

Development *in vitro* of the female germ cells of the polychaete *Ophryotrocha labronica*

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SUMMARY

In the polychaete *Ophryotrocha labronica* each oocyte is during its growth period associated with a single nurse cell. The fact that the oocyte–nurse cell pairs occur isolated in the female coelom makes them easily removable for analysis of their developmental ability *in vitro*. Using Dulbecco's modified Eagle medium supplemented with amino acids, nucleosides, foetal calf serum and sea water, we have managed to support development *in vitro* of germ cell pairs from early and mid-oogenesis until maturation of the oocyte, when the nurse cell degenerates and the oocyte enters meiotic metaphase. Radiolabelling of germ cells in mid-oogenesis with tritiated amino acids and uridine during the first day of incubation indicates normal development with synthesis of RNA and protein, and pulses two days later verify a continued normal protein synthesis and yolk formation. The investigation confirms autotranslation of yolk proteins in the germ cells of this species and indicates a leading role of the nurse cell in the process.

INTRODUCTION

It is generally recognized that yolk granule proteins (vitellins) in ova of non-mammalian vertebrates originate as vitellin precursors (vitellogenins) in somatic cells outside the ovary. From their site of synthesis the vitellogenins are transported to the ovary, where they are incorporated and sequestered in the growing oocyte (reviewed by Tata & Smith, 1979). The corresponding mode of yolk formation may be valid for invertebrates as well, and in some instances, e.g. in the nematode *Caenorhabditis elegans*, an extra-ovarian origin of the yolk proteins is confirmed (Kimble & Sharrock, 1983). However, in the fruitfly *Drosophila melanogaster*, yolk proteins are synthesized not only in the somatic cells, but also in the ovary (Gutzeit, 1980). In the polychaete *Nereis virens*, synthesis of yolk proteins may be focussed to the ovary (Bertout & Dhainaut, 1971) but there are also strong indications that yolk precursors are produced in somatic tissue (Heacox, Fischer & Frangenberg, 1983).

Analyses of the incorporation of labelled amino acids during oogenesis in the polychaete *Ophryotrocha labronica* have indicated that in this species yolk synthesis is confined to the germ cells (Emanuelsson, 1985). Up to the termination

Key words: Cell culture, polychaete, oogenesis, yolk synthesis, autoradiography.

of oogenesis each oocyte in *Ophryotrocha* is connected with a single nurse cell which is considered to have originated from the same oogonium as the oocyte, and from the nurse cell metabolites and organelles are transferred to the oocyte via an intercellular canal, a fusome (Emanuelsson, 1969). Apparently the nurse cell plays a leading role in the yolk protein synthesis. However, as the labelling studies were performed on intact females a transfer of yolk protein from somatic cells cannot be excluded. Any doubts about autosynthesis will of course be dissipated if it can be demonstrated that the germ cells are able to maintain normal development and yolk synthesis in growth medium *in vitro*.

Therefore, the principal aim of this study has been to create an artificial growth medium that can support normal development of the isolated oocyte–nurse cell complex *in vitro*. We have achieved this and have also monitored the synthesizing ability of the cultured germ cells with tritium-labelled amino acids and uridine. The experiments confirm an extensive yolk protein and RNA synthesis in the germ cells of *Ophryotrocha* and emphasize the importance of the nurse cell in vitellogenesis.

MATERIALS AND METHODS

Animal material

The marine polychaete *Ophryotrocha labronica* LaGreca and Bacci (from the Mediterranean, Naples) is kept in sea water cultures at room temperature (18–20°C) in our laboratory. Under these conditions the polychaete breeds all through the year, and the interval between successive generations is about 30 days. In the female polychaetes the first obvious signs of germ cell growth appear at about 20 days of development. It is completed by day 30. During the oocyte growth period each oocyte is connected with a single nurse cell, which originally is larger than the oocyte, but degenerates in the final phase of oocyte maturation. The oocyte–nurse cell complexes, which develop as free cell pairs in the female coelom, are reasonably synchronous in development. Upon mating the mature female produces an egg pack containing about 300 eggs, all of which develop synchronously. For this investigation we mainly used 21- to 23- and 26- to 28-day-old females, i.e. individuals in early and mid-oogenesis.

The selected females were anaesthetized in 3.5 % MgCl_2 . The immobilized worms were ripped up with metal needles, and the oocyte–nurse cells sucked up in a fine pipette and rapidly transferred into sterile sea water. Following repeated washes in sterile sea water and control in the microscope, the germ cell complexes were finally transferred into 35 mm plastic petri dishes with culture medium (1 ml) and cultured in a thermostatically controlled incubator at $20.0 \pm 1.0^\circ\text{C}$ in a humid atmosphere. Some of the germ cell complexes had unintentionally been torn apart during handling. They usually made up less than 10 % of the total, and no attempts were made to remove them. Each culture dish regularly contained about 200 intact germ cell complexes.

Culture medium

As a result of systematic testing of various combinations of growth media and sea water, the oocyte–nurse cell complexes were cultured in a growth medium consisting of Dulbecco's modified Eagle medium (DMEM) (from Gibco Europe), supplemented with pyruvate (1 mM), serine (1 mM), proline (1 mM), adenosine (30 μM), guanosine (30 μM), cytidine (30 μM), uridine (30 μM), thymidine (10 μM), non-essential amino acids (1 mM), penicillin (50 i.u./ml), streptomycin (50 μg /ml), and 10 % foetal calf serum. One part of this medium was mixed with

one part of boiled, sterile filtered sea water. To avoid growth of micro-organisms the medium was supplemented with extra antibiotics: penicillin (1000 i.u./ml) and streptomycin (100 µg/ml) prior to culture. Since the oocytes were incubated in air without CO₂, the medium was buffered with Tricine buffer (25 mM). The osmolarity of the final medium was determined to 625 mOsm/kg (sea water: 908 mOsm/kg), using an Advanced osmometer m3D (Advanced instruments, U.S.A.).

The germ cell complexes were cultured for one week maximum. The mortality was generally below 20 %. Addition of fungicide to the normal culture medium usually proved unnecessary. However, culture series incubated for more than 5 days usually included dishes, containing Fungizone (Gibco Europe, 0.25 µg/ml) in addition to the normal ones lacking the fungicide.

For examination in the light microscope the cultured germ cells were first transferred to a slide, covered with a layer of 1 % gelatin in phosphate-buffered saline and then fixed on the slide in a mixture of absolute alcohol–glacial acetic acid (3:1). The preparation was stained in 1 % toluidine blue and mounted in DePeX.

For examination in the electron microscope the cell material was prepared as described for the autoradiographic material.

Labelling experiments

Labelling with [*5-³H]uridine (The Radiochemical Centre, Amersham, specific activity 1.04 TBq/mmol; final activity 18.5 MBq/ml), with *L*-[*4,5-³H]leucine (The Radiochemical Centre, Amersham, specific activity 1.74 TBq/mmol; final activity 18.5 MBq/ml) and with *L*-[*5-³H]tryptophan (The Radiochemical Centre, Amersham, specific activity 888 GBq/mmol; final activity 18.5 MBq/ml) was performed using the culture medium described above (DMEM). In all cases the isotope solution was first evaporated to dryness in an air or nitrogen stream at room temperature, and the residue was then dissolved in the medium (0.25 ml). The oocyte–nurse cell complexes were pulse-labelled for 0.5 and 2 h. After that they were chased in isotope-free medium for 1 h and then immediately fixed for light and electron microscope autoradiography.***

Due to the comparatively high leucine content of the ordinary culture medium (DMEM) some of the labelling experiments with *L*-[*4,5-³H]leucine were for control purposes performed using a less leucine-rich growth medium than DMEM, viz. Ham's nutrient mixture F12 (Gibco Europe), supplemented with penicillin (50 i.u./ml), streptomycin (50 µg/ml) and 10 % foetal calf serum, and buffered with Tricine buffer (50 mM). One part of this medium was mixed with one part of boiled and sterile filtered sea water, and supplemented with extra antibiotics (penicillin, 1000 i.u./ml; streptomycin 100 µg/ml). So far the observed labelling in this medium was found identical with that in DMEM.*

Autoradiography

The cultured germ cells were fixed in 2 % glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3, 1 h, 4°C) and postfixed in 1 % osmium tetroxide in the same buffer. After rinsing in buffer they were dehydrated and stained in ethanol containing 1 % phosphotungstic acid and 0.5 % uranyl acetate. They were embedded in Epon (Polarbed 812) and sectioned for light microscope autoradiography (1 µm sections) and for electron microscope autoradiography (ultrathin sections). The 1 µm sections were covered with Ilford K 2 liquid nuclear emulsion by dipping. After exposure for 1–2 weeks at 4°C the autoradiographs were developed in Kodak D 19 (5 min, 18°C), briefly rinsed in distilled water, and fixed in Kodak F 24 (6 min, 18°C). They were finally stained in a solution containing Richardson's azure II and methylene blue.

The ultrathin sections for electron microscope autoradiography were first given a protective carbon coat and were then covered with a monolayer of Ilford L 4 liquid nuclear emulsion according to the loop method. After exposure for 1–2 months the autoradiographs were developed in Kodak D 19 (2 min, 20°C), rinsed in distilled water (30 sec), fixed in newly made 15 % Na₂S₂O₃ (3 min, 20°C) and finally washed in distilled water. They were examined in a Jeol 100CX electron microscope at the Unit of Electron Microscopy, Department of Zoology, University of Lund, Sweden.

RESULTS

The *Ophryotrocha* females reared in our laboratory usually reach maturation by day 30. Although all the oocyte–nurse cell complexes of a female are not strictly synchronous in development the vast majority of the oocytes has by that time lost the nurse cell, and has entered the first meiotic metaphase.

Germ cell complexes cultured *in vitro* (Fig. 1) were found to develop at the same rate. Thus, most oocytes from 21- to 23-day-old females had attained maturation after 7 days in culture, and maturation was reached after 3–4 days in culture by oocytes from 26- to 28-day-old females. Usually growth and development of invertebrate oocytes is monitored by measuring the oocyte diameter increase. In *Ophryotrocha* such measurements in fact turned out to be unnecessary, as the presence and magnitude of the adherent nurse cell clearly indicated the progress in oogenesis. The non-developing germ cell complexes in the cultures were easily spotted, as they gradually showed a characteristic wrinkling of the cell surface, first in the nurse cell, later on also in the oocyte. They also showed a marked aggregation of the cell contents. So far we have not observed any structural differences between the germ cells developing in the intact female, and those developing *in vitro*.

However, as morphology and spatial distribution of organelles is much more easily controlled in the cultured cells, some previously unnoticed interactions between the oocyte and the nurse cell have been disclosed in this material. For example, we noted that shortly before the elimination of the nurse cell, the oocyte nucleus moves from its earlier, permanent position distally from the nurse cell to a site close to the intercellular canal (fusome) between the two cells (Fig. 3). Correspondingly, large parts of the voluminous endoplasmic reticulum from the degenerating nurse cell move into the oocyte region, abandoned by the oocyte nucleus. During its movement to the new position, the oocyte nucleus is observed

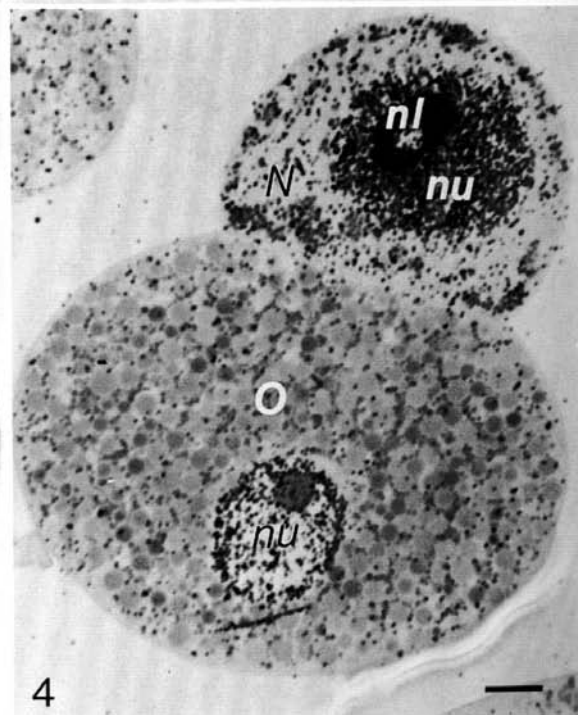
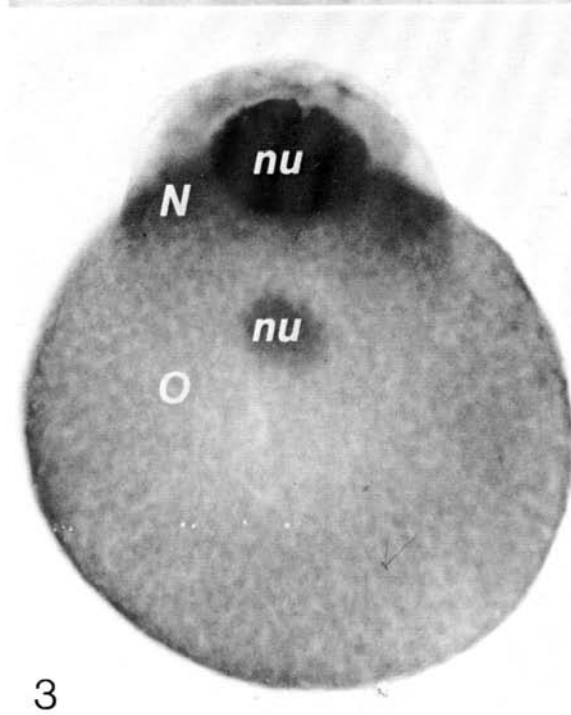
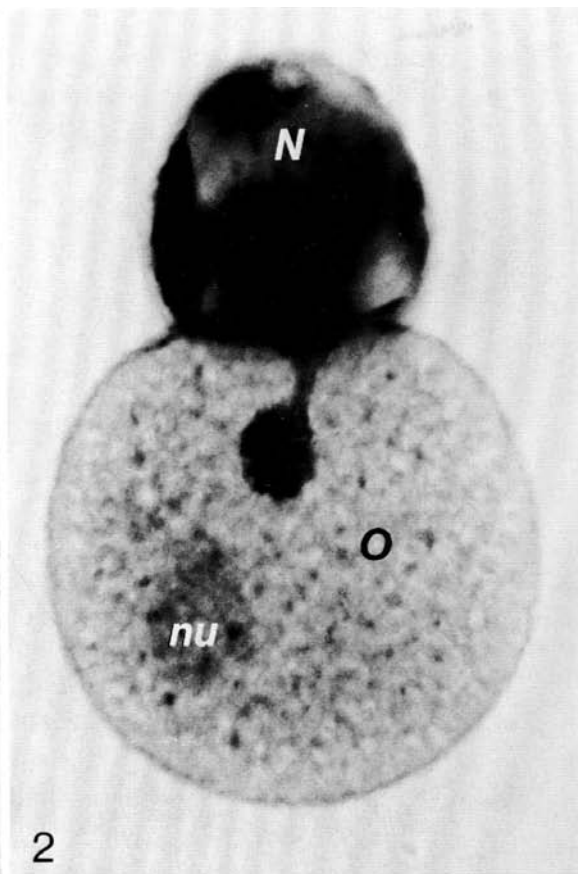
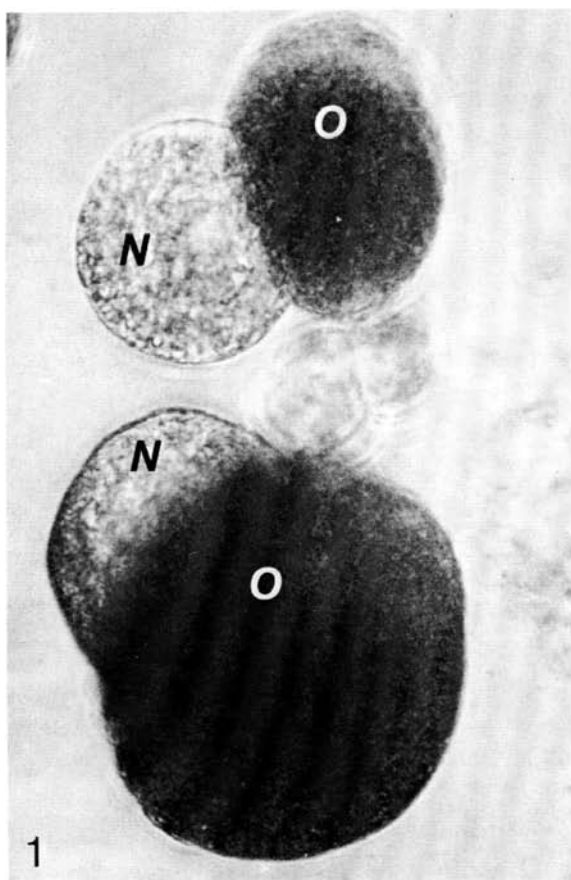
Fig. 1. Oocyte–nurse cell pairs isolated from an *Ophryotrocha labronica* female in mid-oogenesis. The cell pair in the upper part of the figure represents an early stage at which the components are of about the same size. The cell pair in the lower part of the figure has reached a more advanced stage, the oocyte being now the dominating component. *N*, nurse cell; *O*, oocyte.

Fig. 2. *Ophryotrocha* oocyte–nurse cell pair in late oogenesis. Notice the nurse cell material projecting into the oocyte. *N*, nurse cell; *O*, oocyte; *nu*, nucleus. Stain: toluidine blue.

Fig. 3. *Ophryotrocha* oocyte–nurse cell pair shortly before elimination of the nurse cell. At this stage the oocyte nucleus has taken up a position close to the nurse cell. Notice the large, polyploid nucleus of the nurse cell. *N*, nurse cell; *O*, oocyte; *nu*, nucleus. Stain: haematoxylin.

Fig. 4. Light microscope autoradiograph demonstrating incorporation of [³H]uridine in an isolated *Ophryotrocha* oocyte–nurse cell pair, cultured *in vitro*. The cell pair was radiolabelled for 2 h during the first day of *in vitro* culture. Notice the strong labelling of the nurse cell nucleus and nucleolus. *N*, nurse cell; *O*, oocyte; *nu*, nucleus; *nl*, nucleolus.

Magnification is the same in Figs 1–4. Bar equals 10 µm.



to be in close contact with basophil material, probably of nurse cell origin, projecting from the fusome region (Fig. 2).

In *Ophryotrocha* the oocyte, but not the nurse cell, is supplied with a protective chorion (Fig. 6). This difference is even more apparent in isolated cell pairs than in cell pairs, crowded in the coelom. Although the oocyte plasma membrane is provided with a multitude of cytoplasmic processes penetrating the chorion, its surface against the surrounding medium appears to be considerably smaller than that of the chorion-free nurse cell which, in addition, is supplied with numerous small cell projections.

To all appearances the development of the *in vitro* cultured germ cells was identical with that *in vivo*, which obviously should imply that also synthesis of RNA and proteins have continued in a normal way in the cultured cells. To control this, RNA and protein synthesis in the cultured cells was monitored with the aid of [^3H]uridine and [^3H]leucine. Also [^3H]tryptophan was utilized, as previous observations have demonstrated that *in vivo* this substance is preferentially accumulated in the typical yolk granules of the oocyte (Emanuelsson, 1985). The cells were pulsed for 0.5 and 2 h respectively during the first day of culture, and for some cultures during the third day.

The labelling experiments demonstrate that there is substantial incorporation of tritiated uridine and tritiated amino acids both in germ cell complexes pulsed during the first day of culture and in complexes pulsed after previous culture for three days. The observed labelling patterns correspond to those earlier found in germ cell complexes labelled *in vivo*. Short pulses (0.5 h) with [^3H]uridine, or with [^3H]leucine (Fig. 5), or [^3H]tryptophan result in labelling which in all cases is more extensive in the nurse cell than in the oocyte. For [^3H]leucine the cytoplasmic label is concentrated to the endoplasmic reticulum, the Golgi apparatus, and to such yolk bodies that are recognized as precursor material to the typical yolk granules. Within the endoplasmic reticulum it is notably the small, peripheral aggregates, that are heavily labelled, the large perinuclear aggregates usually show a less-intense labelling.

Also after longer pulses (2 h) incorporation of [^3H]uridine is stronger in the nurse cell than in the oocyte (Fig. 4), and cytoplasmic labelling in the latter is weak only. Extended (2 h) pulses with [^3H]leucine result in impressive cytoplasmic incorporation in both germ cells, but is still stronger in the nurse cell. After extended pulses with [^3H]tryptophan (Fig. 6) incorporation into the 4 μm oocyte

Fig. 5. Electron microscope autoradiograph and (inset) light microscope autoradiograph demonstrating incorporation of [^3H]leucine in an isolated oocyte–nurse cell pair cultured *in vitro*. The cell pair was pulse labelled for 0.5 h during the third day of *in vitro* culture. In the electron microscope autoradiograph only the nurse cell is shown. The light microscope autoradiograph demonstrates that after a short pulse the uptake is essentially restricted to the nurse cell (N). In the latter strong labelling is found in the endoplasmic reticulum (er) and in yolk bodies (yb), recognized as precursor material to the typical 4 μm oocyte yolk granules. O, oocyte; nu, nucleus. Bar equals 1 μm (inset 10 μm).

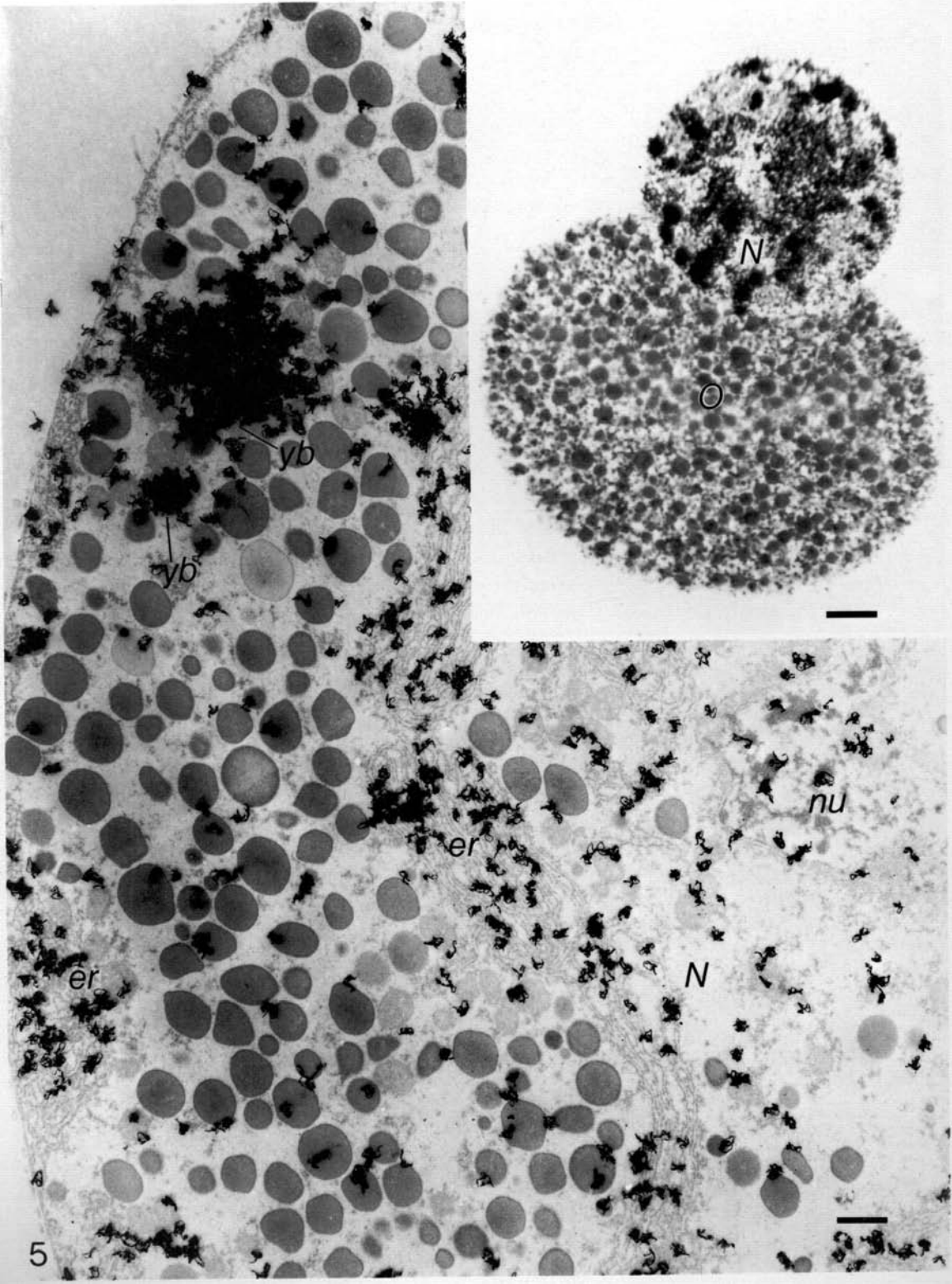


Fig. 5.

yolk granules is particularly manifest, other cytoplasmic labelling being roughly the same in the two germ cells.

DISCUSSION

Studies of the endocrine control of oogenesis in polychaetes have shown that in the nereids the presence of brain hormones is necessary only for the completion of the earlier stages of oogenesis (Schroeder & Hermans, 1975). The positive outcome of the present culture experiments suggests basically similar conditions in *Ophryotrocha labronica*. It may be noted, however, that in the protandric species *Ophryotrocha puerilis* oogenesis was not accomplished after brain extirpation (Pfannenstiel, 1973). In *Nereis* brain extirpation does not prevent the synthesis of yolk proteins, but protein incorporation into the yolk is reported to be disturbed (Bertout & Dhainaut, 1971). The experiments by Heacox *et al.* (1983) demonstrate, however, that *Nereis* oocytes can be successfully cultured *in vitro* in the absence of brain hormone. So far, the present labelling experiments on isolated oocyte–nurse cell pairs from *O. labronica* have demonstrated an incorporation of amino acids into the oocyte yolk granules identical with that observed *in vivo* (Emanuelsson, 1985). Our control experiments have also shown, that it made no difference to the development of the germ cell pairs, if isolated heads from the females were included in the cultures.

In *Ophryotrocha* nurse cell metabolites and organelles are, as already stated, transferred to the oocyte via an intercellular canal, a fusome (Emanuelsson, 1969), and except for the polyploid nucleus the whole nurse cell content is ultimately exported to the oocyte in this way. The existence of the canal and the frequent observation of nurse cell yolk bodies passing through it, already at early stages of the vitellogenesis period, demonstrates that nurse cell products are designed for the oocyte, not for export outside the germ cell complex. In accordance, exocytosis from the nurse cell surface, indicating secretion into the culture medium, was never observed. Therefore, the alternative possibility that nurse cells might be secreting something else while oocytes are making yolk proteins seems entirely excluded.

As the chase following the radioactive pulse was kept at 1 h in the present investigation a more widespread transfer of label from the nurse cell to the oocyte was not to be expected. That label actually moves from the nurse cell to the oocyte is evident, however, and has been observed in comparable labelling experiments

Fig. 6. Electron microscope autoradiograph and (inset) light microscope autoradiograph demonstrating incorporation of [^3H]tryptophan in an isolated oocyte–nurse cell pair cultured *in vitro*. The cell pair was radiolabelled for 2 h during the first day of *in vitro* culture. Notice the protective chorion (*ch*) surrounding the oocyte (*O*), and the strong labelling of the oocyte yolk granules (*yg*). Labelling of the nurse cell (*N*) is focussed to the endoplasmic reticulum (*er*) and to that type of yolk bodies (*yb*) that will be incorporated into the oocyte yolk granules. *nu*, nucleus. Bar equals 1 μm (inset 10 μm).

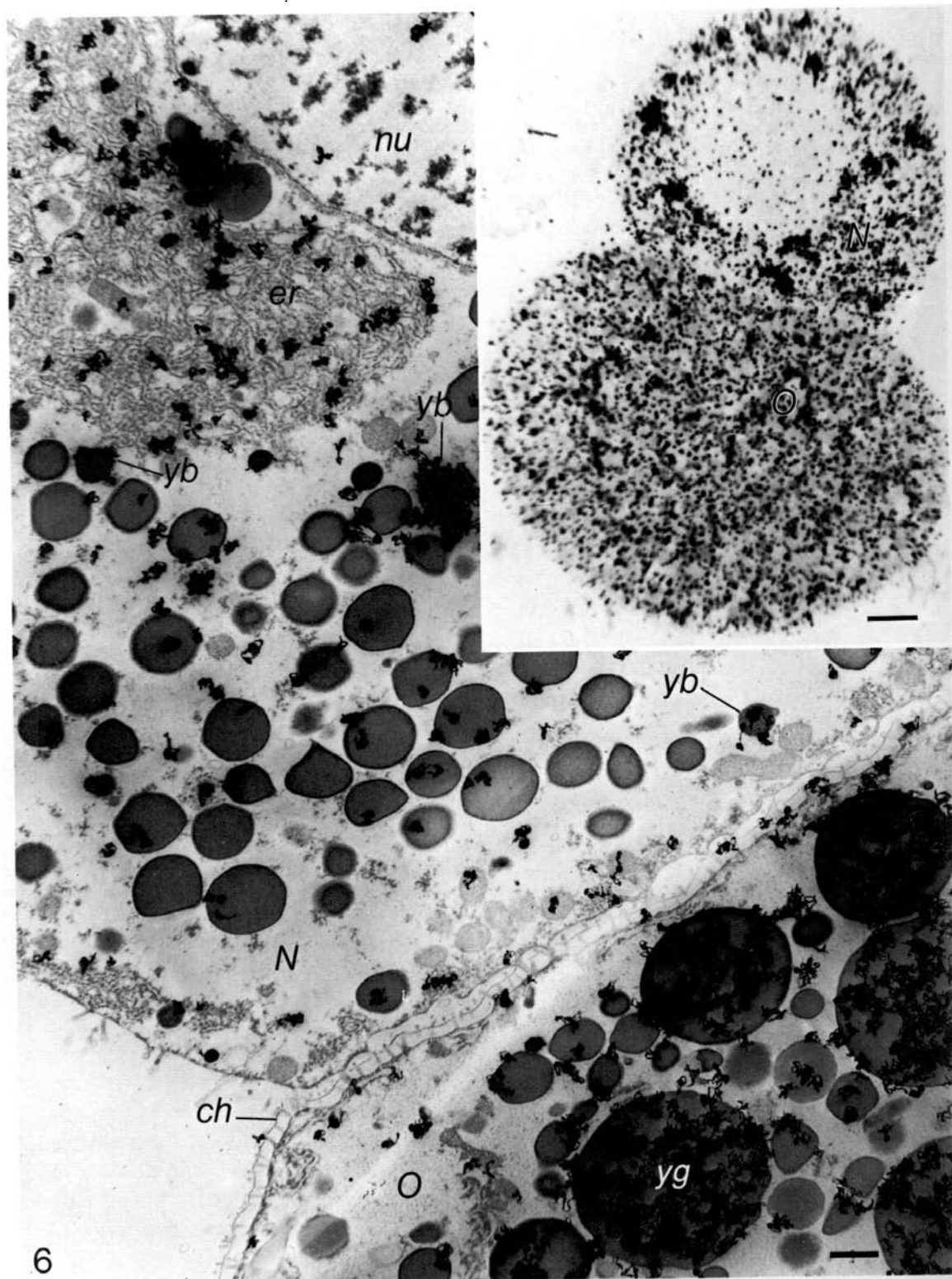


Fig. 6.

on intact *Ophryotrocha* females in mid-oogenesis. When these females were pulsed with [^3H]leucine or [^3H]tryptophan for 2 h, chased for 1 h and then autoradiographed the females showed conspicuous labelling of the nurse cells and weak labelling of the oocytes, whereas the same pulse followed by a 24 h chase resulted in prominent labelling of the oocytes but only weak labelling of the nurse cells (Emanuelsson, unpublished).

Considering the fact that the protective chorion of the oocyte restricts oocyte incorporation of labelled substances to occur via projecting cell processes, it is understandable that in cultured germ cell pairs incorporation after short pulses with labelled amino acids is less intense in the oocyte than in the chorion-free nurse cell. However, since an essentially similar labelling pattern is found after longer pulses as well, we may conclude that the main part of the protein synthesis during vitellogenesis actually occurs in the nurse cell. This impression is also supported by the fact that the endoplasmic reticulum is much more extensive in the nurse cell than in the oocyte. Admittedly we do not know the pool sizes of the amino acids in the two cell types concerned, but possibly the existence of the intercellular canal will prevent major differences between the two cells.

Obviously, the labelling of yolk granules observed in oocyte–nurse cell pairs, pulsed with tritiated amino acids, represents autosynthesis of proteins in the germ cell complex, as a transfer of labelled macromolecules from an extracellular site is completely excluded. An autosynthesis is also clearly indicated by the manifest labelling of such organelles as the endoplasmic reticulum and the Golgi apparatus, particularly those of the nurse cell. This autosynthesis of yolk in the germ cells of *Ophryotrocha* is in apparent contrast to conditions in *Nereis*, in which a vitellogenin–vitellin system is indicated (Heacox *et al.* 1983). Thus for proper growth and development of *Nereis* oocytes in culture vitellin is required in the culture medium. Only large oocytes show some growth without vitellin, but they probably do not synthesize yolk any more.

This difference between the polychaete species seems less strange, however, if one considers the supporting role of the nurse cell in *Ophryotrocha*. The present study has pointed to a leading role for the nurse cell in yolk protein synthesis. Interestingly this synthesis includes not only yolk bodies that are built into the typical 4 μm granules of the oocyte (Emanuelsson, 1969) but also 1 μm granules which appear to be identical with the cortical granules of the mature oocyte. Due to the direct intercellular connection between the nurse cell and the oocyte in *Ophryotrocha* it is not possible to decide from our present study to what extent yolk proteins actually are synthesized in the oocyte itself. For such information we need observations on oocytes, from which the nurse cell has been experimentally removed, experiments that so far are at the planning stage only. Admittedly, one cannot exclude therefore, that in *Ophryotrocha* the nurse cell and the oocyte between them form a vitellogenin–vitellin system, which would mean that the difference between *Ophryotrocha* and *Nereis* would be far less than it appears. Such an explanation is attractive, but remains to be confirmed.

The fact that yolk protein synthesis and yolk granule completion seem to be divided among the nurse cell and the oocyte obviously facilitates a discrimination between the various phases in yolk formation, and makes the germ cell complex of *Ophryotrocha* very useful in extended analyses of yolk formation in invertebrates. This usefulness is further increased by our finding that the oocyte–nurse cell complex can be successfully cultured *in vitro* up to maturation of the oocyte. Accordingly, the present system offers new opportunities to follow the effects of various agents direct on the developing germ cells, and makes the cells accessible to micro-injections and micro-surgery during development. Moreover, the *in vitro* cultured oocyte–nurse cell complex should also be an attractive research object for investigators of the intracellular organization of the oocyte. It is generally recognized that the early development of animals with spirally cleaving eggs, e.g. polychaetes, molluscs, is strictly controlled by cytoplasmic factors, deposited already during oogenesis. Since oocyte development of *Ophryotrocha* can be followed *in vitro* this material should offer good opportunities to localize and identify such factors.

This investigation was supported by the Swedish Natural Science Research Council. The excellent technical assistance of Mrs Lena Olsson and Mrs Annagreta Petersen is gratefully acknowledged.

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(Accepted 27 September 1984)