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Selective protein incorporation by vitellogenic *Salmo gairdneri* oocytes *in vitro*

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Summary. An *in vitro* method is presented for investigation of protein incorporation into the ovarian follicles of rainbow trout (*Salmo gairdneri*).

A protein induced into serum of *S. gairdneri*, after estrogen treatment, was incorporated into vitellogenic follicles to a greater extent than other serum proteins of *S. gairdneri* or bovine serum albumin (BSA) which served as controls. A large proportion of the incorporated BSA and other non-vitellogenic proteins is probably associated with follicular tissues other than the oocyte.

These results offer further evidence that the estradiol-induced serum protein found in teleosts is vitellogenin, the precursor of oocyte yolk proteins.

Introduction.

In the model proposed for vitellogenesis of amphibia and birds, a yolk protein precursor, vitellogenin, is synthesised by the liver, transported by the blood, and incorporated into vitellogenic oocytes (Follett, Nicholls and Redshaw, 1968 ; Wallace and Dumont, 1968 and Bergink *et al.*, 1974).

Support for a similar model for vitellogenesis in teleosts is now accumulating. Numerous workers have demonstrated changes in serum constituents associated with vitellogenesis in female teleosts and Utter and Ridgeway (1967), Plack *et al.* (1971) and Amirante (1972) characterized a serum protein in *Parophyrys vetulus*, *Hippoglossus stenolepis*, *Gadus morhua* and *Salmo gairdneri* as a probable yolk precursor by its cross-reactivity with antibodies to gonadal extracts. Plack and Frazer (1971), Emmersen and Peterson (1976) and Campbell and Idler (1976) presented data to indicate that vitellogenin is synthesised in the teleost liver and Campbell and Idler (1976), Campbell (1978) Ng and Idler (1978) found that incorporation of the precursor into vitellogenic ovaries is stimulated by teleost pituitary gonadotropins which exhibit no affinity to concanavalin A.

Wallace *et al.* (1970, 1976) have studied the amphibian mechanisms of protein

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incorporation into oocytes using a system of *in vitro* incubation of oocytes from *Xenopus laevis*. This system was adopted to study the incorporation of protein into oocytes of *Salmo gairdneri* and investigate the selectivity of protein incorporation.

Materials and methods.

Donor fish and preparation of oocytes.

Hatchery reared *S. gairdneri* were maintained in a recirculated water raceway system. Follicles (> 3 mm in diameter) were obtained from the ovaries of females undergoing active vitellogenesis between September and early December.

Incorporation assays were carried out on different systems :

1. Individual follicles separated from ovaries using small forceps : Follicles were isolated and incubated in trout balanced salts solution (TBSS) buffered at pH 8.0 by HEPES-NaOH (Jalabert, 1976) : NaCl 133 mM ; KCl 3.1 mM ; $MgSO_4$ 0.3 mM ; $MgCl_2$ 1.0 mM ; $CaCl_2$ 3.4 mM ; glucose 5.6 mM ; HEPES-NaOH 40 mM, penicilin 100 000 U/l ; streptomicin sulphate 100 000 μg/l.

2. Individual follicles isolated by mild collagenase treatment using BSA to neutralize non-specific proteolytic effects. Small chunks of ovary were actively agitated (1.7 Hz) for 1-2 h at 15 °C in TBSS containing 2 mg ml^{-1} collagenase (Sigma, type II) and 5 mg ml^{-1} BSA, resulting in rupture of the basement membrane between thecal layers and ovarian tissue.

3. Individual oocytes surrounded only by granulosa cells : Following stage 2, follicles were agitated very gently (< 0.5 HZ in 2 mg ml^{-1} collagenase, 15 mg ml^{-1} BSA), for a further 2 hrs. This treatment resulted in the rupture of the basement membrane between granulosa and thecal layers.

4. Individual naked oocytes : When follicles prepared to stage 3 were aspirated several times into a closely fitting pipette the granulosa layer was usually scraped off. Occasionally exposure for 10 min to Ca, Mg-free TBSS + 5 mM EDTA was required before mechanical treatment.

Follicles were sorted using a dissecting microscope in order to begin incubation within 5-10 hrs of the death of the donor fish. Those selected retained the appearance of follicles from a freshly killed fish.

Individual follicles were placed in the wells of Cooke microtiter plates and excess medium was aspirated and replaced with 50 μl of experimental incubation medium. Experimental lots of 12 or 24 individual oocytes from each fish were incubated for 18-25 hrs at 12.5 or 15° under an O_2/N_2 atmosphere (50:50) saturated with water to prevent problems of evaporation of medium.

At the end of incubation the wells were rinsed three times with saline to wash any unabsorbed proteins from the follicles. The oocyte was then examined using a dissecting microscope and the diameters of those having retained a similar appearance as at the beginning of incubation were recorded. These oocytes were dissolved in Soluene (Packard Instruments) and radioactivity counted. Disintegrations per minute and per mm^2 of oocyte surface were calculated and the mass of protein incorporated calculated using the specific activity of the labelled protein determined using Lowry protein quantification.

Protein labelling.

Ten mg of test protein in 0.2-1.0 ml of 0.5 M NaCl, 0.02 M $CaCl_2$ was adjusted to pH 9.0 with NaOH at 0 °C. 100 μCi of ^{14}C-formaldehyde (CEA France — 30 mCi mM^{-1}) was added and the solution mixed. Sodium borohydride (20 μl : 100 μg) was added four times at 30 second intervals, followed by a final 100 μl (500 μg), with mixing each time. After 16 hrs at 0 °C the labelled protein mixture was freed from reaction products using Sephadex G-25 equilibrated with TBSS (without glucose and antibiotics). Labelled proteins were stored at —70° at concentrations of 5-10 mg/ml. This method, based on Rice and Means (1970) yielded proteins with specific activities of 1 000 to 7 000 DPM μg^{-1}.

Protein incorporation experiments.

The proteins described in table 1 and figure 1 were labelled by reductive methylation. The incorporation of preparations which were essentially free of the estrogen-induced serum protein was compared with incorporation of vitellogenic proteins.

TABLE I

Description of test proteins

Symbol	Source	Isolation
Control		
BSA	Bovine Serum Albumin	Sigma ; Fraction V
C_1	Serum from ♂ *S. gairdneri*	Untreated
C_2	—	Principle peak in TEAE chromatography
C_3	Serum from estrogen-treated ♀ *S. gairdneri*	—
C_4	Serum from non-vitellogenic ♀ *S. gairdneri*	Second peak area in TEAE chromatography
Vitellogenic		
V_1	Serum from estrogen-treated ♂ *S. gairdneri*	Untreated
V_2	—	Second peak in TEAE chromatography
V_3	—	—

The effect of varying concentration of these proteins on incorporation into oocytes in the presence and absence of 2 mg ml^{-1} BSA was tested. The effect of varying BSA concentration on incorporation of ^{14}C-labelled V_1, V_2, C_1 and BSA was evaluated.

The incorporation of ^{14}C-vitellogenin and ^{14}C-BSA by follicles which were prepared enzymatically or mechanically was tested in order to characterize any interference by other ovarian tissues.

Results.

Preliminary trials showed that addition of BSA to the collagenase solution resulted in preparation of follicles which retained the morphological characteristics of those excised from ovaries of freshly killed animals. Without BSA few collagenase treated

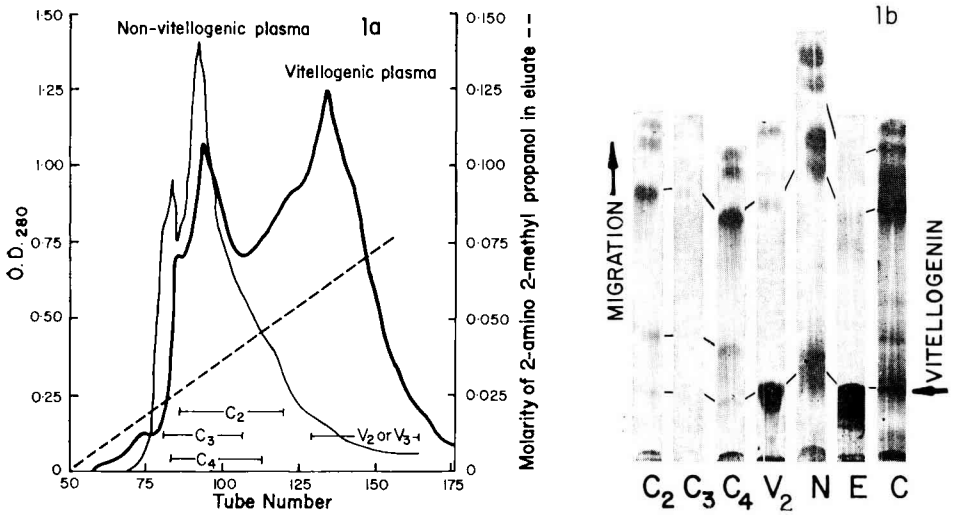

FIG. 1. — (a) *Typical profiles of serum proteins eluted from a* 2.6 cm × 40 cm *column of TEAE cellulose by a linear gradient of* 0.015 M 2 *amino-2 methyl-1 propanol* ; 0.002 5 M *citric acid to* 0.125 M 2 *amino-2 methyl-1 propanol* ; 0.04 M *citric acid.* Bars show elution positions of partially purified test fractions.

(b) *Polyacrylamide gels* (6 p. 100 *Tris-glycine system) showing estrogen induced protein (V) and comparison of protein mixtures tested.* C : serum from a previtellogenic adult ♀ ; E : serum from estradiol-treated ♀ ; N : serum from a normal vitellogenic ♀.

oocytes were viable after incubation. Seventy-five percent of follicles separated enzymatically retained these characteristics till the end of the incubation period but only 45 p. 100 of those isolated manually were similar.

TABLE 2

ng mm^{-2} *of* ^{14}*C-labelled protein in follicles or oocytes after* 21 *hrs incubation with* 1 *mg ml*$^{-1}$ *test protein,* 2 *mg ml*$^{-1}$ *BSA (fish* 1*) or* 2 *mg ml*$^{-1}$ *test protein only (fish* 2*)*
Mean ± SE ; numbers in parenthesis indicate number of viable oocytes remaining (*a, b, c* indicate $P \leqslant 0.001$ for comparaison by t-test).
Groups 2, 3 and 4 were all prepared by collagenase treatment

Treatment of oocyte before incubation	Test protein		
	^{14}C-vitellogenin (V_2)		^{14}C-BSA
	Fish 1	Fish 2	Fish 2
1. Manual separation	40 ± 1 (22)^a	59 ± 3 (14)^b	20 ± 1 (13)^c
2. Collagenase separation....	67 ± 6 (22)^a	78 ± 3 (18)^b	13 ± 1 (21)^c
3. Theca removed	92 ± 6 (24)	68 ± 3 (9)	17 ± 2 (10)
4. Granulosa removed	61 ± 4 (19)	43 ± 2 (13)	—

Significantly ($P < 0.001$) greater quantities of ^{14}C-vitellogenin (V_2, V_3) were found associated with oocytes which had been prepared by collagenase dissociation (table 2) and conversely the quantity of ^{14}C-BSA was greatest ($P < 0.001$) in manually dissociated oocytes though there was always less BSA than vitellogenin incorporation. In a

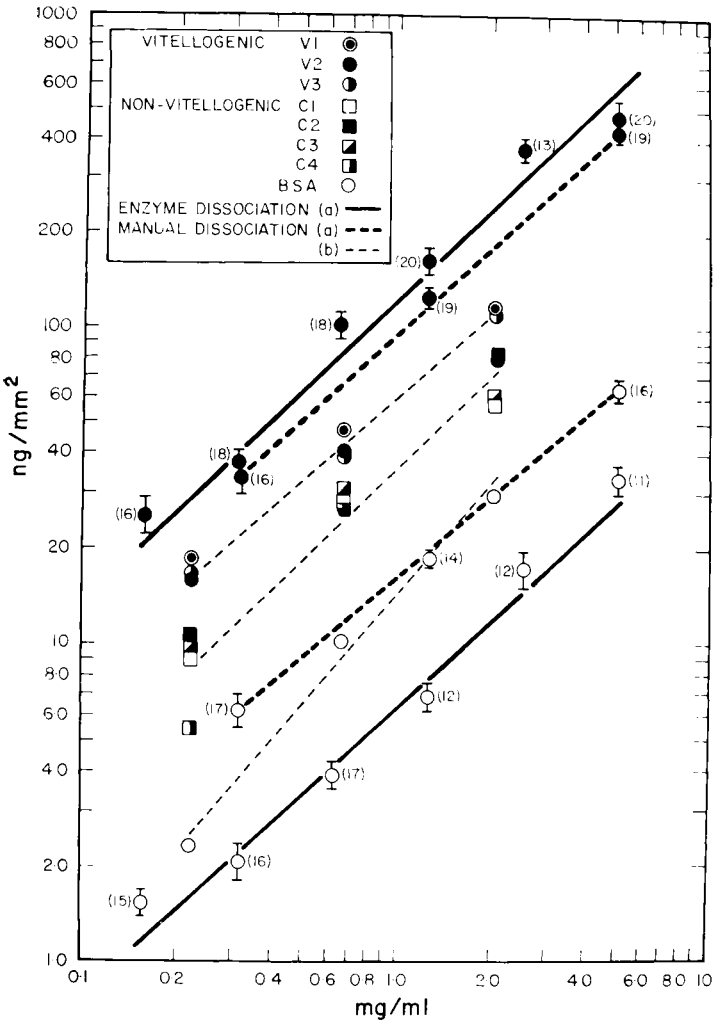

FIG. 2. — (a) *Comparison of protein (^{14}C-V_2, ^{14}C-BSA) incorporation into oocytes dissociated manually or by collagenase incubated with various concentrations of ^{14}C-V_2 in the absence of unlabelled* BSA.

(b) *Comparison of incorporation of ^{14}C-labelled-vitellogenic and non-vitellogenic proteins and ^{14}C-BSA at different concentrations in the presence of* 2 mg ml^{-1} BSA *into manually dissociated oocytes.*

Data points represent mean incorporation ± standard error for (n) viable oocytes after 20 hrs. Lines are least squares fit.

further experiment (fig. 2) it was found that these phenomena were reproduced similarly at different concentrations for vitellogenic and non-vitellogenic proteins.

In each of the above cases less ^{14}C-labelled BSA or control proteins than vitellogenic protein was found incorporated into oocytes. Analysis of variance shows that vitellogenic proteins (V_1, V_2, V_3) were incorporated significantly ($P < 0.001$) more actively than all non-vitellogenic test proteins into manually separated follicles (table 3). At different protein concentrations the same tendency was found (fig. 2).

TABLE 3

ng mm^{-2} of ^{14}C-labelled protein in manually separated follicles after incubation with 2 mg ml^{-1} test protein in the presence of 2 mg ml^{-1} BSA.
Mean ± SE for (n) surviving oocytes from the initial 12 (fish 1) or 24 (fish 2) placed in culture (*a* : no significant difference ; *b* : P ⩽ 0.001).

	Test protein						
	V_1	V_2	V_3	C_1	C_2	C_3	C_4
Fish 1[a] ...	62 ± 3 (4)	78 ± 2 (4)	80 ± 12 (4)	35 ± 2 (5)	37 ± 1 (4)	42.6 ± 4 (4)	30 ± 1 (5)
Fish 2[a] ...	116 ± 8 (12)	110 ± 7 (20) *b*	107 ± 7 (20)	56 ± 3 (21)	44 ± 2 (23)	55 ± 2 (19) *b*	47 ± 2.6 (20)

A single experiment showed that increasing BSA concentrations up to 9 mg/ml^{-1} did not affect the incorporation of vitellogenic (V_1, V_3) or non-vitellogenic (C_1) ^{14}C-labelled proteins but higher concentrations may inhibit incorporation of non-vitellogenic serum proteins (fig. 3). However the incorporation of ^{14}C BSA decreased as a function of dilution of ^{14}C labelled BSA by non-labelled protein. Incubation performed in the presence of 27 mg ml^{-1} BSA (normal plasma protein concentrations Ca 50 mg ml^{-1}) showed a marked reduction of incorporation of male serum protein (C_1) also.

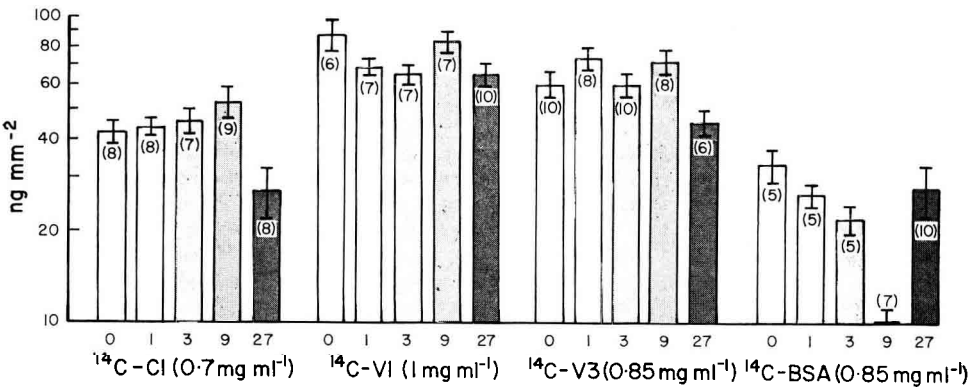

FIG. 3. — *Incorporation of labelled proteins by manually separated oocytes during incubation with* 1, 3, 9 *or* 27 *mg ml^{-1} of unlabelled BSA* (mean and standard error for (N) viable oocytes after 19 hrs).

Two attempts to incubate oocytes from which thecal or all follicular tissues had been removed yielded conflicting data, perhaps due to the extensive mortality of oocytes from one fish (table 2). Removal of thecal tissues may facilitate incorporation but it is probable that the totally naked oocytes are too fragile for the results to be conclusive.

Discussion.

Immunological studies have demonstrated cross reactivity of the protein, induced into the serum of estradiol treated fish, or present in vitellogenic females, with antibodies to the yolk protein (Utter and Ridgeway (1967), Plack *et al.* (1971). In the present study, it was found that the incorporation of this type of estradiol-induced protein into vitellogenic oocytes was always much greater than for BSA (fig. 2, fig. 3, tables 2 and 3) and the « non-vitellogenic » proteins (isolated from male serum, non-vitellogenic and vitellogenic female serum) were generally associated with follicles in a similar manner to BSA. These data support the hypothesis that this estrogen-induced serum protein is vitellogenin, the yolk precursor.

Wallace and Jared (1976) showed that protein incorporation by the vitellogenic oocytes of *Xenopus Laevis* is highly specific for *Xenopus* vitellogenin which was accumulated in greater quantities than other (non-amphibian and non-vitellogenic) test proteins. In the present work no absolute specificity has been shown but it is clear that BSA was unable to inhibit the incorporation of vitellogenic protein (fig. 3). It does however competitively inhibit the incorporation of ^{14}C-BSA (fig. 3) as evidenced by lower levels of tracer incorporation in the presence of higher concentrations of unlabelled BSA. The effect of competition of unlabelled BSA with ^{14}C-BSA (fig. 2) results in a different response (greater slope) to increasing the concentration of ^{14}C-labelled protein, whereas the incorporation responses to changes in concentration were similar for all proteins including ^{14}C-BSA in the absence of competing unlabelled BSA. This suggests that the association of each protein with incubated follicles is to some degree site specific.

Wallace and Jared (1976) found that there was no appreciable competition between BSA and ^{14}C-vitellogenin for partially denuded oocytes of *X. laevis* and they felt justified in applying the concept of enzyme kinetics to describe the incorporation of proteins. We have not attempted to make similar interpretations since we used entire follicles with the attendent problems of non-specific association of protein to thecal tissues.

It is probable that a part of the ^{14}C-labelled protein represents incorporation into or association with follicular tissues rather than incorporation into oocytes. Mechanically isolated oocytes are surrounded by granulosa and thecal layers and also interstitial tissues whereas when oocytes are separated enzymatically they are only invested by granulosa and thecal layers. The vitellogenin « incorporation » is higher for enzymatically treated oocytes suggesting that the basement membrane between interstitial tissues and thecal layers may act as a barrier to uptake of vitellogenin. Wallace *et al.* (1970, 1973) found that oocytes from *X. laevis* must be divested of epithelial and thecal layers prior to incubation in order to facilitate incorporation of vitellogenin. The lower ^{14}C-BSA incorporation by follicles incubated without interstitial tissues (after enzyme dissociation) compared to entire follicles (manual dissociation) suggests that there may be extensive association of non-vitellogenic proteins with these tissues which are not removed by washing after incubation before counting (table 2, fig. 2). In fact, Campbell (1978) showed by removal of follicular tissues after incubation that, whereas only 15 p. 100 of total incorporated follicular vitellogenin was found in

follicle cells and 85 p. 100 in the oocyte, 60 p. 100 of incorporated non-vitellogenic trout serum protein was associated with the follicles cells and only 40 p. 100 in the oocyte. Thus it is probable that the specificity of true *incorporation* into the oocytes is greater than is suggested by results presented here.

The methods of oocyte preparation and incubation have proven equally satisfactory for oocytes (0.75-1.5 mm diam) from trout which had not begun active vitellogenesis (Campbell, 1978). The addition of BSA to the incubation medium is apparently unnecessary though addition during collagenase treatment is the key to successful use of this technique.

Labelling of the proteins by reductive methylation seems not to extensively damage the proteins. Wallace and Jared (1976) found that chemically labelled vitellogenin was incorporated by oocytes of *X. laevis* with similar kinetics to vitellogenin labelled by incorporation of labelled amino acid *in vivo*. Busby *et al.* (1977) showed that biological activities and half-lives were unchanged when three enzymes were labelled by this *in vitro* method.

This approach to the study of vitellogenesis lends itself to investigations of the activity of vitellogenic gonadotropins (Campbell, 1978) and may enable evaluation of the mode of action of these gonadotropins as well as exploration of the mechanisms of yolk incorporation by studies similar to those of Wallace *et al.* (1970-1976) made in amphibia.

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Résumé. Une méthode d'étude de l'incorporation de protéines *in vitro* dans le follicule ovarien de la truite arc-en-ciel (*Salmo gairdneri*) est décrite.

Une protéine sérique dont l'apparition est induite par traitement œstrogénique *in vivo*, s'est révélée être mieux incorporée *in vitro* dans les follicules en vitellogenèse que les autres protéines sériques ou la BSA qui servent de témoins. Une proportion importante de celles-ci est d'ailleurs probablement associée aux tissus folliculaires, plutôt qu'aux ovocytes eux-mêmes.

Ces résultats contribuent à démontrer que la protéine sérique induite par traitement œstrogénique chez les téléostéens est bien la vitellogénine, précurseur de protéines vitellines de l'ovocyte.

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