Egg Proteins in Cod Serum

NATURAL OCCURRENCE AND INDUCTION BY INJECTIONS OF OESTRADIOL 3-BENZOATE

By P. A. PLACK, D. J. PRITCHARD AND N. W. FRASER

Natural Environment Research Council Fisheries Biochemical Research Unit, University of Aberdeen,
St Fittick's Road, Aberdeen AB1 3RA, U.K.

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1. Before the uptake of water that precedes spawning, eggs of cod (*Gadus morhua* L.) contained 30% dry matter, of which 80% was protein. Some 75% of this protein was soluble in 0.5 M-sodium chloride. The major components in the extract were two similar lipoproteins, of molecular weight about 400 000, containing 21% lipid, some two-thirds of which was phospholipid, and about 0.5% protein phosphorus. 2. These lipoproteins were identified by immunochemical methods in the serum of female cod with developing ovaries, but not in the serum of male or of immature female fish. 3. The concentrations of egg proteins in the serum of female cod were determined by a serial-dilution double-diffusion immunological method, and were shown to increase with development of the ovaries, reaching a value of about 32 mg/ml when the weight of the ovaries was 10% of the weight of the fish. 4. Immature male and female cod were injected intramuscularly with a solution of oestradiol-17 β 3-benzoate in oil and the concentration of egg proteins in their serum was measured by the immuno-diffusion method. The serum contained no detectable egg proteins before injection of the fish, but 30 μ g of oestradiol benzoate/kg gave rise to detectable amounts of egg proteins in 10 days, and with 300 μ g or 1 mg of oestradiol benzoate/kg the concentration of egg proteins rose to 32 mg/ml. The values for male and female cod were similar and represented about one-half of the total serum protein. 5. With a dose of 1 mg of oestradiol benzoate/kg, egg proteins were first detected in the serum 2 days after injection and the concentration increased up to 10 days. 6. Serum samples taken before and 10 days after an injection of 1 mg of oestradiol benzoate/kg were fractionated by gel-filtration on Sephadex G-200. The difference curves obtained from fractionation curves after and before injection confirmed the values of the concentrations of egg proteins obtained from the immunodiffusion test and showed that the concentrations of the normal serum components fell by 20-50% of the initial value, the high-molecular-weight globulins showing the most marked fall. 7. Egg proteins were detected in the liver and testes of the injected fish, but not in the ovaries.

The ovaries of a mature female cod just before spawning contain several million eggs and represent some 20% of the weight of the fish. Each egg contains a supply of nutrients sufficient not only to enable the fertilized egg to develop into an embryo, but also to nourish the hatched fish, via its attached yolk sac, until it is able to catch sufficient food. The development of the eggs in the adult female must involve a major change in the balance of protein synthesis and there is evidence that the female sex hormones may be concerned in its control.

Gottfried, Hunt, Simpson & Wright (1962) found low concentrations of oestradiol-17 β and of oestrone in mature ova of cod, and in earlier experiments with a minor component of eggs, retinal, we found that injections of oestradiol into

immature male or female cod gave rise to concentrations of retinal in the plasma similar to those found in female fish with maturing gonads (Plack & Pritchard, 1968). The effects of oestrogen injections on the serum proteins of other fish have been investigated by Bailey (1957) and Ho & Vanstone (1961), who found increases in total protein and protein phosphorus, and by Urist & Schjeide (1960-61) and Utter & Ridgway (1967), who showed the appearance of egg protein. A number of other studies with amphibia, reptiles and birds have shown that injections of oestrogens into males or immature females give rise to egg proteins in the serum.

In this paper we show that the major protein constituents of cod eggs are present in the serum of

female fish with developing ovaries and can be induced in the serum of immature male or female cod by injections of oestradiol.

A preliminary account of some of these results has been given (Plack & Fraser, 1970).

MATERIALS AND METHODS

Chemicals. M.S.222 (*m*-ethoxycarbonylanilinium methanesulphonate) was obtained from Sandoz Products Ltd., London W.1, U.K. Thiomersal (sodium ethylmercurithiosalicylate) and 2-amino-2-methylpropan-1-ol (Monol) were from British Drug Houses Ltd., Poole, Dorset, U.K. and crystallized bovine plasma albumin from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Oestradiol-17 β 3-benzoate dissolved in arachis oil-ethyl oleate (4:1, v/v) (Oestroform, British Drug Houses Ltd., London N.1, U.K.) was used for injection as supplied, at concentrations of 5 mg/ml or 1 mg/ml, or diluted with sterile solvent.

Fish. Cod (*Gadus morhua* L.) were caught by seine net in shallow water off Aberdeen and transferred to tanks of aerated sea water at 10°C in the laboratory. This stock of cod spawns in February–April, and fish were caught in the months November–March. Uninjected fish were killed the day after capture by a blow on the head, the heart was exposed and a blood sample of 5–10 ml was taken by syringe. The fish were weighed and the gonads were removed and weighed to give the sex and maturation coefficient (100 $\times$ wt. of gonads/wt. of whole fish).

For the oestradiol experiments, fish estimated to be 1.5–2.0 kg were chosen to ensure that they were sexually immature. They were anaesthetized in sea water containing 1 part of M.S.222 in 10 000, and a blood sample of about 2 ml was taken by heart puncture. Each fish was then weighed and the dose of oestradiol, based on the weight of the fish, was injected intramuscularly into the epaxial muscle behind the head. The volume of oestradiol solution injected was kept at 0.2 ml/kg and its concentration was adjusted to give the appropriate dose. After tagging with coloured plastic tubing, the fish was allowed to recover in a small tank of aerated sea water before transfer to the holding tank. Each fish was anaesthetized a second time and a blood sample of 2 ml taken by heart puncture. This second sample was usually taken 5 days after the oestradiol injection but, with the time experiment, sometimes at 1 or 2 days. At the end of the experiment, the fish was killed by a blow on the head, the heart was exposed and a blood sample taken from it.

Blood samples were allowed to clot at room temperature; the serum was removed and centrifuged to clear. Two 5 μ l samples of serum were taken and each was added to 5 ml of 0.9% NaCl; two 1 ml samples from each dilution were used for the determination of protein. To the remaining serum, a 1% (w/v) solution of thiomersal was added to give a final concentration of 0.01%, and twofold serial dilutions were prepared in 0.9% NaCl containing 0.01% of thiomersal.

Homogenates of liver and gonads were prepared in a Potter–Elvehjem homogenizer with 1 g of tissue and 4 ml of 0.5 M-NaCl containing 0.01% of thiomersal, and were left to extract for 2 h. They were centrifuged at 100 000 *g* for 45 min and the clear liquid between the insoluble

material at the bottom of the tube and the lipid layer at the surface was used for twofold serial dilutions in 0.5 M-NaCl containing 0.01% of thiomersal.

Ovaries containing ripe eggs, before the uptake of water that precedes spawning, were obtained from the fish in the aquarium or from local fishermen. They were stored at –20°C until used. One of the fish from the aquarium was spawning and from it a sample of spawned eggs was obtained.

Preparation of egg proteins. Tissue, consisting primarily of eggs, was removed from the inside of the ovaries and homogenized with 3 or 4 times its weight of 0.5 M-NaCl containing 5 mM-EDTA in a Potter–Elvehjem homogenizer. The homogenate was left for 2 h at room temperature with occasional stirring to extract the proteins and then centrifuged at 75 000 *g* for 45 min. The supernatant liquid contained some floating particles, which were removed by filtration through Whatman no. 1 paper. In one instance, a similar extraction was made with 5 mM-EDTA in place of 0.5 M-NaCl–5 mM-EDTA.

Dilution of the saline extract of cod eggs with 10 vol. of water gave a precipitate, called ‘phosvitin–lipovitellin’ following the nomenclature for hen’s egg proteins. ‘Phosvitin–lipovitellin’, dissolved in 1 M-NaCl, was treated at 6°C with 2 vol. of a saturated solution of (NH₄)₂SO₄ to precipitate lipovitellin. In these preparations the methods of Wallace, Jared & Eisen (1966) were followed. The lipovitellin precipitate was dissolved in 1 M-NaCl and dialysed at 6°C against four changes of 1 M-NaCl. With preparations A and B of lipovitellin, the (NH₄)₂SO₄ precipitation and dialysis were repeated and the dialysed solution was centrifuged at 75 000 *g* for 1 h to remove small particles.

Analytical methods. Dry matter was determined by drying to constant weight at 105°C. Lipid in the spawned cod eggs was extracted by the method of Folch, Lees & Sloane-Stanley (1957) and determined by weighing. With extracts of eggs from ovaries and with preparations of ‘phosvitin–lipovitellin’ and lipovitellin, lipid and protein contents were determined by weighing, the procedures of Wallace (1965) being followed.

Kjeldhal nitrogen determinations were made by the method of Guston & Ogg (1965) and other protein determinations were made, without heating, by the Miller (1959) modification of the procedure of Lowry, Rosebrough, Farr & Randall (1951); with samples derived from cod eggs, bovine plasma albumin was used as a secondary standard and lipovitellin preparations A and B as the primary standard, but with serum samples, bovine plasma albumin was used as the primary standard. Total phosphorus was determined by the method of Allen (1940) and alkali-labile phosphorus by the procedure of Martin & Doty (1949) after hydrolysis in 1 M-NaOH for 15 min at 100°C and neutralization.

Immunological methods. Antiserum to cod eggs was prepared by injecting New Zealand White rabbits with a 10% (w/v) homogenate of cod eggs (taken from the inside of an ovary) in 0.9% NaCl mixed with an equal volume of Freund’s complete (first injection) or incomplete (second and third injections) adjuvant, by the procedure of Darcy (1966). A 1% solution of thiomersal was added to the immune serum to give a final concentration of 0.01%, and the serum was stored in 2 ml portions at –20°C. Before use in the immunodiffusion and immunoelectrophoresis tests

it was thawed and diluted with an equal volume of 0.9% NaCl containing 0.01% of thiomersal.

For immunodiffusion, buffered agar was prepared as described by Darcy (1966) and 9 ml was pipetted into 6 cm-diameter petri dishes. Wells were cut as shown in Fig. 1 and samples were placed in the outer wells and the diluted rabbit antiserum in the inner well. Double diffusion was allowed to proceed for 65 h at room temperature in a moist chamber and the plates were then examined for precipitin lines. The highest dilution of the sample giving a positive precipitin line was noted. This test was given a semi-quantitative basis by calibration with preparations of lipovitellin of known protein content. For tests of identity, dilutions of the samples previously found to give the sharpest lines were used, and the diffusion was allowed to proceed for 7 or 8 days.

Immunoelectrophoresis on microscope slides (76 mm × 26 mm) coated with agar gel was carried out as described by Hirschfeld (1960), except that calcium lactate was omitted from the two buffers and electrophoresis was carried out for 1 h. The slides were left for 40 h at the immunodiffusion stage and, after removal of unprecipitated protein by drying and washing, the precipitin arcs were stained with nigrosine (0.05% in water-methanol-acetic acid, 5:5:1, by vol.) for protein or with Oil Red O (0.05% in methanol-water-trichloroacetic acid, 5:4:1, by wt.) for lipid.

Chromatographic methods. Sephadex G-200 was allowed to swell in 1 M-NaCl containing 0.05 M-tris and 0.02% NaN₃, and adjusted to pH 8.0 at 6°C with 2 M-HCl. A column (87 cm × 1.5 cm) was prepared and run in the cold-room at 6°C, and the above buffered 1 M-NaCl was used for elution. Fractions of 50 drops were collected and their extinctions at 280 nm read on a Unicam SP.500 spectrophotometer with 1 cm light-path cells. The volumes of the fractions were measured and the partition coefficient (K_{av}) of the eluted material was calculated from $K_{av} = (\text{elution volume} - \text{void volume}) / (\text{volume of gel bed} - \text{void volume})$. The column was calibrated with sucrose, glucagon, cytochrome *c*, bovine plasma albumin, catalase and

Blue Dextran 2000 as described by Andrews (1965), except that elution was with the buffered 1 M-NaCl, and with glucagon and catalase NaN₃ was omitted. For the egg-protein-fractionation curve, a 25% (w/v) homogenate of cod eggs, from the inside of a deep-frozen ovary, was prepared in 0.5 M-NaCl and left to extract for 2 h. After centrifugation at 75 000 *g* for 45 min, 0.5 ml of the supernatant liquid was applied to the Sephadex G-200 column and 10 ml was used to determine the protein content, by extracting the lipid and weighing the protein precipitate (Wallace, 1965).

Chromatography on TEAE-cellulose* (Cellex-T, Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was by the procedures of Wallace (1965) with the modification of Jared & Wallace (1968), except that the final size of the column (27 cm × 1.8 cm) was slightly less. Fractions (10 ml) were collected, beginning with connexion of the gradient, and E_{280} was measured against the starting buffer. Laboratory grade Monol contained an impurity with an absorption maximum at 278 nm. It was removed by adding 400 ml of water to 500 ml of Monol and extracting with three 500 ml portions of light petroleum (b.p. 40–60°C). Monol was recovered from the aqueous layer by fractional distillation.

The capacity of the TEAE-cellulose columns for cod egg proteins was low, so that purification of the two main egg proteins on a large scale was not possible by this method. However, the material in the first peak (see the Results section) from one run was concentrated by ultrafiltration, chromatographed again on TEAE-cellulose and material in the first peak again concentrated by ultrafiltration and dialysed against 0.5 M-NaCl. The protein concentration of the solution was determined by the Miller-Lowry method and the partially purified lipovitellin was used for a calibration of the immunodiffusion test.

* Abbreviation: TEAE-cellulose, triethylaminoethyl-cellulose.

Fig. 1. Composite diagram of immunodiffusion lines obtained with an antiserum to cod eggs in the centre well and in the outer wells (a) extract of cod eggs; (b) serum from a mature female cod; same pattern given by serum from an oestradiol-injected immature cod; (c) as (a); (d) as (b); (e) extract of liver from an oestradiol-injected immature cod; (f) extract of ovaries from an oestradiol-injected immature cod; (g) extract of testes from an oestradiol-injected immature cod. With (a) and (b) diffusion was for 65 h and with the remainder for 8 days, at room temperature.

RESULTS

Proteins of cod eggs

Protein content. Cod eggs obtained from a spawning fish contained 7.3% dry matter and 92.7% water. Of the dry matter, 12.8% was lipid and 79% protein (nitrogen content $\times 6.25$). Before the uptake of water that precedes spawning, eggs taken from the inside of cod ovaries contained 30% dry matter, of which about 80% was protein.

Properties. Of the protein present in eggs from the ovaries of mature female cod, some 15% was extracted with water but 75% with 0.5M-sodium chloride. These sodium chloride extracts, at concentrations greater than 1mg of protein/ml, gave two precipitin lines close together in the immunodiffusion test (Fig. 1a). At lower protein concentrations the two lines were not resolved and a single diffuse line was just visible at a protein concentration of 100–150 $\mu\text{g}/\text{ml}$. With longer periods of diffusion an additional faint line appeared closer to the centre well (Fig. 1c).

Immunoelectrophoresis of a sodium chloride extract gave the pattern shown in Fig. 2 (a). These precipitin arcs stained with Oil Red O showing the presence of lipid. The positions of the arcs varied slightly with the concentration of egg protein applied.

Separation of 'phosvitin-lipovitellin' and lipovitellin from a saline extract by dilution and ammonium sulphate fractionation gave products

Fig. 2. Precipitin arcs stained with nigrosine after immunoelectrophoresis of (a) extract of cod eggs; (b) serum from a mature female cod; serum from an oestradiol-injected immature cod gave the same pattern.

with immunochemical properties similar to those of the crude extract and no evidence from the compositions (Table 1) for any substantial quantity of the phosphorus-rich protein phosvitin. The substances giving the two main precipitin lines on immunodiffusion and immunoelectrophoresis therefore appear to be two similar lipovitellins, containing 21% of lipid, most of which was phospholipid.

Gel-filtration of an extract of cod eggs on Sephadex G-200 gave a fractionation curve with two peaks of E_{280} (Fig. 3). The first (K_{av} 0.11) was due to a protein of molecular weight about 400 000, which gave similar results to the crude extract in the immunodiffusion and immunoelectrophoresis tests. The second peak (K_{av} 1.0) was due to a material of low molecular weight. Lipovitellin prepared from the extract gave the first peak on gel filtration but not the second. No resolution of the two main components found in the immunological tests was obtained by this procedure.

Chromatography of a sodium chloride extract on TEAE-cellulose also gave a fractionation curve with two peaks of E_{280} (Fig. 4), corresponding to 310ml and 540ml of the eluting mixture, together with some material in the void volume. As judged by the intensity of the precipitin lines on immunodiffusion, fractions from the first peak were rich in

Fig. 3. Fractionation curve obtained by gel filtration of an extract of cod eggs on Sephadex G-200.

Table 1. *Composition of cod egg proteins*

Percentages of phospholipids were calculated from 25 $\times$ percentage of lipid P.

	Protein (%)	Lipid (%)	Protein N (%)	Protein P (%)	Lipid P (%)	Phospholipid in lipid (%)
'Phosvitin-lipovitellin'	77.9	22.1	14.8	0.75	2.58	64
Lipovitellin	79.8	20.2	15.3	0.60	2.64	66
Lipovitellin, prep. A	78.9	21.1	17.0	0.47	—	—
Lipovitellin, prep. B	78.2	21.8	16.6	0.41	—	—

Fig. 4. Fractionation curves obtained by the chromatography of an extract of cod eggs on TEAE-cellulose. ●, E_{280} ; ■, concentration of phosphorus.

the component giving the outer precipitin line of the pair and those from the second peak were rich in the component giving the inner precipitin line of the pair, but separation of the two components by TEAE-cellulose chromatography was only partial. Determinations of alkali-labile phosphorus in the fractions gave maxima corresponding to the E_{280} peaks (Fig. 4) but no maximum in the expected position of phosphovitin.

Quantitation of the immunodiffusion test. With the material purified by TEAE-cellulose chromatography, the last visible precipitin line in the immunodiffusion test was given by a solution containing 102 µg of protein/ml. Corresponding values for preparations A and B of lipovitellin, the protein contents being determined by weighing, were 145 and 127 µg/ml respectively. A mean value of 125 µg/ml has been used to convert the results in the immunodiffusion tests into concentrations of egg proteins, which are given in terms of the protein component. These values are similar to those obtained with a crude sodium chloride extract (see above) and indicate that the main components in the extract are lipovitellins.

Egg proteins in the serum of normal cod

Identification. Serum from female cod of maturation coefficient greater than 0.75 gave the immunodiffusion pattern shown in Fig. 1(b), and more prolonged diffusion with an extract of cod eggs in an adjacent well showed that the corresponding lines given by the serum and the extract were confluent (Figs. 1c and 1d). Further dilution of the serum before testing resulted in a single diffuse precipitin line in place of the outer two lines and no inner line. Serum from immature female fish and from immature or maturing male fish gave no precipitin lines in this test.

Immunoelectrophoresis of serum from mature female fish gave the pattern shown in Fig. 2(b). The

Fig. 5. Extinction at 280nm of fractions obtained by gel filtration on Sephadex G-200 of serum from an immature male cod. (a) Curve A (●), before and curve B (▲), 10 days after an intramuscular injection of 1mg of oestradiol benzoate/kg. (b) Curve C (○) is the difference curve (B-A), curve D (Δ), a theoretical curve for egg proteins with the same maximum extinction as curve C (see the text) and curve E (□) is the difference curve (C-D), showing the loss of normal serum proteins.

two strong arcs corresponded in shape and approximately in position to those obtained with an extract of cod eggs, and also stained for lipid. These two principal arcs were absent when serum from immature female or from immature or maturing male fish were tested, but the fainter arc nearer to the anode could sometimes just be seen. Female fish of intermediate maturity gave essentially the same results as were obtained with females of high maturation coefficient but, as with the proteins from cod eggs, the main arcs were nearer to the anode with the low concentrations of egg proteins present in these fish.

Gel-filtration of serum from immature cod on Sephadex G-200 gave the fractionation curve shown in Fig. 5(a), curve A. The peak at the void volume

Fig. 6. Concentrations of egg proteins in the serum of female cod with developing ovaries. Maturation coefficient = $100 \times \text{wt. of gonads/wt. of whole fish}$. Logarithmic scales are used on both axes.

(fraction 19) and the inflexion at fraction 31 (K_{av} 0.33) were due to components precipitated by half-saturation with ammonium sulphate, presumably globulins, whereas the main peak, K_{av} 0.17 corresponding to a molecular weight of about 250 000, was due to a component soluble in half-saturated ammonium sulphate, presumably an albumin. Serum from female cod of high maturation coefficient gave a fractionation curve similar to Fig. 5(a) curve B. Egg proteins in the fractions were detected by immunodiffusion, and serial dilution showed that the highest concentrations were present in fractions 21 and 22, corresponding to the position of the egg-protein peak found with extracts of cod eggs and to the minimum between the two main peaks obtained with serum from immature cod.

Concentration. The concentration of egg proteins in the serum of female cod of various maturation coefficients is shown in Fig. 6. The sera from 20 immature female fish, of maturation coefficient <0.5 , were tested and gave no precipitin line in the immunodiffusion test, indicating an egg-protein concentration $<0.125 \text{ mg/ml}$. With mature female cod, an egg-protein concentration of about 32 mg/ml was found.

Egg proteins in the serum of immature, oestradiol-injected cod

Identification. Samples of serum obtained from immature male or female cod 5 or 10 days after an intramuscular injection of 1 mg of oestradiol benzoate/kg body wt. gave the immunodiffusion pattern shown in Fig. 1(b) and the pattern shown in Fig. 2(b) after immunoelectrophoresis, the same as those found for the serum of normal female cod with developing eggs. Blood samples taken before injection or after an injection of carrier oil, gave a

Fig. 7. Concentrations of egg proteins in the serum of immature cod (on a logarithmic scale) at different times after an intramuscular injection of 1 mg of oestradiol benzoate/kg. Values with arrows indicate that egg proteins were not detectable, and their concentration, if present, would be $<0.125 \text{ mg/ml}$.

negative immunodiffusion test, but with immunoelectrophoresis the faint arc nearer to the anode was sometimes found. Gel filtration of the serum gave the curves shown in Fig. 5(a), curves A and B, before and after the oestradiol injection. The extinction maximum of the difference curve C (Fig. 5b) corresponded in position to that found with an extract of cod eggs (Fig. 3) thus confirming the presence of egg proteins in the serum of injected fish.

Concentration and days after injection. Fig. 7 shows the increase in concentration of egg proteins in the sera of immature cod with time after injections of 1 mg of oestradiol benzoate/kg. Blood samples taken before injection gave no reaction in the immunodiffusion test. One day after injection, one positive result was obtained from four fish tested. Two days after injection and thereafter the serum of all fish contained egg proteins, the maximum concentration, 32 mg/ml , being found 10 days after injection, with indications that slightly higher values might be found after longer time-intervals. Values for immature male and female cod are included in Fig. 7 since, within the accuracy of the immunodiffusion test, there was no difference between the sexes.

Concentration and dose of oestradiol. Fig. 8 gives the concentrations of egg proteins in the sera of immature fish 10 days after they were given injections of various quantities of oestradiol benzoate. Blood samples taken before administration of the hormone gave no indication of the presence of egg proteins. With $10 \mu\text{g}$ of oestradiol

extinction at the peak is the same as that at the peak of curve *C*, we obtain curve *D* (Fig. 5b), a theoretical curve for egg proteins present in the serum. It can then be seen that the lower extinctions on each side of the peak in curve *C* are due to a fall in the normal serum components with peaks in the fractionation curve at fractions 19 and 24, since the difference curve *C* - *D*, drawn as curve *E* (Fig. 5b) shows peaks at these two positions. Based on the extinctions at the peaks of curves *E* and *A* for the six fish, about 50% of the high-molecular-weight globulin (peak at fraction 19) is lost after oestradiol injection and about 20% of the albumin (peak at fraction 24). This result is contrary to the assumption made in obtaining curve *C*, and the extinction values of this curve should be higher. However, the peak of curve *C* corresponds to a minimum in curve *A*, thus decreasing the error, and no further corrections were made in calculating the concentrations of egg proteins given in Table 2.

From the difference curves *C* (Fig. 5b) for nine cod, before correction to the true shape for egg proteins, seven out of nine results showed a negative difference of extinction at the first globulin peak, indicating a decrease in concentration after oestradiol treatment, one result gave a positive difference and one result no difference. With the extinction at the albumin peak (fractions 24 and 25) oestradiol treatment resulted in a lower extinction in three instances, a higher extinction in five and no change in one, again with no correction for the true shape of the curve for egg proteins.

Egg proteins in liver, ovaries and testes

The livers of six immature control cod, together with four ovaries and two testes, gave no indication of the presence of egg proteins in the immunodiffusion test. Ten days after an injection of oestradiol benzoate (1 mg/kg), egg proteins were detected in the livers of all the six cod of Table 2 at a concentration of about 2.5 mg/g, and in the testes of two of them at one-half this concentration. The ovaries of the other four fish did not give the precipitin lines corresponding to the principal egg proteins, but gave lines nearer to the centre well, which were also found with liver and testes. With a longer time for diffusion, the main precipitin lines of liver and testes (Figs. 1e and 1g) joined with the lines obtained with 10-day serum samples from the same fish and with a saline extract of cod eggs, whereas with ovary (Fig. 1f) the pattern of lines obtained with serum and egg samples in adjacent wells indicated the absence of the main components. The inner precipitin lines obtained with liver, ovary and testes joined with similar lines from serum and an extract of eggs (Fig. 1).

Livers from two normal mature female cod were also found to contain egg proteins by the immuno-

diffusion test, the concentrations being about 2.5 mg/g.

DISCUSSION

The values given here for dry matter and protein content of the ovaries show that for a female cod of 4 kg wet wt., containing about 800 g of dry matter and with a maturation coefficient of 10, some 100 g of egg proteins are present in the ovary. These specialized proteins are synthesized over a period of 3 or 4 months, and biochemically the system is noteworthy for the control mechanisms involved, in which the gonadotrophins and female sex hormones are no doubt involved.

Most of the proteins of cod eggs can be extracted with 0.5 M-sodium chloride, but a small proportion is also extracted with water. Immunochemical methods show the presence of two major components with very similar properties, that were not resolved by gel filtration on Sephadex G-200 but were partially resolved on TEAE-cellulose. Jared & Wallace (1968) had previously examined the yolk proteins of 17 species of teleost fish by chromatography on TEAE-cellulose and obtained useful separations with about one-half of the samples. Since this method did not prove suitable for a large-scale purification of the cod egg proteins the precipitation procedure of Wallace *et al.* (1966) for trout eggs was used. Dilution of the saline extracts of eggs with water is reported to give a co-precipitate of phosvitin-lipovitellin, the two components being separated by ammonium sulphate fractionation (Wallace *et al.* 1966). With cod eggs, however, we found little or no evidence of the phosphorus-rich protein phosvitin. It did not appear in the chromatography on TEAE-cellulose nor in the solution remaining after precipitation of lipovitellin. Jared & Wallace (1968) also found relatively small amounts of phosvitin in a number of marine fish. Mano & Lipmann (1966) have prepared phosvitin from cod roe, but examination of their values indicates that it is a very minor component, comprising less than 1% of the egg proteins.

The two major components of cod egg proteins are therefore lipoproteins with a molecular weight of about 400 000, containing about 21% lipid, some two-thirds of which is phospholipid, and about 0.5% of protein phosphorus.

Differences in the serum proteins of male and female salmon have been detected by electrophoresis (Vanstone & Ho, 1961; Drilhon & Fine, 1963) and by immunological methods (Ridgway, Klontz & Matsumoto, 1962; Fine & Drilhon, 1963) and have been related to the presence of egg proteins in the serum of female fish with developing ovaries. Utter & Ridgway (1967) made an immunological study of the occurrence of egg proteins in kidney fluid and blood serum of English sole (*Parophrys*

vetulus) and of Pacific halibut (*Hippoglossus stenolepis*) and related the frequency of occurrence to the size of the fish and the weight of the ovaries. The occurrence of egg proteins in the serum of mature female cod with developing ovaries is established here by the immunological similarity to the proteins derived from cod eggs and by the similar behaviour on gel filtration. There were some slight differences in the position of the precipitin arcs obtained after immunoelectrophoresis of serum and an extract of cod eggs, but we have already mentioned that the position of these arcs is dependent on the concentration of the protein solutions used, and may be related to the solubility of the high-molecular-weight lipoproteins in the electrophoretic medium of low ionic strength. It has also been suggested (Jared & Wallace, 1968) that a lower phosphate content of egg proteins in the serum than in the eggs may account for their solubility in serum. The semi-quantitative determination of the egg proteins in serum used here could be refined, but it is sufficient to show that the concentration of egg proteins in the serum of cod with maturing ovaries increases with increase in maturation coefficient. By the use of small blood samples from live fish, the method could be used to establish the sex, and the state of development of the ovaries, of maturing cod.

We used an intramuscular injection of oestradiol benzoate in oil to give a slow release of hormone over a period of time. Parkes (1937) has shown with capons that a similar injection of 1 mg of oestradiol 3-benzoate gave a release of hormone over 9 days. If this is so with cod, and our experiments show a higher concentration of egg proteins in the serum of injected fish 10 days after injection than at 5 days after injection, then the dose of 300 μ g of oestradiol benzoate/kg is roughly equivalent to 33 μ g of oestradiol benzoate/kg per day and to the dose of 10 μ g/day for an adult rat commonly used by others (Hamilton, 1968). The minimum dose of hormone to produce detectable egg proteins in the serum after 10 days was 30 μ g of oestradiol benzoate/kg, and the maximum concentration of egg proteins was given by a dose of 300 μ g/kg, 1 mg/kg giving similar results.

With an injection of 1 mg of oestradiol benzoate/kg, egg proteins were detected in the serum of cod after 2 days and their concentration increased rapidly during the next 3 days and more slowly over the following 5 days. The time lag before appearance of the egg proteins in serum is probably dependent on the mode of administration. Munday, Ansari, Oldroyd & Akhtar (1968) gave an intramuscular injection of oestradiol to toads and found a new calcium-binding protein in the serum after 4 days, and with cod injected intramuscularly, Plack & Pritchard (1968) found an increase in plasma retinal

3 days after injection. An intraperitoneal injection into goldfish gave increases in a number of serum components after 1 day (Bailey, 1957) and a subcutaneous injection of diethylstilboestrol into male chickens gave rise to a plasma phosphoprotein after a lag of 1 day (Greengard, Gordon, Smith & Acs, 1964).

By the use of oestradiol injections, we establish a system in which the normal hormonal sequence of secretion of hypothalamic hormones, controlling pituitary gonadotrophins, which in turn control secretion of oestrogens, is missing. If we apply our experimental results to normal fish, then the oestrogens, or an unknown subsequent hormone or metabolite, are responsible for the control of the synthesis of egg proteins. In the experimental fish, in the absence of the gonadotrophins, there is no uptake of egg proteins by the ovary. It might therefore be expected that the concentration of egg proteins in the serum of injected immature fish would be higher than in mature female fish, as was found with retinal (Plack & Pritchard, 1968), but the values obtained were similar to those found in normal mature female cod and of the order of 32 mg of protein/ml. This concentration of egg proteins seems high in relation to the total serum proteins after injection with oestradiol (Table 2) and represents about one-half of the total, as was found by Wallace & Jared (1968) for the toad *Xenopus laevis*. The high concentration of egg proteins in the serum was confirmed by the difference curve from gel filtration of serum before and after injection of oestradiol (Table 2). Since the immunodiffusion test is only accurate to a factor of two, the results from the difference curves are in good agreement.

The occurrence of egg proteins in the liver of oestradiol-injected fish but not in the ovaries suggests that egg proteins are synthesized in the liver and transported in the serum to the ovaries. Experiments with liver slices (Plack & Fraser, 1971) confirm that the liver of oestradiol-injected fish is able to synthesize egg proteins. Munday *et al.* (1968) showed that hepatectomy of *Xenopus laevis* prevented the synthesis of a calcium-binding protein after oestradiol injections.

The presence of egg proteins in the testes of injected fish is puzzling and it may be due to the serum content of the tissue. Ronald, Macnab, Stewart & Beaton (1964) found that blood plasma constituted 1.8% of the weight of Atlantic cod. If the serum contained 32 mg of egg proteins/ml and was equally distributed throughout the tissues, then 1 g of tissue would contain 0.576 mg of egg proteins. Extraction of the proteins by making a 20% homogenate would give a solution in which the concentration of egg proteins would be at the limit of detectability by the immunodiffusion test. However, the white muscle of cod contains very

little blood, and it is conceivable that the testes contain twice the average content of serum, which would be sufficient to explain the results obtained. By the same reasoning, however, the ovaries might be expected to contain egg proteins due to their content of serum, but they were not found.

The fall in concentration of the albumin and globulin components of the serum of oestradiol-injected fish with the appearance of egg proteins suggests that oestradiol is directing the synthesis of specific proteins rather than acting as a general protein stimulant. However, another control mechanism other than the rate of synthesis may be interposed to regulate the concentration of serum proteins.

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