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Characterization of an Estradiol-Induced Protein from Rainbow Trout Serum as Vitellogenin by the Composition and Radioimmunological Cross Reactivity to Ovarian Yolk Fractions[1]

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ABSTRACT

An estrogen-induced protein has been isolated by a simple method from plasma of rainbow trout *(Salmo gairdneri)* and an antibody against it was raised in a rabbit. Three fractions were isolated from trout ovaries using the method for isolation of lipovitellin and phosvitin of Wallace et al. (1966). Two factors crossreacted with the antibody and could be displaced by serum from vitellogenic trout only, suggesting that the isolated serum fraction was trout vitellogenin. Biochemical analysis established similarities between vitellogenin, lipovitellin and phosvitin from trout and other oviparous species. A radioimmunoassay for vitellogenin has been developed for rainbow trout using vitellogenin as standard and gonad lipovitellin or β' component as radioligand. In this system little cross reactivity was found with vitellogenin rich sera from closely related *Salmo salar*.

INTRODUCTION

The presence of serum protein, specific to reproductive female fish, has often been detected (Ho and Vanstone, 1961; Fine and Drilhon, 1963; Thurston, 1967; Utter and Ridgeway, 1967; Aida et al., 1973; Heesen and Engels, 1973) and the origin of this protein has been shown to be the liver (Plack and Frazer, 1971).

The hypothesis of hepatic synthesis of vitellogenin and subsequent transport to the ovaries has been advanced for Amphibia (Follett et al., 1968; Wallace and Dumont, 1968) and for Aves (for review see Bergink and Wallace, 1974). Support for a similar hypothesis applicable to teleosts has come from the work of Campbell and Idler (1976), Ng and Idler (1978) and Campbell (1978), who investigated the incorporation of estrogen-induced serum protein into oocytes, and from the study of estrogen effects on teleost liver (Kobayashi, 1953; Egami, 1955 and Upadhyay, 1978) and estrogen induction of vitellogenin synthesis (Plack and Frazer, 1971; Emmersen and Petersen, 1976; Hara and Hirai, 1978). A response to estradiol was generally found in both sexes.

Immunological cross reactivity between ovarian extracts and the female specific serum protein was shown by Utter and Ridgeway (1967), Plack et al. (1971), Amirante (1972), Idler et al. (1979) and Hara and Hirai (1978) but for the most part "vitellogenin" has been quantified using alkali labile phosphorus or some other index of phosphoprotein content.

The present study was undertaken to isolate, in its native form, the vitellogenin and gonad yolk fractions from rainbow trout. This preparation has already been evaluated physiologically (Campbell, 1978) and was used radioimmunologically to confirm the relationship of the female specific serum protein, vitellogenin, to these ovarian yolk proteins. The development of a radioimmunoassay for rainbow trout vitellogenin was deemed necessary since the system previously described (Idler et al., 1979) for salmon yolk did not yield parallelism of displacement with rainbow trout plasma. The assay for trout vitellogenin was used to examine immunological cross reactivity with vitellogenin in other species.

MATERIALS AND METHODS

Induction of Vitellogenesis

Ten male rainbow trout (∿1 kg BW) were maintained in a partial recirculating raceway system at

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20°C. These fish were injected at 3 day intervals into the belly musculature or subdermally with 5 mg/kg/0.5 ml estradiol [E_2, 1,3,5(10)-Estratrien-3,17β diol] dissolved in peanut oil.

Blood samples were taken at the time of each injection; after 19 days a large volume of blood was collected from a caudal blood vessel or by cannulating the dorsal aorta. After clotting and centrifugation, serum was collected and $CaCl_2$ to 20 mM was added before storage at −20°C. Redshaw and Follett (1976) found that addition of calcium to chicken serum was essential for preservation of vitellogenin intact.

A total of 95 rainbow trout (20–25 g) were acclimated to water at 11.5°C and each injected i.p. with 110 μg estradiol benzoate in 50 μl peanut oil. Injections were performed every 3 days with new groups of fish being added so that 4 groups of fish could be killed simultaneously to give samples for fish 24 h after treatment, 24 h after a second injection, 24 h after a third and 24 h after a fifth injection. Serum was collected, diluted (1:2) with Vg buffer (0.25 M NaCl, 20 mM $CaCl_2$, 50 mM Tris, pH 8.0) and stored at −20°C.

Isolation of Vitellogenin

Twenty-five ml Ca^{2+} treated plasma were thawed, centrifuged and filtered and 20 ml of this added to 600 ml cold (4°C) distilled H_2O saturated with phenyl methylsulfonylfluoride. This mixture was stirred overnight before centrifugation. The supernatant was concentrated for examination by electrophoresis. The precipitate was dissolved in 10 ml Vg buffer and the soluble fraction was diluted with cold distilled water to reduce NaCl to 0.005 M. After 10 min the precipitate was collected by centrifugation and redissolved in Vg buffer. When the extract was run on TEAE-cellulose in a linear gradient of citric acid-2 amino propanol (Campbell and Jalabert, 1979), a single adsorbed protein peak resulted. The eluate was adjusted to 20 mM $CaCl_2$, concentrated on an Amicon XM 100 membrane, applied to a 2.6 × 127 cm column of Ultrogen AcA 22 and eluted with Vg buffer. In subsequent preparations the TEAE cellulose step was eliminated to reduce losses due to reduced solubility of the product. Elution of protein was followed by monitoring absorbance at 280 nM.

Isolation of Lipovitellin and Phosvitin and β′ Component

Ovaries which contained oocytes undergoing germinal vesicle migration were collected from 4 rainbow trout. After storage at −20°C for 1 year, 5 g of each were extracted with 0.5 M NaCl and 0.005 M EDTA and the yolk complex was precipitated by dilution to 0.005 M NaCl. The precipitate was redissolved, precipitated once more and then treated with anmonium sulphate (Wallace et al., 1966) to separate phosvitin and lipovitellin. Lipovitellin was dissolved and precipitated a second time from $(NH_4)_2SO_4$.

Phosvitin was chromatographed on a 1.6 × 70 cm column of Ultrogel AcA 54 and lipovitellin on a 2.6 × 80 cm column of Ultrogen AcA 22, using Vg buffer. The low molecular weight peak material obtained in this last stage was rechromatographed on AcA 54 to prepare the β′ component.

Electrophoresis

A comparative approach was used to follow the induction of yolk protein into the serum of trout. Disc electrophoresis in a 6% gel, using Tris-glycine was used to examine 4 μl serum diluted to 40 μl. Isolated protein fractions were tested at concentrations of ∿100 μg. Proteins (with the exception of phosvitin) were stained by amido black or Coomassie Blue. The molecular weight of vitellogenin was determined using a polyacrylamide gradient gel slab (PAA 4/30, Pharmacia Fine Chemicals) and high molecular weight calibration kit.

Phosphorus Determination

Alkali labile phosphorus concentration was determined on 50, 100 and 200 μg aliquots of vitellogenin and lipovitellin and on 5, 10 and 20 μg aliquots of phosvitin; the methods were based on Chen et al. (1956).

Protein Determination

Protein concentrations were measured by the Hartree (1972) method employing an overnight trichloracetic acid protein precipitation for all samples, except isolated phosvitin which was determined without precipitation. Bovine serum albumin was used for the reference standard.

Lipid Determination

Total lipids were extracted from 200 μg lipovitellin or vitellogenin and from 1 mg phosvitin using a modification of the Bligh and Dyer (1959) method. Quantification was performed using the charring method of Marsh and Weinstein (1966). The standard was tripalmitolein.

Analytical Ultracentrifugation and Amino Acid Analysis

A preparation of vitellogenin was tested in an analytical ultracentrifuge (Spinco, Model E, AN-D rotor) using 20 mM NaCl, 3 mM $CaCl_2$ as solvent (Chervenka, 1969). Samples of vitellogenin, lipovitellin, phosvitin and β′ component were hydrolyzed in 6N HCl and the amino acid analysis performed on a Beckman analyzer 121 MB and computed by a systems amino acid integrator. Vitellogenin was analyzed after hydrolysis for 24, 36, 48 and 72 h, lipovitellin and phosvitin after 24 and 48 h and β′ component after 24 h.

Radioimmunological Procedures

Rainbow trout. A rabbit was injected subdermally with 475 μg vitellogenin plus Freund's complete adjuvant. Three monthly injections of 2 mg were given after an initial 2 month wait. Six weeks later, a booster injection of 2 mg with Freund's incomplete adjuvant was given i.m. and the final bleeding was performed

after 4 weeks. The antibody titer was checked periodically using [^{131}I]-labeled lipovitellin.

Since vitellogenin has proved difficult to iodinate (Redshaw and Follett, 1976; Idler et al., 1979) attempts were made to iodinate vitellogenin using the microelectrolytic method of Donabedian et al. (1972) in a platinum crucible (50 μl) using a platimum cathode as stirrer and current of 20 μA for 45 min. The method of Greenwood et al. (1963) was attempted for iodination of lipovitellin, phosvitin, vitellogenin and β′ component.

Radioimmunological testing was carried out in Vg buffer containing 0.5% BSA, 0.01% thiomersal (ethylmercurithiosalycilate). Assay volume was normally 300 μl: 100 μl sample, 100 μl containing 10,000 cpm iodinated protein and 100 μl antibody diluted 1: 65,000. After 48 h at 4°C, 100 μl normal rabbit serum and goat anti-rabbit γ-globulin antibody was added and 17 h later the precipitated complex was counted. Vitellogenin (or occasionally lipovitellin) was used as a standard from 1 ng to 1 μg in routine radioimmunoassay using [^{131}I]-labeled lipovitellin or β component.

Salmon. The radioimmunoassay for salmon *(Salmo salar sebago)* vitellogenin was described by Idler et al. (1979). A low molecular weight gonad yolk fraction was labeled and reacted with antisera to preparation of salmon vitellogenin and egg yolk.

RESULTS

Induction of Vitellogenin Synthesis

The data presented in Table 1 shows that upon treatment with estradiol, there was a rapid increase in total serum protein. Disc electrophoresis of constant volumes of serum (4 μl) showed that there was 1 band of protein which increased during the course of estradiol treatment at the expense of other proteins to the extent that faster migrating proteins were strongly reduced in absolute concentrations (Fig. 1A).

Isolation of Vitellogenin

A serum pool, which was heavily enriched in vitellogenin, containing 2625 mg protein yielded 2500 mg protein precipitate upon dilution with distilled water; 70 mg protein was left in the supernatant and 8 mg was not extracted from the pellet with Vg buffer. When the Vg buffer solubilized-precipitate was diluted a second time, the quantity of protein remaining in the supernatant was 41 mg and the residue upon reextraction contained only 4 mg. When half of the redissolved precipitate was chromatographed on TEAE cellulose followed by Ultrogel AcA 22, 485 mg vitellogenin was isolated. This represents a recovery of 57% of original protein as vitellogenin with no estimation of procedural losses. Figure 1B shows disc electrophoresis gels which demonstrate clearly that the first precipitation is an extremely effective purification step since most serum proteins remained in solution and vitellogenin was the principal component precipitated (a vs b).

Vitellogenin was not effectively purified during TEAE chromatography using the present conditions when starting with the fraction enriched by precipitation. Upon Ultrogel AcA 22 chromatography, a small higher molecular weight peak preceded elution of vitellogenin at MW 440,000 daltons.

Isolation of Lipovitellin and Phosvitin

Starting from frozen tissue approximately

TABLE 1. Changes in serum protein concentrations after treatment with estradiol. A) 2 mg/kg/2 days, i.p. and subdermal; B) 5 mg/kg/3 days, subdermal; all ♂ fish.

	Serum protein concentration (mg/ml)						
	Day						Vitellogenin isolated
	1	2	4	6	8	10	
A)							
Peanum oil injected (1 ♂ + 2 ♀)	39 ± 3	41 ± 1	36 ± 1	49 ± 1	48 ± 2	56 ± 6	1 mg/ml
E_2 injected (2 ♂ + 1 ♀)	43 ± 2	43 ± 2	48 ± 4	54 ± 6	67 ± 9	82 ± 8	17 mg/ml
	Day						
	1		6	9		12	18
B)							
Protein (mg/ml)	55 ± 3 (10)[a]		77 ± 2 (7)	103 ± 6 (7)		117 ± 6 (6)	105 ± 14 (4)

[a]Numbers of fish in parentheses.

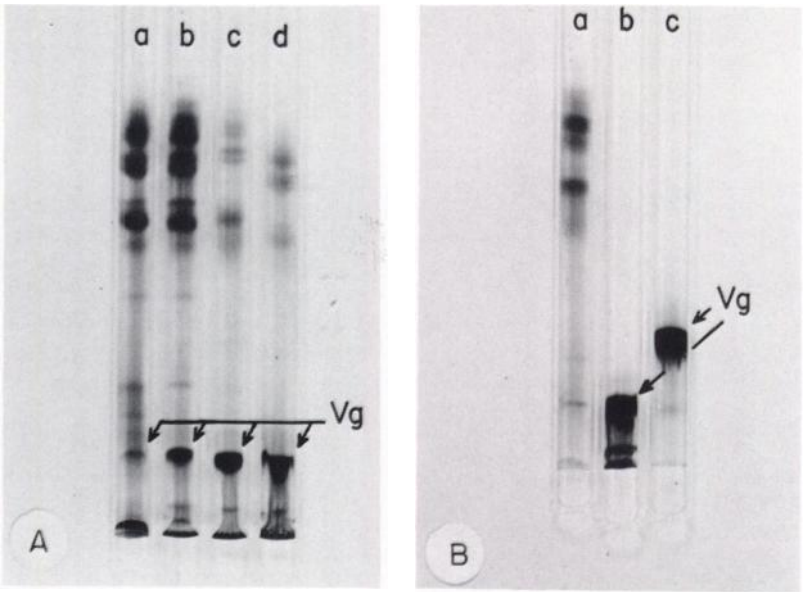

FIG. 1. A) Disc electrophoresis of 4 μl sera from a typical estrogen treated male trout (Table 1B) sampled a) before injection, b) 6 days, c) 12 days and d) 19 days after initiation of treatment. (Vg, vitellogenin).

B) Disc electrophoresis of samples of: a) a concentrate of the supernatant left after precipitation of vitellogenin by 30-fold dilution; b) the precipitate dissolved in Vg buffer; and c) vitellogenin isolated from this precipitate by TEAE cellulose and Ultrogel chromatography. Samples (a) and (b) were performed in one electrophoresis; (c) was developed separately.

equivalent to 8 g dry weight from ovaries of 4 fish, a total of 2.9 g lipovitellin enriched powder was produced and ∿100 mg phosvitin fraction. During chromatography on Ultrogel AcA 22 a principal lipovitellin peak (290,000 daltons) was followed by a small peak of lower molecular weight. This small peak isolated as a byproduct during chromatography of lipovitellin was essentially homogeneous when rechromatographed on AcA 54; it has a molecular weight of 21,000 daltons and corresponded to the β' component of Markert and Vanstone (1971). The phosvitin fraction yielded 1 protein peak upon elution from AcA 54 (MW 45,000 daltons). After electrophoresis no contaminating protein bands were observed when gels on which phosvitin had migrated were stained with Coomassie Blue or amido black.

Biochemical Characterization of Fractions Isolated as Vitellogenin, Lipovitellin and Phosvitin

Both vitellogenin and the total ovarian yolk were insoluble when extracts were diluted to 5 mM NaCl and 0.4 mM $CaCl_2$, but after $(NH_4)_2SO_4$ precipitation of lipovitellin, to separate the ovarian yolk complex into lipovitellin and phosvitin, the lipovitellin fraction became soluble in distilled water. On one occasion 20 mM $CaCl_2$ was used to make a 30-fold dilution of serum in the hope of obtaining a vitellogenin enriched precipitate, but no precipitate formed even after sufficient EDTA was added to reduce the effective Ca^{2+} concentrations to 5 mM. Phosvitin was soluble in 12% trichloracetic acid.

Lipid and phosphorus contents (Table 2) show the phosvitin fraction was rich in phosphorus and very poor in lipid and conversely the lipovitellin was rich in lipid and poor in organic phosphorus. Calculations based on phosphorus concentration of phosvitin suggested that vitellogenin contained 4% phosvitin and the lipid concentration of lipovitellin suggests that lipovitellin could account for 85% of the vitellogenin molecule. The phosvitin recovered from the ovarian yolk complex was 3% of protein isolated.

Analytical ultracentrifugation gave peaks at 11.6 S, 18.9 S and 23.8 S for vitellogenin. After hydrolysis the amino acid analysis of vitellogenin, lipovitellin and phosvitin yielded data for

TABLE 2. Lipid and phosphorus concentration in isolated serum fraction corresponding to vitellogenin and yolk fractions lipovitellin and phosvitin.

Protein	Total lipid			Alkali labile phosphorus		
	Protein (μg)	Lipid (μg)	%	Protein (μg)	P (μg)	%
Vitellogenin	188	40.4	21.5	154	0.9	0.6
Lipovitellin	288	57.9	25.4	2800	0.19	0.007
Phosvitin	1000	ND[a]		10.8	1.7	15.8

[a]ND, not detectable.

comparison with these proteins from other species (Table 3).

The molecular weight of vitellogenin was determined as 470,000 dalton by gradient gel electrophoresis.

Radioimmunological Data

During the period that the rabbit received monthly injections of vitellogenin, 50% binding (of 10,000 cpm of [^{131}I]-labeled lipovitellin) was given by an antibody dilution of ∿1:20,000. Ten weeks of recovery from primary immunization followed by a single booster injection resulted in titers as high as 70% binding at 1:160,000 dilution but, at final bleeding 1 month after boosting, the titer had declined to 50% binding at 1:80,000 antibody dilution.

Attempts to iodinate vitellogenin with ^{131}I using electrolytic reduction in a platinum microcrucible yielded a small amount of poorly labeled material which exhibited no cross reaction with the antibody, nor was vitellogenin successfully labeled using the Chloramine T oxidation method. Phosvitin was poorly labeled and showed no cross reactivity with vitellogenin but lipovitellin labeled well, giving up to 94% binding with an antibody dilution of 1:5000 and nonspecific binding in the absence of antibody of ∿10%. Some preparations of lipovitellin labeled well but the nonspecific binding was too high for use in a radioimmunoassay.

A preparation of β' component was successfully labeled using the Greenwood technique; excess antibody bound 90% of this label and in the absence of antibody nonspecific binding was 10%.

Passage of [^{131}I]-labeled lipovitellin on a "clean up" column of AcA 34 yielded a major peak of radioactivity followed by a small peak. No difference in binding was observed when aliquots of labeled material from throughout the principal peak were tested radioimmunologically. No difference in binding was found if the label was kept unfrozen, or frozen and thawed, for up to 1 week.

In the radioimmunoassay system of Idler et al. (1979) for salmon vitellogenin using a labeled yolk fraction as tracer and antibody against the ovarian yolk complex, no parallelism could be shown for rainbow trout vitellogenin or serum from vitellogenic rainbow trout (Fig. 2A). Using the same label, but with an antibody against a salmon plasma yolk component, a similar lack of parallelism was found (Fig. 2B). The trout phosvitin fraction was evaluated in the system used for Fig. 2A and no displacement of the labeled salmon material was found.

In the assay system for trout vitellogenin using [^{131}I]-trout lipovitellin and antibody to trout vitellogenin both lipovitellin and vitellogenin displaced the label but no displacement was given by phosvitin. Figure 3 shows that known quantities of one preparation of lipovitellin added to 3 trout vitellogenin radioimmunoassays were detected quantitatively from 1 to 250 ng but with an overestimation of 80% when expressed as vitellogenin. A second preparation of lipovitellin had similar characteristics of displacement but would be underestimated in the vitellogenin radioimmunoassay (Fig. 4).

When vitellogenin was used as standard from 4 ng to 1 μg, log/logit lines ($r^2 > 0.95$) were obtained with a slope of -2.1 ± 0.2 and an intercept of 3.9 ± 0.5 (mean ± SEM for 6 assays). Figure 4 shows that displacement curves for β' component, vitellogenin and lipovitellin were parallel when both labeled lipovitellin and labeled β' component were used. A sample assayed 5 times in duplicate in a

TABLE 3. Amino acid composition of rainbow trout vitellogenin, lipovitellin and phosvitin compared to published analyses (residues/100).†

	Vitellogenin				Lipovitellin		
	S. gairdneri		*X. laevis*	*G. domesticus*	*S. gairdneri*		*X. laevis*
	(a)	(b)	(c)	(d)	(a)	(b)	(c)
Aspartic acid	8.8	8.4	8.4	9.0	7.6	7.7	8.8
Threonine	5.1	5.0	5.5	5.0	5.4	5.3	5.6
Serine	7.4	7.5	11.7	19.0	5.0	4.8	8.1
Proline	4.8	5.2	4.8	4.9	5.4	5.4	5.0
Glutamic acid	9.2	11.5	13.2	10.3	9.3	11.6	13.8
Glycine	4.1	4.2	5.1	4.9	4.0	4.1	5.3
Alanine	11.3	11.7	8.5	7.4	12.7	14.2	10.2
1/2 Cysteine	1.6	1.2	1.3		1.2		0.9
Valine	7.9	7.1	4.6	6.3	8.3	7.7	5.1
Methionine	2.6	2.6	2.3	2.2	2.8	2.6	2.0
Isoleucine	5.7	5.5	3.6	5.3	6.0	5.9	4.1
Leucine	9.1	9.5	8.1	8.0	9.8	10.4	8.5
Tryrosine	2.9	3.0	3.0	3.0	2.8	2.9	3.0
Phenylalanine	4.0	4.0	3.6	3.1	4.4	4.6	4.2
Lysine	7.6	7.1	7.9	6.7	7.0	6.3	7.3
Histidine	2.3	2.1	2.9	3.2	2.3	2.2	2.9
Arginine	4.3	4.5	5.3	5.5	4.3	4.4	5.6
% Lipid	21.5		12.3		25.4		20.0
% Phosphorus	0.6		1.35	3.4	0.007		0.5
Molecular weight (Dalton X 10^{-3})	455	600	390	235	290	300	240

	Phosvitin							E_2	β'	Vitellin
	S. gairdneri		*S. trutta*		*O. keta*	*X. laevis*	*G. domesticus*	*S. gairdneri*		*L. migratoria*
	(a)	(e)	(f)	(g)	(h)	(c)	(h)	(b)	(a)	(i)
Aspartic acid	9.6	10	8	10	9	5.0	6.0	15.7	15.3	11
Threonine	1.8	2	2	2	1	0.7	0.9	4.0	4.5	4.8
Serine	42.0	54	57	50	57	56	57.9	10.9	7.9	7.5
Proline	4.6	3	3	5	3	3.0	1.4	3.2	3.1	5.9
Glutamic acid	3.7	3	3	4	3	10.1	5.6	10.0	7.8	12.6
Glycine	4.2	4	3	3	3	2.8	2.3	5.7	6.0	5.0
Alanine	2.4	4	3	2	3	2.2	3.7	3.5	3.5	8.4
1/2 Cysteine	0.2							2.7	0.9	1.0
Valine	0.2					0.6	1.4	7.8	9.4	7.7
Methionine	0						0.5	3.0	2.7	1.7
Isoleucine	2.3	2	2	2	3	0.3	0.9	5.0	5.8	5.1
Leucine	0.2	0.4				1.4	1.4	7.0	8.2	10.1
Tryrosine	1.0	2	1	2	1	0.9	0.5	3.9	6.0	5.0
Phenylalanine		1		1		0.7	0.5	1.7	2.1	2.9
Lysine	4.9	3	4	5	4	7.6	6.9	10.6	12.2	5.6
Histidine						2.8	5.6	2.3	2.6	1.5
Arginine	15.6	10	14	14	12	6.0	4.6	3.2	2.2	4.0
% Lipid	ND					Trace				
% Phosphorus	15.8	4		8.5		9.5				9.6
Molecular weight (Dalton X 10^{-3})	43		19	19		38	29,33	30	21	550

[†]a) This study; b) Hara and Hirai (1978); c) Redshaw and Follett (1971); d) Christmann et al. (1977); e) Ito et al. (1966); f) Mano and Yoshida (1969); g) Schmidt et al. (1965); h) Clark and Joubert (1974); i) Chen et al. (1978).

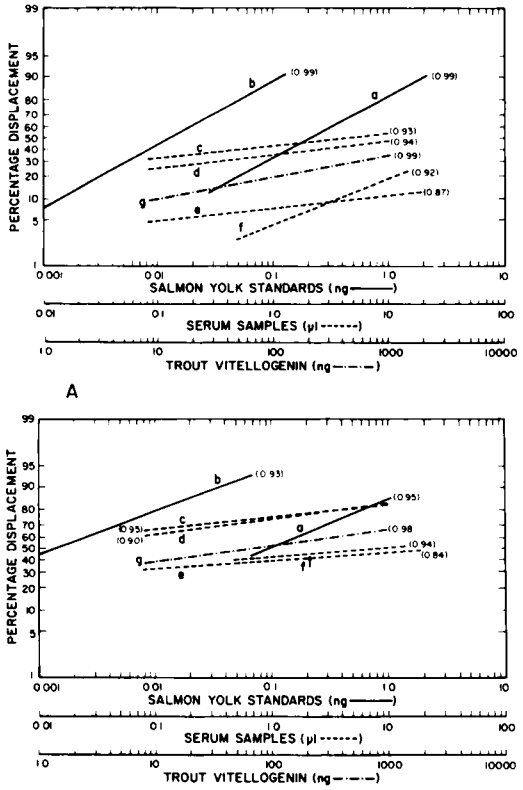

FIG. 2. A) Log dose/logit transformed displacement plots of serial dilutions: a) salmon gonad yolk standard; b) salmon plasma fraction "Biogel II" (Idler et al., 1979); c) serial dilutions of serum from estrogen treated rainbow trout ♀; d) dilutions of serum from a vitellogenic ♀ rainbow trout; e) dilutions of sera from nonvitellogenic ♀ trout; f) dilutions of plasma from vitellogenic ♀ flounder; g) trout vitellogenin. Assayed in the salmon vitellogenin assay of Idler et al. (1979). *Salmo salar* yolk fraction is radioligand and standard with an antibody to crude ovarian yolk; maximum binding 75%.

B) Log dose/logit transformed displacement plots of serial dilutions (see above) in the salmon vitellogenin assay system using an antibody against the salmon plasma Biogel II fraction (Idler et al., 1979). Maximum binding 50%.

Displacement curves for Figs. 2, 4 and 5 were established using 1:2 dilutions and numbers in parentheses are coefficients of determination for unweighted least squares fit of the data. The lines shown are least squares fit after 5 iterations to reduce the effect of outliers and show the range over which the transformed displacements were linear.

single assay was determined with a coefficient of variation of 0.12. The interassay coefficient of variation for 11 samples determined in 3 assays was 0.27.

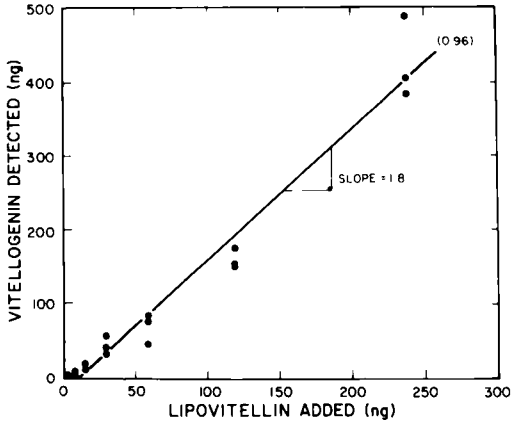

FIG. 3. Plot of "vitellogenin" detected when known amounts of lipovitellin were assayed in RIA using antivitellogenin, [^{131}I]-lipovitellin and vitellogenin as standard.

The radioimmunoassay system was attempted using 0.08 M barbital diluent, Vg diluent and low salt (0.15 M NaCl, 3 mM $CaCl_2$, 10 mM Tris, pH 8.0) diluent (buffer + 0.5% BSA, 0.01% Thiomersal). No marked differences in sensitivity or slope of the displacement curves was found. When the antibody and standards were preincubated for 24 h before addition of radioiodinated lipovitellin, 50% displacement was given by approximately a 7-fold lower concentration of standard, making the assay more sensitive.

When serum samples from 3 immature trout treated with a salmon pituitary gland extract for 2 weeks were assayed at dilutions corresponding to 4, 2, 1, 0.5, 0.25 and 0.125 µl, parallelism with displacement by standards was evident. Four dilutions of an internal standard serum from vitellogenic trout are shown parallel to vitellogenin standards in Fig. 4. Two µl serum from immature males treated similarly gave no detectable displacement.

Samples of serum from vitellogenic Pacific and Atlantic salmon were checked in the trout vitellogenin radioimmunoassay and near parallelism was found for samples from sockeye *(Oncorhynchus nerka)* and chinook *(O. tsawytscha)*. Samples from coho *(O. kisutch)* did did not yield parallel displacement and neither did those from Atlantic salmon sampled from a bay about 4 months before spawning and at a spawning channel (Fig. 5).

Radioimmunological determination of vitellogenin in serum from rainbow trout treated

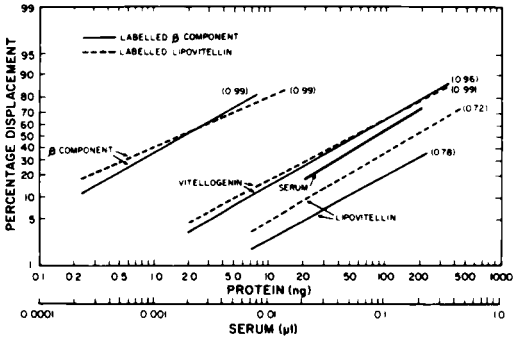

FIG. 4. Log dose/logit transformed displacement plots for displacement by vitellogenin, lipovitellin, and β' component in the assay systems anti-vitellogenin/[^{131}I]-lipovitellin component (– – –) and anti-vitellogenin/[^{131}I]-β' component (——). Maximum binding: β' component, 37%; lipovitellin, 36%.

with estradiol benzoate shows that immunoreactive vitellogenin increased with increasing length of hormonal treatment for both male and female fish (Fig. 6).

When female rainbow trout over 3 years old were sampled in October and 10 µl serum screened in this radioimmunoassay system, 9 fish which had serum vitellogenin concentration in excess of 100 µg/ml proceeded to ovulate normal eggs by February, 1 failed to ovulate and 4 fish with a mean vitellogenin content of only 36 µg/ml did not complete vitellogenesis.

In August, serum vitellogenin in 7 of these animals which ovulated the previous year was >>4.7 mg/ml, samples from 4 fish being in excess of 5.4 mg/ml. In early November, 5 vitellogenic fish had vitellogenin contents of 37, 22, 12, 13 and 2 mg/ml and in late December 7 similar fish had 13.2 ± 3.5 mg/ml circulating vitellogenin.

DISCUSSION

By using serum from heavily estrogenized trout, it was possible to isolate the serum vitellogenin complex in an apparently undenatured condition and high purity. Preliminary work had shown that use of the 0.5 M NaCl, 0.005 M EDTA (Plack et al., 1971) and the citric acid-monol buffers of Jared and Wallace (1968) tended to result in isolation of a barely soluble protein after purification. Redshaw and Follett (1976) added Ca^{2+} to plasma up to 20 mg/ml before precipitating chicken vitellogenin by dilution. In the present work, it was found that 3 mg/ml was adequate to avoid denaturation of vitellogenin. The integrity of this purified vitellogen was suggested by its use in experiments to study incorporation into vitellogenic oocytes (Campbell, 1978; Campbell and Jalabert, 1979) in which incorporation rates were similar to those found for *X. laevis* when vitellogenin-rich sera were used (Wiley and Dumont, 1978) and also by the finding that dilutions of standard vitellogenin produced displacement curves in a radioimmunoassay which paralleled those given by dilutions of serum from estrogenized fish (Fig. 4). Previous immunological studies have shown cross reaction between a serum factor, specific to reproductive females, and extracts of ovarian protein. Campbell and Jalabert (1979) showed that the antigen used in the present study was preferentially incorporated into vitellogenic oocytes confirming identity of this fraction as vitellogenin. Comparison of amino acid composition of this vitellogenin and the FS fraction (Hara and Hirai, 1978) shows them to be presumably the same protein (Table 3). Similarly the fraction extracted from gonads as lipovitellin in the present work was found to have amino acid compositions similar to the fraction E_1 prepared from rainbow trout eggs by Hara and Hirai (1978). The radioimmunological cross reactivity of this fraction with the antibody confirms the antigen to be vitellogenin, a yolk precursor.

The phosvitin fraction isolated in the present work was a nonlipidic, highly phosphorylated (15% P) peptide, characterized by an extremely

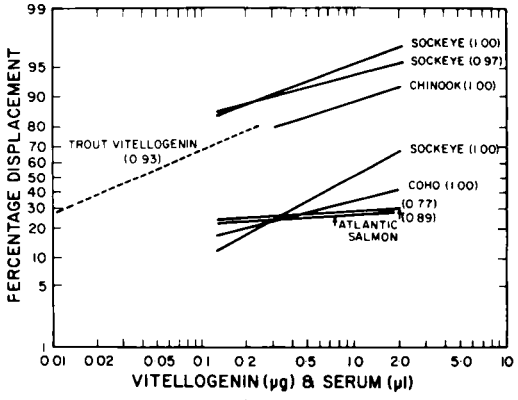

FIG. 5. Log dose/logit transformed displacement plots for displacement by samples of Pacific and Atlantic salmon plasma compared with trout vitellogenin in the assay system anti-trout vitellogenin/[^{131}I]-β' component. Maximum binding 36%.

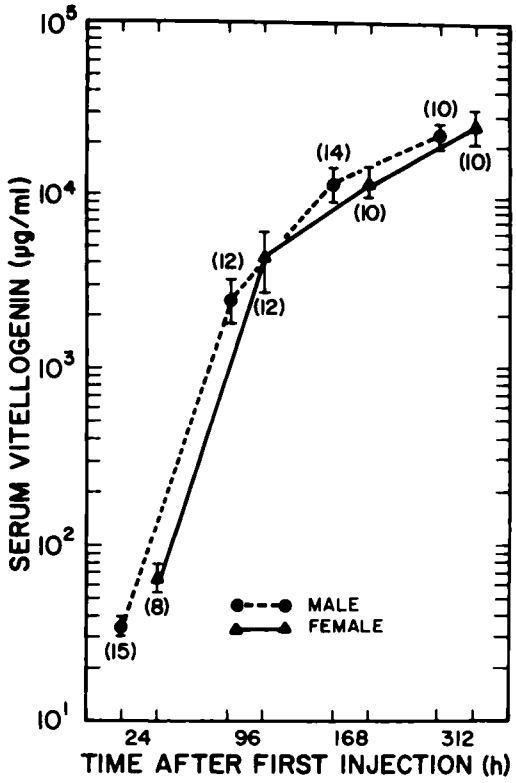

FIG. 6. Profile of induction of serum vitellogenin in immature ♂ and ♀ rainbow trout treated with estradiol benzoate at 0, 72, 14, 216 and 288 h.

high serine content (42%). The amino acid analysis (Table 3) was closely comparable to that established for rainbow trout (Ito et al., 1966; Mano and Yoshida, 1969), brown trout (Schmidt et al., 1965) and for *Oncorhynchus keta* (Mano and Yoshida, 1969). This fraction did not crossreact with the vitellogenin antibody. When the amino acid composition was compared with that found for fraction E_2 by Hara and Hirai (1978), little homology was found indicating that the fraction E_2 is not phosvitin but another yolk protein which had immunological cross reactivity with antisera to vitellogenin. Jared and Wallace (1968) isolated 3 principal yolk proteins from teleost ovaries using TEAE cellulose chromatography. The α and β components absorbed UV light at 280 nM and were low in phosphorus while γ had no extinction of UV 280 nM but was rich in phosphorus. The phosvitin fraction isolated in the present study absorbed UV light at 280 nM and in fact the phosvitin preparations for *X. laevis* (Redshaw and Follett, 1971), chicken, turtle, frog and trout (Wallace et al., 1966) exhibited some UV absorbance.

Markert and Vanstone (1971) isolated a β' component from *O. kisutch* which differed from the β component of Jared and Wallace (1968) by its solubility in low salt concentrations. The procedures used by Hara and Hirai (1978) would not have separated the β' component and phosvitin which may explain why fraction E_2 cross reacted with an antibody to female specific serum proteins. In this study we have isolated a fraction from vitellogenic oocytes which corresponded with β' component and crossreacted with an antibody raised against purified vitellogenin of rainbow trout plasma. Markert and Vanstone (1971) concluded from their immunological results that β' component must be a serum protein specific to vitellogenic salmon. The radioimmunological data support a hypothesis that vitellogenin contained lipovitellin and β' component and the phosphorus content of phosvitin could account for the phosphorus in vitellogenin. Recently Penning et al. (1977) suggested that vitellogenin contained a heavy and a light lipovitellin peptide. Perhaps the β' component could be such a light lipovitellin.

Amino acid analysis showed similar compositions for vitellogenin and lipovitellin but phosvitin and β' component were clearly different from each other and from the bigger molecules. This might suggest that β' component is a discrete peptide which is transported to the ovary or synthesized in situ during vitellogenesis and is immunologically related to vitellogenin/lipovitellin. The precise relationship between these proteins must be defined by further study.

The concentrations of total lipid in vitellogenin and lipovitellin isolated in this study are similar to those found for cod (Plack et al., 1971) coho salmon (Markert and Vanstone, 1971) and rainbow trout (Wallace and Jared, 1968) and the phosphorus content of lipovitellin was even lower than that previously determined for trout.

The phosphorus content (16%) of phosvitin isolated in this study was higher than values previously obtained (4%, Mano and Lipman, 1966; 9.6%, Wallace et al., 1966). This might be due to some residual phospholipid contamination; however, the lipid determination ($\cong$0 µg/mg) would not indicate a significant contamination. It is possible that the lower phosphorus values were determined for preparations

of phosvitin which were contaminated with β' component.

When purified vitellogenin was subjected to analytical ultracentrifugation in 20 mM NaCl, 3 mM $CaCl_2$, 3 peaks were observed: S°_{20w} 11.6, 18.9 and 23.8 in descending order of abundance. In similar studies of vitellogenin, from *Xenopus laevis*, Redshaw and Follett (1971) found S°_{20w} of 17.5, 25.0 and 35.0 suggesting that polymerization may be common at least during experimental manipulation. By using the partial specific volume and diffusion coefficients derived by Redshaw and Follett (1971) from the data of Wallace (1970) for *Xenopus* vitellogenin, the principal peak of trout vitellogenin corresponded to a molecular weight of 342,000. Molecular weights determined by gel chromatography and gradient electrophoresis were 440,000 and 470,000, respectively. These values are lower than the estimates of 600,000 for rainbow trout (Hara and Hirai, 1978) and 550,000 for flounder (Emmersen and Petersen, 1976) and correspond closely to the values of 400,000 for cod vitellogenin (Plack et al., 1971) and 390,000 for coho salmon (Markert and Vanstone, 1971).

In the salmon yolk radioimmunoassay system used by Crim and Idler (1978) and Idler et al. (1979) as well as for Fig. 2 of this work, a protein from salmon egg yolk with a molecular weight similar to that of β' component as standard in the trout vitellogenin radioimmunoassay would result in a 31-fold (Fig. 4) underestimation of vitellogenin concentrations. Idler et al. (1979) found this difference to be 49-fold for the salmon assay. For this reason the results of Crim and Idler (1978) should not be considered as representing the absolute vitellogenin levels but rather the relative differences among samples.

Redshaw and Follett (1976) drew attention to the possibility that immunological cross reactivity of the biochemically complex vitellogenin might be lost during purification. Thus, though the purified vitellogenin was employed as standard, we are unable to state that the vitellogenin concentrations measured are absolute although they are in the range when compared with other fish (Idler et al., 1979), reptile (Gapp et al., 1979) and bird (Redshaw and Follett, 1976). Relative comparisons are valid and absolute values are probably close to assay values. Concentrations of greater than 5 mg/ml seem reasonable in light of the yolk accumulation during ovarian growth of up to 15% of body weight during ~6 months of vitellogenesis, necessitating growth of the ovaries by ~1 g/day. The value of 32 mg/ml reported by Plack et al. (1971) using quantitative immunodiffusion, corresponds to the higher values found for rainbow trout. Lipovitellin is clearly recognized quantitatively in the vitellogenin radioimmunoassay (Figs. 3, 4) but the concentration of the preparations used may not be precise because of properties of the specific preparations. The lipovitellin that was used for labeling in routine assays was in its turn overestimated in the assay, while the preparation, which when labeled resulted in high nonspecific binding in the absence of antibody, was underestimated though in each case displacement curves were parallel to the vitellogenin standard. Current trout vitellogenin radioimmunoassays use labeled β' component for greater reliability and vitellogenin as standard. It is recommended that a plasma sample containing vitellogenin be frozen in small aliquots and put into each assay as a control; experience has shown that such a standard has a shelf life of at least 1 year at -80°C.

Figure 1 shows the relative increase in one serum protein component when trout were treated with estradiol. Estrogen effects on serum proteins identified as yolk precursors have previously been described for amphibia (Follett et al., 1968; Wallace et al., 1968) and in teleosts (Ho and Vanstone, 1961; Thurston, 1967; Utter and Ridgeway, 1967; Plack et al., 1971; Aida et al., 1973; Heesen and Engels, 1973; Campbell and Idler, 1976; Emmersen and Petersen, 1976; Crim and Idler, 1978). In another experiment there was (Fig. 6) an increase of greater than 400-fold in the plasma vitellogenin of immature (0+) rainbow trout between 24–312 h after commencement of treatment. These levels are presumably supraphysiological because of the high levels of estradiol benzoate administered and the probability that the clearance rate of vitellogenin from blood in the absence of any gonadotropic stimulation of the gonad, is slow. Campbell and Idler (1976) found that a gonadotropin was necessary to stimulate incorporation into oocytes of flounder and Emmersen and Kjaer (1974) showed that estrogen treatment of *Bufo bufo* induced high levels of serum phosphoprotein which decreased if the animal was treated with gonadotropin.

The term "vitellogenin" has come to be used for all yolk precursors found in sera of vitello-

genic animals and indeed among vertebrates there is an apparent similarity between the yolk transportation and accumulation systems. However, Redshaw and Follett (1976) found that antiserum to fowl vitellogenin showed negligible cross reaction to sera from vitellogenic fish, amphibians or reptiles. Gapp et al. (1979) showed cross reactivity of only a few turtle species in the turtle vitellogenin radioimmunoassay and it was found that displacement was not parallel even for these. We have presented evidence that even within the genus *Salmo* there is poor immunological cross reactivity between vitellogenins. The assay system developed for *Salmo salar* does not quantitatively recognize trout vitellogenin or sera from vitellogenic trout or flounder *(Pseudopleuronectes americanus)* and the assay for vitellogenin from *S. gairdneri* cannot quantify the vitellogenin in serum from *S. salar* and *O. kisutch.* It is difficult to speculate how vitellogenins from such closely related species (genus, *Salmo*) might differ since it seems probable that the scheme of vitellogenesis involving vitellogenin incorporation into oocytes and conversion to lipovitellin and phosvitin is common to all oviparous vertebrates. This is suggested by the fact that the vitellogenin, lipovitellin and phosvitin fractions isolated from trout and from other vertebrates all have similar lipid, phosphorus and amino acid contents (Table 3). The fact that the amino acid composition of vitellin from *Locusta migratoria* (Chen et al., 1978) is also very similar to the vertebrate vitellogenins implies that the term vitellogenin can justifiably be applied to proteins found in the circulation of oviparous species throughout the animal kingdom which have a common function in supplying lipophosphoprotein to the gametes for use in embryogenesis.

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REFERENCES

Aida, K., Ngan, P.-V. and Hibiya, T. (1973). Physiological studies on oocyte maturation of fishes. I. Sexual difference in composition of plasma protein of ayu in relation to gonad maturation. Bull. Jap. Soc. Sci. Fish. 39, 1091–1106.

Amirante, G. A. (1972). Immunochemical studies on rainbow trout (*Salmo gairdneri* Rich) lipovitellin. Acta Embryol. Exp. (Suppl.), 373–383.

Bergink, E. W., Wallace, R. A., Van de Berg, J., Bos, E. S., Gruber, M., Geert, A. B. (1974). Estrogen induced synthesis of yolk proteins in roosters. Amer. Zool. 14, 1177–1193.

Bligh, E. G., Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Campbell, C. M. (1978). *In vitro* stimulation of vitellogenin incorporation into trout oocytes by salmon pituitary extracts. Ann. Biol. Anim. Bioch. Biophys. 18, 1013–1018.

Campbell, C. M. and Idler, D. R. (1976). Hormonal control of vitellogenesis in hypophysectomized winter flounder (*Pseudopleuronectes americanus* Walbaum). Gen. Comp. Endocrinol. 28, 143–150.

Campbell, C. M. and Jalabert, B. (1979). Selective protein incorporation by vitellogenic *(Salmo gairdneri)* oocytes *in vitro.* Ann. Biol. Biochim. Biophys. 19, 429–437.

Chan, P. S., Toribara, T. Y. and Warner, H. (1956). Microdetermination of phosphorus. Anal. Chem. 28, 1756–1758.

Chen, T. T., Strahlendorf, P. W. and Wyatt, G. R. (1978). Vitellin and vitellogenin from locusts *(Locusta migratoria).* J. Biol. Chem. 253, 5325–5331.

Chervenka, C. H. (1969). Manual of methods for the analytical ultracentrifuge. Spinco Division, Beckman Instruments, Palo Alto, CA.

Christmann, J. L., Grayson, M. J. and Hwang, R.C.C. (1977). Comparative study of hen yolk phosvitin and plasma vitellogenin. Biochemistry 16, 3250–3256.

Clark, R. C. and Joubert, F. J. (1971). The composition of cyanogen bromide cleavage products from Len's egg phosvitin. FEBS Letters 13, 225–228.

Crim, L. W. and Idler, D. R. (1978). Plasma gonadotropin, estradiol and vitellogenin and gonad phosvitin levels in relation to the seasonal reproductive cycles of female brown trout. Ann. Biol. Anim. Bioch. Biophys. 18, 1001–1005.

Donabedian, R. K., Levine, R. A., Seligson, D. (1972). Micro-electrolytic iodination of polypeptide hormones for radioimmunoassay. Clin. Chim. Acta 36, 517–520.

Egami, N. (1955). Effect of estrogen and androgen on the weight and structure of the liver of the fish, *Oryzias latipas.* Annot. Zool. Japon. 28, 79–85.

Emmersen, B. and Kjaer, K. J. (1974). Seasonally and hormonally induced changes in the serum level of the precursor protein vitellogenin in relation to ovarian vitellogenic growth in the toad, *Bufo bufo bufo* (L.). Gen. Comp. Endocrinol. 22, 261–267.

Emmersen, B. K. and Petersen, I. M. (1976). Natural occurrence and experimental induction by estradiol-17β of a lipophosphoprotein (vitellogenin) in flounder (*Platichthys flesus* L.). Comp. Biochem. and Physiol. 54B, 443–446.

Fine, J. M. and Drilhon, A. (1963). Etude immunolique des protéines du sérum de *Salmo salar:* Etude par immunodiffusion. C.R. Soc. Biol. 157, 1937–1940.

Follett, B. K. and Redshaw, M. R. (1968). The vitellogenic response in the south African clawed toad

(*Xenopus laevis* Daudin). J. Cell. Physiol. 72 (Suppl.), 91–102.

Gapp, D. A., Ho, S. M. and Callard, I. P. (1979). Plasma levels of vitellogenin in *Chrysemys picta* during the annual gonadal cycle: Measurement by specific radioimmunoassay. Endocrinology 104, 784–790.

Greenwood, F. C., Hunter, W. M. and Glover, J. S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114–123.

Hara, A. and Hirai, H. (1978). Comparative studies on immunochemical properties of female-specific serum protein and egg yolk proteins in rainbow trout *(Salmo gairdneri)*. Comp. Bioch. Physiol. 59B, 339–343.

Hartree, E. F. (1972). Determination of protein: Modification of the Lowry method that gives a linear photometric response. Analyt. Bioch. 48, 422–427.

Heesen, P. T. and Engels, W. (1973). Electrophoretic studies on vitellogenesis in *Brachydanio rerio*. Whilhelm Roux Arch. Entw. Merch. Org. 173, 45–59.

Ho, F.C.W. and Vanstone, W. E. (1961). Effect of estradiol monobenzoate on some serum constituents of maturing sockeye salmon *(Oncorbynchus nerka)*. Fish. Res. Bd. Can. 18, 859–864.

Idler, D. R., Hwang, S. J. and Crim, L. W. (1979). Quantification of vitellogenin in Atlantic salmon plasma by radioimmunoassay. J. Fish. Res. Bd. Can. 36, 574–578.

Ito, Y., Fuji, T., Kanamori, M., Hattori, T. and Yoshioka, R. (1966). Phosvitin of the trout roe. J. Biol. Chem. 60, 726–728.

Jared, D. W. and Wallace, R. A. (1968). Comparative chromatography of the yolk proteins of teleosts. Comp. Biochem. Physiol. 24, 437–443.

Kobayashi, H. (1953). Effect of estrone upon the structure, weight and fat content of the liver in the fish, *Misgurnus anguillicaudatus*. Annot. Zool. Japan 26, 213–216.

Mano, Y. and Lipmann, F. (1966). Characteristics of phosphoproteins (Phosvitins) from a variety of fish roes. J. Biol. Chem. 241, 3822–3833.

Mano, Y. and Yoshida, M. (1969). A novel composition of phosvitins from salmon and trout roe. J. Biol. Chem. 66, 105–111.

Markert, J. R. and Vanstone, W. E. (1971). Egg proteins of coho salmon *(Oncorbynchus kisutch)*: Chromatographic separation and molecular weights of the major proteins in the high density fraction and their presence in salmon plasma. J. Fish. Res. Bd. Can. 28, 1853–1856.

Marsh, J. B. and Weinstein, D. B. (1966). Simple charring method for determination of lipids. J. Lipid. Res. 7, 574–576.

Ng, T. B. and Idler, D. R. (1978). "Big" and "Little" forms of plaice vitellogenic and maturational hormones. Gen. Comp. Endocrinol. 34, 408–420.

Penning, T. N., Merry, A. H., Munday, K. A. and Akhtar, M. (1977). Studies on the biosynthesis, assembly, and secretion of vitellogenin, and estrogen-induced multicomponent protein. Biochem. J. 162, 157–170.

Plack, P. A. and Frazer, N. W. (1971). Incorporation of L-(^{14}C) leucine into egg proteins by liver slices from cod. Biochem. J. 121, 857–862.

Plack, P. A., Pritchard, D. J. and Frazer, N. W. (1971). Egg proteins in cod serum. Biochem. J. 121, 847–856.

Redshaw, M. R. and Follett, B. K. (1971). The crystalline yolk–platelet proteins and their soluble plasma precursor in an amphibian *Xenopus laevis*. Biochem. J. 124, 759–766.

Redshaw, M. R. and Follett, B. K. (1976). Physiology of egg yolk production by the fowl: The measurements of circulating levels of vitellogenin employing a specific radioimmunoassay. Comp. Bioch. Physiol. 55A, 399–405.

Schmidt, G., Bartsch, G., Kitagarva, T., Fujisawa, F., Knolls, T., Joseph, J., DeMoreo, P., Liss, M. and Hashemeyer, R. (1965). Isolation of a phosphoprotein of high phosphorus content from the eggs of brown trout. Bioch. Biophys. Res. Comm. 18, 60–65.

Thurston, R. (1967). Electrophoretic patterns of blood serum proteins from rainbow trout (*Salmo gairdneri*). J. Fish Res. Bd. Can. 24, 2169–2188.

Upadhyay, S. N., Breton, B. and Billard, R. (1978). Ultrastructural studies on experimental induced vitellogenesis in juvenile rainbow trout. Ann. Biol. Anim. Biochim. Biophys. 18, 1019–1025.

Utter, F. M. and Ridgeway, G. J. (1967). A serologically detected serum factor associated with maturity in English Sole, *Parophyrys vitelus* and Pacific Halibut, *Hippoglossus stenolepis*. Fish. Bull. U.S. Fish and Wildl. Serv. 6, 47–58.

Wallace, R. A. (1970). Studies on amphibian yolk. IX. *Xenopus* vitellogenin. Biochem. Biophys. Acta 215, 176–183.

Wallace, R. A., Jared, D. W. and Eisen, A. Z. (1966). A general method for isolation and purification of phosvitin from vertebrate eggs. Can. J. Biochem. 44, 1647–1656.

Wallace, R. A. and Dumont, J. N. (1968). The induced synthesis and transport of yolk proteins and their accumulation by the oocyte in *Xenopus laevis*. J. Cell. Physiol. 72 (Suppl.), 73–90.

Wiley, H. S. and Dumont, J. N. (1978). Stimulation of vitellogenin uptake in stage IV *Xenopus* oocytes by treatment with chorionic gonadotropin in vitro. Biol. Reprod. 17, 762–771.