

## The fate and rôle of macromolecules synthesized during mammalian oocyte meiotic maturation.

### II. — Autoradiographic topography of [<sup>3</sup>H]-fucose incorporation in pig oocytes cultured *in vitro*

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**Summary.** Pig oocytes in different maturational stages — germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) — were cultured *in vitro* with [<sup>3</sup>H]-fucose. The incorporation of the precursor was followed by LM or EM autoradiography on air-dried preparations and on semithin or thin sections. The cumulus cells connected with the oocytes at the GV stage were intensely labelled, while the labelling of the cumulus of MI and MII oocytes was lower. The cytoplasm of oocytes in the GV stage was characterized by nests of silver grains located mainly in a juxtannuclear position. The accumulation of label in the cortical region, observed in oocytes cultured with an intact cumulus, was less evident in cumulus-deprived oocytes. Lower labelling of the ooplasm, together with uniform distribution of the grains, was observed in later stages of meiosis. EM autoradiographs demonstrated the main localization, at the GV stage, of label in the Golgi apparatus and near the cell surface of oocytes and cumulus cells, as well as in the cytoplasmic processes of corona radiata cells. It is concluded that a relatively intense glycoprotein synthesis takes place in pig oocytes and cumulus cells during resumption of meiosis, at least before GV breakdown. Metabolic cooperation may occur as long as oocytes and cumulus cells keep membrane junctions.

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### Introduction.

Autoradiography has already been used to detect synthetic activities of the oocyte before and during the resumption of meiosis ; topography and dynamics of RNA and protein syntheses were studied in mouse (Bloom and Mukherjee, 1972 ; Rodman and Bachvarova, 1976 ; Wassarman and Letourneau, 1976a, b), rabbit and pig (Motlik, Kopečný and Pivko, 1978) oocytes. A further step was the demonstration of the involvement of proteins, synthesized during oocyte maturation, in the formation of zygote pronuclei and the nuclei of early cleaving

embryos (Motlik *et al.*, 1980). The aim of this work was to study glycoprotein synthesis during the resumption of oocyte maturation.

Pig oocytes, isolated at a well-defined stage of meiotic maturation (Motlik and Fulka, 1976), were cultured *in vitro* in the presence of tritiated fucose, the most specific glycoprotein precursor, at least for fucose-containing glycoproteins (Sandoz and Roland, 1976 ; Corfield and Schauer, 1979), and the sites of its incorporation were investigated by light (LM) and electron (EM) microscope autoradiography (ARG).

### Material and methods.

Ovarian oocytes were obtained by dissecting the follicle of superovulated miniature pig gilts (crosses of Minnesota and Göttingen strains). All gilts were injected with 500 IU PMSG (Antex, Leo, Copenhagen) on day 16 of the oestrous cycle, and some of them were given 500 IU HCG (Praedyn, Spofa, Praha) 72 h later. The oocytes from preovulatory follicles were isolated 0, 24 or 36 h after HCG, and fixed at least 3 h later (see below), that is at the germinal vesicle (GV) stage and around metaphase I and II (MI and MII), respectively. GV breakdown is generally completed 24 h after HCG (Motlik and Fulka, 1976), and in the two later stages the cumulus is already expanded and mucified. Two series of experiments were run, the first to detect changes between stages or treatments, the second to quantitate and localize at the EM level the labelling found at the critical stages.

In the first series of experiments, GV stage oocytes were divided into 3 groups and incubated with the precursor either (i) with the intact cumulus (C+), (ii) after removal of the cumulus and corona radiata cells (C-) by repeated pipetting or (iii) after removal of the cumulus and zona pellucida (Z-) with 0.25 p. 100 pronase (Koch-Light). Oocytes in MI and MII were incubated with or without cumulus cells. They were labelled for 3 h in a medium, according to Motlik and Fulka (1974), enriched by L- [1-<sup>3</sup>H]-fucose (The Radiochemical Centre, Amersham, UK ; specific activity : 1,4 Ci/mmol) to a final concentration of 100 µCi per ml. After washing in cold medium, the oocytes were prepared for autoradiography as air-dried spreads fixed by phosphate-buffered (pH 7.2) 4 p. 100 formol with cetyltrimethyl ammonium bromide added. The preparations were treated routinely with cold 5 p. 100 trichloroacetic acid and extracted with alcohol-ether (Motlik *et al.*, 1978). Cumulus cells were superimposed on oocytes in only a few cases.

In the second series of experiments, 16 oocytes (C±) in the GV stage and 15 (C±) in MI were cultured for 3 h again under the same conditions and with the same precursor ; at the end of incubation, the oocytes were thoroughly washed and prepared for autoradiography on thin or semithin sections after fixation according to Szöllösi and Hunter (1973) and embedding in Durcupan (Fulka). Air-dried oocytes and Durcupan semithin sections were coated with Ilford K.5 nuclear liquid emulsion and exposed for one month in evacuated containers at 4 °C. After development, the air-dried preparations were stained with Toluidin Blue (Gurr, 1 p. 100 ; pH = 4.2). After the emulsion was fixed by 5 p. 100 formalin, the

Durcupan semithin sections were stained with 1 p. 100 Methylene Blue or Safranin 0 (all stains from Gurr). The preparations were mounted in Clearmount (Gurr). The preparations for EM ARG were coated on grids with Ilford L.4 nuclear emulsion using the loop method (Bouteille, 1976), and were exposed for 2 to 5 months. The EM autoradiograms were developed in Orwo A 49, contrasted with 1 p. 100 uranyl acetate and lead citrate and observed in a Zeiss AM 10.

We attempted to make a semi-quantitative evaluation of the labelling intensity of air-dried preparations, using an arbitrary scale (table 1). Moreover, Durcupan semithin sections were submitted to quantitative analysis of grain distribution using an automatic scanning densitometer (Mignot, 1978). The relative area of silver grains was measured on different regions of large oocyte sections. Variance analysis and the Duncan multiple range test were applied to compare different categories of oocytes. The block method was used to compare the different regions at the same stage (table 2). Densitometric analysis of labelling on the GV and the nucleoli was not possible as some nucleoli were over-stained (figs. 3, 4).

## Results.

For clarity, the results of experiments 1 and 2 will be given simultaneously for each successive stage or treatment; the (semi)-quantitative results of the separate experiments are analyzed in tables 1 and 2.

TABLE 1

*Incorporation of [<sup>3</sup>H]-fucose on air-dried preparations of pig oocytes at different stages of meiotic maturation*

Source of oocytes (1)	Conditions of culture (2)	No. of oocytes	Labelling (3)						
			cumulus cells			ooplasm		GV (4)	
			+	++	+++	+	++	+++	
GV	C+	31	31			14	17	15	3
	C-	27				2	25	18	3
	ZP-	32				6	26	16	6
MI	C+	20	20			20			
	C-	25				25			
MII	C+	24	24			24			
	C-	24				24			

(1) GV, MI, MII : Oocytes were isolated from PMSG-stimulated gilts 0, 24 and 36 h after HCG and cultured 3 h with the precursor. (2) C+ oocytes cultured with intact cumulus; C- oocytes cultured after removal of cumulus cells; ZP- oocytes after removal of cumulus cells and zona pellucida. (3) To evaluate oocytes on air-dried preparations the following scale was used: + + + + very intense labelling, + + + intense labelling, + + medium labelling, + just over background. (4) The labelling of all the GV could not be estimated.

TABLE 2

Statistical significance of labelling levels on semithin sections

Categories	Regions				Difference between regions
	Ground ooplasm (O)	Cortical ooplasm (C)	Nests in ooplasm (N)	Cumulus cells (CC)	
GV (+ C)	(4) 4.03 ± 0.4 <sub>x</sub>	(6) 20.2 ± 1.2	(6) 18.4 ± 3.1	(6) 30.5 ± 1.0	C/N : NS 0 ≪ C, N < CC
GV (- C)	(6) 3.18 ± 0.4	(6) 10.3 ± 1.3	(6) 14.6 ± 2.0		C/N : NS 0 ≪ C, N
MI (+ C)	(7) 1.60 ± 0.4			(7) 10.3 ± 2.5 <sub>Δ</sub>	0 ≪ CC
Difference between categories	GV(+C)/GV(-C) : NS MI < GV(-C)    GV(-C) ≪ GV(+C)		GV(+C)/GV(-C) : NS    MI ≪ GV		

The numbers in brackets correspond to sections counted, each from a different oocyte. The values are the arithmetic means and standard errors of the percentage of the area covered by silver grains.

Significance : ≪ P < 0.01 ; < 0.05 ; NS : not significant.

x : The four values are only compared with the values of the other regions of corresponding oocytes.

Δ : Some differences of cumulus labelling were found among MI oocytes.

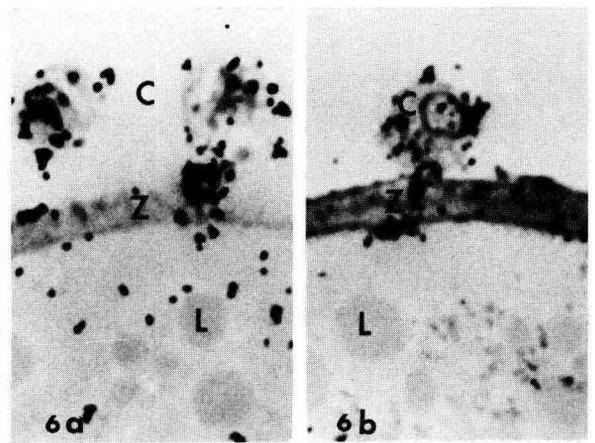
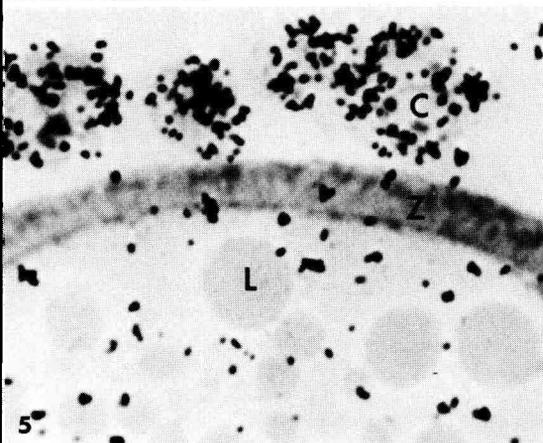
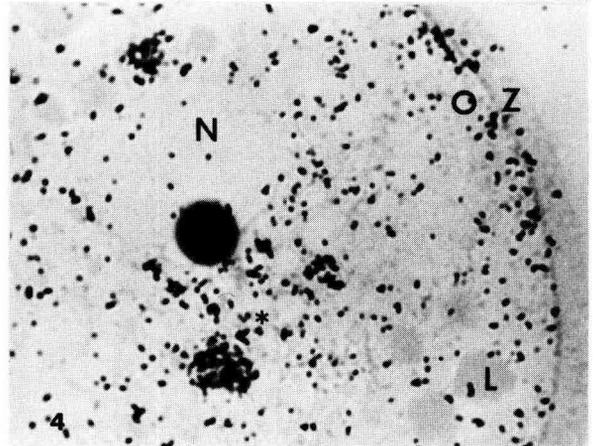
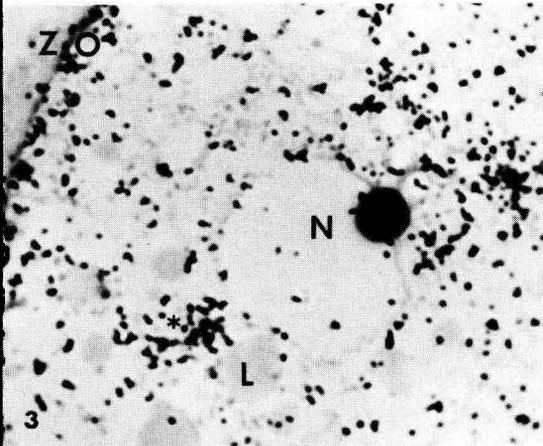
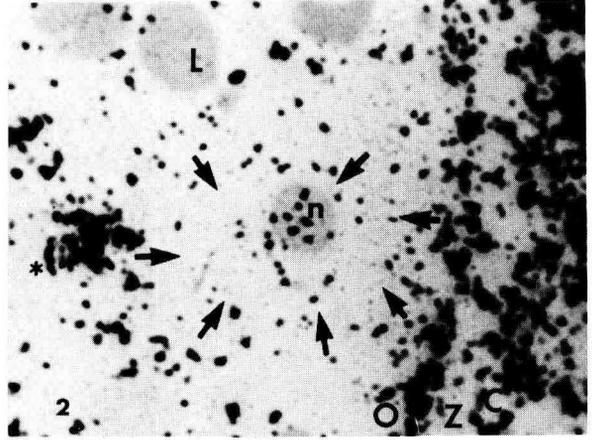
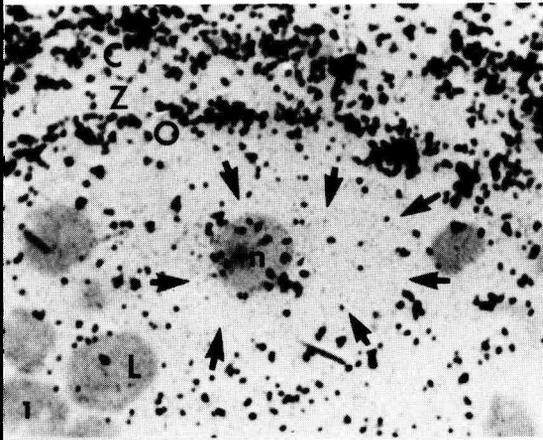
PLATE I

LM-ARG on semithin sections of pig oocytes labelled in vitro with [<sup>3</sup>H]-fucose.

FIGS. 1, 2. — *Labelled GV stage oocytes with intact cumulus.* Both pictures show very intense cumulus cell labelling (C) as well as prominent accumulation of label in the cortical ooplasm (O). In Fig. 1 the labelling increases from the nucleoplasm and ground ooplasm to the nucleolus (N). Fig. 2 shows a « nest » of silver grains (\*) in a juxtannuclear position ; the nucleolus (n) is also labelled. Safranin-Blue staining : the lipid inclusions (L) are contrasted as well as the nucleolus ; the nuclear envelope is hardly visible (arrows). The zona pellucida (Z) is not stained. × 600.

FIGS. 3, 4. — *Labelled GV stage oocytes with the cumulus cells removed.* Both pictures show a moderate accumulation of label in the cortical region (O) and « nests » of grains in a juxtannuclear position (\*). Few grains are visible on the nucleoplasm (N). Methylene Blue staining : the nucleolus is dark (some associated grains are difficult to see) ; the nuclear envelope and the zona (Z) are clearly visible ; the lipid droplets (L) are more lightly stained. × 600.

FIGS. 5, 6a,b. — *Labelled MI oocytes with cumulus cells.* All the cumulus cells (C) are labelled and the labelling of the cytoplasmic processes is still distinct in some. The ooplasm is more lightly and uniformly labelled (no accumulation of grains in any particular area). Same staining as for Figs. 3, 4. L : lipid droplets ; Z : zona pellucida. × 700.



*GV stage oocytes with cumulus.* — In the cumulus cells of GV stage oocytes, the cytoplasm and cell processes crossing the zona were very intensely labelled (figs. 1, 2). On thin sections, silver grains were mainly found over the Golgi apparatus and the cell processes and in the proximity of the plasma membrane (figs. 7, 8, 9).

The GV stage ooplasm showed an intense or medium labelling. On semithin and thin sections, the label was not uniformly distributed; grains accumulated in « nests » usually located in a juxtannuclear position (fig. 2). An intense incorporation occurred in the cortical ooplasm, particularly in the vicinity of the end of the labelled corona cell processes and on the profiles of the Golgi apparatus (figs. 1, 2, 8, 9). Germinal vesicles were labelled at a similar or lower level than the ooplasm. A higher labelling of the nucleolus over the nucleoplasm was found on semithin sections of 6/16 oocytes (figs. 1, 2).

*GV stage oocytes without cumulus or zona.* — In C- or Z- oocytes at the GV stage, ooplasm labelling did not reach the level obtained in the presence of the cumulus. Fewer grains were concentrated on the cortical ooplasm, whereas « nests » were still present on the perinuclear ooplasm (figs. 3, 4). Germinal vesicles were similarly or less labelled than in C+ oocytes, and no comparable nucleolar labelling was found. Briefly, there was no difference between C- and Z- oocytes, and *the main effect on the oocytes of cumulus removal was to lower the labelling in the cortical ooplasm.*

*MI and MII stage oocytes with cumulus.* — Instead of being very intense as at the GV stage, the labelling of cumulus cells was intense and medium at MI and MII stages, respectively. At MI stage, the cytoplasm and cell processes still crossing the zona were labelled (figs. 5, 6). Ooplasm labelling was the same at MI and MII stages and much lower than at the GV stage. There was no concentration of grains on semithin sections in the cortical and perinuclear regions of the ooplasm (figs. 5, 6).

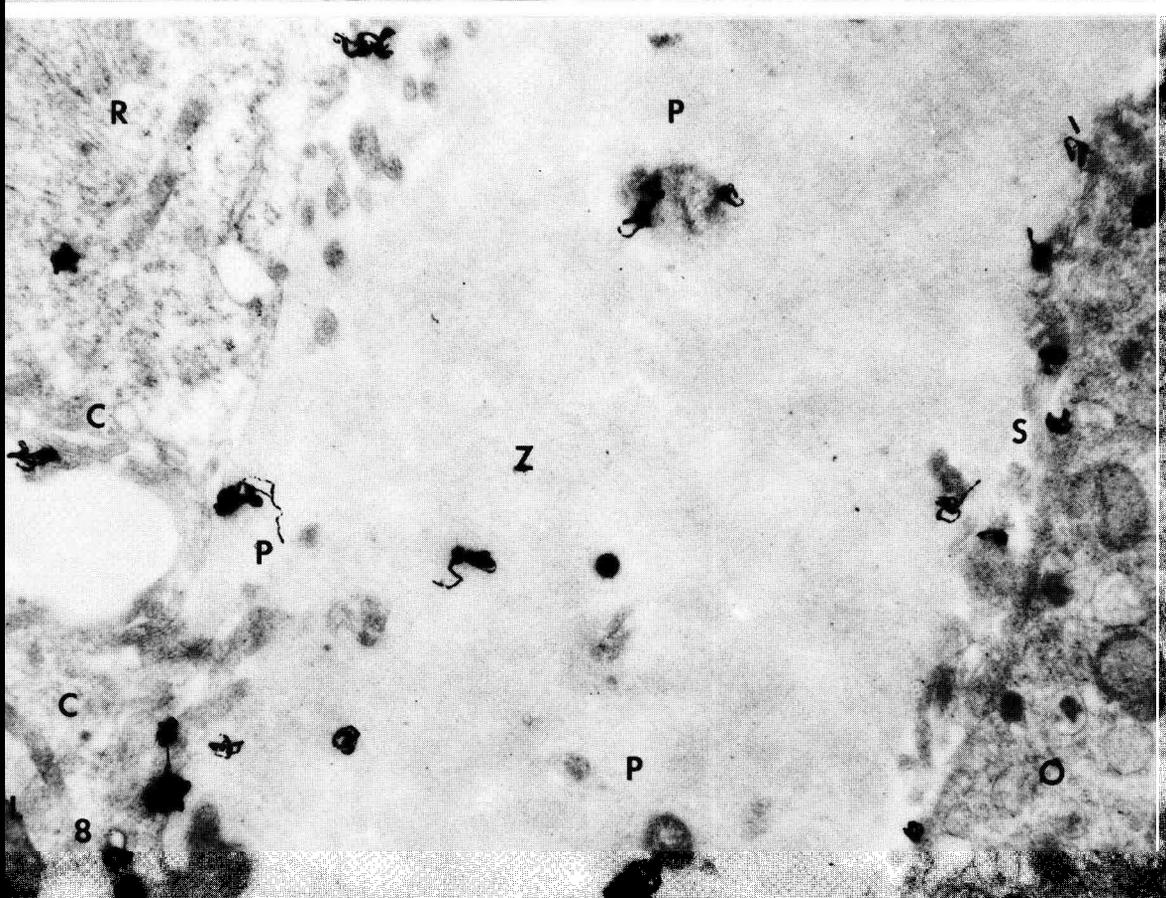
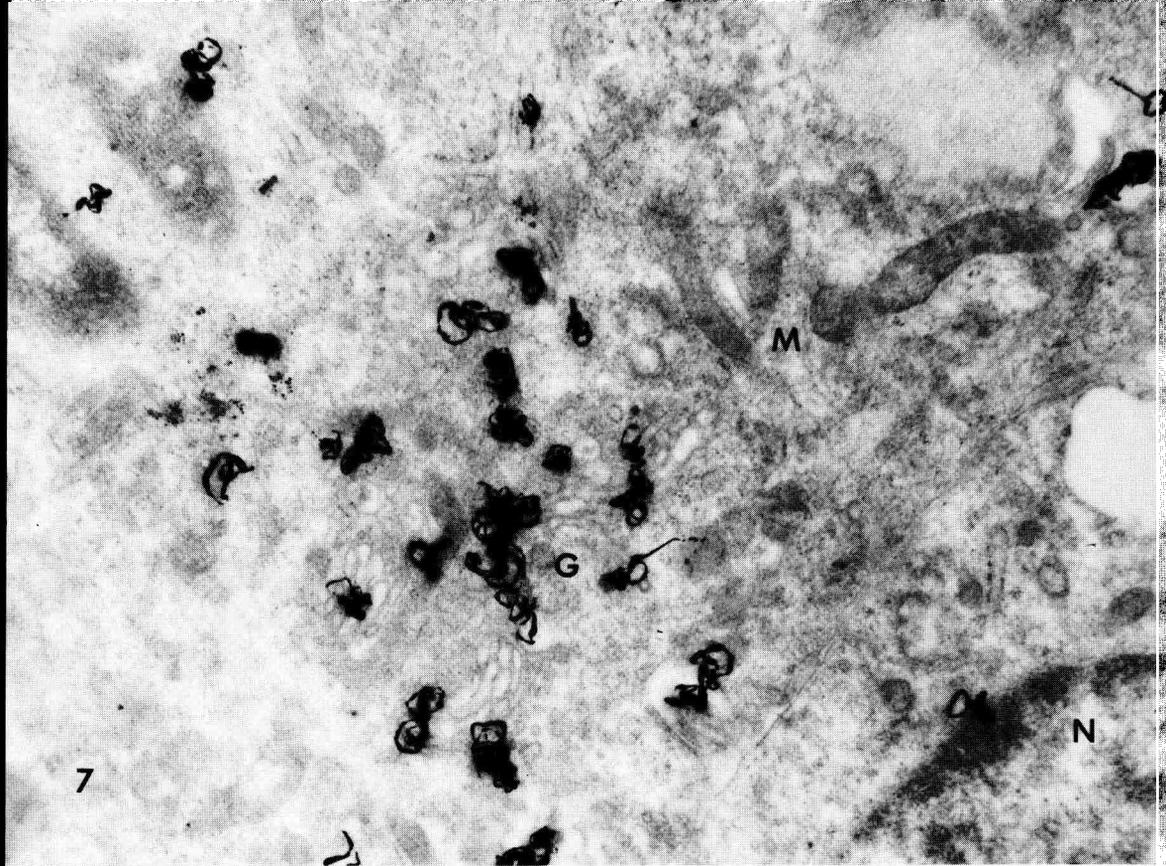
*MI and MII stage oocytes without cumulus.* — There was no difference between C+ and C- oocytes at MI and MII stages. At the EM level, a low labelling was found in the ooplasm (fig. 10). So, no noticeable change in incorporation occurred between the MI and MII stages in either C+ or C-

## PLATE II

*EM autoradiographs of GV stage material cultured for 3 h with [<sup>3</sup>H]-fucose in the presence of cumulus cells.*

FIG. 7. — *Labelling of a cumulus cell Golgi area (G). No accumulation of silver grains is seen on other organelles such as the mitochondria (M) or the nucleus (N). × 25 000.*

FIG. 8. — *Labelling is seen on the corona cell surface (C) and on cell processes crossing the zona pellucida (Z) and ending in the perivitellin space (S). It is also present on the cortical ooplasm (O). Rough endoplasmic reticulum (R) and lipid droplets (L) are not labelled. × 23 000.*



ocytes. The main modification took place between the GV and MI stages, i.e. *ooplasm labelling was less intense and more homogeneous from the MI stage on.*

Quantitative analysis of labelling on semithin sections (T2) confirmed the semi-quantitative data independently obtained previously on air-dried preparations (T1). The resolution on the semithin sections was much better, and there was no risk of cell superposition on such preparations. Oocytes did incorporate [<sup>3</sup>H]-fucose during resumption of meiosis and labelling was significantly higher before GV breakdown than near the MI stage in both oocytes and cumulus cells. At the GV stage, cortical ooplasm labelling was significantly higher in the presence of cumulus cells.

## Discussion.

*Interpretation of the quantitative results.* — The evident interpretation of our quantitative results is that [<sup>3</sup>H]-fucose incorporation by cumulus cells and the oocyte appears more intense when the cumulus is present and not expanded; synthetic activity would then decline in the cumulus cells and the oocyte when the gap junctions between those cells and between the corona cell processes and the oocyte (review in Szöllösi, 1980) disappear during maturation (Gilula, Epstein and Beers, 1978; Szöllösi *et al.*, 1978) or are mechanically disrupted. This coincidence confirms the hypothesis of a metabolic coordination and cooperation between the follicular cells and the oocyte through gap junctions (Moor, Smith and Dawson, 1980; Heller, Cahill and Schultz, 1981).

The granulosa cells can be regarded as supporting cells for oocytes, and junctional association is necessary for oocyte growth *in vitro* (Eppig, 1979; Bachvarova, Baran and Tejblum, 1980). Fully-grown oocytes still need cumulus cells in order to achieve their maturation *in vitro* (Nicosia and Mikhail, 1975; Sato, Iritani and Nishikawa, 1977a; Fukui and Sakuma, 1980).

A favorable effect of the granulosa cells on the incorporation of ribonucleic acid and protein precursors in oocytes was also reported (Wassarman and Letourneau, 1976a; Eppig, 1979; Bachvarova, Baran and Tejblum, 1980). It is possible that direct or metabolized precursors are supplied by the cumulus cells as

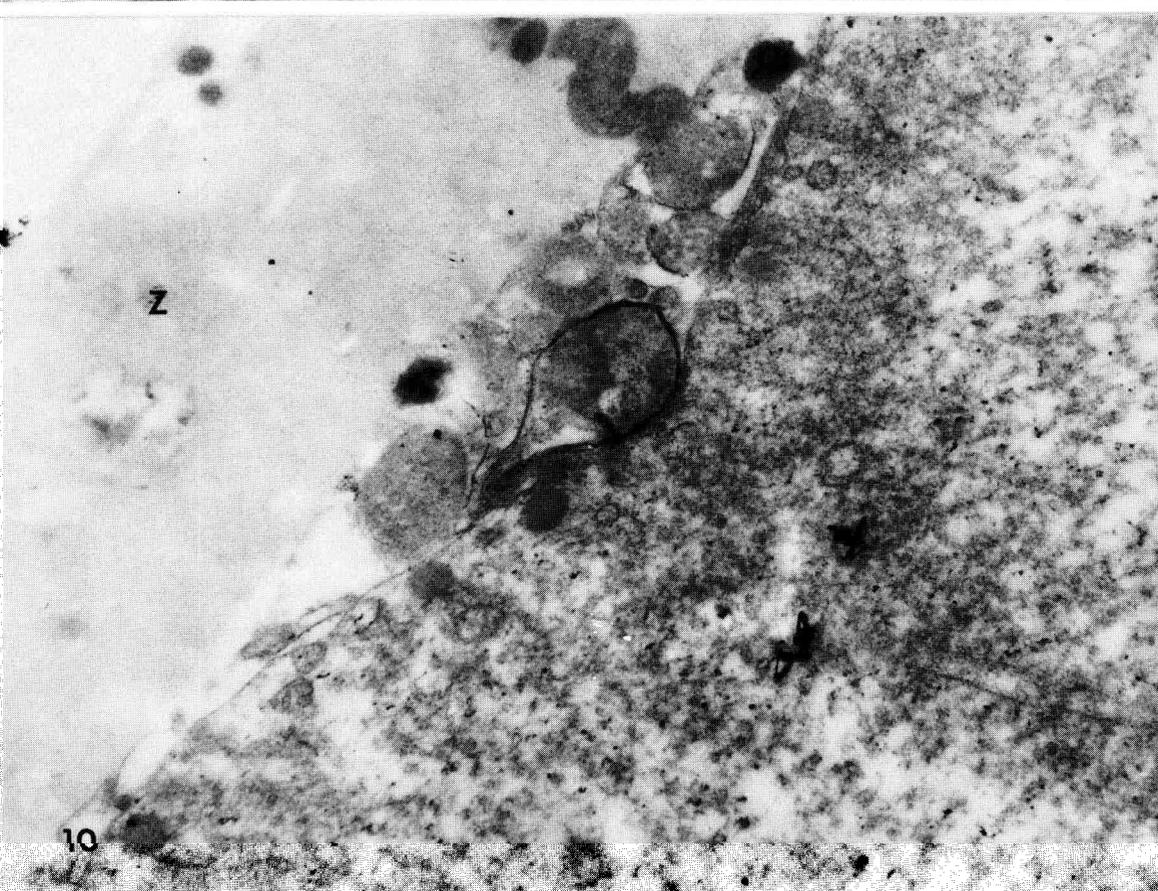
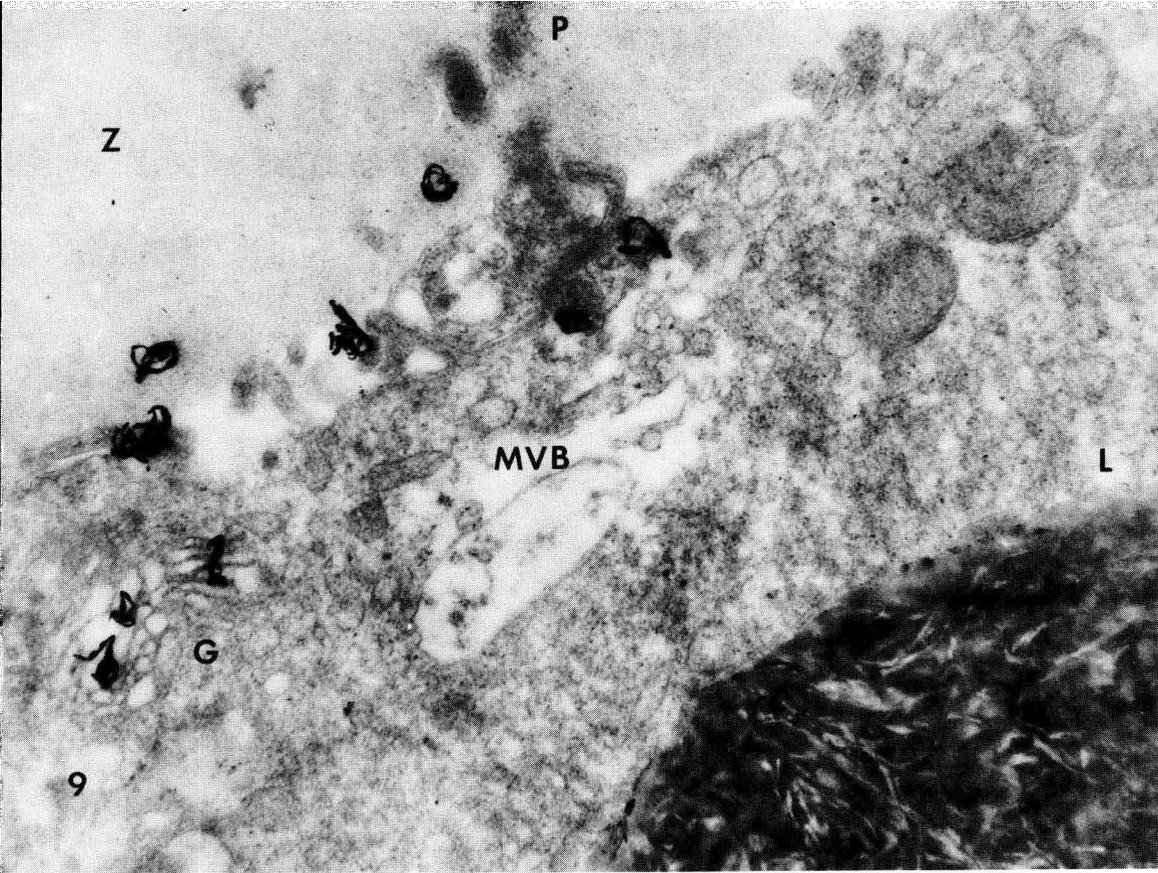
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### PLATE III

*EM autoradiographs of GV or MI stage oocytes cultured for 3 h with [<sup>3</sup>H]-fucose in the presence or absence of cumulus cells.*

FIG. 9. — *GV stage, C+*. The labelling is visible on the end of the cytoplasmic process (P) of a corona cell and on Golgi cisternae (G) in the cortical ooplasm. MVB: multivesicular body; L: lipid droplet. × 28 000.

FIG. 10. — *MI stage, C-*. An example of the low labelling obtained on the ooplasm. Z: zona pellucida. × 28 000.



different kinds of small molecules enter the immature oocyte through those cells (Gilula, Epstein and Beers, 1978 ; Moor, Smith and Dawson, 1980). It is also well known that granulosa and/or cumulus cells are able to furnish necessary energy substrates like pyruvate to the oocyte (Donahue and Stern, 1968 ; Sato, Iritani and Nishikawa, 1977b ; Bachvarova, Baran and Tejblum, 1980), and, in fact, the oxygen consumption of oocytes maturing *in vitro* increases only if the cumulus is present (Magnusson, 1980). The lower follicular cell activity after cumulus expansion may also be related to the decreased oxygen consumption of those cells (Ahren *et al.*, 1978).

Another possibility is oocyte uptake of larger macromolecules produced by cumulus cells. However, oocytes without cumulus show at least a basal level of glycoprotein synthesis (our study) as well as of protein synthesis (Lawrence, Beers and Gilula, 1978). Lastly, hormonal stimulation(s), which would indirectly regulate oocyte metabolism, may be mediated by intercellular communication (Lawrence, Beers and Gilula, 1978). The acquisition of the male pronucleus growth factor apparently requires connections in the intact follicle (Thibault, Gérard and Ménéz, 1975).

The modification of [<sup>3</sup>H]-fucose labelling in pig oocytes during resumption of meiosis is not an isolated phenomenon, as qualitative changes in protein synthesis were reported during that period in the same and other species (see review by van Blerkōm and McGaughey, 1978). The decrease observed in our material parallels the diminution of the absolute rate of protein synthesis from dictyate stage to metaphase II measured in mouse oocytes (Schultz, La Marca and Wasserman, 1978), although an increase in amino acid uptake was reported in sheep oocytes at MII (Moor and Smith, 1979).

*Localization and nature of labelled material.* — Few data are available on the synthesis of glycoproteins in the mammalian ovary ; glycosyl-transferases were found in ovarian homogenates (French and Bahl, 1980), and glycoprotein incorporation into the ovarian plasma membrane was obtained *in vitro* (Pandian, Bahl and Segal, 1975). The only previous autoradiographic work reported was done after *in vivo* injection of [<sup>3</sup>H]-fucose and therefore the labelling was very low (Haddad and Nagai, 1977). One of our aims was to localize labelled material at the LM and EM level and to make assumptions as to its nature and rôle. The localization of [<sup>3</sup>H]-fucose incorporation was much less uniform than that obtained with amino acids (Motlik, Kopečný and Pivko, 1978). This observation is in favor of specific glycoprotein labelling, as grains, for example, accumulated as expected on the Golgi apparatus of oocytes and cumulus cells and were not above the background on rough endoplasmic reticulum and lipid droplets. As the labelling found in our material was not in either the acid-soluble and liposoluble fractions or the glycosaminoglycans (see below), we conclude, following Bennet *et al.* (1974), that [<sup>3</sup>H]-fucose was effectively incorporated into glycoproteins. Biochemical studies beyond our scope would be required to characterize the material synthesized.

— In cumulus cells, the elaborated glycoproteins may either contribute to the formation of lysosomal enzymes, as lysosomes occur in the cytoplasm of

these cells (Anderson, 1972), or to the renewal of the cell coat since the other site of rather intense labelling was the cell surface. The relatively long incubation period with [ $^3\text{H}$ ]-fucose explains the labelling of both the Golgi apparatus and the cell surface.  $^{35}\text{S}$  was also incorporated into the Golgi apparatus of follicular cells (Young, 1973), and labelling was found on the cytoplasmic processes of the corona cells (Morocard and Gothié, 1955).  $^{35}\text{S}$  incorporation probably corresponds to the elaboration of mucosubstances. However, we shall not discuss here the synthesis of the hyaluronidase-sensitive sticky mucus material embedding the expanding cumulus oophorus and both faces of the zona (Fléchon, 1974) since it is produced around 16-20 h after HCG. Moreover, fucose is not a good precursor for glycosaminoglycans (Sandoz and Roland, 1976).

— The heterogeneous labelling (cortical labelling and « nests ») in the ooplasm at the GV stage is related to the Golgi cisternae which are distributed either around the GV or mainly in the cortex, close to the corona cells process, as described by Norberg (1972); at the MI stage, a lower labelling and fewer Golgi profiles were found in the cortex. As for the cumulus cells, the labelled oocyte glycoproteins may correspond to either lysosomal enzymes or cell surface material. Lysosomes are present in the ooplasm (Anderson, 1972; Štašná, 1978) and their distribution is modified during GV breakdown (Ezzel and Szego, 1979). Changes may occur in the cell coat of oocytes during meiotic resumption (de Felici and Siracusa, 1980). The synthesis of new fucose-containing surface glycoproteins could be related to the high fusion rate of mature versus immature oocytes with capacitated sperm (see review in Gwatkin, 1977). The zona, which is synthesized at early stages of follicular development, and the cortical granules, present in large numbers before resumption of meiosis, were not labelled.

The low labelling in the GV and nucleolus is intriguing. Only relatively old cytochemical studies indicate the possible presence of glycoproteins in oocyte nucleoli (see reviews in Austin, 1961; Raven, 1961; Graumann, 1964; Hay, 1968; Busch and Smetana, 1970). However, more recent studies suggest that glycoproteins (Hozier and Furcht, 1980) and enzymes involved in carbohydrate metabolism (Siebert and Humphrey, 1965; Iijima, Shanta and Bourne, 1967; Kean, 1970; Berthillier, Benedetto and Got, 1980) are actually present in the nuclei and nucleoli.

As a logical extension of our present and previous studies (Motlik *et al.*, 1980), it would be interesting to improve our knowledge of the synthesis of the enzymes — some of which may be glycoproteins — present in the GV and of their rôle in the nuclear events occurring during maturation and after fertilization.

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**Résumé.** Des ovocytes de truie à différents stades physiologiques, vésicule germinative (VG), métaphase I (MI) et métaphase II (MII), ont été cultivés *in vitro* en présence de fucose [<sup>3</sup>H]. L'incorporation de précurseur a été observée par autoradiographie en microscopie photonique (MPH) et électronique (ME) sur des préparations séchées à l'air et des coupes semi-fines et fines, respectivement. Au stade VG, les cellules du cumulus oophorus, connectées aux ovocytes, sont très intensément marquées, tandis que l'incorporation est plus faible aux stades MI et MII. Le marquage du cytoplasme ovocytaire au stade VG est intense et hétérogène : en MPH, les grains d'argent sont concentrés en amas, notamment autour de la VG et dans le cytoplasme cortical. En ME, la localisation principale des grains d'argent est sur les appareils de Golgi des cellules du cumulus et des ovocytes, les surfaces cellulaires et les prolongements cytoplasmiques des cellules de la corona radiata. L'élimination du cumulus avant incubation provoque une diminution du marquage cortical de l'ovocyte. Après la rupture de la VG, le cytoplasme ovocytaire est marqué d'une manière plus faible et homogène. L'incorporation est interprétée comme une synthèse de glycoprotéines assez intense dans les cellules du cumulus et les ovocytes en reprise de méiose, au moins avant la rupture de la VG. Une coopération métabolique semble exister entre ovocytes et cellules du cumulus tant que leurs connections existent.

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