

## A method for a selective local application of radioactive precursors of nucleic acids to the cow ovary

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**Abstract** — Local application of  $^3\text{H}$ -uridine or  $^3\text{H}$ -thymidine to the cow ovary was obtained by the instillation of the radioactive solution into a rubber balloon ('bursa') tightly enclosing the organ exposed by laparotomy. After 30–90 min the exteriorized ovary was replaced in the abdominal cavity. Ovaries were recovered 1–8 days later and processed for autoradiography. After  $^3\text{H}$ -uridine application the peripheral layer of the ovary, containing the follicles in different stages of development was most intensely labelled. The labelling by  $^3\text{H}$ -uridine could be attributed prevalingly to the cytoplasm of different cells constituting the ovary but in some cases the labelling was higher in nuclei. The highest level of labelling was seen in the cytoplasm of follicular cells of follicles composed of one or two layers of cuboidal cells. Labelling was also detected in the ooplasm of oocytes with the maximal number of grains being seen in the first phase of their growth. On the other hand, no labelling was detected in the ooplasm of oocytes isolated from antral follicles the growth of which had been induced previously by hormonal treatment. It is believed that the labelling obtained by the described procedure represents to a significant degree RNA. A similar local application of  $^3\text{H}$ -thymidine allowed us to obtain labelling restricted specifically to nuclei of different cells composing the ovarian tissue. In the presented results, the entry of flattened follicular cells of primordial follicles in vivo in the S-phase of the cell cycle was demonstrated as well as an evident different proliferation rate in the successive stages of follicle development. The method proposed here may probably be the way for testing the effects of different substances, available only in small quantities, on the cow ovary. © Inra/Elsevier, Paris.

**cow ovary / autoradiography / RNA / DNA / folliculogenesis**

**Résumé** — Une technique pour l'application locale à l'ovaire de vache de précurseurs radioactifs d'acides nucléiques. L'application locale de précurseurs ( $^3\text{H}$ -uridine ou  $^3\text{H}$ -thymidine) à l'ovaire de vache exposé par laparotomie est effectuée à l'aide d'un ballon de caoutchouc renfermant étroitement l'organe. La solution radioactive est injectée dans cette cavité où l'incubation dure

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30 à 90 min. L'ovaire est ensuite replacé dans la cavité abdominale. Après l'application de  $^3\text{H}$ -uridine, les autoradiogrammes sur coupes en paraffine d'ovaires montrent un marquage périphérique. Le marquage, surtout cytoplasmique et parfois nucléaire intense, est observé principalement dans les cellules somatiques de follicules formés d'une ou deux couches de cellules cuboïdales. Dans les ovocytes, l'ooplasm est marqué le plus intensément au début de la phase de croissance. Au contraire, aucun marquage n'est détectable dans des ovocytes isolés de follicules à antrum après traitement hormonal pour induire leur croissance. Le marquage détecté après incorporation de  $^3\text{H}$ -uridine représente vraisemblablement essentiellement une synthèse d'ARN. Après application de  $^3\text{H}$ -thymidine, le marquage est restreint au noyau de différents types cellulaires. Par exemple entrée en phase S des noyaux de cellules somatiques aplaties des follicules primordiaux. Le taux de prolifération des cellules folliculaires au cours des phases successives de développement a été obtenu. Cette technique permet l'étude de la dynamique cellulaire *in vivo* et pourrait aussi être utilisée pour tester l'effet de différentes substances sur l'ovaire. © Inra/Elsevier, Paris.

**ovaire / bovin / autoradiographie / ADN / ARN / folliculogénèse**

## 1. INTRODUCTION

In some small rodents such as mice or hamsters the ovary is surrounded by a complete bursa ovarica which is continuous with the oviduct. This fact made it possible to study to a great extent either RNA synthesis and fate in oocytes in the mouse ovary and in ovulated oocytes by radiobiological methods after injecting a small amount of tritiated adenosine or uridine into the bursal cavity [1, 15], or zona pellucida formation after a similar application of tritiated glucosamine [6]. Intrabursal injection procedure has also been developed in the hamster for the study of the effects of different drugs or chemicals inducing ovulation [10]. In the latter study it was found that compounds with molecular weight of less than 1 000 daltons penetrate rapidly through all the interposed cellular layers and reach the follicles.

Our experiments described in this study aimed at developing a method of local application of tritiated precursors of nucleic acids to the cow ovary based on artificial formation of a temporal 'ovarian bursa' into which a relatively small amount of a radioactive precursor would be introduced but which would allow, at the same time, an intensive bathing of the ovary in the radioactive solution adequate for a sufficient labelling of the cortical part of the ovarian tissue con-

taining the follicles. A similar approach has been already used by Zeleznik et al. [16] in Rhesus monkey.

In this way, the detection of cells in the S-phase would permit different studies of cellular dynamics in the cow ovary *in vivo*, similar to that performed with radioisotopic methods in small laboratory animals and the labelling of 'maternal' RNAs in the oocyte would allow the study of their fate in the early preimplantation embryo.

## 2. MATERIALS AND METHODS

### 2.1. $^3\text{H}$ -Uridine

Three Holstein-Friesian heifers aged 6–8 months and weighing from 120 to 400 kg were used (*table 1*). Before operation, the animals were fasted for 24 h and deprived of water for 12 h. The operation leading to the application of the 'bursa' was performed on animals lying in the dorsal position, after fixation on a hydraulic operation table after a total anaesthesia. The animals were premedicated first by atropine sulphate (Atropin injections SPOFA, Biotika, Slovenská Lupca, Slovak Republic) in a dose of 40 mg injected subcutaneously together with propionyl promasin hydrochlorid (COMBELEN, Bayer, Leverkusen, Germany) in a dose of 0.5 mL/100 kg intravenously and then fixed to the operation table. General anaesthesia was introduced and maintained using an anaesthesional apparatus ANESTAR N 7 (Chrirana, Stará Turá, Slovak

Republic) and in a closed circuit the animals were insufflated by halothanum (NARCOTAN liquidum, Léciva, Praha, Czech Republic) in a mixture with medicinal oxygen. After additional local anaesthesia a perpendicular incision of about 15 cm in length beginning at 3–5 cm under the tuber coxae was made which provided the access to the ovary. After intramuscular application of about 100 mg of isoxsuprinlaktat (UTERUSRELAXANS, Wirtschaftsgenossenschaft deutscher Tierärzte, Hannover, Germany) the ovary was exposed and immobilized by two sutures through the mesovarium to the skin. Around the immobilised ovary a thin rubber balloon was applied which, in its turn, was fixed by a suture to the hilus ovarii. In this way an artificial 'ovarian bursa' surrounding the ovary has been formed and then filled, by means of a syringe provided with a plastic tube, with approximately 2 mL of the radioactive solution in PBS (figure 1). Using this system a constant level of the solution in the 'bursa' and its movement from time to time were ensured. A 20 MBq per mL isotonic solution of  $^3\text{H}$ -uridine (UVVVR Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic, specific activity of 740 GBq/mmol) was used as radiolabelled precursor (table I). Two millilitres

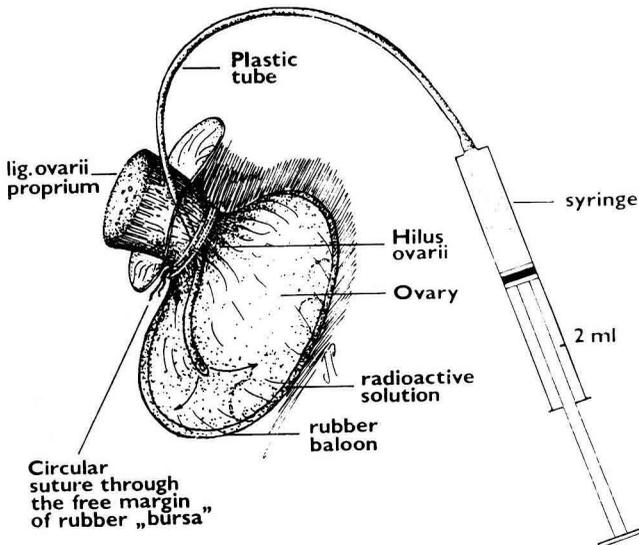
of this solution filled up the space between the ovary and the rubber balloon enclosing it.

In one animal (Animal C, table I) follicular growth was stimulated for 4 d by eight injections of a total dose of 13 mg of FSH (FOLICOTROPIN, SPOFA, Léciva Praha, Czech Republic). The last injection was administered 2 d before operation.

The ovarian tissue as well as the harvested oocytes in animal C (table I) was isolated at 6 or 8 d, respectively, after 'bursa' application (table I) and the distribution of the persisting incorporated radioactivity was detected by autoradiography.

## 2.2. $^3\text{H}$ -Thymidine

Four Pinzgau heifers weighing 120–150 kg were used (table II). The procedure for bursa application was the same as described for  $^3\text{H}$ -uridine, but  $^3\text{H}$ -thymidine (UVVVR, Prague spec. activity 900 GBq/mmol, 0.2–2 MBq·mL<sup>-1</sup>) was administered (table II). Again the procedure followed that used for  $^3\text{H}$ -uridine. In one animal (animal F, table II)



**Figure 1.** A schema (drawing J.P.) showing the procedure of selective application of  $^3\text{H}$ -uridine or  $^3\text{H}$ -thymidine to cow ovary. The ovary of a living animal was put in a rubber balloon which, in its turn, was filled by means of a syringe provided with a plastic tube with the radioactive solution in which the ovary was bathed, cf. tables I and II.

follicular growth was stimulated by a single injection of 3 000 I.U. of PMSG (Antex Leo, Pharmaceutical Products, Copenhagen, Denmark) 5 h before bursa application.

### 2.3. Autoradiography

Whole ovaries were fixed for several days by neutralized 4 % formol. During fixation, the ovaries were cut into two halves along their longer axis. Afterwards the tissues were embedded in paraffin and sections were cut in thicknesses of 2–5  $\mu\text{m}$  and placed on slides. After deparaffinization, they were coated with nuclear liquid emulsion Ilford K.5. From some of the formol pre-fixed ovarian halves, smaller pieces were cut and fixed by glutaraldehyde/osmium

and embedded in Epon. Semi-thin sections of about 1  $\mu\text{m}$  were cut and subjected to autoradiography in the same way. Isolated oocytes, obtained after hormonal stimulation in animal C (table I) were treated identically. After an exposure of 1–3 months the autoradiograms were developed in D 19 and stained by Hematoxylin (Harris) Eosin or Methylene Blue, respectively.

## 3. RESULTS

### 3.1. $^3\text{H}$ -uridine

Radioactivity was detected in all samples from ovaries to which tritiated uridine was applied by immersion of the whole

**Table I.** Duration of  $^3\text{H}$ -uridine exposure to the ovarian tissue via an artificial 'bursa' and the successive time interval to ovary isolation.

Code of heifer	Weight in kg	Duration of 'bursa' application to ovaries in min		Number of days between uridine exposure and isolation of ovary
A	400	L	90	6
		R <sup>1</sup>	0	
B	150	L	30	8
		R <sup>2</sup>	0	
C	120	L	30	8 <sup>3</sup>
		R	30	

<sup>1</sup> Intrastromal injection.

<sup>2</sup> Ovary intact.

<sup>3</sup> On the 2nd day after the radioactive precursor application the induction of follicular growth was stimulated by FSH.

**Table II.** Duration of  $^3\text{H}$ -thymidine exposure to the ovarian tissue via an artificial 'bursa' and the successive time interval to ovary isolation.

Code of heifer	Weight in kg	Duration of 'bursa' application to ovaries in min		Number of days between thymidine exposure and isolation of ovary
D	150	L	30	1 (24 h)
		R	30	1 (24 h)
E	150	L	30	7
		R	30	7
F	120	L	45 <sup>1</sup>	1 (24 h)
		R	45 <sup>1</sup>	1 (24 h)
G	120	L	45	7
		R	45	7

<sup>1</sup> Five hours before bursa application an injection of PMSG was administered.

organ into the solution of the radioactive precursor. On the other hand, no significant radioactivity was detected either in ovaries injected directly into their stroma by the same radioactive solution or in contralateral intact ovaries.

In autoradiograms of cross-sections of whole ovaries embedded in paraffin most of the radioactivity was detected in the ovarian periphery as a band reaching to one-third of the radius of the section (*figure 2*). The difference in arbitrary assessed overall radioactivity among different ovaries corresponded apparently to the length of the interval of radioactive precursor availability during 'bursa' application but no important decrease in radioactivity was seen among ovaries isolated at 6 or 8 d after operation. At the cellular level (*figures 3-8*), a distinct labelling was seen mostly in the cytoplasm of different cells forming the ovary. On the other hand, a significantly higher labelling was detected in some cell nuclei (*figures 3-6*).

The highest labelling was seen in both cytoplasm and nuclei of follicular cells of follicles formed by one or two layers of cuboidal cells (*figures 5-7*) in contrast to lower levels of labelling in the cytoplasm of squamous follicular cells of earliest follicles (*figure 5* - upper left follicle) and in cuboidal follicular cells of follicles with already formed theca (*figure 8*). In some cases, the intensity of labelling in some follicles distinguished them clearly from the much less labelled surrounding ovarian tissue (*figure 6*).

Labelling was also seen in oocytes (marked in suitable sections by two contralateral arrows). The labelling was seen mostly in their ooplasm, the oocyte nuclei (between empty arrows) were usually not labelled. The highest labelling in ooplasm was attributed to oocytes in the first phase of their growth (*figures 5-7*). A lower level of labelling was seen in the ooplasm of other categories of oocytes (*figure 8*) but no labelling was detected in those oocytes

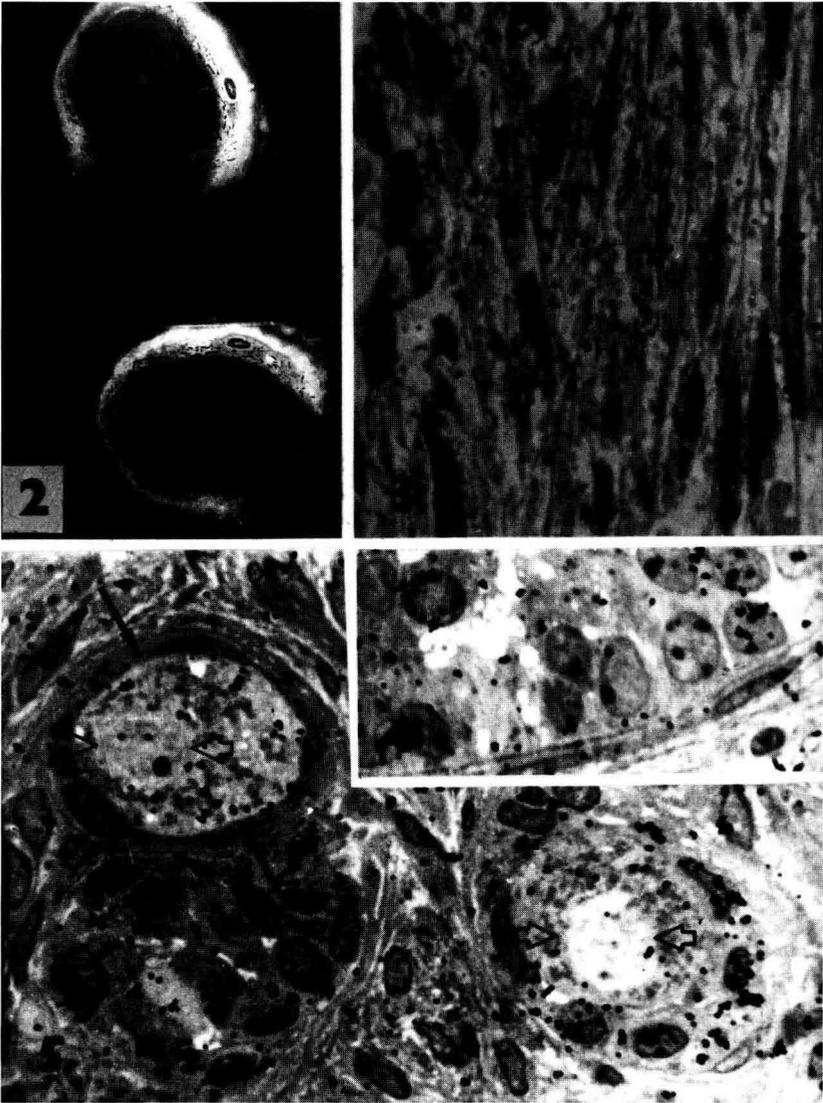
which were isolated separately in addition to ovarian tissue by puncture from antral follicles developed after a previous hormonal stimulation (*table 1*, animal C).

### 3.2. /6-<sup>3</sup>H/thymidine

Using identical procedures as for 5-<sup>3</sup>H/uridine, clear-cut results were obtained in most cases, the label being concentrated, as expected, in all cases and intervals in nuclei (*figures 9-11*). Note the strict localization of the autoradiographic reaction to the nuclear DNA contrasting with the labelling pattern after tritiated uridine labelling - cf. *figures 3-8*.

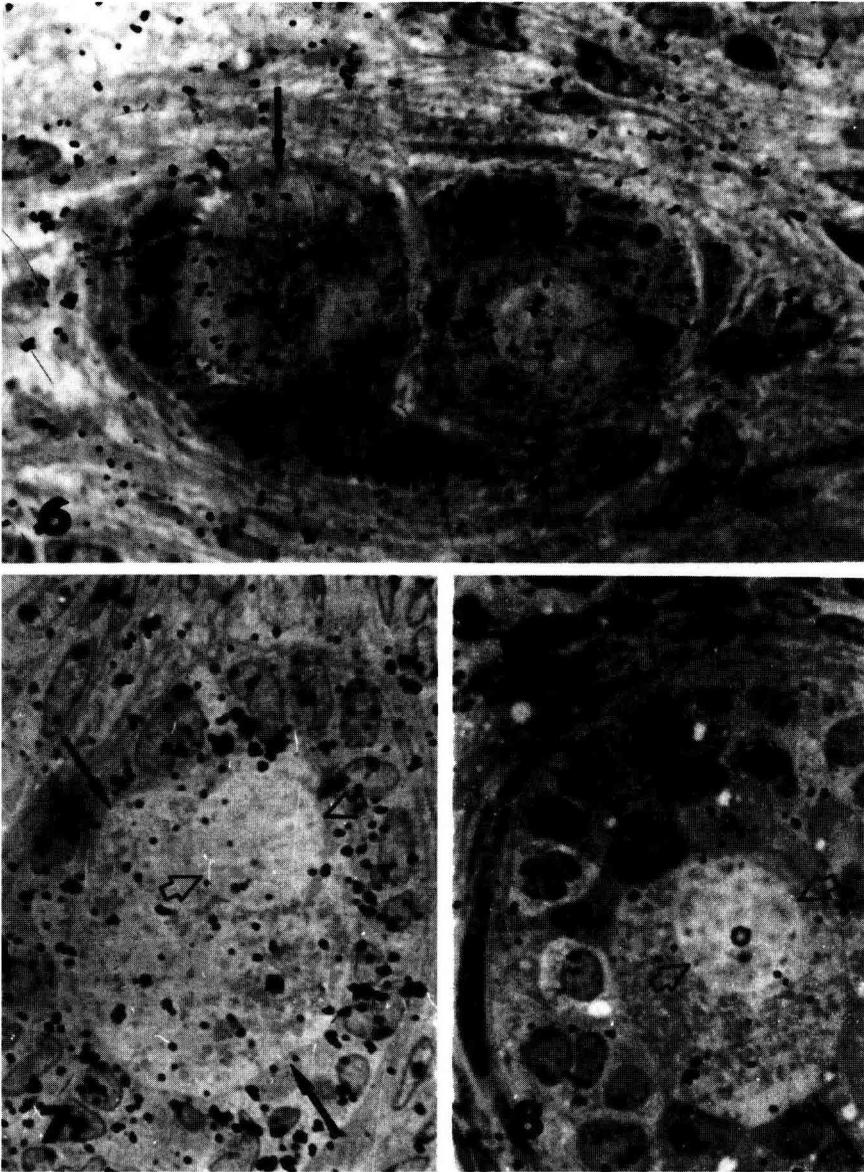
## 4. DISCUSSION

Tracer techniques based on application of radioactively labelled precursors of nucleic acids or (glyco)proteins 'in vivo' have been extensively used in studies of cell synthetic activities and/or proliferation (cell kinetics) in different cellular systems. In the mammalian ovary, the analysis of follicular kinetics has often been studied by intraperitoneal <sup>3</sup>H/thymidine administration followed by autoradiography (e.g. [12]). These studies were therefore limited to small laboratory animals in which the radiobiological approach was feasible from an economical and safe handling point of view after whole body administration or in vitro culture of the ovarian tissue with radioisotopes. In cattle, however, mostly morphological and morphometrical approaches have been used in studies concerning ovarian follicular growth and atresia [14]. Up to the present time, since the success of bovine preantral follicle culture in vitro remains limited, the autoradiographic detection of cells in the S-phase for kinetic studies in this species remains scarce [4, 11]. On the other hand, the technique of local ovarian application of different radioactive precursors, as described in this study may allow



**Figure 2.** A direct photographic projection from a slide bearing an autoradiogram from two paraffin sections through the whole cow ovary across its longer axis. The ovary was treated as in *figure 1* and isolated 6 d after  $^3\text{H}$ -uridine application (animal A, *table 1*). The silver grains in the autoradiogram are demonstrated in this way in the negative print as a white band reaching approximately to one third of the ovarian radius and represent the radioactivity from the incorporated tritiated uridine. Nuclear liquid emulsion Ilford K.5, exposure 30 d,  $\times 4$ .

**Figures 3–5.** Light-microscopy autoradiograms ( $\times 1\,250$ ) of ovarian tissues from ovaries treated as shown in *figure 1* with a solution containing  $^3\text{H}$ -uridine. (Epon semi-thin sections, nuclear liquid emulsion K.5 Ilford.) **3**, and **4**, Examples of an increased nuclear labelling after  $^3\text{H}$ -uridine in some of the ovarian tissues. **5**, Labelling in oocytes was seen clearly at the beginning of their growth. Animal C, exposure 100 d. Oocytes: between contralateral black arrows; oocyte nuclei: between empty arrows.

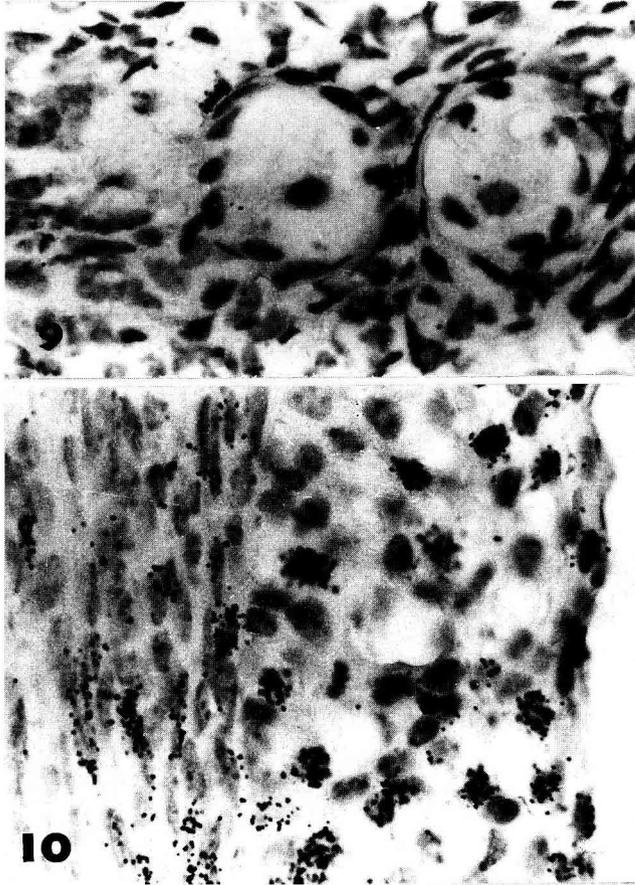


**Figures 6–8.** Autoradiograms of different type follicles from ovaries pulsed by  $^3\text{H}$ -uridine. (Epon sections,  $\times 1\ 250$ .) Oocytes and oocyte nuclei marked by arrows as in *figure 5*. **6**, An intensive labelling of both cytoplasm and nuclei of follicular cells distinguishes here the transitory type primordial follicle against the less labelled surrounding tissues. Note also an intensive labelling in two oocytes' ooplasm. Animal C, exposure 100 d. **7**, High level of labelling in the ooplasm of an oocyte in a similar type follicle as in *figure 6* from ovary of animal B. Exposure 100 d. **8**, The ooplasmal labelling of oocytes decreased in multilaminar follicles in which theca formation began. The labelling in follicular cells was also diminished. Note a vacuolated nucleolus in the oocyte nucleus. Animal C, exposure 100 d.

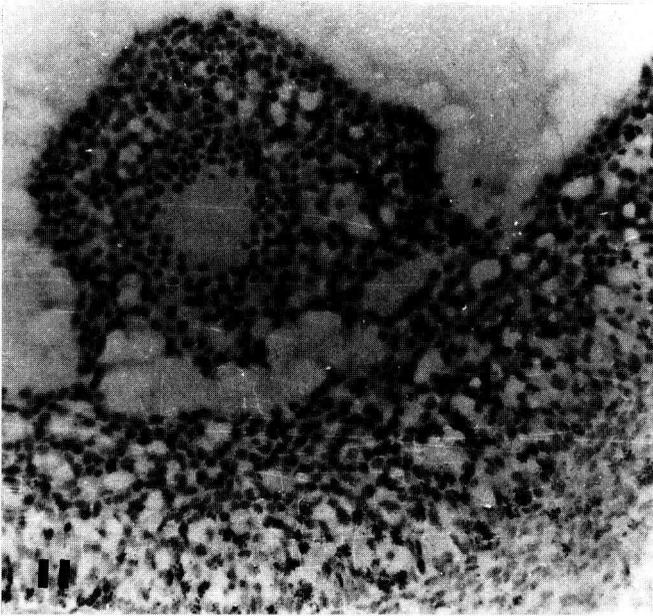
the extension of radiobiological and radio-histochemical approaches to large domestic animal ovarian function. Such methodology may help to better understand follicle development, especially during the early folliculogenesis since one of the main events linked with the initiation of follicle growth is the proliferation of granulosa cells, most efficiently signaled by the detection of the nuclei in the S-phase, i.e. in the phase of DNA replication.

Our original interest, however, which inspired the described experiments, was to investigate the possibility of labelling maternal RNA in growing ovarian bovine oocytes as previously performed in the mouse and other laboratory animals [2].

Early bovine embryo differs substantially from the mouse embryo by the cleavage stage at which its own embryonic genome is switched on. According to the general agreement, major RNA synthesis does occur, in



**Figures 9 and 10.** Light microscopy autoradigrams of cow ovarian tissue after  $^6\text{-}^3\text{H}$ /thymidine labelling by intrabursal instillation of the radioactive precursor. Paraffin sections, nuclear liquid emulsion Ilford K.5, exposure 1 month. **9**, A single cell nucleus labelled among non-labelled squamous follicular cell nuclei of a cross-section of a unilaminar follicle signalize the beginning of the follicular growth. Animal D,  $\times 1\ 000$ . **10**, In contrast to *figure 9*, many nuclei were labelled in intensely proliferating ovarian tissues, as shown here on the wall of a graafian follicle. Animal D,  $\times 1\ 000$ .



**Figure 11.** Both the wall of the follicle as well as cumulus oophorus in a graafian follicle showed the high rate of proliferation, respectively, of the cells forming them by the share of the  $^6\text{-}^3\text{H}$ /thymidine labelled cells in an autoradiogram of a paraffin section exposed for 1 month. Animal D,  $\times 220$ .

the mouse, at the 2-cell stage [8]. In the bovine embryo tritiated uridine incorporation specific for pre-mRNA and that linked with nucleolar transformation were both detected only during the late 8-cell stage when the major onset of embryonic RNA transcription is localized [3, 9]. On the other hand, there is a low-level transcription detectable even from the late 1-cell stage onwards (for a review see Schultz [13]). The role of maternal RNA in early mouse embryo is limited and is closely related to the activation of the embryonic genome [2]. The study of the fate of maternal RNA in the early bovine embryo may be enriched by the methodology presented here. The labelling in our autoradiograms of cow ovarian tissue after the application of  $^5\text{-}^3\text{H}$ /uridine and analysed after longer time intervals represents probably to a significant degree RNA. It was shown that, after longer pulses of  $^5\text{-}^3\text{H}$ /uridine in vitro, there is still a high proportion of radioactivity detectable in

RNA [5] with the minor unspecific label mostly being present in DNA. A critical approach to the labelling specificity as shown by autoradiography would include digestion of the tissue sections by specific nucleases. Nevertheless, the specific labelling in DNA after  $^6\text{-}^3\text{H}$ /thymidine reported also in this paper showed a basically different pattern of the autoradiographic reaction, the latter labelling being strictly limited to the nuclei.

In conclusion, it is expected that the devised technique of local radioisotopic precursor application to the cow ovary may allow the extension of radioisotopic tracer techniques also to this species. In a similar way the application of other substances would allow the testing of their effect on the cow ovary.

The main goal for the development of the presented methodology remains the evidence of a possibility to introduce radioac-

tivity representing very probably RNA into cow ovarian oocytes ooplasm as a basis for a biochemical analysis of cow maternal RNA in the mature oocyte, as performed by Bachvarova [2] in the mouse. However, since a preliminary publication of our results obtained after  $^3\text{H}$ -uridine application [7] there was, to our knowledge, no report in the literature using this approach, we extended our experiments to the most commonly used  $^3\text{H}$ -thymidine in tissue kinetics studies. The application of this selective precursor of DNA led to clear-cut results showing that this method may be used for the study of cow ovarian tissue proliferation kinetics *in vivo* under the actual situation of humoral regulation. This is not the case in experiments using the *in vitro* culture of the ovarian tissues.

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