ON THE PERSISTENCE OF OOCYTE NUCLEI FROM FETUS TO MATURITY IN THE LABORATORY MOUSE

GEORGE T. RUDKIN, Ph.D., and HELEN A. GRIECH

From the Institute for Cancer Research, Philadelphia

ABSTRACT

Tritiated thymidine injected intraperitoneally into female mice midway through the gestation period was demonstrated by autoradiographic methods to be incorporated into the nuclei of oocytes of female embryos, observed at the pachytene stage of meiosis 2 to 4 days after the injection. The tritium label was also demonstrated in the oocyte nuclei of the daughters of similarly treated females at maturity (6 weeks post partum). It was also found that some follicle cells, likewise labeled with H^3-thymidine in mid-fetal life, persisted to maturity with few or no intervening mitoses. The observations are presented in support of the prevailing view that individual oocytes which arise from germ cell primordia in fetal stages become the egg cells of the adult female mammal.

Controversy raised early in the century over the origin of germ cells in the adult female mammal has continued to the present time in the face of an imposing and ever growing mass of observation. The argument centers around the general question whether or not the cells of somatic origin in the ovary can give rise to sex cells; the negative and now much the stronger view holds that they cannot, and regards the primordial germ cells as the sole progenitors of the gametes. The present status of the problem has been the subject of recent reviews, *e.g.*, in 1959 by Mintz (7) and in 1960 by Zuckerman (13).

The main evidence that the primordial germ cells are both necessary and sufficient to account for the fertility of the female mouse rests in part on the detailed analysis of their development in normal embryos by Chiquoine (4) and by Mintz and Russell (8), but most heavily on the correlation between their elimination by mutant genes (8) or x-rays (7) and infertility. In the latter studies the absence of virtually the entire population of germ cell primordia in the fetal stage was found in conditions known to lead to sterility of adults, but it was not possible to trace individual cells from the primordial stage through to the oocyte stage of the adult in fertile individuals. Thus direct evidence is lacking for continuity between the fetal oocytes seen to enter into pachytene during the last week of gestation and the oocytes present in the follicles of the mature female.

It was thought that a direct approach to the problem might be achieved in the normal mouse by the use of tritiated thymidine to mark individual cell nuclei in fetal life so that they could be sought for individually at later stages of development. Only those cells which underwent few or no divisions between marking and scoring would be identified; those which underwent extensive mitosis would escape detection because of dilution of the label. Since the early stages of meiosis are entered before birth (see, *e.g.*, 2, 7, 10) no divisions are expected in the oocytes; prolific mitosis is, on the other hand, characteristic of the ovarian epithelial tissues and has, indeed, been in part responsible for the idea that germ cells might be derived from that source of somatic cells.

The results show that oocyte nuclei can be

found labeled in the adult after the (proved) introduction of tritiated thymidine in the fetal stage, in accordance with the prevailing view that they do persist. In addition, it was also noted that some epithelial cells, labeled at the same time, also retained the label in their nuclei into the adult stage.

EXPERIMENTAL

Six female mice of the ICR albino strain were the source material. Litter sizes of 9 to 15 are typical for this strain; 6 to 9 daughters per insemination were found in the series reported here. Gestation was dated from the last litter, and so the developmental ages of the fetuses are subject to some uncertainty, probably no more than 1 day in this strain.

Each of the females (weighing 48 to 58 grams) was given a single intraperitoneal injection of 250 μc of H^3-thymidine (Schwartz) (specific activity 1.6 c/mM) near the middle of the 21 day gestation period, as follows: 1 mouse on the 12th and 1 on the 13th day, 2 mice on the 14th and 2 on the 15th day. Preliminary experiments in which older mice were given only 1 μc/gm of material had shown that incorporation into fetal oocyte nuclei could be obtained between the 12th and 15th days. The larger dose used in the present experiment was necessary in order to assure unequivocal evidence of label after dilution by nuclear growth in mature stages. (A fetal oocyte nucleus 7 μ in diameter squashed to a thickness of 1 μ has approximately 100 times the autoradiographic effectivness of a dictyate oocyte nucleus 20 μ in diameter and sectioned at 5 μ.)[1]

[1] Autoradiographic effectiveness as used here refers to number of grains produced per unit of area per unit of time. The effective range of tritium beta rays is taken as 1 micron. The calculation compares a nucleus 7 μ in diameter, just labeled in embryo, with the same nucleus after it has developed to the dictyate stage and grown to 20 μ in diameter, a 22-fold increase in volume. The 7 μ nucleus was squashed to a thickness of about 1 μ so all of its incorporated H^3 was within effective range of the emulsion; the 20 μ nucleus was sectioned at 5 μ so only about $\frac{1}{5}$ of its H^3 was equally effective. The product of the 22-fold "dilution" factor and 5-fold sectioning factor gives a factor of about 100 for the relative grain densities in the two experimental situations for the same exposure time.

FIGURE 1

Autoradiograph of a section through the ovary of a 17 day fetus from a female injected with H^3-thymidine on the 14th day. The scale line represents 50 μ. × 300.

FIGURES 2 AND 3

Autoradiographs of pachytene nuclei from ovaries of 17 day fetuses from females injected with H^3-thymidine on the 13th (Fig. 2) or the 15th day (Fig. 3). Squash preparations, fixed in 50 per cent acetic acid, stained and squashed in lactic acetic orcein (see text). × 820.

FIGURES 4 TO 11

Autoradiographs of sections through ovaries, 6 weeks post partum, of offspring of females injected with H^3-thymidine on the 14th or 15th day. The letters *a* and *b*, where they occur, refer to different focal levels chosen to show more clearly the grains above and tissue below. Sections of individual follicles including the centrally placed oocyte nucleus are shown; the follicles are arranged in order of increasing size.

FIGURE 4 *a* AND *b*

H^3-thymidine injected on the 15th day. Oocyte nucleus labeled, follicle cell nuclei unlabeled. Other unidentified (follicle cell?) labeled nuclei near by. × 820.

FIGURE 5 *a* AND *b*

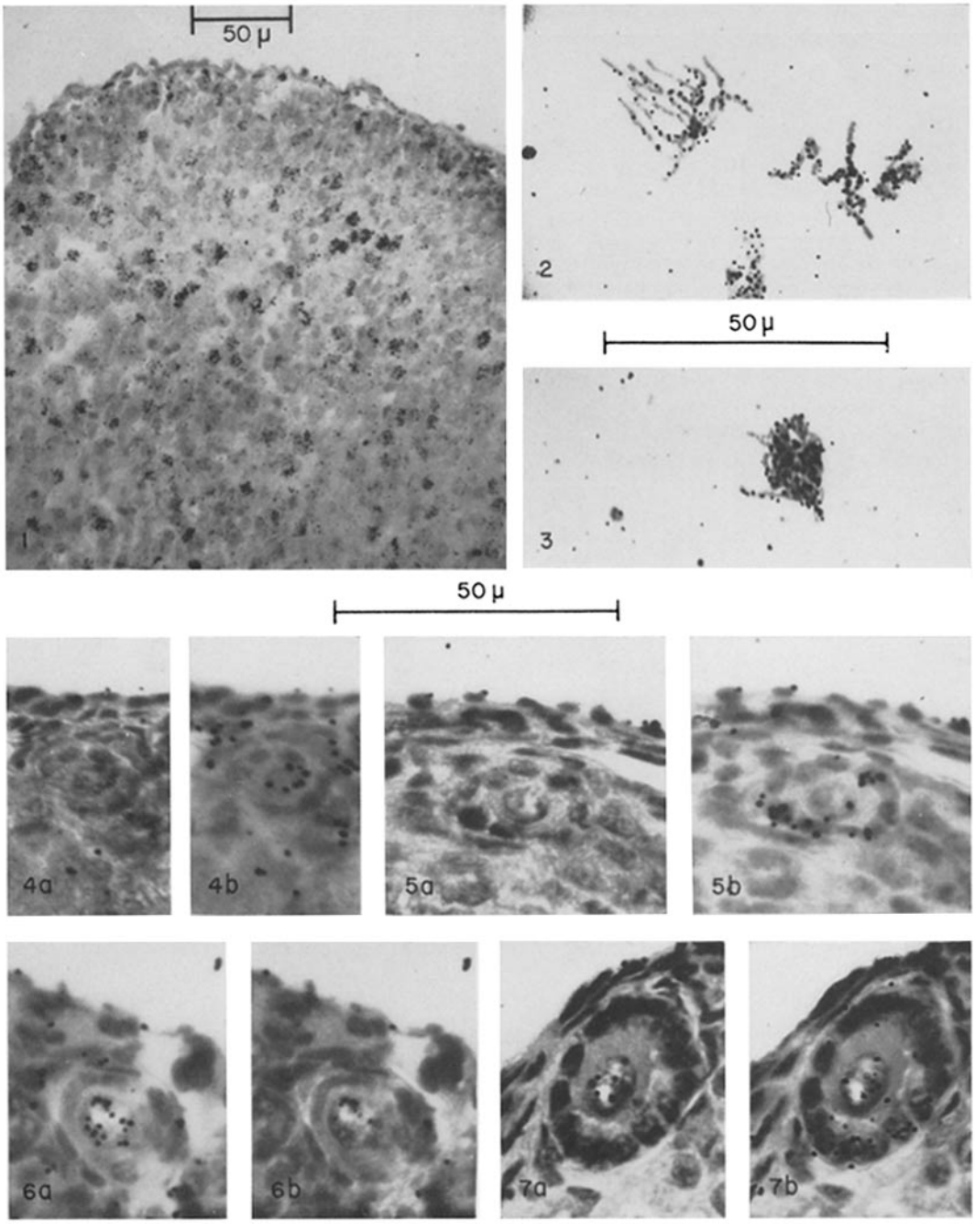
Label injected on the 14th day. Follicle cell nuclei labeled, oocyte nucleus not labeled. × 820.

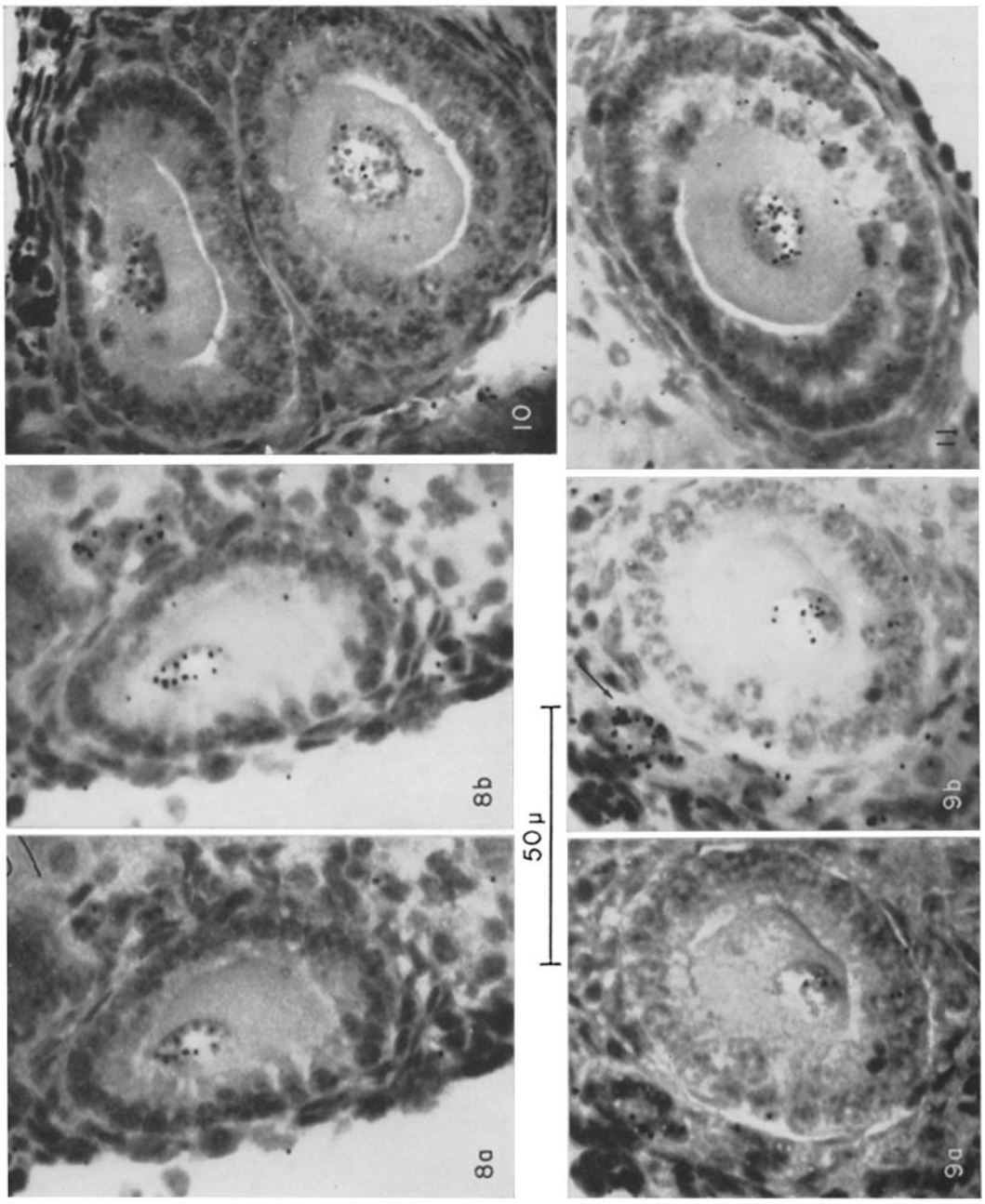
FIGURE 6 *a* AND *b*

As Fig. 4.

FIGURE 7 *a* AND *b*

As Fig. 4, except that both follicle cell and oocyte nuclei are labeled.


50 µ
1
2
50 µ
3
50 µ
4a
4b
5a
5b
6a
6b
7a
7b


10
11
8b
9b
50 μ
8a
9a

Four of the females (one from each injection date) were sacrificed on the 17th day of gestation, and the gonads and other tissues of the female fetuses and some maternal tissues were fixed either in alcohol–acetic acid (3:1) or in 50 per cent aqueous acetic acid. Squash preparations of the gonads were made after staining in a 1 per cent solution of orcein in a mixture of lactic acid and acetic acid (1:1) or in a saturated solution of orcein in 50 per cent acetic acid. An ovary from one fetus from each "litter," fixed in alcohol–acetic acid (3:1), was embedded and sectioned as described below.

The remaining 2 females (injected 14 and 15 days after insemination) were allowed to raise their litters until the 7th week post partum. Maternal and daughter tissues were then fixed in alcohol–acetic acid (3:1), embedded in paraffin, and sectioned at 5 μ.

Autoradiographs were prepared either by dipping slides in Kodak NTB-3 nuclear track emulsion or by covering them with AR-10 stripping film. 30 days' exposure was adequate for the embryonic tissues; the slides from 6-week-old mice were exposed for 30 or 60 days. Sections were stained with Harris' hematoxylin after photographic processing. No attempt was made to render the autoradiographic data quantitative; unequivocal labeling was taken as a positive result.

FIGURE 8 *a* AND *b*

As Fig. 4. × 740.

FIGURE 9 *a* AND *b*

H^3-thymidine injected on the 15th day. Two follicles are shown. In the larger one, only the oocyte nucleus is clearly labeled; in the smaller one (arrow) only follicle cell nuclei show autoradiographs, but the oocyte nucleus is not in the section. × 740.

FIGURE 10

As Fig. 4. × 740.

FIGURE 11

H^3-thymidine injected on the 15th day. Only the oocyte nucleus is clearly labeled; grains over other areas are not significantly above background (one or two grains per nucleus). × 740.

RESULTS

Incorporation into Fetal Germ Cell Nuclei

The high frequency of pachytene stages on day 17 facilitated the identification of oocyte nuclei, many of which were found to be labeled in all the squash preparations (see *e.g.* Figs. 2 and 3). Other nuclei not in division were also labeled, as were some somatic cells in mitosis. That not all cells took up the precursor is not surprising, for it is known that injected thymidine is available for incorporation for less than an hour (5) and could mark only those cells in which DNA synthesis was taking place during that time. In one experiment, 106 out of 467 nuclei of all types counted at random in a squash preparation were labeled.

Since it is known that increase in number of germ cells ceases at about the 12th day (7), it is considered likely that in most cases the last premeiotic synthesis of DNA was taking place when the label was introduced. Inhomogeneous distribution of silver grains over the chromosomes of some nuclei (see Figs. 2 and 3) is probably an expression of asynchrony of synthesis in different chromosome regions, as reported in other forms by Lima-de-Faria (6) for meiosis, and by Taylor (11) and Wimber (12) for mitosis. The possibility that one or two mitoses intervened between labeling and the onset of meiosis cannot, however, be excluded in any individual case.

Ovaries cut in 5 μ sections showed a uniform distribution of labeled nuclei (see Fig. 1). It was so difficult to identify the cell types through the developed autoradiographic film that quantitative procedures were not employed. It was noted that, as in the squashes, some unequivocally pachytene nuclei were labeled; some other nuclei in small cells, presumed to be of epithelial type, were labeled as well.

Somatic tissues in other fetal organs were found to be rich in labeled nuclei, as would be expected in populations of rapidly dividing cells. Liver, lung, and gut were among those examined in aceto orcein squash preparations. Atlas, Bond, and Cronkite (1) in a time study between the 6th and 17th days of gestation also reported extensive

uptake in many somatic tissues after the 10th day, but made no mention of ovaries.

Occurrence of Labeled Nuclei at Maturity

The expectation that thymidine incorporated into fetal oocytes would lead to labeled oocyte nuclei in sexually mature females was realized in the two litters allowed to develop to 6 weeks of age. Examples are shown in Figs. 4 to 11 which illustrate follicles at different stages of maturation in sections which cut through the centrally placed oocyte nucleus. The two litters differed in the stages of follicle development at which label was found. Only very early or primary follicles were clearly labeled in oocyte nuclei in the daughters of one mouse (M2). As Table I shows, the largest follicle so labeled was only 69 μ in diameter, and of 221 easily recognized follicles (28 to 306 μ in diameter) only 6.8 per cent were so labeled. It was clear that many more oocyte nuclei in the primary follicle stage were covered by silver grains which prevented confident classification of cell type in the preparations. Oocyte nuclei of more advanced follicles were labeled in the daughters in the other litter (M22), the largest follicle being 228 μ in diameter, and fully half of 211 easily recognizable follicles showed clear nuclear autoradiographs. As in the daughters of M2, more primitive follicles were also labeled positive but were identified with less confidence than those scored in Table I.

Many of the less advanced follicles revealed the presence of tritium in nuclei of follicle cells. Figs. 5 and 9 (arrow) illustrate cases in which only those nuclei are labeled; Figs. 4, 6, 7, and 8 show label over both germinal and somatic nuclei. In Figs. 4 and 8 to 11 follicles are shown with label over only the oocyte nucleus. The frequency with which somatic nuclear label occurred was not measured; it was common among follicles less than 50 μ in diameter, *i.e.*, those with a single layer of follicle cells (see ref. 3), but rare or absent in later stages, *i.e.*, after the second layer had begun to appear. Dilution of the label by mitosis is an adequate explanation for the absence of grains after proliferation in the granulosa.

By contrast with the ovarian tissues, no labeled nuclei were found in sections of livers of the same animals.

The results presented above leave no doubt that few or no cell divisions intervene between the middle of fetal life and maturity in the oocyte of the mouse. The simplest interpretation, and the one most consistent with other recent evidence, cited earlier, is that the label found in oocyte nuclei at maturity was incorporated into primordial germ cells, identified as such in the fetal stages. The identity of some mature germ cells with their embryonic primordia is thus confirmed.

TABLE I

Oocyte Nuclei in Adult (Six-Week-Old) Mice Labeled by H^3-Thymidine in a Fetal Stage

Litter*	Day of H^3 injection‡	Oocyte nuclei scored			Diameters of follicles			
			Labeled		Labeled		Unlabeled	
		Total	No.	Per cent	Smallest	Largest	Smallest	Largest
					μ	μ	μ	μ
M2	14	221	15	6.8	21	69	24	306
M22	15	211	106	50.2	28	228	36	157

* Two ovaries from each litter scored.

‡ Intraperitoneally into mother; number of days after last litter.

DISCUSSION

The view taken here, now shared by most workers in the field, is that the primordial germ cells not only are a source of oocytes at maturity, as confirmed by the present experiment, but are the only source. Zuckerman (13) has rightly said that it will probably be impossible to prove that no other cell type can ever enter the germ line. Negative evidence could, however, be built up by the radioactive tracer method. If the view is correct, then it should be impossible to label oocyte nuclei with thymidine after the last premeiotic synthesis of DNA. The time of that synthesis can be set near the middle of fetal life, as

determined from the observation that proliferation ceases about then (7) and is shortly followed by meiotic prophase; the present results show that it does, indeed, occur at that time. Reports of attempts to introduce labeled thymidine, as yet meager, have to date given negative results at stages later than parturition. Sirlin and Edwards (9) observed no incorporation at 6 hours post partum; no uptake into oocyte nuclei has been observed in the ovaries of the females in the present series injected at 13 weeks of age (14 to 15 days after insemination), and sacrificed about 4 weeks later. A survey extending over the life span and reproductive cycles would be a formidable undertaking even in the female mouse, but experiments in which DNA synthesis after parturition could have been demonstrated in oocyte nuclei, if it occurred, should be put on record.

This work was aided by grant C1613 from the National Institutes of Health, United States Public Health Service, and by grants from the American Cancer Society.

We wish to acknowledge the technical assistance of Mr. John Grohsman and Miss Barbara Valulis and to express appreciation to Dr. B. Mintz for invaluable discussions of the manuscript.

Received for publication, July 20, 1961.

LITERATURE CITED

1. ATLAS, M., BOND, V. P., and CRONKITE, E. P., *J. Histochem. and Cytochem.*, 1960, **8**, 171.
2. BRAMBELL, F. W. R., *Proc. Roy. Soc. London, series B*, 1927, **101**, 391.
3. BRAMBELL, F. W. R., *Proc. Roy. Soc. London, series B*, 1928, **103**, 258.
4. CHIQUOINE, A. D., *Anat. Rec.*, 1954, **118**, 135.
5. CRONKITE, E. P., BOND, V. P., FLIEDNER, T. M., and RUBINI, J. R., *Lab. Invest.*, 1959, **8**, 263.
6. LIMA-DE-FARIA, A., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 457.
7. MINTZ, B., *Arch. anat. micr. et morphol. exp.*, 1959, **48**, 155.
8. MINTZ, B., and RUSSELL, E. S., *J. Exp. Zool.* 1957, **134**, 207.
9. SIRLIN, J. L., and EDWARDS, R. G., *Exp. Cell Research*, 1959, **18**, 190.
10. SLIZYNSKI, B. M., *Nature*, 1961, **189**, 683.
11. TAYLOR, J. H., *in* Cell Physiology of Neoplasia, *14th Ann. Symp. Fundamental Cancer Research*, Austin, University of Texas Press, 1960, 547.
12. WIMBER, D. E., *Exp. Cell Research* 1961, **23**, 402.
13. ZUCKERMAN, S., *in* Sex Differentiation and Development, (C. R. Austin, editor), Cambridge University Press, 1960, 63.