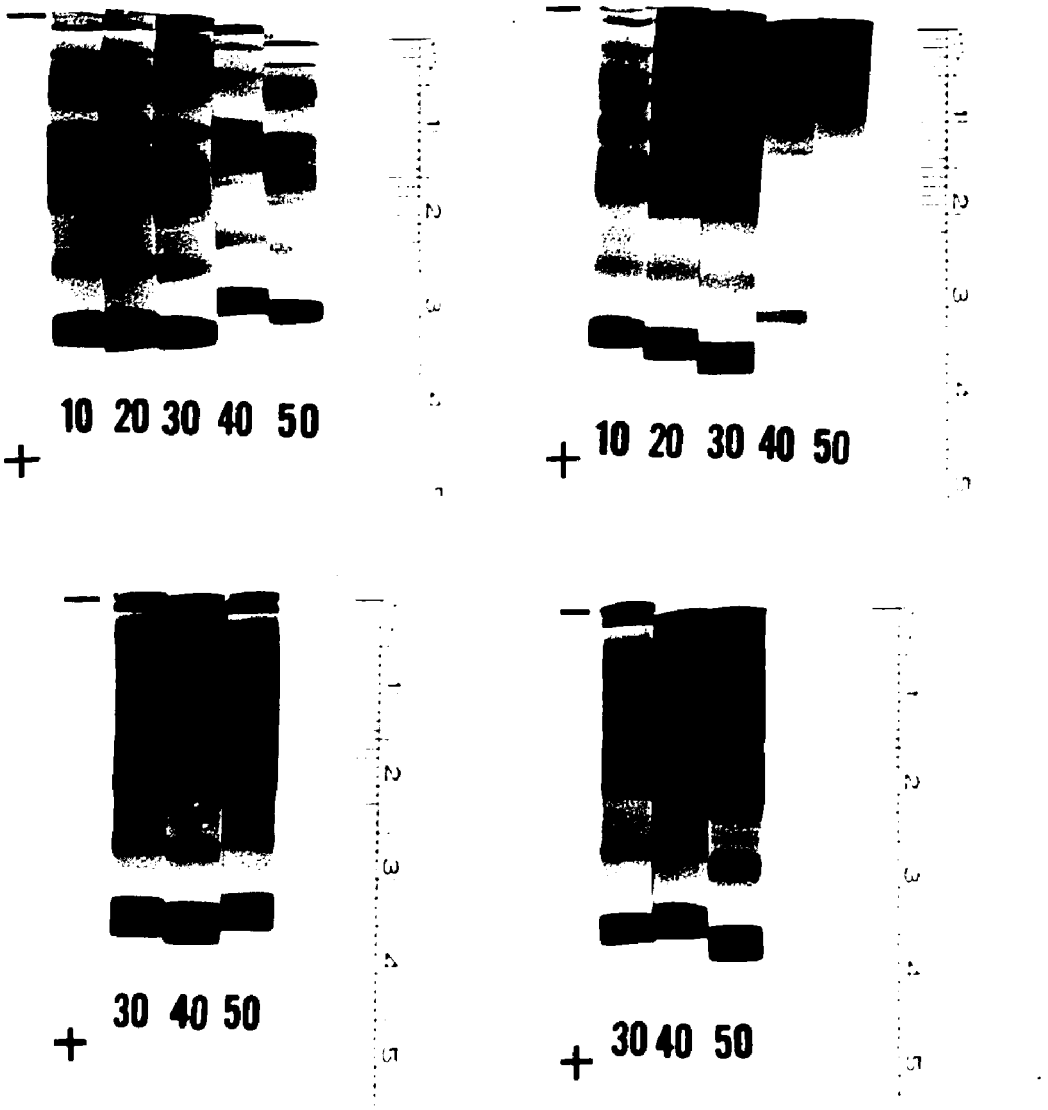


Fig. 19 Effect of steroid hormones on male serum proteins. The numbers on gels represent days post-injection.

a. Control.

b. Effect of 100 µg of estrogen.

c. Effect of 500 µg of testosterone.

d. Effect of 500 µg of deoxycorticosterone.

as suggested by Figs. 18 and 19b, then it might have been expected that vitellogenic oocytes were mobilized in an attempt to replenish the circulating anodal components.

Skin color changed from green or brown to bronze, and the gall bladder turned yellow after adminstration of estrogen. Adults which received more than 250.0 µg and juveniles which received more than 100.0 µg of estrogen died within 60 days after injection.

Adult males and females were also treated with testosterone and corticosterone to test the influence of other steroids on serum patterns. Neither of these hormones noticeably affected the serum patterns (Figs. 19c and 19d), demonstrating that only estrogen and not testosterone or corticosterone influences the electrophoretic patterns of the serum proteins.

α-FSP and Ovulation

Females collected from nature in late September just prior to hibernation are capable of ovulation. Of these, only 10% contained detectable quantities of α-FSP. This suggested that the disappearance of α-FSP might serve as an indicator for the readiness of laboratory-maintained females to ovulate. However, when this was tested (Table 2), α-FSP was absent from only two of four room-temperature females which ovulated completely (Frogs 63, 65, 26, and 36), and was detectable in all the females containing overripe oocytes. Thus, though α-FSP seems to disappear with the maturation of oocytes in the wild, in the laboratory

TABLE 2. Test of correlation between α-FSP and ovulation of frogs maintained in the laboratory.

Frog #	Days from ovulation[1]	α-FSP[2]	Ovulation[3]	Ovary condition[4]	Development (to hatching)
63	90	0	Complete (2000)	only Y_o oocytes	>75%
65	90	0	Complete (300)	1 over-ripe egg and Y_o oocytes	>75%
54	95	+	None (jelly only)	over-ripe eggs & Y_o oocytes	--[5]
60	95	+	Partial (600)	over-ripe eggs and Y_o oocytes	>75%
55	100	+	Partial (100)	over-ripe eggs and Y_o oocytes	--
56	110	+	Partial (200)	over-ripe eggs and Y_o oocytes	--
67	110	+	None	over-ripe eggs and Y_o oocytes	--
66	110	+	None	Y_o through Y_4^o oocytes	--
26	120	+	Complete (2000)	Y_o oocytes	>75%
36	110	+	Complete (3000)	Y_o oocytes	>75%

[1]Animals were received from the wild and immediately ovulated. Ovulation was complete as indicated by laparotomy.

[2]Presence or absence of α-FSP on day indicated.

[3]Approximate number of eggs ovulated on day indicated.

[4]Based on autopsy after ovulation. Eggs classified as indicated in text and Table 1.

[5]No eggs, or development not tested.

it must rapidly reappear with the resorption of the mature oocytes and the growth of the new clutch. This renders the use of -FSP as an indicator of readiness for ovulation unsuitable for practical application in the laboratory.

Serum Proteins in the Egg

Immunochemical Analyses of Oocyte Extracts

Electrophoretic bands with mobilities identical to those of albumin, α-FSP, β-FSP, and γ-globulins of maternal serum were found in oocyte supernatants. Maternal serum bands were eluted from the acrylamide gels and placed separately in agar diffusion gels adjacent to bands of equivalent electrophoretic mobilities eluted from patterns produced by oocyte supernatants. When tested against Anti-Adult Frog Serum (A.AFS), lines of fusion were observed between those serum and oocyte fractions of equivalent mobilities (Fig. 20). Thus, they were antigenically cross reacting if not identical molecules.

Immunodiffusion tests with A.FSA and A.FSG showed albumin and γ-globulin antigens to be present in the saline-soluble extracts of oocytes at selected stages (Fig. 21). In the earliest oocyte stage tested (Y_o oocytes, wells a and b, Fig. 21), albumin was more readily detected than γ-globulin. The position and density of the precipitin lines suggested a marked accumulation of both of these antigens during vitellogenesis (Y_{1-4} oocytes, wells c and d, Fig. 21). In the mature oocyte extracts (Y_5 oocytes, well e, Fig. 21), as many as four albumin and two globulin antigens were detected. Comparision of precipitin line patterns

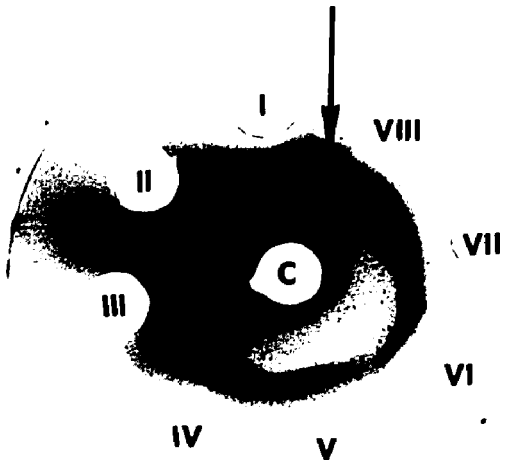


Fig. 20 Antigenic similarities between β-FSP and proteins from the ovary and liver. These proteins were eluted from gels following electrophoresis.

Center well: A.AFS
Wells I and II: β-FSP from serum of two females.
Wells III and IV: Proteins from liver extracts of two females with identical mobility as that of β-FSP
Wells V, VI, VII, VIII: Proteins from ovary extract of four females with identical mobility as that of β-FSP.

Note the identity of the antigens from serum, liver, and ovary extracts. Also note the spur (arrow) showing that β-FSP of serum consists of more than one antigen and has a serum specific component not found in the ovary.

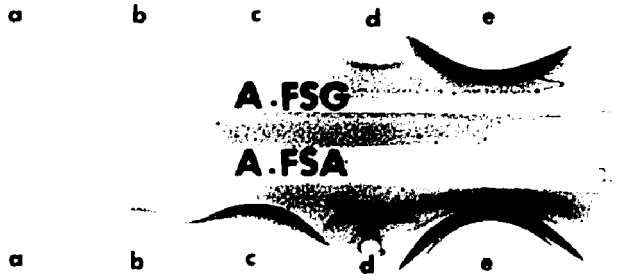


Fig. 21 Albumin and globulin antigens in the egg.

Top trough: A.FSG, same as Fig. 5b.
Bottom trough: A.FSA, same as Fig. 5b.

Wells a and b: Supernatant of pre-vitellogenic oocytes. Two frogs tested individually.
Wells c and d: Supernatant of vitellogenic oocytes. Two frogs tested individually.
Well e: Supernatant of mature oocytes.

Protein concentration of antigen preparations: 5 mg/ml.

The arch between the A.FSG and A.FSA troughs at "a" resulted from the juxtaposition of two diffusion patterns in this figure.

produced by individuals with oocytes in identical stages (Fig. 21, wells a, b and c, d) showed what seem to be individual differences.

To test for the presence of albumin and γ-globulin antigens in the yolk platelets, A.FSA and A.FSG were absorbed with yolk platelets from mature oocytes. These absorbed antisera did not react with their homologous antigens, indicating the occurrence of several albumin and γ-globulin antigens on the yolk platelets. Treatment with 10% NaCl solubilized one albumin and one γ-globulin components from yolk platelets. Different concentrations of Triton-X failed to solubilize any components. This demonstrated that the other albumin and γ-globulin antigens could not be solubilized by the method used here.

Thus, the egg contains several albumin and γ-globulin components in both saline soluble and yolk platelet fractions. Among the components in the yolk platelets, only one albumin and one γ-globulin could be solubilized by treatment with 10% NaCl.

Immunohistology of Oocytes

Table 3 tabulates immunohistological observations on eggs at each stage of oogenesis. Each observation represents the examination of at least three ovaries. In all cases, the antiserum preparations were those used for the immunodiffusion tests. A.FSA and A.FSG produced identical localization patterns suggesting that, at these stages, albumin and globulin antigens are handled identically by

TABLE 3. Immunohistology of serum albumin and γ-globulin antigens in the frog ovary[1,2].

Oocyte stage[3] (mm)	Follicle-thecal complex	Zona radiata	Cytoplasm	Yolk nuclei	Yolk platelets	Nuclear membrane	Nucleoplasm	Nucleoli
Early Y_o (0.09)	-	0	++++[4]	0	0	-	±	-
Mid Y_o (0.10)	-	0	++[4]	+	0	-	+++	-
Late Y_o (0.14)	++	0	+[4]	++	0	±	++	-
Y_1 (0.30)	++	-	++[5]	+	-	±	+	±
Y_2 (0.65)	++	-	++[5]	±	-	-	+	±
Y_3 (0.99)	+++	-	++[5]	±	+	-	+	±
Y_4 (7.91)	+++	-	+[4]	-	+	-	+	±
Y_5 (13.56)	+	-	+[4]	-	++	-	+	-

[1]Data from three or more females per stage.

[2]Fluorescence intensity subjectively graded from "++++" for very bright fluorescence to "+" for fluorescence on some preparations. "-" indicates no fluorescence, and "0" indicates the organelle was not present.

[3]Stages according to Kemp, 1953. See Table 4 for volumes, except for mid Y_o which was calculated from fresh oocytes (100μ in diameter).

[4]Throughout the cytoplasm.

[5]Limited to the zone of yolk deposition.

the oocytes. Since it was not possible to distinguish between follicular, thecal, and serosal cells, they were identified together as the follicle-thecal complex in accordance with the procedure used by Glass (1959).

Localization in Pre-Vitellogenic Oocytes. The cytoplasm of early Y_o oocytes fluoresced brightly (Fig. 22, Table 3) with the intensity of fluorescence decreasing at mid (Fig. 23) and late (Fig. 24) Y_o stages.

The perinuclear yolk nuclei were fluorescent at mid-Y_o stage. These fluorescent perinuclear yolk nuclei migrated away from the nucleus until they occupied a position equidistant from the nuclear and cytoplasmic membranes at late Y_o stage (Fig. 24).

The nuclei of the early Y_o oocytes were weakly to negatively fluorescent (Fig. 22), becoming brightly fluorescent in mid (Fig. 23) and late (Fig. 24) Y_o oocytes. The nucleoli were negative at all Y_o stages (Figs. 22, 23, and 24).

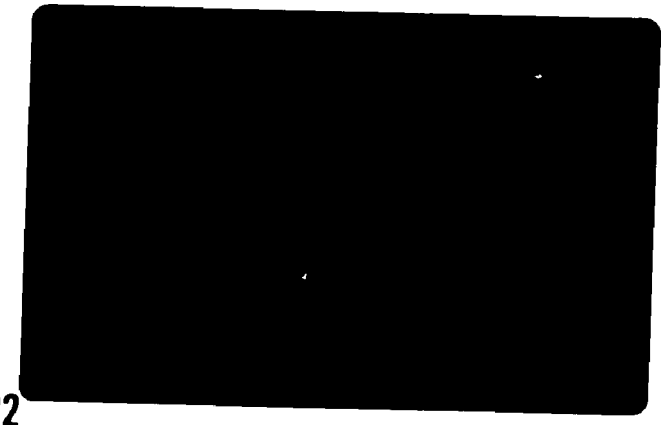
While the follicle-thecal complex could not be discerned at the early Y_o stage, examination of this maternal tissue surrounding the egg did reveal staining at the mid and late Y_o stage. This indicated the presence of albumin and globulin antigens in it.

Thus, albumin and globulin antigens were present in the follicle-thecal complex and in the pre-vitellogenic oocytes. The distribution of these antigens changed with the growth of these oocytes.

Fig. 22 Localization of albumin and globulin antigens on early pre-vitellogenic (Y_o) oocytes. 150X. Fluorescein as fluorochrome. Dark areas in the cytoplasm are fixation artifacts.

Fig. 23 Localization of albumin and globulin antigens on mid pre-vitellogenic (Y_o) oocytes. 150X. Fluorescein as fluorochrome. The intense yellow color is a result of over development. Actual fluorescence was less intense than that seen in Fig. 22.

Fig. 24 Localization of albumin and globulin antigens on late pre-vitellogenic (Y_o) oocytes. 300X. Fixation has caused the nucleus to shrink leaving an area of artifact. Note the ring of fluorescent yolk nuclei. The surface of the cell is visible at the right.



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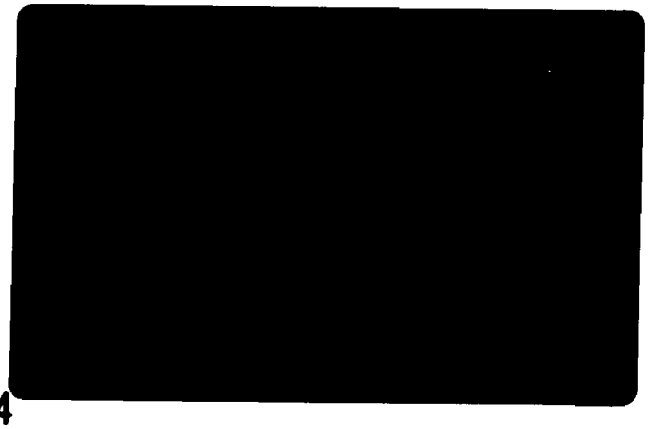
23



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Localization in Vitellogenic Oocytes. The cytoplasm in areas of yolk deposition was brightly stained in Y_1 through Y_3 stages (Fig. 25a, Table 2). The intensity of staining decreased at the Y_4 stage (Fig. 26a). While the cytoplasm internal to the area of yolk deposition was weakly fluorescent, the cytoplasm surrounding the large yolk platelets was brightly stained from the beginning of yolk deposition. Few if any of the yolk platelets were stained until Y_3 and Y_4 stages. The small yolk platelets were either stained intensely or were negative, showing the heterogeneity amongst yolk platelets. On the large yolk platelets, the staining seemed to be most prominent on the granular or superficial layer surrounding the crystalline body.

The nuclei showed weak fluorescence while the nucleoli were occasionally stained at Y_1 through Y_4 stages (Figs. 25a and 26a).

The cytoplasm of the follicle cells was stained throughout vitellogenesis, being brightest at Y_3 and Y_4 stages (Figs. 25a and 26a). A non-fluorescent zone, similar to one described by Glass (1959), between the follicle-thecal complex and the cortex of the egg, was present throughout vitellogenesis (Figs. 25a and 26a). This corresponds to the zona radiata (Kemp, 1956).

The bright fluorescence, indicating the presence of albumin and γ-globulin antigens in the areas of yolk deposition peripheral to the nucleus, and the centripetal accumulation of these antigens concurrent with vitellogenesis

Fig. 25

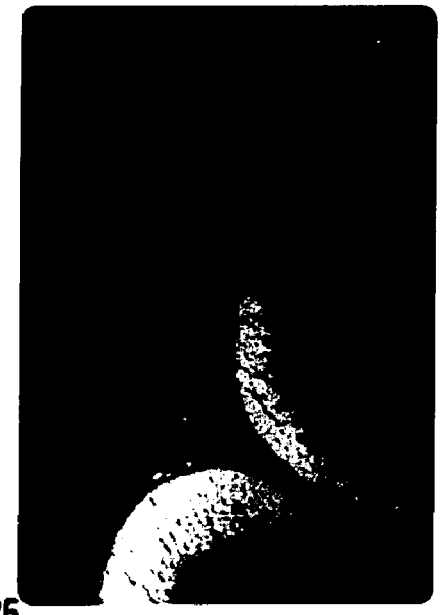
a. Localization of albumin and globulin antigens on vitellogenic (Y_1, Y_2, and Y_3) oocytes. 150X. Rhodamine as fluorochrome.

b. Same oocytes (mirror image) stained with Heidenhain's triple stain. 150X. Differential shrinkage renders point for point comparision difficult.

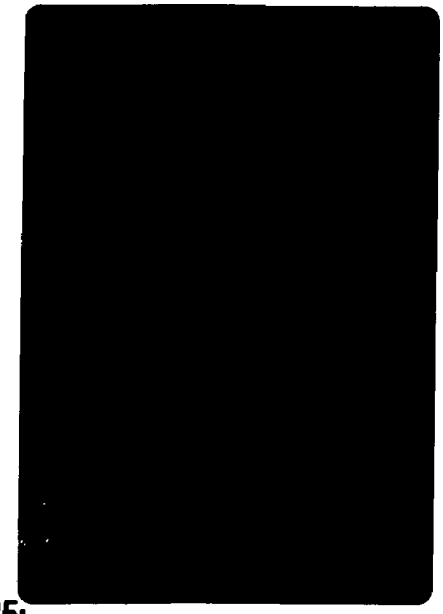
Fig. 26

a. Localization of albumin and globulin antigens on late vitellogenic (Y_4) oocytes. 300X. Rhodamine as fluorochrome.

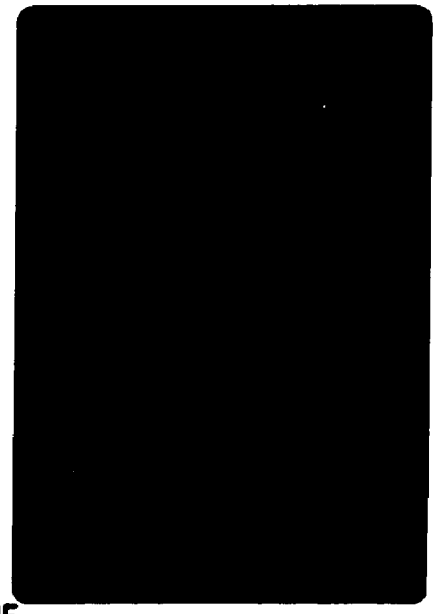
b. Same oocytes (different view) stained with hematoxylin and eosin. 300X.



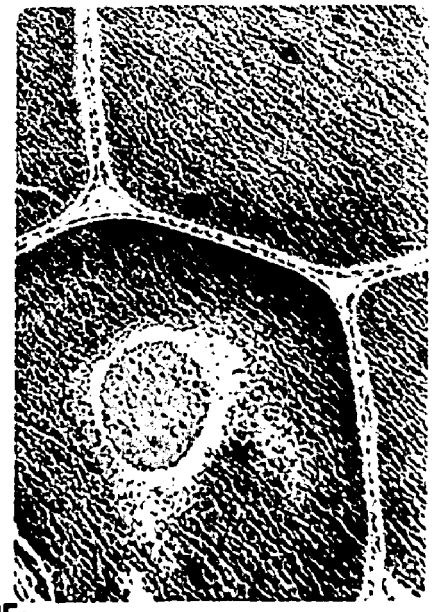
25a



25b



25a



25b

suggested that these antigens may be transferred from the maternal circulation into the periphery of the oocytes. The presence of the antigens in the follicle cells indicated that the transfer may take place through these cells into the oocytes. The absence of the antigens in the zona radiata did not mean the absence of transfer, but only the failure to accumulate, probably because of the rate of transfer.

Localization in Mature Oocytes. The cytoplasm of the mature oocytes was stained. Albumin and globulin antigens could be detected on some small yolk platelets. On the large yolk platelets the staining was more prominent on the outer granular or superficial layer surrounding the crystalline body (Fig. 27). Nuclei were stained but the nucleoli were either weakly stained or negative. The follicle-thecal complex lost most of its earlier fluorescence, becoming only weakly stainable. The non-fluorescent zone corresponding to the zona radiata persisted.

The uniform distribution of the antigens in the oocytes and the decrease in the fluorescence in the follicle cells suggested that the transfer was completed concurrent with maturation of the oocytes.

Administration of Foreign Serum Proteins

Supernatants prepared from vitellogenic ovaries of frogs injected with Bovine Serum Albumin (BSA), Normal Rabbit Serum (NRS), or Human Serum (HS) were examined in



Fig. 27 Localization of albumin and globulin antigens on yolk platelets. 300X.

radial agar diffusion tests with antisera directed against each of these foreign serum preparations. The agar diffusion plates shown in Fig. 28 reveal that each of the foreign antigens was detected in eggs. This demonstrates that the antigenically intact foreign serum proteins were transferred from the maternal circulation into the oocytes. That the positive reactions resulted from the presence of the foreign sera in the oocytes, rather than in the follicle-thecal complex, was confirmed by the positive reaction of Y_{1-2} and Y_3 oocyte extracts (Fig. 29) which were dissected out of their follicles and were punctured to extract the internal contents. Immunohistological observations showed that the foreign serum proteins were present in the cytoplasm and yolk platelets.

Oocytes from those frogs injected with BSA conjugated with Evan's blue or rhodamine were examined under UV and white light. The color of the dyes could be seen in the capillaries of the skin within five minutes after injection. Evan's blue proved to be unsuitable for histological studies. The rhodamine showed clearly under UV light with both bright and dark field illuminations (Fig. 30).

Only Y_1, Y_2, and Y_3 vitellogenic oocytes took up the stain more readily than the pre-vitellogenic Y_o, and "almost mature" Y_4 oocytes. There was a centripetal accumulation of rhodamine-labelled material in the cytoplasm. The yolk nuclei and the nucleus (Fig. 30) were free of rhodamine.

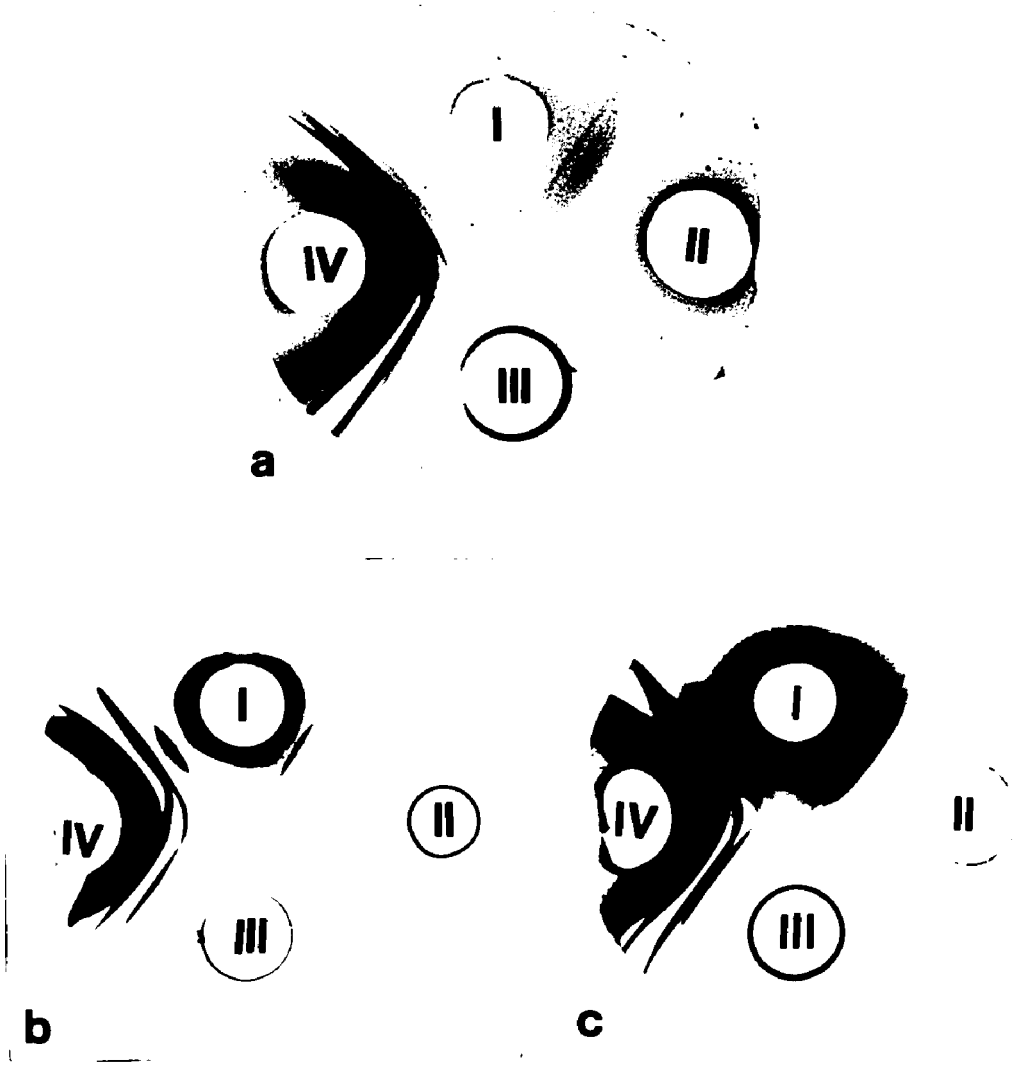


Fig. 28 Detection of foreign serum proteins in an oocyte fraction.

a. Well I: VOS from a female injection with BSA.
Well II: A.BSA.
Well III. VOS from control female.
Well IV: A.LOS.

b. Well I: VOS from female injected with NRS.
Well II: A.RY.
Rest the same as Fig. 28a.

c. Well I: VOS from a female injected with HS.
Well II: A.HS.
Rest the same as Fig. 28a.

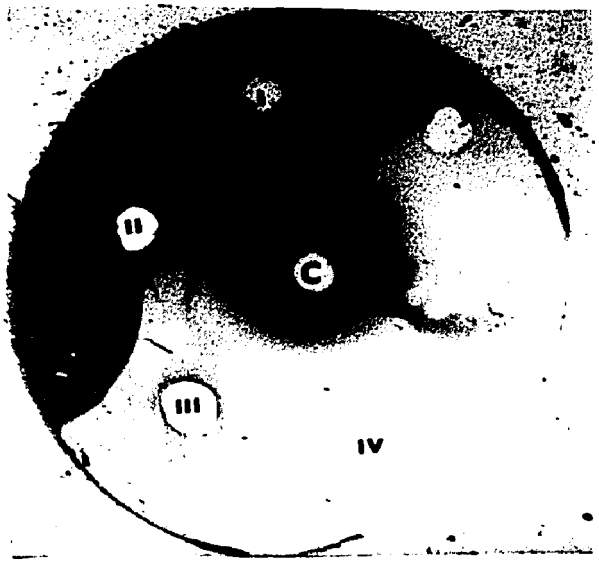


Fig. 29 Detection of foreign serum protein in vitellogenic oocytes manually removed from the ovary.

Center well: Supernatant from Y_3 (gray) oocytes. Wells I, II, III, and IV: 1/4, 1/8, 1/16, and and 1/32 dilutions of A.BSA, respectively.



Fig. 30 Localization of rhodamine labelled BSA in vitellogenic (Y_3) oocytes. Deep blue area represents the nucleus and the light blue patch represents part of the yolk nuclei. 300X.

Immunodiffusion tests showed that the foreign serum proteins were present in the serum of recipient females at least until ninety days after administration. This is consistent with the observations of Kaster and Schechtman (1957). Since this treatment, most of these frogs have survived for a year and look healthy.

Recapitulation of Serum Protein Data on the Egg

Proteins which are electrophoretically and antigenically similar to those of albumin, α, β, and γ-globulins were present in oocyte extracts (Fig. 20).

The immunodiffusion analyses with A.AFS showed that albumin, α-FSP, β-FSP, and γ-globulin antigens were present in the oocytes throughout oogenesis. Similar tests with A.FSA and A.FSG demonstrated that albumin antigens were more readily detected than the globulin antigens in the pre-vitellogenic oocytes (Fig. 21). Semi-quantitative evaluation suggested a progressive increase in the antigens during oogenesis. One albumin and one globulin antigen were detected in pre-vitellogenic and vitellogenic oocytes. Four albumin and two globulin determinants were detected in mature oocytes.

The staining of the ovary by fluorescent antisera demonstrated albumin and globulin determinants in identical locations in the oocytes (Table 3). The cytoplasm was fluorescent throughout oogenesis. In late pre-vitellogenic oocytes the yolk nuclei were fluorescent. In the vitellogenic oocytes the fluorescence in the cytoplasm in the areas

of yolk depostion was brighter than the part of the cytoplasm internal to the areas of yolk deposition. Most of the small yolk platelets fluoresced with the beginning of vitellogenesis, but a few small yolk platelets were negative. Initially, the large yolk platelets were not fluorescent, but later became fluorescent.

The nucleoplasm, which was negative at early pre-vitellogenic stages, became fluorescent at the mid-pre-vitellogenic stage, and remained fluorescent during and after maturation of the eggs. The nucleoli were negative during the pre-vitellogenic stage, but were occasionally fluorescent during maturation of the oocytes.

The follicle-thecal complex was fluorescent from mid-pre-vitellogenic to mature stages. The fluorescence was brightest at vitellogenic stages.

Foreign serum proteins were transferred, antigenically intact, into oocytes of stages Y_o, Y_{1-2}, Y_3, and Y_4.

The distribution of serum proteins during oogenesis suggested that transfer of serum proteins might take place. The transfer of antigenically intact foreign serum proteins into the oocytes suggested that transfer of homologous proteins must take place.

Serum Proteins in the Embryo

Immunochemical Analyses of Embryo Extracts

Extracts of five early developmental stages were tested for albumin and γ-globulin antigens (Fig. 31). These

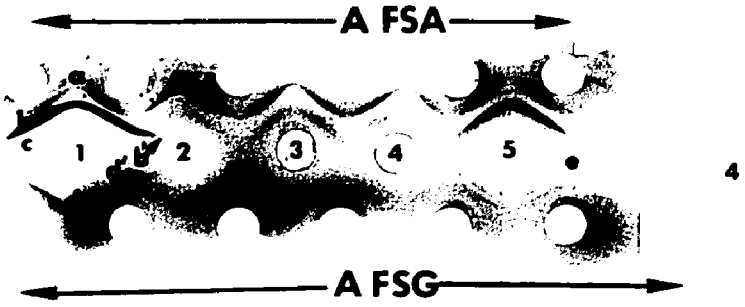


Fig. 31 Albumin and globulin antigens in the embryo.

Top row: A.FSA.
Bottom row: A.FSG.

Well 1: Stage 7 - blastula.
Well 2: Stage 11 - mid-gastrula.
Well 3: Stage 14 - neural fold.
Well 4: Stage 17 - tailbud.
Well 5: Stage 20 - gill circulation, hatching.

Protein concentration of antigen preparations:
5 mg/ml.

were the blastula (Shumway Stage 7), the mid-gastrula (SS 11), the neural fold (SS 14), the tailbud (SS 17), and the gill circulation-hatching (SS 20) stages. Note that the initiation of heart beat occurs at SS 19, there being only rudimentary development of elements of the circulatory system prior to this stage.

There was a stage-specific distribution of antigens which reacted specifically with A.FSA and not with A.FSG. At SS 7 there were four albumin antigens. In immunodiffusion tests against A.FSA, there was fusion between each of these four lines with the corresponding four antigens in mature oocyte extracts, demonstrating that these four antigens in the embryo were identical to the four albumin antigens present in mature oocytes. These four determinants are hereafter designated a, b, b', and c (Well 1, Fig. 31). The third antigen was labelled b' because it is a spur from b, demonstrating that it shares reactive sites with b but has its own distinct features. Two antigens, b' and c, were lost between SS 7 and 11 (compare Wells 1 and 2, Fig. 31). Only two were present at SS 11 through 17 (Wells 2 to 4, Fig. 31), and b showed a differential increase between SS 17 and 20 (Wells 4 and 5, Fig. 31). An albumin component, perhaps b', appeared as a spur from b at SS 20 in two out of ten tests. It is not illustrated here.

There was a stage-specific distribution of antigens which reacted specifically with A.FSG and not with A.FSA, and therefore were γ-globulin antigens. These are labelled d, d', and e (Fig. 31). At SS 7 two globulin reaction sites

were identified. Tests similar to those used for albumin demonstrated that these were identical to those detected in the mature oocytes. Since one was a spur from the other, demonstrating common as well as different identity with reactive sites, they are referred to as d and d'. Antigen d was present from SS 7 to 20, but antigen d' was lost between SS 7 and 11. The other globulin antigen, e, appeared at SS 20. In three out of ten tests, component e was detected as early as SS 17 (see insert in Fig. 31).

Immunohistology of Embryos

The cytoplasm and small and large yolk platelets of all the cells were positively stained by both fluorescein and rhodamine-labelled A.FSA and A.FSG from SS 7 through 20. A few small yolk platelets were not stained. Note that this pattern of staining was identical to the pattern of staining of yolk platelets in vitellogenic and mature oocytes. The nuclei were negative at all stages. No significant differences were seen between different areas of the same embryo or between the different developmental stages in spite of the presence, absence, or state of degradation of the yolk platelets. Since this fluorescence was observed even in cells in which the yolk had been "utilized", it must be concluded that the "serum protein" antigens they contained remained intact, even though the platelets were degraded. This continual presence of these macromolecules suggests that they might be used intact even though some among them might be degraded and utilized.

Effect of Parenterally Administered Foreign Serum Proteins on Development

Evidence for the direct utilization of transferred macromolecules came from examining the development of eggs which contained foreign serum proteins as a result of parenteral administration to the females. Among these females and the saline-injected controls, the number of eggs ovulated was between 40 and 200, compared to 1,500 to 2,000 under normal conditions. However, among the control groups 100% of the cleaving eggs hatched, whereas, among the experimental groups, none of the cleaving eggs advanced beyond the late blastula stage. This was true for the eggs which had received Bovine Serum Albumin (BSA), Human Serum (HS), or Rabbit Serum γ-globulin (γRS).

Recapitualation of Serum Proteins in the Embryo

The results showed that several albumin and γ-globulin antigens could be detected at all stages from the blastula through the hatching stage (SS 20). However, there were differences between stages. All the albumin and γ-globulin antigens of the mature oocytes were present in saline extracts at the blastula stage. Several of these antigens were absent from the saline extracts prepared from embryos after the blasula and mid-gastrula stage. However, these same albumin and globulin antigens reappeared in saline extracts after the tailbud stage. Foreign serum proteins interfered with the normal development of the embryo and caused development to stop at the late blastula stage.

DISCUSSION

The Distribution of Serum Proteins in Females and their Oocytes

The distribution of serum proteins in female frogs and their oocytes was analyzed by disc electrophoresis and by the use of Anti-Frog Serum Albumin (A.FSA) and γ-globulin (A.FSG). This section will summarize those results and develop a concept for the transfer of these proteins from maternal serum to the oocytes.

Although A.FSA and A.FSG distinguished unique sets of antigens, each was capable of reacting with several antigens falling within the serum protein class against which they were directed. Thus, while the A.FSA sera could distinguish albumins from other serum proteins, they could not distinguish among the several albumin antigens (Fig. 5). The same was true for the A.FSG sera (Fig. 5). It is within this frame of reference that the albumins and the γ-globulin antigens are treated in the following discussion.

Albumin and γ-globulin antigens were detected in R. pipiens oocytes throughout oogenesis. This confirms previous reports (Cooper, 1948, 1950; Flickinger and Nace, 1952; Flickinger and Rounds, 1956; and Glass, 1958, 1959) that serum-like antigens are present in frog oocytes, and extends them by demonstrating that the serum-like antigens are represented by both albumins and globulins.

Immunodiffusion analyses of oocyte extracts by the

use of A.FSA and A.FSG showed that at least one albumin and one γ-globulin antigen was common to pre-vitellogenic, vitellogenic, and mature oocytes (Fig. 21). However, in mature oocytes two additional albumin and one additional γ-globulin components were found. Semi-quantatative evaluation of the reactions suggested that the concentration of albumin and γ-globulin antigens in oocyte extracts increased with the growth of the oocytes.

Immunohistological observations showed identical localization of the albumin and γ-globulin antigens in the oocytes. At each stage of oogenesis the distribution of both antigens showed identical patterns which were similar to those previously described in studies using antisera against whole adult frog serum (Glass, 1958). This suggested that both albumin and γ-globulin antigens were processed in similar manner during oogenesis. Their presence in the same cellular compartments in pre-vitellogenic, vitellogenic, and mature oocyte stages (Table 3) pointed toward a common method of transfer from the exterior, rather than local synthesis which one would not expect to occur identically for both types of constituents.

In early pre-vitellogenic oocyte stages when a 55% increase in volume occurs (Table 4), both groups of antigens were present in the cytoplasm. There are three main possibilities for the origin of these antigens: autosynthesis, follicle cells, and maternal serum. These antigens could not have been transferred from follicle cells because

TABLE 4. Increase in oocyte volume during maturation.

Oocyte stage	Diameters[1] in mm		Volume in mm^3	% final volume	% increase in vol over previous stage
	A	B			
Early Y_o	.150	.150	.093	0.68	
Late Y_o	.312	.354	.144	1.06	55.0
Y_1	.382	.483	.295	2.18	105.0
Y_2	.506	.607	.650	4.79	119.0
Y_3	.589	.681	.986	7.27	52.0
Y_4	.824	.926	7.913	58.36	703.0
Y_5	1.390	1.675	13.558	100.	71.0

[1]Diameters based on measurements published by Kemp (1953), with the exception of the early Y_o stage which was published by Kessel (1969). A: minor axis; B: major axis.

of the poor development of the follicular layer at this stage. Therefore, the antigens were either transferred from the maternal serum or were synthesized de novo. The latter possibility seems unlikely because of the absence of a rough endoplasmic reticulum (Kemp, 1956). Thus it is more reasonable to conclude that the early pre-vitellogenic oocytes receive their albumin and γ-globulin components from maternal serum. This view is supported by the demonstration that the similarities between the soluble proteins of the newt liver and oocyte are maximal at pre-vitellogenic stage (Panje and Kessel, 1968).

In late pre-vitellogenic oocytes albumin and γ-globulin antigens were localized in the cytoplasm and the nucleus (Table 3). At this stage rough endoplasmic reticulum is present, but lacks the parallel arrangement indicative of active protein synthesis (Kemp, 1956, 1957). Thus, these antigens were probably transferred from either follicle cells or from the maternal serum. Since these follicle cells also have little rough endoplasmic reticulum (Figs. 1 and 4, Kemp, 1956), transfer from maternal serum is the most likely source. However, the localization of the antigens in the follicle cells indicated their probable transfer through the follicular epithelium. The specific localization of these antigens in the nucleus of oocytes may result from the transfer of these antigens from the cytoplasm through nucleopores (Kemp, 1956).

The accumulation of these antigens in the yolk nuclei may be the result of their binding by acidic components.

One can suggest that the antigens are degraded and the degradation products are used for autosynthesis. The absence of these antigens in the yolk nuclei at later stages could result from the degradation or the displacement of the antigens by more basic proteins.

The distribution of albumin and γ-globulin antigens in the oocytes during vitellogenesis was also consistent with the concept of transfer from the maternal serum. With the initiation of vitellogenesis, the periphery of the cytoplasm where yolk deposition occurs immediately internal to the zona radiata was more fluorescent than was the internal cytoplasm where yolk deposition was not yet apparent. As yolk deposition continued, this brightly fluorescent zone moved centripetally, a movement coinciding with the appearance of yolk platelets. During vitellogenesis the oocyte increases 650 times in volume (Table 4). If the oocyte synthesizes serum proteins in addition to the structural proteins necessary for growth, one would expect extensive rough endoplasmic reticulum. However, neither the cortex (Figs. 5 and 7, Kemp, 1956) nor the sub-surface cytoplasm (Figs. 9 and 10, Kemp, 1956) of the vitellogenic oocytes contain the necessary rough endoplasmic reticulum. In addition, the surface area of the oocyte increases as a result of the development of microvilli and pinocytotic vesicles which are characteristic adaptations of absorptive cells (see discussion, Kemp, 1956). Thus, the vitellogenic oocytes show adaptations for absorption, rather than

autosynthesis. The conclusion is that serum proteins in the oocytes most probably were transferred into these cells.

Synthesis of these antigens in the follicle cells during vitellogenesis is not probable, since, as at the earlier stage, these cells have little rough endoplasmic reticulum (Figs. 5 and 7, Kemp, 1956). The follicle-thecal complex, was fluorescent in late pre-vitellogenic oocyte stages. This fluorescence increased in intensity with the initiation of vitellogenesis and corresponded with bright fluorescence in the periphery of the cytoplasm of the vitellogenic oocytes. This suggested a transfer of antigens from maternal serum into the oocytes through the follicle-thecal complex. The lack of localization of the antigens in the zona radiata could have resulted from the rapidity of this transfer.

Most of the small yolk platelets fluoresced from the beginning of yolk deposition, suggesting a direct incorporation of the antigens. Subsequently, the large yolk platelets became fluorescent, giving the impression that they absorbed the antigens from the already fluorescent cytoplasmic areas, an impression consistent with the observations of Glass (1958). The follicle-thecal complex of mature oocytes was still fluorescent, indicating the continued presence of these antigens in this complex. The cytoplasm of mature oocytes was uniformly fluorescent, possibly as a result of continued transfer and retention from earlier stages.

Concurrent with changes in the ovarian tissues, changes

occurred in the maternal serum. These changes were consistent with an interpretation of transfer. Two proteins, α- and β-FSP, were identified as essentially unique to female serum. They were present in trace amounts in juveniles, males, and hibernating females, but increased in the maternal serum during vitellogenesis and decreased as the oocytes matured (Figs. 11-16). In addition, the synthesis of β-FSP could be selectively induced in females, males, and juveniles by the administration of estrogen.

The transitional increase of the two FSP's during vitellogenesis in R. pipiens was comparable to the behavior of female serum proteins reported in insects (Telfer, 1965), fishes (Booke, 1964), and birds (Schjeide et al., 1963). Recently, a similar female serum protein, SLPP, was described in Xenopus (Wallace and Jared, 1968). Transfer into the oocytes results in a decrease in the concentration of these proteins in the female serum after vitellogenesis in insects (Telfer, 1965), fishes (Booke, 1964), Xenopus (Wallace and Jared, 1968; and Follett, Nicholls and Redshaw, 1968), and birds (Schjeide et al., 1963). Therefore, it seems reasonable to assume that the decrease in the level of the two FSP's in the females in the current study probably resulted from the transfer of these proteins into the oocytes. This was supported by the fact that proteins with identical mobilities and antigenicities to those of the two FSP's were found in the mature oocytes.

This assumption might, however, be attacked on the basis that the presence of these proteins with identical

mobilities and antigenicities could also result from the simultaeous synthesis of these proteins in oocytes as well as tissues synthesizing serum proteins. However, ovariectomy resulted in the increase in all serum proteins, including the two FSP's, in the maternal serum (Fig. 17) which was consistent with the view of extraovarian origin of all serum proteins.

Direct evidence for this transfer of serum proteins was obtained by the transfer of antigenically intact foreign serum proteins such as bovine serum albumin, human serum proteins and rabbit globulins into the oocytes. These results showed that transfer of homologous serum proteins probably could occur from maternal circulation into oocytes.

The demonstration of transfer of foreign serum proteins into the oocytes in the present study contradicts the results of Kaster and Schechtman (1957). Using a dosage comparable to that used in the present study, they failed to show a transfer of human serum albumin from maternal serum into the oocytes in R. pipiens. Since they did not mention the condition of the ovary, or the maintainance and nutritional conditions of the recipient females, it is possible that their females were not vitellogenic and were not maintained under optimal conditions.

In the present study the foreign serum proteins were more readily localized in the vitellogenic oocytes than in pre-vitellogenic oocytes. This can be explained by the fact that, in contrast to the surface of the vitellogenic oocytes, the surface of the pre-vitellogenic oocytes is

smooth, and therefore the pre-vitellogenic oocytes cannot absorb macromolecules as rapidly as can the vitellogenic oocytes. Support for this view comes from the demonstration that uptake of H^3-labelled protein is fifteen times more rapid in vitellogenic oocytes than non-vitellogenic oocytes of Xenopus (Jared and Wallace, 1969).

The evidence presented above demonstrates that homologous serum albumin, α-FSP, β-FSP, and γ-globulins and heterologous serum proteins are transferred from the maternal circulation to the oocytes in R. pipiens. It also shows that there is a variable transfer into the oocytes and a variable localization of albumin and γ-globulin antigens within them depending on their stage of growth.

Fate of Maternal Serum Proteins in Development

There were qualitative and quantitative changes in the albumin and γ-globulin antigens during development. The changes were most prominent between cleavage and early tailbud stages (SS 7 and 17). The disappearance of two albumins and one γ-globulin antigen between the 32-cell and mid-gastrula stages (SS 7 and 11, respectively) coincided with rapid cell division and early differentiation. The reappearance and increase of albumin and γ-globulin antigens coincided with the establishment of circulation between tailbud and gill circulation stages (SS 17 and 20).

The development of eggs in females which had received the foreign serum proteins was arrested at the blastula stage.

These results showed that maternal albumin and γ-globulin antigens were not completely degraded, but remained intact in the embryo during development. The significance of the qualitative and quantitative changes of the antigens is discussed later.

Histology and Mechanism of Transfer

Among the several species which have been examined, there appear to be two anatomical adaptations to facilitate the pinocytotic transfer of macromolecules from the maternal circulation to the oocytes. One is the development of villi on the surface of the oocyte which interdigitate with villi of the follicle cells, and the other is the appearance of interfollicular spaces during vitellogenesis. During vitellogenesis the transfer of both homologous and heterologous macromolecules has been demonstrated in insects (Telfer, 1965) and birds (Knight and Schechtman, 1954). Interfollicular spaces as found in the ovary of some amphibians (Hope, et al., 1963; Wischnitzer, 1966; Wallace and Dumont, 1968; and Kessel and Panje, 1968) are not conspicuous in R. pipiens (Kemp, 1956). It is possible that transfer of macromolecules occurs through the follicular cells (Glass, 1958; Nace and Lavin, 1963; and Wartenberg, 1964), but it is also possible that such molecules reach the oocyte by migration between the follicular cells.

Several workers have demonstrated that there is a selective transfer of specific proteins into the oocytes in Cecropia (Telfer, 1965), in Xenopus (Wallace and Jared,

1969), in mouse (Glass, 1968), and across the placenta into foetal rabbits (Brambell, 1966). However, demonstration of the transfer does not necessarily explain the mechanism of transfer, especially that of selective transfer.

Two major hypotheses have been proposed for selective transfer. The first, based on studies on insects (Telfer and Melius, 1963) and amphibians (Wallace and Dumont, 1968), proposes that selection is dependent upon the avidity of "receptor sites" for various macromolecules at the oocyte surface. It should be kept in mind that most of the data on which selection at the surface of the oocyte is based comes from the analyses of materials inside the oocyte, i.e. the end product of transfer. Thus, the process is inferred from the nature of the end product. The second hypothesis, based on studies on transfer through the placenta in mammals, suggests non-selective transfer along with selective protection of the macromolecules by association with "intracellular" receptor sites (Brambell, 1966).

Evidence against any selective transfer at the cell surface in mammals comes from the demonstration that maternal cells can be transferred into the embryo (Tuffrey, Bishun and Barnes, 1969). Using transplanted blastocysts of CFW mice to CBA/T_6T_6 foster mothers, they showed the transfer of maternal cells into the embryos. As mentioned in the introduction, transfer of immunoglobulins occurs mostly after birth in mice. Therefore, it is hard to imagine a placenta which is porous to maternal cells being impervious to small immunoglobulins. But, it is possible that immuno-

globulins are transferred across the placenta, but are immediately degraded because of the lack of receptor sites as proposed in the second hypothesis on selective transfer.

Thus, a consideration of the mechanism of transfer suggests that, although there is a general agreement about transfer of macromolecules by pinocytosis into the oocytes or embryos, the mechanism of selective transfer is not clear. Even the possibility of different mechanisms operating in different species is complicated by contradicting results on transfer. Thus, the mechanism of selective transfer is still an open question.

Role of Maternal Albumin and γ-Globulin in the Oocytes

The albumin and γ-globulin antigens in pre-vitellogenic oocytes probably maintain osmotic pressure and help regulate ionic concentration. The localization of the antigens in the nuclei of mid-pre-vitellogenic oocytes is significant because, following this stage, growth of the nucleus and formation of lampbrush chromosomes takes place (Duryee, 1950). It is possible that degradation products of some of these antigens contribute to the growth of the nucleus and the elaboration of the chromosomes. Such a possibility exists also for the saline-soluble antigens in the cytoplasm throughout oogenesis. Since yolk platelets are utilized only during development, the antigens associated with yolk platelets probably represent the reserve to be used later during embryonic development.

Role of Maternal Albumin and γ-Globulin During Embryogenesis

Source of Amino Acids and Polypeptides

Two albumin and one γ-globulin antigens disappeared from the saline-soluble extracts between the 32-cell and mid-gastrula stages. This is a period of early differentiation and rapid cell division, and the embryo has to make structural as well as functional proteins. It is likely that the degradation of these antigens into amino acids or peptides provides some of the raw materials needed by the embryo. This is especially significant since, in a study of protein synthesis during cleavage stages, Ecker and Smith (1968) showed that the amino acid pool compares poorly with the known composition of the yolk proteins. They concluded that yolk platelets were probably not the only source of the amino acids. They probably understated the case, however, because yolk utilization does not start until the blastula stage (Karasaki, 1963b). Thus, the yolk platelets, which also contain serum proteins, cannot be the source of amino acids during cleavage stages. A more likely source of amino acids in the embryo would be the saline-soluble proteins such as the "serum" proteins. Hence, it is proposed that some of the degraded serum proteins present in saline-soluble form in the oocytes contribute to the pool. Their contribution to such a pool would explain the further decrease in the concentration of saline-soluble albumin and globulin antigens between mid-gastrula (SS 11) and neural fold (SS 14) stages.

Serum Proteins in Early Development

The increase of one albumin antigen and the appearance of one globulin antigen between stages 17 and 20 coincided with the solubilization of the yolk platelets, and specifically of their granular layer (Karasaki, 1963b), a shift of protein from particulate to soluble fractions, and the establishment of circulation (Brown and Caston, 1962). As solubilization of yolk platelets containing albumin and γ-globulin occurred immediately before circulation is established (SS 19) and when organs which synthesize serum proteins are rudimentary, these antigens, which represent maternal serum constituents sequestered during earlier embryogenesis, must contribute to the circulation of the embryo as in birds and mammals (Nace, 1970; also see Introduction and Appendix).

In R. pipiens immunocompetence develops around Taylor-Kollros stages IV-V (Volpe, 1964; and Bovbjerg, 1966), i.e. several weeks after hatching. Therefore, the embryo needs immunological protection during its development, both before and after hatching. It is likely that transferred maternal immunoglobulins provide this protection, just as in birds and mammals (Nace, 1970; also see Introduction and Appendix).

The amphibian embryo is exposed to the environment even before the initiation of development, and therefore needs immunity much earlier than either the bird embryo developing in a more impervious case, or the viviparous

mammalian embryo. It may be argued that jelly, the vitelline membrane, and lysozyme may provide this protection. However, the effectiveness of jelly (Rugh, 1951), the vitelline membrane, and lysozyme (Nace, Suyama, and Iwata, 1965; and Ostrovsky, 1970) in the protection of the embryo from micro-organisms, though it has been discussed, has not been demonstrated.

The role of the γ-globulin antigens in the soluble fraction can be easily tested by actively or passively immunizing the mother against a microbial organism during vitellogenesis and determining the susceptibility of the embryo at various stages during development. Similar experiments have been done in birds and mammals.

Differentiation and Development

The development of the embryo was arrested at the blastula stage as a result of the presence of heterologous serum proteins. There are two possible explanations for this. One is that, contrary to the classical concept that the embryo degrades all macromolecules in the egg to make its own proteins, these proteins were not degraded during embryogenesis, and competed with homologous proteins used in the macromolecular form. But, because of their foreign nature, they could not serve the necessary specific functions, and thus caused the arrest. The other is that these foreign proteins were degraded into amino acids, but the amino acid ratios were not appropriate for the continuation of autosynthesis. Thus, these heterologous serum proteins affected

the development drastically and adversely, showing their active or passive involvement in the control of differentiation.

Recently, several workers have emphasized the importance of components in the cytoplasm in the control of gene expression (Gurdon and Woodland, 1969). Although the nature of these components or the mechanism of their action in this control has not been specified, transferred proteins such as the serum proteins, would seem, on the basis of the data presented here, to be good candidates for such a function. Other evidence supports this. For example, the injection of macromolecular extracts of liver into ovulated eggs of R. pipiens leads to blastular arrest and chromosomal abnormality (Markert and Ursprung, 1963). From similar experiments with homologous protein derived from liver and heterologous proteins, Melton and Kimmel (1967) have commented that most closely examined cases of blastular arrest were accompanied by chromosomal anomalies. If this is true, it is probable that one can induce minor developmental defects with replicable changes at the chromosomal level by administration of a controlled dose of specific homologous or heterologous proteins. This could help in the identification of chromosomal sites functioning at specific stages of development. Note also that the extracts used by these authors were prepared from those organs whose products contribute most significantly to the serum protein composition. The detection of serum proteins within the nuclei and nucleoli of growing oocytes (Table 3) does not detract

from this suggested role for transferred serum proteins. Glass (1968) has added to this argument by localizing serum proteins in oocyte nuclei and nucleoli of the mouse, and has speculated that these serum proteins may affect genetic and synthetic control.

After studying serum proteins in the hen's egg, Nace (1953) postulated that a variety of functional macromolecules may be transferred into the ovum from the maternal organism, and suggested that some of them may have regulatory action. Later, Glass (1968) expanded this to include other functional molecules that may be carried into the ovum by albumin. In the present study it was demonstrated that both albumin and γ-globulins were transferred into the oocytes from the maternal circulation. It is possible that in addition to albumin, other serum proteins serve vehicular functions of importance to the embryo.

General Considerations on the Effects of Estrogen

The results showed that estrogen can stimulate the synthesis of β-FSP. Therefore, it is reasonable to conclude that the increase in β-FSP during vitellogenesis under normal conditions reflects a higher endogenous level of estrogen, and conversely, that the low level of β-FSP in females during hibernation and post-hibernation results from a low level of estrogen. However, passage of estrogen from cytoplasm to nucleus in the target tissue, a critical step in the mechanism of action of estrogen, does not take place at low temperatures (Jensen et al., 1969). Hence,

it is possible that, instead of low level, the failure of estrogen to get into the nucleus of the target tissue prevents the synthesis of β-FSP at the cold temperature of hibernation.

Other effects of estrogen, though they are not directly pertinent to the objectives of this study, do warrant consideration because they open avenues for future studies.

Parallel to the studies done on birds and mammals, estrogen-mediated β-FSP synthesis could be used to study the mechanism of action of estrogen and the resulting protein synthesis in amphibians. Birds have two target organs for estrogen action, the oviduct and the liver; estrogen induces the synthesis of different proteins in these two organs (Schjeide and Lai, 1970). In amphibians, estrogen acts on liver (Rudack and Wallace, 1968), and since the oviducts were hypertrophied in both estrogenized males and females in the current study, there is a possibility that amphibians also have two target organs and may also synthesize different proteins in liver and oviduct.

The reduction in the hematocrit value resulting from high dosage of estrogen is comparable to the effect of estrogen on the hematocrit value in Xenopus (Follett et al., 1968). It is possible that in the mature female under the influence of estrogen, iron is directed from red blood cells and is thus released for mobilization by the growing oocytes (Hegenauer, 1970).

The serum of adult R. pipiens is colorless. However,

the serum of larvae is yellow, since it is colored by bilirubin (Lester and Schmid, 1961). The change of the adult serum color to yellow after estrogen administration suggests a change, possibly a reversal, in the metabolism of bilirubin from the adult type to that of the larva.

The general decrease in serum proteins, including albumin, during the increase of estrogen-induced β-FSP, similar to the results reported in Xenopus (Follett et al., 1968; and Rudack and Wallace, 1968), could have several causes. In mammals it has been reported that a rise in the globulin level, both in pathological and experimental conditions, increases colloid content and depresses albumin synthesis (see discussion in Rothschild, et al., 1969). Since β-FSP is a globulin, a rise in its level could also increase colloid pressure and could have a similar effect on albumin synthesized by the liver. β-FSP could also cause a reduction in the level of fibrinogen synthesized by the liver. This would result in the delay in clotting in estrogenized animals observed in the present study. It is also possible that, as in mammals (Hamashima, 1966), a division of labor exists in the liver of amphibians as a result of which different cells synthesize different serum proteins. This division of labor may be maintained with low levels of estrogen, but a high level of estrogen may convert the cells engaged in the synthesis of other serum proteins to production of estrogen-dependent serum proteins. This proposition could be verified by double fluorescent

antibody methods using labelled anti-β-FSP and anti-albumin on sections of liver.

The accumulation of β-FSP with the disappearance of other serum proteins in males, females, and juveniles is an unusual in vivo method of inducing the synthesis of an enriched serum protein, and could be used to collect estrogen-induced protein from the frogs.

Toxic effects such as anemia, failure of blood clotting, and death, resulting from high dosage of estrogen are comparable to the effects observed on Xenopus (Follett et al., 1968; and Wallace and Jared, 1968). The relatively more severe effects on females as compared to males probably was caused by the higher levels of endogenous estrogen in the former.

Administration of Macromolecules through Maternal Circulation

Many investigators have found it advantageous to design experiments which involve the introduction of macromolecules into amphibian oocytes or eggs (Markert and Ursprung, 1963; and Melton and Kimmel, 1967). To date, however, this has been done by micro-injection. The parenteral administration technique used in this study has several advantages over micro-injection because materials can be introduced simultaneously into many oocytes, and it eliminates mechanical damage to the eggs. The increase in the level of α- and β-EHBPs used as a marker for the time of optimal transfer of macromolecules from the mother to her oocytes.

Summary

Using specific antisera, albumin and γ-globulin antigens were detected in the saline-soluble fraction and yolk platelets of oocytes throughout oogenesis. The distribution of these antigens in the female and her oocytes suggested that these macromolecules are transferred into the oocytes. Further investigations indicated that, concurrent with vitellogenesis, the level of two other serum proteins, α-Female Serum Protein (α-FSP) and β-Female Serum Protein (β-FSP), increased. The levels of these two proteins decreased with the maturation of the oocytes, suggesting their transfer into the oocytes. Ovariectomy led to an increase of circulating serum albumin, α-FSP, β-FSP, and γ-globulins, suggesting that removal of the ovary had deprived the animal of the depot in which these proteins were deposited and, thus, suggesting that these serum proteins are normally accumulated by the oocytes. Direct support for the transfer of maternal serum proteins into oocytes came from observations that parenterally administered heterologous bovine serum albumin, rabbit γ-globulin, and human serum were transferred antigenically intact, into the oocytes.

Two albumin and one γ-globulin antigens were utilized between blastula and gastrula stages. As yolk platelets remain intact until the tailbud stage, it is proposed that these albumin and γ-globulin antigens were degraded to provide the amino acids and polypeptides needed for autosynthesis until yolk is utilized. Two other albumin and one other γ-globulin antigens persisted until the tailbud stage. There was an in-

crease in the pre-existing albumin and γ-globulin antigens and the appearance of one more albumin and one more γ-globulin antigens between the tailbud and gill circulation stages. This increase in albumin and γ-globulin antigens occurs at the time embryonic circulation is established and the organs associated with serum protein synthesis are still rudimentary and therefore probably still nonfunctional. This suggests that these late appearing constituents were of maternal serum origin, that prior to this stage they were sequestered in the yolk and that they function as embryonic serum proteins. It is also proposed that some of the γ-globulin antigens are immunoglobulins which provide the embryo with immunological protection.

When eggs containing heterologous serum were fertilized development was blocked prior to gastrulation. This indicated that the heterologous proteins affect development drastically and adversely.

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APPENDIX

Passage of Maternal Serum Proteins and Their Role in Development

In considering the role of yolk and other materials in the egg, the classical concept, often mentioned in the literature, is that the substances deposited in the oocytes of oviparous animals provide raw materials and free energy for the development of the embryo. This concept has been questioned by several workers (Needham, 1942; Schechtman, 1955; and Waddington, 1957) who offer evidence on which they proposed that some of the macromolecules of the egg are not degraded, but in the intact form play an active role in the development of the embryo. These macromolecules could be synthesized either by the oocyte itself (autosynthesis) or be derived from elsewhere in the maternal body (heterosynthesis) (Schechtman, 1955). Messenger RNA and serum proteins are the best known autosynthetic and heterosynthetic macromolecules, respectively, that illustrate this concept. It is the latter, however, which hold our attention here; thus, the following is a recapitulation of work done on the transfer and fate of serum proteins in various species. It will be obvious from this review that the transfer of serum proteins from the maternal circulation into the oocytes has attracted more attention than the follow up of these proteins during development.

Insects

Transfer of Serum Proteins. The first evidence for the transfer of blood proteins into oocytes came from spectroscopic analyses of blood and egg extracts of Rhodnius, the blood-sucking insect (Wigglesworth, 1943). Both exhibited absorption maxima indistinguishable from those of kathemoglobin, a form of hemoglobin in which the protein moiety has been modified. This was compatible with a concept of transfer, but the additional evidence showing that the amount of pigment in the eggs was increased by injecting laked rabbit blood into the hemocoel more strongly suggested that native proteins could be transported into the egg.

In a series of experiments first reported in 1954, Telfer and coworkers (reviewed by Telfer, 1965) demonstrated that every protein thus far detected in the blood of Cecropia had an antigenic counterpart in the egg. Among these proteins, one, vitellogenin, found in vitellogenic females and only in traces in males, was selectively accumulated (Pan, Bell, and Telfer, 1969). Ovariectomy resulted in a significant buildup in the blood of a protein with the mobility of vitellogenin. In addition, bovine serum albumin, bovine γ-globulin, hen ovalbumin, and ovoverdin from lobster eggs, injected into polyphemus pupae, were transferred into the egg. But the concentrations of these foreign serum proteins in the egg was less than a tenth of their concentration in the blood. Recently, Telfer and Anderson (1968) showed that the transfer is stage specific, terminating 24 hr before chorion formation.

Mechanism of Transfer. Based on fluorescent antibody and autoradiographic studies, Telfer and Melius (1963) suggested that proteins reach the surface of oocytes from the hemocoel by diffusion through the intercellular spaces between the follicle cells and through the basement and vitelline membranes. From the egg surface blood proteins are taken up by pinocytosis and transferred into small yolk spheres which fuse to form large ones.

Fish

Transfer of Serum Proteins. Although changes in serum proteins and their probable mobilization for egg production in fish have been discussed by several workers (reviewed by Booke, 1964), the most conclusive evidence has been presented by Korfsmeier (1966) in zebra fish. Eggs of this fish contain two distinct yolk spheres: autosynthetic intravesicular, and heterosynthetic extravesicular. By isotopic and electron microscopic methods, Korfsmeier presented evidence for liver synthesis, blood transport, and transfer of proteins into oocytes to form the extravesicular yolk spheres.

Mechanism of Transfer. Korfsmeier's cytological observations during vitellogenesis indicated that ooctyes accumulated macromolecules by pinocytosis. Furthermore, administration of trypan blue prevented the formation of the extravesicular yolk spheres by accumulating in the zona radiata and presumably by blocking pinocytosis. This did not affect the formation of the intravesicular yolk spheres.

Amphibians

Transfer of Serum Proteins. The presence of serum-like molecules has been reported at all stages of amphibian development from immature oocytes to larvae (Cooper, 1948, 1950; Flickinger and Nace, 1952; Flickinger and Rounds, 1956; Glass, 1959; Chalumeau-Le-Foulgoc, 1967; and Richmond, 1968). Based on immunochemical, fluorescent antibody, and cytological evidence, several workers (Flickinger and Rounds, 1956; Kemp, 1956; Glass, 1959; Nace and Lavin, 1963; Hope, Humphries and Bourne, 1963; Wischnitzer, 1966; and Kessel and Panje, 1968) suggested that a transfer of macromolecules, especially serum proteins, takes place from mother to oocyte. To obtain direct evidence, Kaster and Schechtman (1957) administered human serum albumin into female Rana pipiens. Since the foreign serum protein remained in the maternal serum and was not detected in the oocytes, they postulated that human serum albumin was too strange a protein to be accepted in such a transfer. Wartenberg (1964), by administration of the iron-dextran complex "myofer" into vitellogenic T. alpestris females, successfully showed the transfer of these macromolecules from the maternal circulation into oocytes, and its incorporation into yolk platelets. Using Xenopus and isotopic and chromatographic methods, several investigators (Follett, Nicholls and Redshaw, 1968; Wallace and Jared, 1969) demonstrated a serum lipophosphoprotein (SLPP) in vitellogenic females which is selectively taken up by the oocytes. They also found traces of this same SLPP in males. In oocytes,

SLPP is transformed into the phosvitin and lipovitellin of the crystalline body of the large yolk platelets by some unknown mechanism.

Mechanism of Transfer. Based on electron microscopic studies, several workers (Kemp, 1956; Hope, Humphries and Bourne, 1963; Wartenberg, 1964; Wischnitzer, 1966; Kemp and Istock, 1967; Kessel and Panje, 1968; and Wallace and Dumont, 1968) have postulated that macromolecules are transferred from the maternal circulation to the oocyte through either interfollicular spaces or through the follicular cells. Again, as in the case of insects, these oocytes take up the macromolecules by pinocytosis. Wallace and Dumont (1968) have proposed that the avidity of receptor sites on the surface of the oocytes is responsible for the selective transfer of SLPP in Xenopus.

Follett, Nicholls and Redshaw (1968) showed that SLPP synthesis was controlled only by estrogen and not by other steroids. They also demonstrated that Follicle Stimulating Hormone (FSH) is responsible for the transfer of SLPP from the maternal serum into the oocytes.

Birds

Transfer of Serum Proteins. The initial evidence for the passage of serum proteins from the maternal circulation into the oocytes was collected as early as 1893 (Klemperer) and came from the demonstration of the transfer of antibodies from the serum of hens to their oocytes (reviewed by Knight

and Schechtman, 1954). That homologous immunoglobulins are transferred more readily than heterologous proteins was shown in the work of Brierly and Hemming (1956). By active immunization of hens with Brucella abortus and Salmonella pullorum and injection of immune sera into the yolk sac, these investigators demonstrated that immunoglobulins from pigeons were transferred less readily than homologous ones, but more readily than the immunoglobulins from mammals. This introduced the concept of species specificity of transfer.

The possibility that the transfer may not be restricted to antibodies was suggested by several workers (Jukes and Kay, 1932a and b; Schechtman, 1947, 1952; and Tyler, 1955). Initial observations by these and other workers showed that chicken serum contains antigens which are similar to some found in egg yolk, egg white, and embryonic serum (Jukes and Kay, 1932a and b; Roepke and Bushnell, 1936; Schechtman, 1947; Nace and Schechtman, 1948; and Marshall and Deutsch, 1950). Subsequently, using serological techniques on the chick, Nace (1953) demonstrated antigenic determinants resembling those of maternal serum albumin on or before the 5th day, those of α- and β-globulins about the 6th day, and those of γ-globulin between the 9th and 12th day. As antigens resembling, but different from, each of these were also detected in the serum, he suggested that the proteins of the yolk form a source of serum proteins available to the embryo until its own synthetic mechanism is established. Since the appearance of α- and β-globulins in the embryonic blood was

coincident with extensive disintegration of blood cells, Schechtman and Hoffman (1952) proposed that these globulins resulted from the disintegration of the blood cells. Williams (1962) and Stratil elaborated on this and have shown the identity of α, β, and γ-livetin and transferrin of the egg with serum albumin, α_2-glycoprotein, γ-globulin, and transferrin, respectively.

Flickinger and Rounds (1956) demonstrated that radioactive phosphate injected into laying hens first appeared in the liver, then in the serum, and finally in the egg. This indicated the liver synthesis, serum transport, and deposition in the egg. Schjeide et al. (1963) expanded on this by showing that comcommitent with the onset of egg production, a series of physiological changes occurs, especially synthesis of serum phosphoprotein and lipovitellin fractions, which can be selectively induced in pullets, roosters, and newly hatched chicks by the administration of estrogenic substances.

Mechanism of Transfer. Electron microscopic observations revealed that the transfer of macromolecules occurs through the zona radiata which is the region of interdigitation between the follicular and oocyte villi (Press, 1959). As in insects and amphibians, the oocyte takes up macromolecules by pinocytosis (Press, 1959; Schjeide et al., 1963).

Fate and Function of Serum Proteins. The most abundant phosphoprotein is almost completely hydrolyzed by the embryo (Marshall and Deutsch, 1950). The immunoglobulins provide

immunological protection, and albumin, α-globulin and β-globulins function as serum proteins until the embryo synthesizes its own (Schechtman, 1955; and Nace, 1970)

Mammals

Transfer of Serum Proteins. In mammals the passage of immunoglobulins and other serum proteins from the mother to the embryo has been demonstrated in a number of species (reviewed by Brambell, 1966). In general, the transfer of antibodies occurs entirely before birth in the rabbit, the guinea pig, and man; entirely after birth in ruminants, the horse, and the pig; and partly before but mostly after birth in the rat and the mouse. Evidence that homologous serum proteins can be more readily transferred than heterologous ones came from comparative studies (reviewed by Brambell, 1966) indicating a selection relative to the different serum protein fractions of the same species as well as to the species of origin of the same serum protein fractions.

In a series of experiments, Glass (reviewed by Glass, 1970) has shown the passage of homologous and heterologous serum proteins to oocytes during oogenesis and before conception in the mouse. Serum proteins are transferred into granulosa cells, small oocytes, and mature oocytes. However, the native serum proteins disappear soon after ovulation and reappear in the ootid in the oviduct after the follicle cells disappear. Based on similar studies with homologous and heterologous sera, Glass concluded that this reappearance of serum protein in the ootid resulted from their transfer

from the oviduct to the ootid. She also demonstrated that there was a differential transfer into the oocytes of the various kinds of serum proteins from the same species, and between the homologous kinds from different species.

Although changes in serum proteins prior to ovulation comparable to those of oviparous species have not been demonstrated in viviparous mammals, a reproduction related protein has been shown in the rat (Heim, 1962). Quantitative changes in serum proteins, however, do take place during pregnancy in mammals (Masters, Bignold and Morgan, 1969).

Mechanism of Transfer. Based on ultrastructural studies, several workers (Anderson and Beams, 1960; and Chiquoine, 1960) suggested that, as villi from ovum and follicle cells extend across the substance of the zona pellucida in the tertiary follicles, materials might diffuse from follicle cells into the zona pellucida, reach the surface of the oocytes, and enter the oocytes by pinocytosis. This was supported by Glass (1968) who showed that maximal transfer coincided with the presence of villi on the follicle cells and the ovum.

The surface of the cells associated with the passage of serum proteins into the mammalian embryo, such as the apical surface of the endodermal cells of the yolk sac in the rabbit and those of the intestine of the young rat (reviewed by Brambell, 1966), are covered with microvilli. As in insects, amphibians, and birds, the serum proteins are transferred by pinocytosis.

Immunoglobulins injected either into the lumen of the uterus of a pregnant rabbit or into the gut of young rats were absorbed completely by the cells of the yolk sac and those of the gut, respectively. However, most of the absorbed immunoglobulins were degraded and only a fraction of the absorbed protein was transmitted intact into the circulation. Brambell (1966) proposed that the absorption of proteins by these cells is non-selective but that the release of proteins by these cells into the circulation of the embryo is selective. Accordingly, selectivity depends on the binding of proteins to receptor sites which protect against proteolytic enzymes. In this scheme, the receptor sites are limited, and therefore protein molecules which cannot attach to these protective receptor sites are degraded. Results on the maternal-foetal transfer of albumin, transferrin, and α-globulin in the rat support this hypothesis (Masters et al., 1969).

Fate and Function of Serum Proteins. Albumin, α-globulin, β-globulin, and γ-globulins function as serum proteins until the embryo synthesizes its own (Solomon, 1965). Immunoglobulins protect the embryo until it becomes immunocompetent.

General Conclusions

Events during vitellogenesis are strikingly similar in various oviparous animals. Concommitant with changes in maternal serum proteins the oocytes undergo morphological changes which are believed to be adaptations to facilitate the transfer of macromolecules into the oocytes. Ovarectomy

leads to the accumulation of vitellogenic serum proteins in the maternal serum. This indicates that vitellogenic serum proteins are normally taken up by the oocytes.

Direct evidence for the transfer of serum proteins into the oocytes in oviparous animals and passage of serum proteins into oocytes as well as into embryos in mammals comes from the demonstration of transfer of heterologous proteins.

However, demonstration of the transfer does not necessarily explain the mechanism of transfer, especially selective transfer. Two major hypotheses for selective transfer have been proposed by several workers. The first, based on studies on insects and amphibians, calls for selection dependent upon the avidity of the receptor sites for various macromolecules at the oocyte surface. It should be kept in mind that most of the data on which selection at the surface of the oocyte is based comes from the analyses of materials inside the oocyte, i.e., the end product of transfer. In other words, the process itself is reconstructed based on the end product. The second hypothesis, based on studies on mammals, calls for non-selective transfer into cells, but selective protection of the macromolecules by association with some intracellular receptor sites.

Evidence against any selective transfer at the cell surface in mammals comes from the demonstration that maternal cells can be transferred into the embryo (Tuffrey, Bishun and Barnes, 1969). Using transplanted blastocysts of CFW mice to CBA/T_6T_6 foster mothers, they showed the transfer

of maternal cells into the embryos. However, as mentioned earlier, transfer of immunoglobulins occurs mostly after birth in mice. It is hard to visualize a placenta which is porous to maternal cells to be impervious to immunoglobulins which are much smaller than cells. But it is possible that immunoglobulins are transferred but are immediately degraded, resulting from the lack of receptor sites, as proposed by the second hypothesis on selective transfer.

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