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In vitro exchange between the follicle and its culture medium

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Summary. Two techniques for culturing bovine de Graaf follicle were compared. The « continuous flow » technique permitting better incorporation of amino acids was used.

As cAMP, puric and pyrimidic bases could pass from the follicle into the culture medium. Glucose consumption and lactate accumulation did not vary significantly in relation to maturation stage or after the addition of HCG into cultures with PMS.

High molecular weight proteins were found to migrate from the follicular fluid into the culture medium if the follicle remained healthy up to the end of culture. Atresia totally suppressed this permeability.

Meiosis may resume *in vitro* in intrafollicular oocytes, depending upon whether or not gonadotropins are added in the culture medium (rat : Tsafrifri *et al.*, 1972 ; rabbit : Thibault and Gérard, 1973 ; monkey and calf : Thibault, Gérard and Ménézo, 1976 ; sheep : Moor and Trounson, 1977). Except for the mouse (Mukherjee, 1972), it is now well established that only these follicle-enclosed mature oocytes are able to support normal embryonic development after fertilization (rabbit : Thibault, Gérard and Ménézo, 1975*b* ; sheep : Moor and Trounson, 1977). However, these results were obtained with large antral follicles, relatively scarce in the ovary.

In order to promote the long-term culture necessary for the maturation of middle-sized follicles, we developed a continuous-flow superfusion technique mimicking the physiological situation in the ovary (Ménézo, Gérard and Thibault, 1976). As *in vivo*, the whole growing follicle is entirely bathed in a continuously flowing medium and metabolic exchange occurs all around the follicle.

This study presents data on metabolic activity and compares the continuous-flow technique with that of organ culture grid.

Material and methods.

Calf and macaque follicles were collected according to our current technique (Thibault *et al.*, 1975*a*). They were cultured either in organ grid conditions or employing the superfusion technique. The gas phase was 57 p. 100 O_2 — 5 p. 100 CO_2 — 38 p. 100 N_2.

The culture medium, based upon cow follicular fluid, was entirely synthetic * (Ménézo, 1976). In some experiments Ficoll 70 (Pharmacia) was substituted for BSA. Different gonadotropins were always added (PMS and PMS + HCG). For biochemical studies, we analyzed the medium before and after 50-hrs culture. Free amino acid analyses were performed using ion-exchange chromatography on the Optica aminolyzer. Puric and pyrimidic bases, cAMP and AMP were detected and identified using HPLC. Glucose was determined using the hexokinase method and lactate using LDH, after precipitation with perchloric acid. Electrophoreses were performed for non-enzymatic proteins using the Pharmacia PAA 4/30 system allowing separation according to molecular weight (MW). Hydrolytic enzymes were detected with the colorimetric method of Monget (1975) and dehydrogenases with a modified method of Altmann (1969).

Results and discussion.

After 52 hrs of culture, follicle diameter increased by 5 to 10 p. 100 when we used superfusion.

Free amino acids (table 1). The uptake of free amino acids is 3 to 39 times higher with superfusion than on grid. The high rate of glycine and glutamic + glutamine

TABLE 1

Comparative study of free AA *consumption* (nMoles/h/follicle)
6 follicles per technique

Amino-acids	Superfusion (m)	Organ culture (m)	Super/org
Asp + AsN ..	29	3.5	8.3
Thre	21	1.7	12
Ser	19	0.5	38.8
Glu-Gin	248	17.7	14
Gly	597	33.8	17.7
Ala	53	4.8	11
Val	22	7.2	3.1
Met	4	1.3	2.9
Ileu	16	2.3	6.8
Leu	18	3.2	4.8
Tyr	15	0.8	18.3
Phe Ala	13	1.1	11.6
Lys	26	3.0	8.4
Hist.....	14	1.2	11.9
Arg	22	3.1	7.2

* API System, 38390 Montalieu, Vercieu, France.

consumption is probably related to their possible utilization for the synthesis of nuclear material. This metabolic increase leads neither to degeneration (Thibault, Gérard and Ménézo, 1975*b*) nor to dedifferentiation, as sometimes observed in immerged cultures.

Bases and nucleotids. Attention has been focussed on the burst of cAMP *in vivo* after LH surge. Effectively, cAMP was identified in culture media of macaque and calf follicles, even when oocytes remained in prometaphase or metaphase 1 stages. However, all puric and pyrimidic bases are also found.

Glucose and lactate (tables 2 and 3). Glucose uptake by 3 to 5 mm calf follicles is about 60 to 80 μg/hr. Lactate accumulation is from 12 to 24 μg/hr. However, glucose uptake and lactate accumulation are statistically unrelated to the oocyte maturation stage reached and to the gonadotropin environment in the culture medium (PMS or PMS + HCG). Similar observations were made for the rat by Tsafriri *et al.* (1976*a*) and Hillensjö (1976).

TABLE 2

Glucose uptake and lactate accumulation according to maturation stage reached in 50-hr culture

Maturation stage reached	Follicles cultured	Glucose uptake μg/hr	(σ)	Lactate accumulation μg/hr	(σ)
Dictyate or prometaphase.	6	61.2	(13.3)	22.1	(5.2)
Met. 1	8	77.9	(13.8)	24.3	(4.2)
Met. 2	6	82.	(13.3)	11.9	(3.8)

TABLE 3

Sugar metabolism according to hormonal treatment in 50-hr culture

Hormonal treatment in culture	Follicles cultured	Glucose uptake μg/hr	(σ)	Lactate accumulation μg/hr	(σ)
PMS + HCG	12	90.3	(12)	19.1	(3.1)
PMS	12	73.0	(13)	21.4	(4.2)

Macromolecules (table 4, fig. 1). Electrophoresis : proteins migrate towards the medium from healthy follicles, whatever maturation stage has been reached. Main proteins transferred from calf follicle have a MW between 80 000 and 100 000, although lower MW proteins also shift. A similar transfer also occurs with macaque follicles, but proteins heavier than 150 000 are also transferred.

Culture in media using Ficoll instead of BSA showed that follicular albumin (MW 65 000) also migrates from the follicle. Comparison of electrophoretic mobilities and ouchterlony immunodiffusion indicate that the proteins found in the culture medium seem to come from the follicular fluid and are not neosynthesized. Our observations may be related to the work of Tsafriri *et al.* (1976*b*) on the inhibitory

TABLE 4

Enzymes found in calf control follicular fluid and in the culture medium after 50-hr culture

	Before culture (follicular fluid)	After culture (medium)
Phosphatase Ak	+	+
Phosphatase Ac	+	+
Leucyl Aryl Amidase	+	+
α glucosidase	+	+
Lipase (Myristate)	0	+
β N. A. glucosaminidase..	+	+
β glucuronidase	+	0
Glucose 6 PD. H.	+	+
L.D.H.	+	+
Isocitrate D.H.	+	0
Malate D.H.	0	+

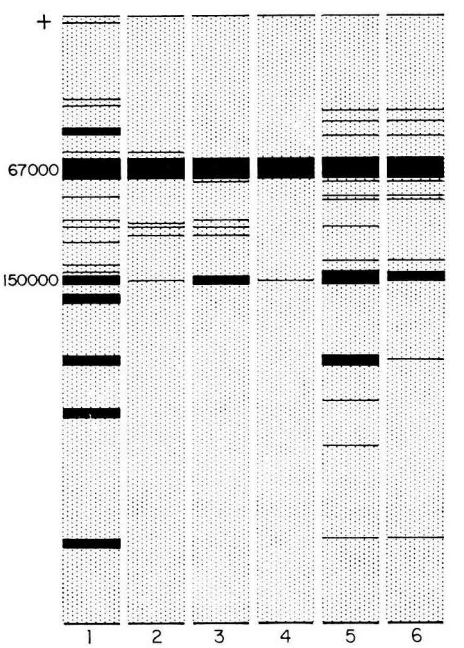

FIG. 1. — *Acrylamid gel electrophoresis of follicular fluid.*

1 : Calf follicular fluid ; 2 : Medium (with BSA) after 50 hrs of calf follicle culture (meiosis reaches M I) ; 3 : Medium with Ficoll after 50 hrs of calf follicle culture (meiosis resumes up to M II) ; 4 : Control medium (with BSA) or medium after culture of an atretic calf follicle ; 5 : Macaque follicular fluid before culture ; 6 : Medium after a 50 hrs-culture of a macaque follicle (meiosis stage : M I).

67,000 : BSA ; 150,000 : BSA dimer.

effect of follicular fluid on meiosis resumption. Before ovulation in healthy follicles, the inhibitor is no longer synthesized and may leave the follicular fluid by the transfer mechanism described.

Malic dehydrogenase, not present in the follicle at the beginning of culture, appears in the medium. Except for the cumulus and the oocyte (Dekel *et al.*, 1976), the presence of this enzyme could confirm that gonadotropins enhance the aerobic pathway. However, a simple release from the theca cells is not completely excluded.

Macromolecular permeability was severely impaired when cultured calf follicles became atretic.

Enzymes. As expected some enzymes pass through the follicular wall ; this is the case of alkaline phosphatase and non-specific esterases. However, the same MW dependence is also observed since β glucuronidase (MW 210 000) was not detected in culture media. Isocitrate dehydrogenase (MW 300 000) shows a similar scheme.

Conclusions.

Continuous-flow superfusion technique was an improvement over the organ culture grid one. There was an increase of mitotic index and of follicle volume related to augmented amino acid consumption. Corona cell reaction was initiated.

However, the percentage of metaphase II at the end of culture remained under 50 p. 100. So, it seems for now that the oocyte maturation process cannot be related to a simple metabolic test or to steroid synthesis (Lieberman *et al.*, 1976). Macromolecular transfer from the follicle, impaired by atresia, could be an intraovarian mechanism for the regulation of ovarian follicle population *in vivo* (Peters, 1973). Very little is known, either *in vivo* or *in vitro*, about normal transfer from the follicle. A signal of complete cytoplasmic and nuclear maturation, which could be detected by metabolite(s) analysis in the culture medium, is yet to be found.

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Résumé. Deux techniques de culture du follicule de de Graaf bovin ont été comparées. La technique « en continu » qui permet une meilleure incorporation des aminoacides a été retenue.

Il apparaît que, comme l'AMP cyclique, les bases puriques et pyrimidiques peuvent passer du follicule vers le milieu de culture. La consommation de glucose et l'accumulation de lactate ne varient significativement ni en fonction du stade de maturation ni par l'addition d'HCG dans les cultures avec PMS.

Des protéines de poids moléculaires élevé peuvent migrer du liquide folliculaire vers le milieu de culture si le follicule demeure sain à la fin de la culture. L'atrésie supprime totalement cette perméabilité.

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