Ann. Biol. anim. Bioch. Biophys., 1978, **18** (4), 1013-1018.

In vitro stimulation of vitellogenin incorporation into trout oocytes by salmon pituitary extracts

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Summary. Follicles, isolated from ovaries of rainbow trout before the onset of active vitellogenesis, incorporate ^{14}C-labelled vitellogenin when incubated *in vitro* for 18 hrs. An aqueous extract of lyophilised salmon pituitaries stimulated the incorporation of vitellogenin, but not other serum proteins.

A pituitary fraction which is not bound to Con A-Sepharose (during two chromatographies) stimulates incorporation in a similar fashion to the total extract.

Less than 20 p. 100 of the total radioactivity incorporated into the follicles was associated with follicular cells and removal of these tissues after incubation showed that hormonal treatment resulted in a real stimulation of vitellogenin incorporation into the oocytes.

The results of this study suggest that the incorporation of vitellogenin by trout oocytes may be under direct control by a pituitary gonadotropin.

Introduction.

In teleosts the process of vitellogenesis apparently involves synthesis of a yolk precursor protein by the liver (Plack and Frazer, 1971 ; Emmersen and Emmersen, 1976 ; Campbell and Idler, 1976) which has been found in the blood by many workers (Ho and Vanstone, 1961 ; Thurston, 1967 ; Utter and Ridgeway, 1967 ; Aida *et al.*, 1973 ; Heesen and Engels, 1973 ; Emmersen and Petersen, 1976). Campbell and Idler (1976) found that a pituitary factor could reinitiate incorporation of this protein into the ovary of hypophysectomized flounder. The use of hypophysectomy precluded the possibility of action of injected preparations via the pituitary but gave no indication whether action was direct or involved a relay by other endocrine tissues.

Wallace *et al.* (1970) began a series of experiments showing that oocytes taken from HCG-treated *Xenopus laevis* continued to incorporate vitellogenin when maintained *in vitro*. This system (Wallace and Jared, 1976) was adopted to examine the direct action of extracts of salmon pituitaries on protein incorporation by isolated trout follicles which had not yet begun active vitellogenesis.

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Materials and methods.

Hatchery reared rainbow trout were maintained in a closed-circuit freshwater raceway system between 10 and 20 °C. Between February and July oocytes in the primary vitellogenic state were isolated from ovaries by treatment with collagenase. Protein incorporation was evaluated by use of proteins labelled with ^{14}C by reductive alkylation (Wallace and Jared, 1976 ; Rice and Means, 1970). The proteins were isolated using Triethyl aminoethyl cellulose (Jared and Wallace, 1968 ; but using a linear gradient) and/or by chromatography on Ultrogel AcA 22 (LKB). Serum from estrogenised trout was extensively purified by distilled water precipitation followed by TEAE and/or Ultrogel chromatography. This preparation contained vitellogenin with two minor contaminants detected by polyacrylamide gel electrophoresis. Serum from non-vitellogenic female trout was chromatographed on TEAE cellulose. The eluate fraction (C6a) which would have contained vitellogenin was selected for use as a control. In electrophoresis C6a contained five principal proteins and a barely detectable amount of a protein with a similar mobility to vitellogenin.

Lyophilised pituitary glands from spawning *Oncorhynchus tsawytscha* were extracted following Idler *et al.* (1975*a*) to yield a soluble total extract (TE) of which a portion was subjected to Con A-Sepharose chromatography. The unbound (Con Al) fraction was chromatographed twice to reduce the contamination by proteins normally bound to the gel (Con All) (Campbell and Idler, 1977). All extracts were tested at concentrations equivalent to 0.07 pituitary glands per incubation, i. e., 200 μg/ml for TE ; 170 μg/ml for Con Al and 30 μg/ml for Con All. When assayed in the immature trout ovarian cyclic AMP assay (Idler *et al.*, 1975*b*) the gonadotropin contents of TE, Con Al and Con All were 80, < 4 and 725 SG units/mg of protein respectively.

Individual oocytes were incubated in 20 μl of trout balanced salt solution (Jalabert, 1975) containing penicillin-streptomycin, labelled protein and pituitary preparation or as control an equal concentration of bovine serum albumin (BSA). Protein concentrations were made up to 200 μg/ml in Con Al and Con All media by addition of BSA. Incubations were performed in microtest plates (Cooke Microtiter) for 18 hours under a water saturated O_2/N_2 atmosphere at a constant temperature appropriate to the current seasonal temperature of the fish (10, 15 and 20 °C ; no attempt was made to evaluate effects of temperature since for each experiment all test treatments were evaluated under the same conditions).

After incubation oocytes were examined using a disecting microscope and those which had maintained their original appearance were rinsed three times with an isotonic balanced salts solution. Post-incubation denuding of oocytes was assured by further collagenase treatment to remove thecal layers followed by mechanical treatment to remove all granulosa cells which could be stained by toluidine blue. Oocytes were dissolved by either Protosol (NEN) or trypsin in preparation for scintillation counting.

Separate experiments using groups of 27 oocytes from each of two or three fish were performed. Data were converted to μg/mm² and combined for comparison by analysis of variance.

Results.

In a preliminary group of experiments treatment of entire follicles with TE increased incorporation of ^{14}C-labelled vitellogenin by 21 p. 100 over BSA controls ($P \leqslant 0.01$, table 1*a*).

Incubation of oocytes of 16 fish with the Con AII subfraction resulted in levels of incorporation similar to control oocytes (table 1*b*). Both Con AI and total extract significantly stimulated ($P \leqslant 0.01$) vitellogenin incorporation. The Con AI fraction was significantly less active than the total extract ($P \leqslant 0.01$). Equal amounts of the Con AI fraction obtained from two separated extractions of salmon pituitary both gave a 17 p. 100 stimulation.

TABLE 1

Total incorporation of ^{14}C-labelled vitellogenin by individual trout follicles incubated with 2 mg/ml vitellogenin and equivalent amounts of total pituitary extract (TE), *Con AI or Con AII fractions with bovine serum albumin* (Control C) *used to adjust to equal protein concentrations for each group* (ng vitellogenin/mm^2 oocyte surface/18 hrs ; mean $\pm$ SE : number of oocytes in parentheses)

	Treatment			
	C	TE	Con AI	Con AII
1*a*	109 $\pm$ 3 (108)	132 $\pm$ 4 (124)	—	—
1*b*	148 $\pm$ 4 (380)	172 $\pm$ 4.0 (395)	164 $\pm$ 4 (376)	153 $\pm$ 4 (368)

In further experiments with five fish the result of treatment with total extract or Con AI was an apparent 13 and 4 p. 100 stimulation ($P \leqslant 0.01$) of incorporation (table 2*a*). Thecal layers removed after incubation contained 14 p. 100 of the total

TABLE 2

ng of vitellogenin incorporated/mm^2 oocyte surface/18 hrs of incubation when oocytes were incubated in the presence of 2 mg/ml vitellogenin and treated with TE, Con AI, Con AII or BSA. 2*a* : incorporated vitellogenin found in entire follicles and follicles from which the thecal cells were removed after incubation ; 2*b* : incorporated vitellogenin found in oocytes from which all follicle cells were removed after incubation (mean $\pm$ SE, number of oocytes in parentheses)

	Treatment			
	C	TE	Con AI	Con AII
2*a* : entire follicle.....	188 $\pm$ 4 (127)	213 $\pm$ 5 (124)	197 $\pm$ 4 (121)	187 $\pm$ 4 (127)
oocyte + granulosa.....	152 $\pm$ 4 (101)	183 $\pm$ 5 (113)	178 $\pm$ 5 (110)	163 $\pm$ 5 (111)
2*b* : naked oocyte.....	218 $\pm$ 4 (64)	246 $\pm$ 6 (65)	242 $\pm$ 6 (69)	223 $\pm$ 6 (66)

protein incorporated partially masking the real stimulation of 21 and 17 p. 100 respectively.

Oocytes of three fish were totally denuded after incubation. An approximately equal 12 p. 100 stimulation ($P \leqslant 0.01$) by total extract or Con Al fraction represents a stimulation of incorporation into the oocyte rather than into the follicle cells (table 2*b*).

In three experiments eight oocytes were damaged during denuding and the chorion alone was taken for counting. Three percent of the radioactivity in a naked oocyte was found associated with the chorion.

Total extract stimulated incorporation of vitellogenin ($P \leqslant 0.01$) but not non-vitellogenic serum protein (C6a) (table 3). The quantity of vitellogenin incorporated was 17-18 times greater than that of C6a and the differences in quantities found in washed follicles and denuded oocytes, representing the protein association with follicular tissues, was only 10 p. 100 for vitellogenin but 60 p. 100 for C6a.

TABLE 3

Incorporated vitellogenin (V25) *and non-vitellogenic serum protein* (C6a) *found in control and total-extract-stimulated oocytes after complete denuding expressed as ng/mm²/18 hrs and also as a percentage of the total incorporation into oocyte and follicular tissues* (1 mg/ml of test protein + 200 µg/ml BSA or TE ; mean of 2 fish ± SE, number of oocytes io parentheses)

Protein	Treatment			
	C		TE	
	ng	p. 100	ng	p. 100
V25	139 ± 5 (36)	86	150 ± 5 (47)	93
C6*a*	8 ± 0.3 (39)	41	8 ± 0.3 (47)	43

Discussion.

The oocytes liberated from the ovarian tissues after experimental incubation were still surrounded by the granulosa and thecal layers and the data show that approximately 14 p. 100 of the total vitellogenin « associated » with the intact follicle is in the thecal tissue (table 2). The difference between vitellogenin incorporated into oocytes which were denuded and the total follicular « association » presented in table 3 approximates to this figure suggesting that little vitellogenin is « associated » with granulosa cells. The association with follicular tissues was greatest for C6a (table 3). For vitellogenin at least, this association may be a non-specific artifact in the system used. Table 2*a* shows that stimulations of incorporation by 21 and 17 p. 100 were masked and detected as only 13 and 4 p. 100 respectively when total follicles were processed.

The denuding of oocytes after incubation has shown that most vitellogenin « associated » with entire follicles, during *in vitro* incubation, was in fact associated with the oocyte. Examination of the chorion of several oocytes has indicated that the vitellogenin was incorporated into the oocyte and very little remained at the cell membrane. Wallace *et al.* (1970) found that vitellogenin incorporation by unstimulated oocytes of

X. laevis was minimal but augmented by five times if the donor had been treated with human chorionic gonadotropin (HCG) 24 hrs in advance. The vitellogenin incorporation by control oocytes suggests that this process might contribute to primary vitellogenic growth. However, this type of oocyte is normally exposed to concentration of < 20 µg vitellogenin/ml of serum at this stage (Crim and Idler, 1978) rather than the 1 or 2 mg/ml used experimentally so that vitellogenin incorporation must be a minor factor. Despite the high basal incorporation by control oocytes, vitellogenin incorporation was increased by treatment of oocytes with pituitary extracts for 18 hrs. The small augmentation seen may be due to some limiting factor in incubation conditions since Wallace *et al.* (1973*a*, *b*) showed that the incorporation of vitellogenin by oocytes from HCG-stimulated *X. laevis* was extremely sensitive to the ionic composition of the incubation media. In addition it is possible that 18 hour incubations are too short to demonstrate a large stimulation of incorporation.

The basal incorporation activity is relatively specific for vitellogenin and the stimulation of protein incorporation by pituitary extract is specific for vitellogenin (table 3). Wallace and Jared (1976), Wallace *et al.* (1970) showed that in HCG stimulated *X. laevis* oocytes vitellogenin was incorporated more actively than several other test proteins.

Treatment of trout oocytes with Con AI fraction or total extract of salmon pituitary glands stimulated vitellogenin incorporation. No such effect was produced by Con AII treatment. These results support a hypothesis of direct action by a Con AI gonadotropin on vitellogenic follicles in the trout. A Con AI preparation from *Hippoglossoides platessoides* pituitaries has already been found to promote yolk incorporation into the gonads of *Pseudopleuronectes americanus in vivo* (Campbell and Idler, 1976). The salmon gonadotropin capable of inducing oocyte maturation and ovulation (Jalabert *et al.*, 1974), having activity in the trout ovary cyclic AMP assay and stimulating ^{33}P-incorporation into chick testes (Idler *et al.*, 1975*a*) was isolated from the Con AII fraction. The results of Campbell and Idler (1977) suggest that the gonadotropin which will induce maturation and ovulation of *P. americanus* oocytes is also found in the Con AII fraction.

For these two species vitellogenin incorporation is stimulated by a gonadotropin which is distinguishable from previously isolated teleost gonadotropins by its lack of affinity to concanavalin A.

Symposium sur la Reproduction des Poissons
Paimpont, France, 19-21 *septembre* 1977.

Acknowledgments. — I thank The Royal Society for their support in the form of a European Fellowship, during this work. This work was partly supported by « Le Ministère de l'Environnement et de la Culture » grant n° 76-37. I am indebted to R. Billard, B. Breton, A. Fostier, B. Jalabert and D. Szöllösi for encouragement and suggestions.

Résumé. Les follicules ovariens isolés, prélevés sur des truites avant le début de la vitellogenèse, accumulent de la vitellogénine marquée au ^{14}C quand ils sont incubés *in vitro* pendant 18 h. L'incorporation de la vitellogénine est augmentée par l'action d'un extrait total d'hypophyse de saumon.

La fraction non liée à la Con A Sépharose (après deux passages) et l'extrait total stimulent l'incorporation de la vitellogénine de la même manière. Les cellules folliculaires

incorporent moins de 20 p. 100 de la vitellogénine ; il y a donc une vraie incorporation dans l'ovocyte. Ces résultats préliminaires montrent que l'incorporation de vitellogénine peut être directement contrôlée par l'hypophyse.

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