Short Contribution

Study of yolk precursor transport in the avian ovary with the use of horseradish peroxidase

KATHARINA D'HERDE[1]* and LUCIEN VAKAET[2]

[1]*Department of Human Anatomy, University of Ghent, Ghent, and* [2]*Laboratory of Experimental Cancerology, University Hospital University of Ghent, Ghent, Belgium*

ABSTRACT Ovaries of adult Japanese quails were exposed *in vivo* to the exogenous protein horseradish peroxidase (HRP) for varying lengths of time to investigate ultrastructurally the permeability of the wall of these follicles, the protein uptake capacity of granulosa and oocyte and the kinetics of protein uptake in different stages. There is a sudden increase in permeability of the follicle wall from previtellogenesis to vitellogenesis. This is not due to a loss of sealing (tight) junctions in the granulosa cell layer, but is probably related to a permeability change in the basement membrane. The transition from the slow growth phase to the rapid growth during vitellogenesis is accompanied by a limited widening of the intercellular channels and the concomitant development of a complex endocytotic apparatus in the ooplasm. The slowing down of yolk deposition during the last day before ovulation is accompanied by a narrowing of the intercellular channel width. The granulosa cells show a high intracellular HRP uptake during intermediary yolk formation. Transcytosis through the granulosa cannot be excluded but is probably a minor pathway at certain stages. The light microscopically detectable uptake of HRP by the oocyte coincides with the start of exogenous vitellogenesis. After 90 sec of exposure to HRP (intravenous injection) the tracer can be found in the intercellular channels of the granulosa and in superficially located yolk spheres. On the other hand it takes 10 min for the tracer to traverse the cortex of the oocyte.

KEY WORDS: *vitellogenesis, horseradish peroxidase, ultrastructure, ovary, quail.*

Hereafter we report the results of the different experiments per stage of folliculogenesis.

Previtellogenic follicles

In the quail ovary follicles in previtellogenesis have a diameter of 50 µm to 1.2 mm. This corresponds to the prelampbrush and lampbrush stage (phase I) of Callebaut (1973, 1975).

Time after injection (TAI) experiments at 90 sec, 5 min, 15 min, 30 min and 60 min showed that in previtellogenic follicles little or no proteins reach the oocyte-granulosa interface. Griffin *et al.* (1984) stated that the permeability of the basement membrane varies during folliculogenesis. A lower permeability of the basement membrane and an underdeveloped microvasculature can explain why so little HRP permeated the granulosa during previtellogenesis. The surface of avian previtellogenic oocytes displays morphological features of pinocytotic uptake. Bellairs (1965) did not succeed in visualizing with colloidal gold labelling the functionality of it. Our experiments showed that the extensive tubular network on the surface of previtellogenic oocytes is not part of a functional endosomal system. These results are in disagreement with the results of Callebaut and Sijens (1985). By using an experimental setup with iterative injections of trypan blue, these authors demonstrated a yolk accumulating system in prelampbrush oocytes. The difference may be due to their setup which was toxic in our hands (unpublished results).

Follicles in intermediary yolk formation

This category comprises the largest lampbrush follicles (1.2 - 1.5 mm) and the early postlampbrush follicles (1.5 - 3 to 4 mm). During intermediary yolk formation the growth of the ovum is slow. The 90 sec experiment revealed that from the start of the peripheral yolk accumulation the intercellular passage through the granulosa is not obstructed by tight junctions (Fig. 1).

Moreover, we see that the endocytosis of HRP in the granulosa is minimal after 90 sec, so transcytosis through the granulosa

Abbreviations used in this paper: BM, basement membrane; GR, granulosa cell layer; HRP, horseradish peroxidase; NUC, nucleus; OOC, oocyte; TAI, time after injection; TI, theca interna; VM, vitelline membrane; VLDL, very low density lipoproteins

***Address for reprints**: Department of Human Anatomy, Ledeganckstraat, 35, B-9000 Ghent, Belgium. FAX: 32-9124.0499.

0214-6282/92/$03.00

Printed in Spain

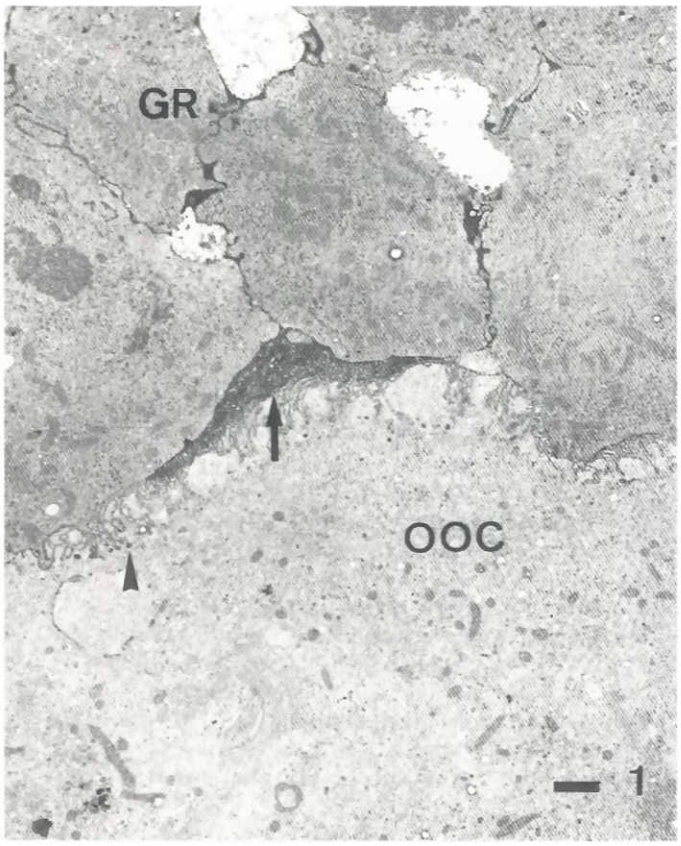

Fig. 1. Lampbr h follicle (phase II), TAI: 90 sec. *60 nm LX-section, not counterstained. Direct mag.: 3000, bar 1 µm. Intercellular positivity in the granulosa. Endocytotic uptake in the granulosa is negligible after 90 sec. Note local accumulation of HRP at the oocytal-granulosal interface (arrow). Some endocytotic vacuoles with HRP at their luminal surface (arrowhead).*

cannot be excluded but is only a minor pathway. In contrast with the previous stage, local accumulations of HRP were found at the oocyte-granulosa interface. Increasing the TAI in this stage revealed some kinetics of the protein uptake mechanism.

After TAI of 90 sec the tracer was confined to the luminal surface of endocytotic vacuoles. After TAI of 5 min the tracer was present in superficially located nascent yolk spheres.

After TAI of 15 min the tracer labelled the whole cortex of the oocyte, suggesting that it takes 10 min (15-5) for a nascent yolk sphere to traverse the cortex.

The passage of the tracer through the cortex of early postlampbrush follicles was previously timed in trypan blue experiments by Callebaut *et al.*, (1981). From their kinetic experiments with subcutaneous injections of TB, these authors concluded it takes the tracer 15 min to traverse the oocyte cortex. However, the intravenous injections in bolus we performed are more accurate for kinetic experiments compared to subcutaneous injections.

The oocyte tubular structures with an average diameter of 50 nm were found loaded with HRP and concentrated around nascent yolk spheres. Lining bodies, the enigmatic organelles only found in avian and reptilian ovaries, are taken up in the oocyte in giant coated vesicles together with HRP. The presence of osmiophilic substance around an invaginated lining body was previously described by Paulson and Rosenberg (1972, 1974). This was interpreted as substance synthesized under the influence of lining bodies. Our results indicate that HRP and probably yolk precursors are taken up together with the lining bodies during the slow growth phase, when intermediary yolk is deposited.

The experiments at TAI of 30 min to 60 min demonstrated that the granulosa cells of follicles in intermediary yolk formation show high endocytotic activity at their apical side in contrast to the other stages: previtellogenic and follicles in yellow yolk formation (Fig. 2). Former tracer studies (Callebaut, 1973, 1975; Nishimura and Urakawa, 1976) failed to give a picture of absorptive capacities of the granulosa during the different stages.

In a review article on vitellogenesis, Anderson (1974) stated that the uptake of large amounts of tracer protein by the follicle cells is typical for the mammalian system, whereas in the insect ovary relatively little is taken up by the follicle cells.

Follicles in yellow yolk formation

This category comprises the postlampbrush follicles between 4 and 19 mm.

The different experiments revealed that the intercellular spaces increase during yellow yolk formation. This is due not so much to the increase in intercellular channel width as to the increase in follicular membrane area as a result of the development of microvilli on the lateral sides of the cells (Fig.3).

Former schematic drawings based on ultrastructural studies of intercellular spaces in the granulosa show large intercellular spaces, exceeding 1 µm (Wyburn *et al.*, 1965, 1966; Jordanov and Boyadjieva-Michailova, 1974; Rothwell and Solomon, 1977; Perry *et al.*, 1978a,b).

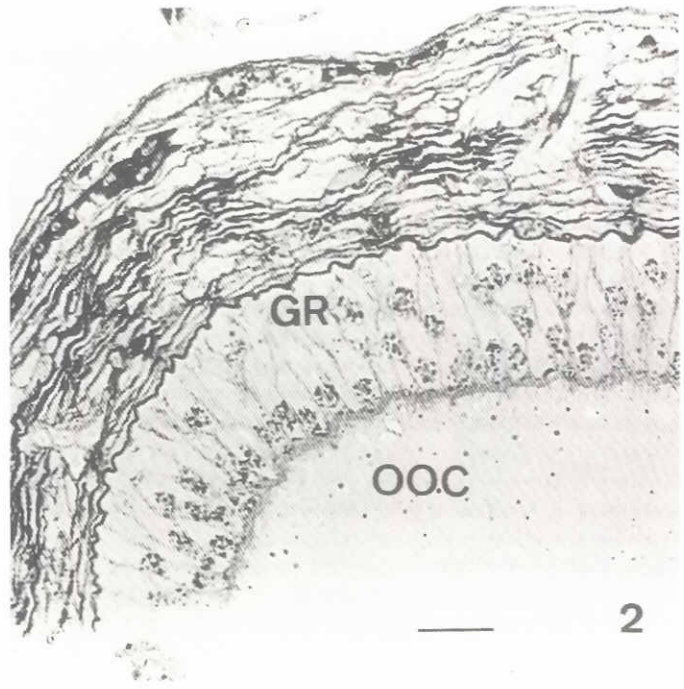

Fig. 2. Early postlampbrush follicle, TAI: 60 min. *2 µm LX-section, not counterstained. Obj. 50, bar 10 µm. The multi-layered granulosa is loaded with HRP (areas containing black dots).*

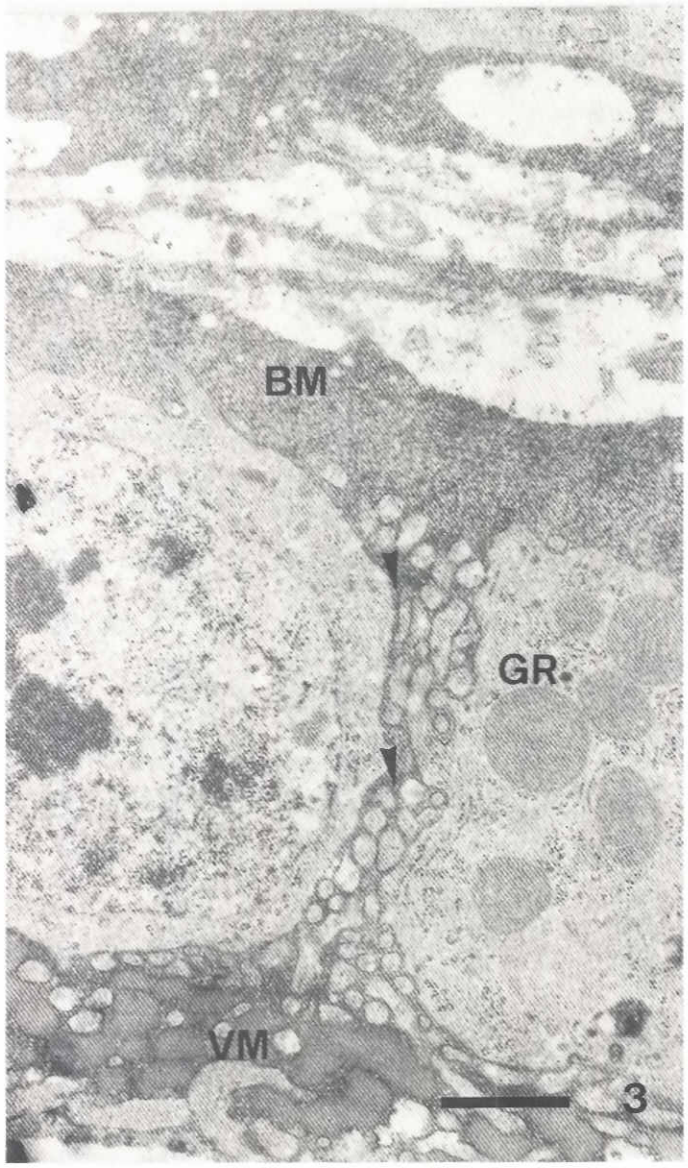

Fig. 3. Postlambrush follicle 2 days before ovulation (F_2) TAI: 90 sec. *60 nm LX-section, counterstained. Direct mag.: 8000, bar 1 µm. Intercellular HRP-positivity (arrowheads) in microvillar channels between the granulosa cells is prominent.*

In the preovulatory follicle, F_1 (one day before ovulation, according to the nomenclature of Gilbert *et al.*, 1982) we found narrow intercellular channels similar to what we have seen during slow growth (Fig. 1) and this in experiments with TAI of 90 sec, 30 and 60 min. This narrowing in concert with the decrease in growth rate was not shown in former tracer studies. Recently it was discovered (Reaven *et al.*, 1990) that so-called microvillar channels can widen through the binding of lipoproteins.

Perry and Gilbert (1979) have shown the presence of very low density lipoproteins (VLDL) in the intercellular spaces of the granulosa during rapid growth.

The narrowing of the microvillar channels the day before ovulation could be due to an alteration of binding characteristics of lipoproteins to the plasma membrane of the microvillar channels. However in *in vitro* experiments Perry *et al.* (1984) could not demonstrate binding of the VLDL to the granulosal plasma membrane. If the modulation of intercellular channel width is not governed by the granulosa itself, then a *vis a tergo* in the follicle wall must change. Scanes *et al.* (1982) found that there is considerable local variation in blood flow within the ovary according to the stage of follicular development. Callebaut *et al.* (1990) ascribe a vitellodynamic function to the follicle wall of oocytes in rapid growth.

After 90 sec TAI we found marking of superficial yolk spheres in the follicles in yellow yolk formation.

Perry and Gilbert (1985) determined with the tracer ferritin that the time needed for the formation of marked yolk spheres was 10 min. This difference (90 sec to 10 min) is probably due to the high sensitivity with which HRP can be localized. Moreover ferritin is segregated in subdroplets, whereas HRP is segregated mainly in the groundplasm.

Experimental Procedures

Horseradish peroxidase (HRP), a mannose-rich glycoprotein of plant origin (the root of *Armoracica rusticana*), has been used as a tracer for studies of protein uptake and epithelial permeability (Graham and Karnovsky, 1966). The advantages of HRP are its sensitivity and the resistance of its enzymic activity to fixation procedures. For a time, HRP tended to be used uncritically as a «fluid phase marker». However, the peroxidase molecules containing mannose can be bound by the mannose-specific endocytotic receptors present on certain cell types (Holtzman, 1989).

Although HRP is unrelated to the natural yolk precursors, we propose that examining different stages after varying injection times gives information concerning the changing permeability of the follicle wall towards proteins in general.

Regularly laying female quails (*Coturnix coturnix Japonica*, n=11) were injected in the brachial vein with HRP. The dose was 30 mg HRP dissolved in 1 ml Ringer (HRP type II Sigma), corresponding to 0.12 mg/g body weight. The animals were killed by decapitation after 90 sec, 5, 15, 30 and 60 min. The interval between injection of the tracer and decapitation is defined as the TAI (time after injection).

As a control experiment one animal received an injection of 1 ml Ringer without addition of the HRP, followed by an incubation as described for the experiments. Non-enzymatic staining of hemoglobin in the derivatives of the red blood cells was present.

A compound-aldehyde fixative containing 3% glutaraldehyde, 2% paraformaldehyde, 1% acroleine and 2.5% DMSO in 0.1 M cacodylate buffer pH 7.2 with 0.01 M calcium chloride was used (after Kalt and Tandler, 1971).

After fixation on ice during 3 h, the tissue was kept overnight at 4°C in the buffer solution (cacodylate with sucrose 0.1 M pH 7.4). Tissue chopper sections of about 60 µm were made (using a Tissue Sectioner TC-2 of Smith and Farquhar, 1963). They were stored in tris buffer solution containing 7.5% sucrose. As incubation medium we used 5 mg diaminobenzidine dissolved in 9 ml Tris buffer (0.05 M, pH 7.6) with 1 ml of freshly prepared H_2O_2 0.1% and 1 mg aminotriazole. The aminotriazole is included to inhibit the endogenous catalase activity.

The incubation performed at 37°C was stopped after 1 h by transferring the tissue in Tris buffer. To obtain a regular staining, we used a mini shaker for the incubation. As control of the incubation H_2O_2 is omitted from the medium.

For the postfixation, we use 2% OsO_4 in cacodylate during 5 h. After the routine dehydration procedure, embedding in LX-resin followed.

Semithin sections were made on a LKB pyramitome. Ultrathin sections were made on a LKB ultratome. The ultrathin sections were examined on a Siemens electron microscope (type Elmiskop 1A), either unstained or

stained automatically in a LKB ultrostainer. The semithin sections were examined on a Leitz Laborlux D and photographed with a Wild photoautomat MPS 45.

References

ANDERSON, E. (1974). Comparative aspects of the ultrastructure of the female gamete. *Int. Rev. Cytol. (Suppl.) 4:* 1-70.

BELLAIRS, R. (1965). The relationship between oocyte and follicle in the hen's ovary as shown by electron microscopy. *J. Embryol. Exp. Morphol. 13:* 215-233.

CALLEBAUT, M. (1973). Correlation between germinal vesicle and oocyte development in the adult Japanese quail *Coturnix coturnix japonica*. A cytochemical autoradiographic study. *J. Embryol. Exp. Morphol. 29:* 145-157.

CALLEBAUT, M. (1975). *Bijdrage tot de studie van de oogenesis van de vogels.* Aggregaatsthesis. Universiteit Gent.

CALLEBAUT, M., HARRISON, F. and VAKAET, L. (1981). Peripheral avian yolk assemblage and its persistence in the blastoderm studied by trypan-blue-induced fluorescence. *Anat. Embryol. 163:* 55-69.

CALLEBAUT, M. and SIJENS, R.J. (1985). Trypan blue induced fluorescence in adult quail oocytes during the prelampbrush and early lampbrush stage. *Anat. Hum. Biol. 13:* 920-921.

CALLEBAUT, M., SIJENS, R.J. and VAN NASSAUW, L. (1990). Evolution of the avian follicle wall during its expansion. *Eur. Arch. Biol. 101:* 77-87.

GILBERT, A.B., HARDIE, M.A. and PERRY, M.M. (1982). The relationship between the weight and diameter of the small ovarian follicles of the domestic hen *(Gallus domesticus). IRCS Med. Sci-Biochem. 10:* 571.

GRAHAM, R.C. and KARNOVSKY, M.J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem. 14:* 291-302.

GRIFFIN, H.D., PERRY, M.M. and GILBERT, A.B. (1984). Yolk formation. *Physiol. Biochem. Dom. Fowl 5:* 345-380.

HOLTZMAN, E. (1989). *Lysosomes* (Ed. P.Siekwitz). Plenum Press, New York and London.

JORDANOV, J. and BOYADJIEVA-MICHAILOVA, A. (1974). Ultrastructural aspects of lipoprotein passage through oocyte envelopes and storage in ooplasm during avian vitellogenesis. *Acta Anat. 89:* 616-632.

KALT, M.R. and TANDLER, B. (1971). A study of fixation of early amphibian embryos for electron microscopy. *J. Ultrastruct. Res. 36:* 633-645.

NISHIMURA, M. and URAKAWA, N. (1976). An electron microscopic study on 203Hg transport in the ovarian tissue of laying Japanese quail. *Jpn. J. Vet. Sci. 38:* 83-92.

PAULSON, J. and ROSENBERG, M.D. (1972). The function and transposition of lining bodies in developing avian oocytes. *J. Ultrastruct. Res. 40:* 25-43.

PAULSON, J.L. and ROSENBERG, M.D. (1974). Formation of lining bodies and oocyte bodies during avian oogenesis. *Dev. Biol. 40:* 366-371.

PERRY, M.M. and GILBERT, A.B. (1985). The structure of yellow yolk in the domestic fowl. *J. Ultrastruct. Res. 90:* 313-322.

PERRY, M.M. and GILBERT, A.B. (1979). Yolk transport in the ovarian follicle of the hen: lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. *J. Cell. Sci. 39:* 257-272.

PERRY, M.M., GILBERT, A.B. and EVANS, A.J. (1978a). The structure of the germinal disc region of the hen's ovarian follicle during the rapid growth phase. *J. Anat. 127:* 379-392.

PERRY, M.M., GILBERT, A.B. and EVANS, A.J. (1978b). Electron microscope observations on the ovarian follicle of the domestic fowl during the rapid growth phase. *J. Anat. 125:* 481-497.

PERRY, M.M., GRIFFIN, H.D. and GILBERT, A.B. (1984). The binding of very low density and low density lipoproteins to the plasma membrane of the hen's oocyte. *Exp. Cell Res. 151:* 433-446.

REAVEN, E., SHI, X.Y. and AZHAR, S. (1990). Interaction of lipoproteins with isolated ovary plasma membranes. *J. Biol. Chem. 265:* 19100-19111.

ROTHWELL, B. and SOLOMON, S.E. (1977). The ultrastructure of the follicle wall of the domestic fowl during the phase of rapid growth. *Br. Poult. Sci. 18:* 605-610.

SCANES, C.G., MOZELIC, E., KAVANAGH, G., MERILL, G. and RABII, J. (1982). Distribution of blood flow in the ovary of domestic fowl *(Gallus domesticus)* and changes after prostaglandin F-2α treatment. *J. Reprod. Fertil. 64:* 227-231.

SMITH, R.E. and FARQUHAR, M.G. (1963). Preparation of thick sections for cytochemistry and electron microscopy by a nonfreezing technique. *Nature 200:* 691.

WYBURN, G.M., AITKEN, R.N.C. and JOHNSTON, H.S. (1965). The ultrastructure of the zona radiata of the ovarian follicle of the domestic fowl. *J. Anat. 99:* 469-484.

WYBURN, G.M., JOHNSTON, H.S. and AITKEN, R.N.C. (1966). Fate of the granulosa cells in the hen's follicle. *Z. Zellforsch. 72:* 53-65

Accepted for publication: June1992