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# Full-grown oocytes from *Xenopus laevis* resume growth when placed in culture

(follicle cells/vitellogenin/nutrient availability/oocyte maturation)

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ABSTRACT **When most full-grown, follicle cell-invested oocytes from *Xenopus laevis* are placed in an appropriate culture medium, they resume growth and remain physiologically healthy for at least 2–3 weeks. Rates of growth by full-grown oocytes *in vitro* generally approximate and can even exceed the most rapid growth rate achieved by vitellogenic oocytes *in vivo*. Resumption of oocyte growth can be correlated with the loss of investing follicle cells, which under normal conditions appear to interfere with vitellogenin and nutrient access to the oocyte. The final size reached by the oocyte within the ovary is thus not an intrinsic property of the oocyte but is extrinsically imposed by the somatic environment.**

Vertebrate oocytes generally grow to a final size that appears to be genetically determined. For nonmammalian vertebrates, oocyte growth is achieved primarily by the sequestration of vitellogenin from the maternal bloodstream and the subsequent proteolytic conversion of vitellogenin into yolk polypeptides (1). In laboratory-maintained females of *Xenopus laevis*, for which this process has been most thoroughly studied, oocytes appear to be continuously growing until they reach a final diameter of about 1.2–1.3 mm [stage VI according to Dumont (2)]. At this stage, the rate of vitellogenin sequestration *in vivo* falls to less than 5% of the maximal rate found for smaller vitellogenic oocytes (figure 1*a* in ref. 3); these full-grown oocytes eventually disappear from the ovarian population, presumably by atresia, if not ovulated within a period of time (3).

We have recently developed a procedure for growing *X. laevis* oocytes in a gonadotropin-free vitellogenin-containing medium (4) and have found among other things that oocytes with a diameter of 1.14 down to 1.02 mm increase in size *in vitro* to a diameter of at least 1.40 mm over a period of 17–24 days, respectively (5). At the time the culture was terminated, there was no indication that the growth rate of the oocytes had reached a plateau. Cytological examination of the oocytes after culture indicated that they appeared normal and that they were essentially free of the adjacent follicle cells by which they are invested both *in vivo* (2, 6) and immediately after being dissected from the ovary (7). Such follicle cells have been shown to dissociate from isolated vitellogenic oocytes at a rate and to an extent that depends upon the individual female from which they were derived (7). Because of these observations, we have examined whether full-grown *X. laevis* oocytes can resume growth *in vitro* and whether this resumption can be correlated with a loss of associated follicle cells.

## MATERIALS AND METHODS

Female *X. laevis* were maintained as described (8) and were not treated with any hormones. Pieces of ovary were surgically removed and placed in solution O-R2 (9). The largest oocytes present were manually dissected from their follicles and individually cultured in serum-supplemented medium containing vitellogenin (10). Immediately after manual dissection from their follicles, oocytes are no longer invested by the principal follicular components—i.e., the theca and surface epithelium—but are still associated with an adjacent layer of squamous "follicle cells" (7). As has been shown (5, 7), these cells gradually slough off the oocyte. For convenience, therefore, such manually dissected preparations will be termed throughout the text as "oocytes" and the extent to which follicle cells are associated with dissected oocytes in culture will be addressed in several experiments below.

For [$^3$H]vitellogenin incorporation, oocytes were placed for 1 hr in solution O-R2 containing [$^3$H]vitellogenin (2.5 mg·ml$^{-1}$; $1.3 \times 10^3$ cpm·$\mu$g$^{-1}$) and subsequently processed for autoradiography (11) or scintillation counting (4). [$^3$H]Vitellogenin was prepared by injecting a female with 4 mg of 17$\beta$-estradiol on day 0 and 5 mCi (1 Ci = $3.7 \times 10^{10}$ becquerels) of [$^3$H]leucine on day 7; [$^3$H]vitellogenin was isolated (12) from plasma on day 8. The ability of oocytes to reinitiate meiosis was determined by placing groups into solution O-R2 containing either progesterone (1 $\mu$g·ml$^{-1}$) or chromatographically pure (13) human chorionic gonadotropin (hCG) [50 international units (U)·ml$^{-1}$] and scoring (14) for germinal vesical breakdown (GVBD) up to 12 hr later.

For protein determination, 7–10 oocytes were washed in solution O-R2, individually dissolved in 150 $\mu$l of 2% sodium dodecyl sulfate/10 mM dithioerythritol (15), and processed on filter paper discs, as described (5, 16).

Protein synthetic patterns were qualitatively assessed by incubating 20 oocytes for 12 hr in 250 $\mu$l of modified Barth's solution (17) containing 250 $\mu$Ci (approximately 100 Ci·mmol$^{-1}$) of a $^3$H-labeled amino acid mixture (New England Nuclear). Oocytes were then homogenized in buffer (18) and centrifuged at $12,000 \times g$ for 15 min. The supernatants were lyophilized and redissolved in 50 $\mu$l of lysis buffer (19); the proteins were separated by isoelectric focusing in the first dimension and sodium dodecyl sulfate/polyacrylamide electrophoresis in the second dimension (20), after which the gels were dried and fluorographed (21).

## RESULTS

**Vitellogenin Uptake by Full-Grown Oocytes After Removal from the Ovary.** Immediately after being dissected from the ovary, full-grown oocytes from three different females were found to sequester [$^3$H]vitellogenin at rates which were 38–46% of the rate indicated for vitellogenic oocytes obtained from corresponding ovaries (Fig. 1*a*). Even though these relative rates

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Abbreviations: GVBD, germinal vesicle breakdown; hCG, human chorionic gonadotropin; U, international unit.

were considerably higher than what has been observed *in vivo* (3), they continued to increase to values approaching 100% by 66 hr for two out of the three groups of oocytes. All oocytes were also tested for their ability to initiate maturation as a response to either hCG or progesterone, which act indirectly via follicle cells or directly on the oocyte, respectively (22). In those two groups that increased [$^3$H]vitellogenin sequestration with time, an initial weak response to hCG was found to disappear rapidly, whereas the third group initially showed a good response which diminished slowly (Fig. 1*b*). The correlation between hCG response and [$^3$H]vitellogenin uptake is complicated somewhat

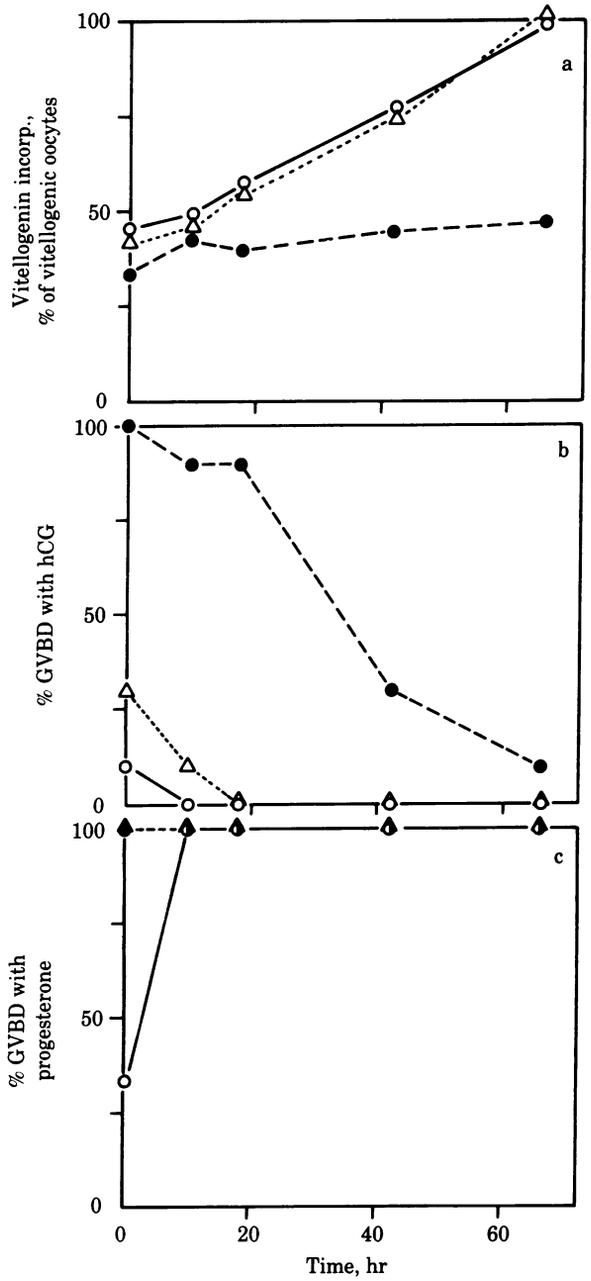


FIG. 1. Vitellogenin uptake (*a*) and either hCG-initiated (*b*) or progesterone-initiated (*c*) resumption of meiosis by full-grown oocytes placed in culture. Full-grown oocytes [mean diameter (±SD) = 1.30 ± 0.02, 1.26 ± 0.02, and 1.20 ± 0.02 mm for groups 1 (○), 2 (△), and 3 (●), respectively] were manually dissected from three different ovaries in solution O-R2 and cultured in 50% L-15 medium with 10% vitellogenin-containing serum for up to 66 hr. Groups of 20 oocytes from each ovary were periodically transferred for 1 hr to solution O-R2 containing [$^3$H]vitellogenin, then washed in solution O-R2; half of each group was then placed in solution O-R2 containing either chromatographically pure hCG (50 U·ml$^{-1}$) or progesterone (1 μg·ml$^{-1}$). Individual oocytes were scored 12 hr later for GVBD and subsequently were prepared for scintillation counting. In *a*, [$^3$H]vitellogenin incorporation is expressed as percent of incorporation found for vitellogenic (0.96 ± 0.02 mm diameter) oocytes at time zero (238, 154, and 164 ng·hr$^{-1}$ per oocyte for groups 1, 2, and 3, respectively).

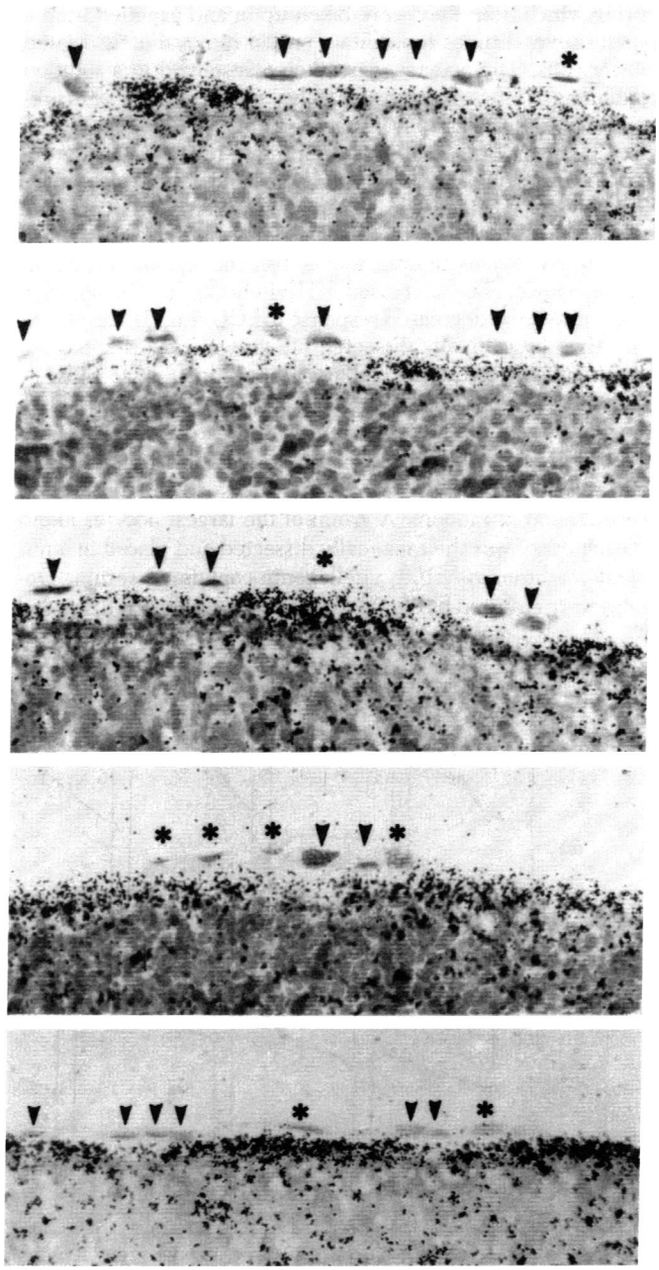

FIG. 2. Autoradiographic examples from five different full-grown oocytes that were incubated in [$^3$H]vitellogenin for 1 hr. Oocytes were manually dissected and placed in solution O-R2 for 6 hr, during which time they were pipetted up and down several times to facilitate partial removal of associated follicle cells. Oocytes were transferred for 1 hr to solution O-R2 containing [$^3$H]vitellogenin and then gently washed in solution O-R2. Labeled oocytes were then fixed by freeze-substitution and processed for autoradiography. Arrows indicate nuclei of follicle cells still associated with the oocyte surface. Subjacent regions of the oocyte cortex are generally less labeled than regions not overlain by follicle cells. Heavy labeling is sometimes observed under a follicle cell when it is somewhat lifted from the oocyte surface (asterisks). (Exposure time: 4 weeks; ×500.)

by uncertainty concerning the functional integrity of follicle cells under our culture conditions. The physiological integrity of the oocytes, on the other hand, was corroborated by the finding that, if anything, the response to progesterone improved with time in culture (Fig. 1*c*).

A further, more direct indication of the relationship between follicle cells and [$^3$H]vitellogenin uptake was obtained by incubating freshly dissected oocytes for 6 hr in a saline medium, during which time they were taken up in and expelled from a pipette several times to facilitate partial removal of associated follicle cells. The oocytes were then transferred to a solution containing [$^3$H]vitellogenin for 1 hr, gently rinsed, and prepared for autoradiography. Oocyte sections generally revealed a patchy incorporation of vitellogenin into the cortex: those regions of the oocyte cortex not overlain by follicle cells were more heavily labeled (Fig. 2; see also figure 2*b* of ref. 7). We conclude from these observations that follicle cells, where present, block or reduce vitellogenin uptake by subjacent regions of the full-grown oocyte. The increased [$^3$H]vitellogenin incorporation (Fig. 1*a*) and the decreased response to hCG (Fig. 1*b*) displayed with time by manually dissected full-grown oocytes appear to be attributable to the gradual loss of investing follicle cells.

**Resumption of Growth by Full-Grown Oocytes *in Vitro*.** The above observations encouraged us to test the growth potential of full-grown oocytes over a longer period of time. Accordingly, 20 *X. laevis* females, previously untreated with any hormones, were chosen at random. A group of the largest oocytes found in each ovary was then manually dissected and placed in a nutrient medium with 10% vitellogenin-containing serum. Oocytes were cultured for 12–22 days, and the group was discarded when more than 10% of the oocytes became abnormal prior to 12 days (five groups). Subsequent mortality in surviving groups was never more than 10%. At periodic intervals, 7–10 oocytes were removed for individual protein determinations. Preliminary experiments had indicated that large oocytes, although otherwise appearing normal, tend to flatten somewhat with time. Thus, we considered protein content rather than oocyte diameter to be a reliable indication of oocyte growth. The relationship between protein content and volume for spherical oocytes larger than 0.1 mm$^3$ (diameter >0.5 mm) has been found to be: $\mu$g of protein = [(329 $\mu$g·mm$^{-3}$) ($V$) − 31 $\mu$g], in which $V$ is the volume in mm$^3$ as determined from the diameter (5).

Growth curves obtained for 15 groups of oocytes are provided in Fig. 3. In several cases, no significant (Fig. 3 *b* and *e*) or very little (Fig. 3*k*) increment in protein content occurred over the culture period. For the majority of cases, however, the protein content increased at average rates ranging from 6.5 (Fig. 3*f*) to 24.3 (Fig. 3*c*) $\mu$g·day$^{-1}$. The last two groups (Fig. 3 *n* and *o*) were grown for up to 22 days, during which time the amount of oocyte protein approximately doubled to values of 639 and 735 $\mu$g per oocyte, respectively (see also Fig. 3*l*). Among the 12 groups that initially responded positively to the culture conditions, only two indicated a possible tapering off in the rate of growth as time progressed (Fig. 3 *i* and *m*). We conclude that full-grown oocytes from most unstimulated *X. laevis* females can resume growth when placed in culture, and some may even double in size if cultured long enough. However, the survival and growth rates of oocytes are subject to considerable variation, depending on the source of oocytes.

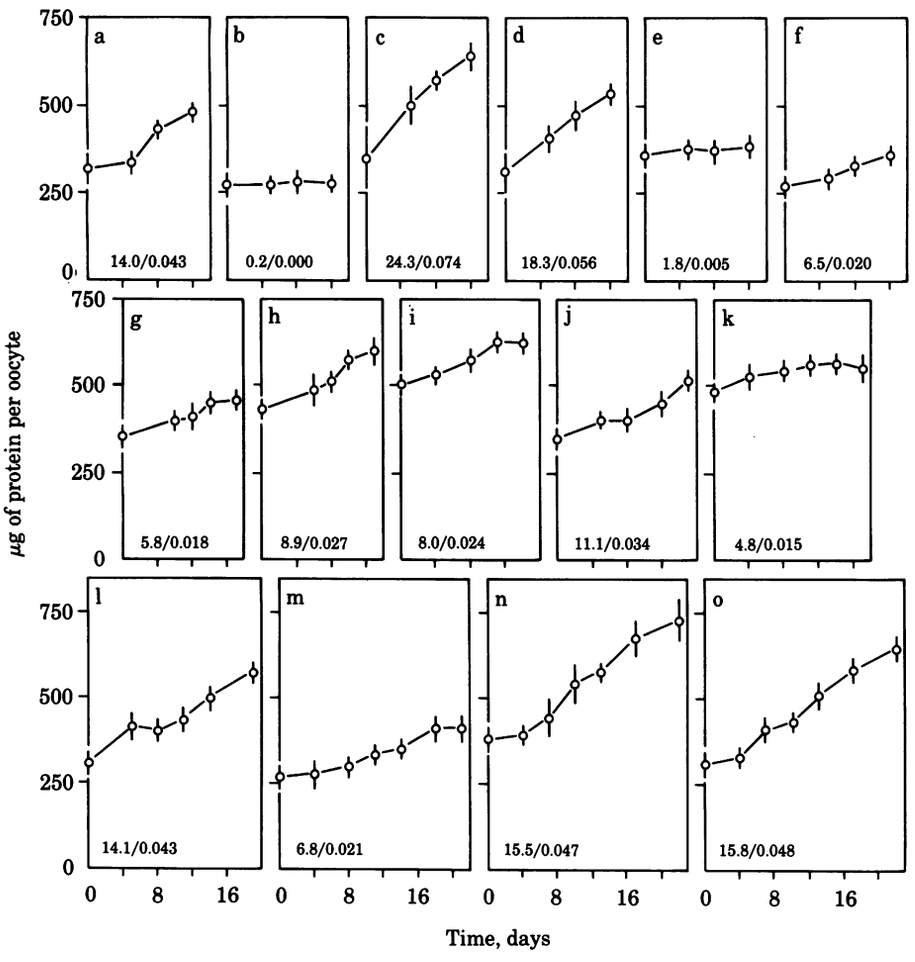


FIG. 3. Growth curves for full-grown oocytes derived from 15 different ovaries. Each point represents the mean value (±SD) found for 7–10 oocytes. The overall growth rates achieved by full-grown oocytes from each of the 15 ovaries are indicated under the curves in two ways: $\mu$g of protein increment per day/mm$^3$ of volume increment per day.

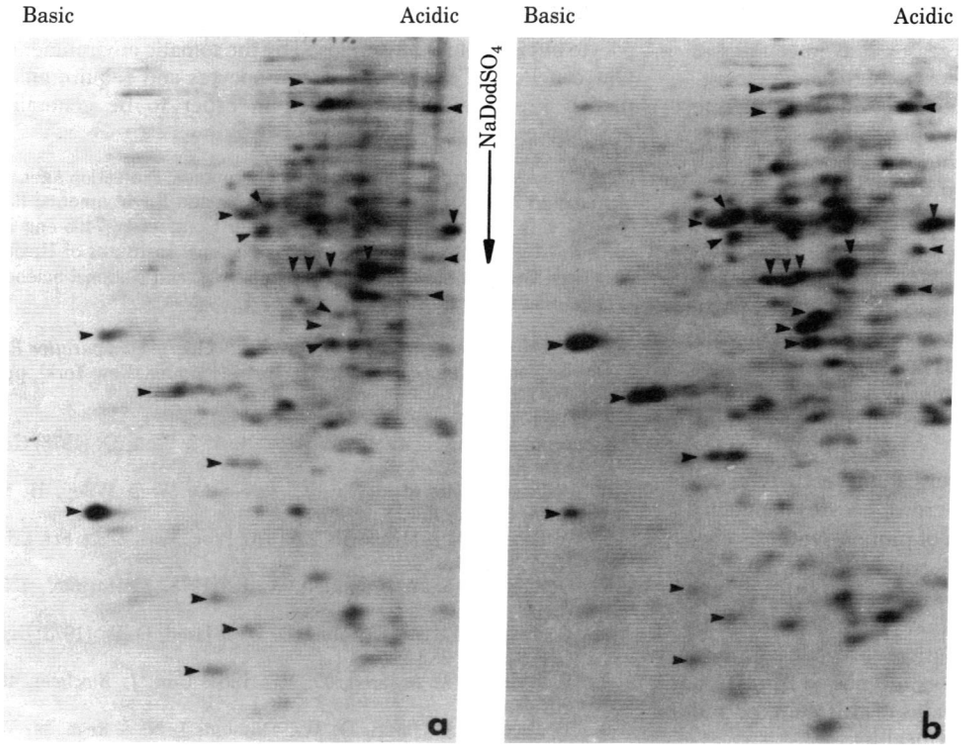


FIG. 4. Fluorographs of two-dimensional gels showing polypeptides newly synthesized by full-grown oocytes from the same ovary after they were dissected from the ovary (*a*) and after dissection and 14 additional days in culture (*b*). Arrows indicate identical peptides on both gels and are inserted as reference points.

**Physiological Properties of Cultured Oocytes.** Profiles of translational activity were compared for full-grown oocytes from the same ovary on day 0 (approximately 1.2 mm diameter) and after 14 days in culture (approximately 1.4–1.5 mm diameter). Fluorographs of two-dimensional gels (Fig. 4) indicated that the patterns of labeled polypeptides were markedly similar in both groups, although certain polypeptides were more heavily labeled on day 0 or day 14. The overall labeling also appeared to be more intense in the fluorograph derived from cultured oocytes. We conclude that some quantitative but not qualitative changes occur in the endogenous protein synthetic pattern once full-grown oocytes are placed in culture.

When full-grown oocytes from unstimulated *X. laevis* females maintained in our laboratory are incubated in a saline solution containing progesterone, most will undergo GVBD within 10 hr, with a 50% response generally scored by 6–9 hr (23). We also routinely tested this response in cultured oocytes by placing them in a progesterone-containing solution prior to protein determinations. Among the 15 surviving groups, including those that grew poorly, the response to progesterone generally became somewhat faster and more synchronous with time. This was more carefully documented in a separate experiment, the results of which are provided in Fig. 5. It thus appears that removal of full-grown oocytes from the ovary and placement in culture eventually establishes a physiologically more responsive and uniform population.

## DISCUSSION

Recent estimates (8) of oocyte growth rates in unstimulated females indicate that the total time for oocytes to progress from a diameter of 0.6 mm (end of stage III) to 1.2 mm (stage VI) is about 16–24 weeks, which corresponds to a volume increment of 0.005–0.007 $mm^3 \cdot day^{-1}$. Oocytes then remain in stage VI for an undetermined length of time. When such full-grown oocytes are manually dissected from their follicles and placed in culture, they initially sequester vitellogenin at rates relatively better than those *in vivo*. Over the next few days, a further increase in vitellogenin uptake occurs in most oocytes, apparently concomitant with the loss of any associated follicle cells (Figs. 1 and 2). These results imply that the follicle cells, which are flattened when associated with full-grown oocytes *in vivo* (11), normally block the access of vitellogenin from the surrounding capillary network to the oocyte. This access is gained when the oocytes are dissected from the ovary and placed in culture, because the follicle cells dissociate from the oocyte surface under these conditions. As a corollary, our results also imply that full-grown oocytes can sequester vitellogenin when it is available. This has already been shown for full-grown oocytes of another amphibian, *Rana pipiens*, in which pinocytotic uptake of vitellogenin is terminated only after maturation is initiated (24, 25).

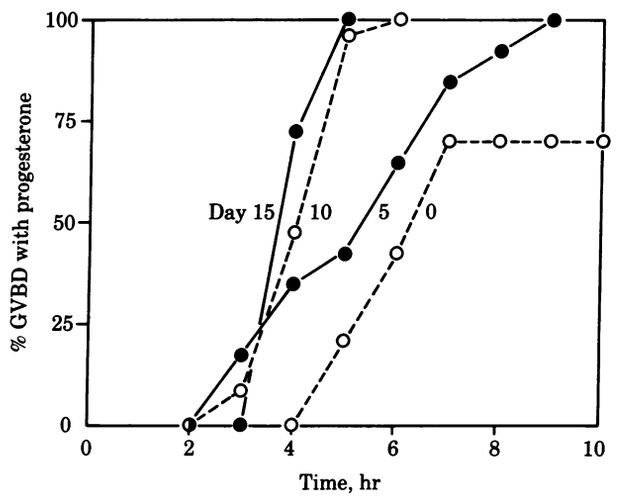


FIG. 5. Time of GVBD among oocytes from the same ovary cultured for up to 15 days. On day 0 and at periodic intervals thereafter, 14 or 15 oocytes were placed in solution O-R2 containing 1 $\mu g \cdot ml^{-1}$ of progesterone and scored for GVBD at hourly intervals up to 10 hr. The overall average growth for this batch of oocytes was 0.030 $mm^3 \cdot day^{-1}$.

Once full-grown oocytes resume growth *in vitro*, the protein content of most groups of oocytes increases at average rates of 6.5–24.3 $\mu g \cdot day^{-1}$ (Fig. 3), which correspond to volume increments of 0.020–0.074 $mm^3 \cdot day^{-1}$ (5). The maximal growth rate normally found for oocytes in gonadotropin-stimulated females is about 0.020–0.025 $mm^3 \cdot day^{-1}$, whereas the fastest growth rate found to date has been measured within partially ovariectomized, gonadotropin-stimulated females (26) and has been calculated to be about 0.050 $mm^3 \cdot day^{-1}$ (table 1 of ref. 3). Thus, most full-grown oocytes can not only resume growth when placed under appropriate culture conditions but also approximate or even exceed the most rapid growth rate of vitellogenic oocytes *in vivo* under extraordinary circumstances. Again, these observations are consistent with the notion that the major limitation to oocyte growth normally involves vitellogenin and nutrient availability.

Two-dimensional gel analysis of translational activity in cultured oocytes has indicated that full-grown oocytes maintain a vigorous qualitatively similar pattern of protein synthesis once they are placed in culture (Fig. 4). Because full-grown oocytes normally appear to have reached a steady state between macromolecular synthesis and degradation (15, 27), it would be interesting to assess further any quantitative changes in these characteristics once full-grown oocytes are placed in culture. For the present, our data at least indicate that cultured full-grown oocytes remain physiologically healthy.

This view is reinforced by the meiotic response of full-grown oocytes in culture, which generally becomes quicker and more synchronous with the passage of time (Fig. 5). Because full-grown oocytes represent a physiologically heterogeneous population composed of those oocytes that have just entered this population through those that are about to leave it via atresia, it is attractive to consider that our culture procedure has rescued the latter group and rendered them highly responsive to progesterone once again. However, the available evidence indicates that growing oocytes *in vivo* become responsive to progesterone prior to reaching stage VI, and that the response improves as the oocytes enlarge (28). The relatively asynchronous response of full-grown oocytes from unstimulated females may be a reflection of this tendency, with long-term residents of the stage VI population responding most quickly. This view presupposes that the trigger for atresia would be the ultimate "spontaneous" maturation of stage VI oocytes without concomitant ovulation (29). The improved response of full-grown oocytes with time in culture would also thus reflect the growth and increased sensitivity of the smallest members.

In summary, we conclude from these studies that: (*i*) the follicle cells investing full-grown oocytes may serve to prevent vitellogenin and nutrient access to full-grown oocytes *in vivo*; (*ii*) full-grown oocytes can resume vitellogenin sequestration and growth when placed in culture, apparently because follicle cells dissociate from the oocyte surface; (*iii*) full-grown oocytes remain physiologically healthy and can at least double in size over a 2- to 3-week period *in vitro*; (*iv*) the final size reached by full-grown oocytes *in vivo* is not an intrinsic property of the oocyte but is extrinsically imposed by the somatic environment. Our conclusions apply to *X. laevis* oocytes and require affirmation in other species as well in order to be generally acceptable.

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1. Wallace, R. A. (1978) in *The Vertebrate Ovary: Comparative Biology and Evolution*, ed. Jones, R. E. (Plenum, New York), pp. 469–502.
2. Dumont, J. N. (1972) *J. Morphol.* **136,** 153–179.
3. Keem, K., Smith, L. D., Wallace, R. A. & Wolf, D. (1978) *Gamete Res.* **2,** 125–135.
4. Wallace, R. A., Misulovin, Z., Jared, D. W. & Wiley, H. S. (1978) *Gamete Res.* **1,** 269–280.
5. Wallace, R. A. & Misulovin, Z. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5534–5538.
6. Dumont, J. N. & Brummett, A. R. (1978) *J. Morphol.* **155,** 73–97.
7. Wallace, R. A., Ho, T., Salter, D. W. & Jared, D. W. (1973) *Exp. Cell Res.* **82,** 287–295.
8. Wallace, R. A. & Jared, D. W. (1968) *Can. J. Biochem.* **46,** 953–959.
9. Wallace, R. A., Jared, D. W., Dumont, J. N. & Sega, M. W. (1973) *J. Exp. Zool.* **184,** 321–334.
10. Wallace, R. A., Misulovin, Z. & Wiley, H. S. (1980) *Reprod. Nutr. Dév.* **20,** 699–708.
11. Wallace, R. A., Nickol, J. M., Ho, T. & Jared, D. W. (1972) *Dev. Biol.* **29,** 255–272.
12. Wiley, H. S., Opresko, L. & Wallace, R. A. (1979) *Anal. Biochem.* **97,** 145–152.
13. Canfield, R. E., Morgan, F. J., Kammerman, S., Bell, J. J. & Agosto, G. M. (1971) *Recent Prog. Horm Res.* **27,** 121–156.
14. Merriam, R. W. (1972) *J. Exp. Zool.* **180,** 421–426.
15. Wallace, R. A. & Hollinger, T. G. (1979) *Exp. Cell Res.* **119,** 277–287.
16. Bramhall, S., Noack, N., Wu, M. & Loewenberg, J. R. (1969) *Anal. Biochem.* **31,** 146–148.
17. Gurdon, J. B. (1968) *J. Embryol. Exp. Morphol.* **20,** 401–414.
18. De Robertis, E. M., Partington, G. A., Longthorne, R. F. & Gurdon, J. B. (1977) *J. Embryol. Exp. Morphol.* **40,** 199–214.
19. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
20. O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12,** 1133–1142.
21. Laskey, R. A. & Miller, A. D. (1975) *Eur. J. Biochem.* **56,** 335–341.
22. Masui, Y. & Clarke, M. J. (1979) *Int. Rev. Cytol.* **57,** 185–282.
23. Vitto, A. & Wallace, R. A. (1976) *Exp. Cell Res.* **97,** 56–62.
24. Schuetz, A. W., Wallace, R. A. & Dumont, J. N. (1974) *J. Cell Biol.* **61,** 26–34.
25. Schuetz, A. W., Hollinger, T. G., Wallace, R. A. & Samson, D. A. (1977) *Dev. Biol.* **58,** 428–433.
26. Scheer, U. (1973) *Dev. Biol.* **30,** 13–28.
27. Dolecki, G. J. & Smith, L. D. (1979) *Dev. Biol.* **69,** 217–236.
28. Reynhout, J. K., Taddei, C., Smith, L. D. & LaMarca, M. J. (1975) *Dev. Biol.* **44,** 375–379.
29. Schuetz, A. W. (1974) *Biol. Reprod.* **10,** 150–178.