

A NOVEL TECHNIQUE FOR ISOLATING AND CULTURING MOUSE OVARIAN FOLLICLES.

COLIN TORRANCE,
Postgraduate Student,
Department of Physiology,
University Medical School,
Teviot Place.

(Submitted for the Ellis Prize in Physiology June 1987)



Abstract

A method has been developed which permits the early stages of follicular development to be investigated *in vitro*. Ovaries from 8-11 day CBA/C57BL6 F₁ hybrids were used. Follicles were isolated by enzymatic treatment followed by repeated pipetting. They were cultured in Medium 199, supplemented with 10% donor calf serum, at 37°C in 5% CO₂. The isolated follicles consisted of an oocyte with 1 to 3 layers of epithelial cells, generally stripped of adhering stromal cells. When the follicles were cultured on a plastic substrate the granulosa cells soon became adherent and began to spread, disrupting the follicle. The oocyte was shed within 1-3 days. To maintain the structural integrity of the oocyte-granulosa unit the follicles were embedded in a matrix of collagen extracted from rat tail tendons. 20µl samples were transferred to the wells of a Terasaki plate, excess fluid was removed and 10µl of collagen gel solution added. The gel was set and transferred to a second well containing more collagen gel solution. The final gels were set and cultured as above. The collagen gels can be fixed and processed for assessment by histology and autoradiography. Preliminary results indicate that the follicles can be maintained in these conditions for several days and may continue to progress to later stages of development. Follicles with 4 or more intact layers of granulosa cells have been identified after 8 days in culture.

INTRODUCTION

A preliminary report is presented of work undertaken as a postgraduate student in the Department of Physiology. The main aim of the project is to develop techniques which will allow the developmental biology of small ovarian follicles to be investigated. The work is primarily directed at the granulosa cells of murine follicles, ranging up to the antral stage. It has involved establishing a system for isolating and culturing follicles *in vitro*. A range of supplementary techniques including autoradiography and *in vivo* transplantation are also being developed.

The follicle is the functional unit of the mammalian ovary. It has a dual function - it provides the microclimate for oocyte maturation and it is the site of steroidogenesis. The steroids produced are necessary for follicle growth, oocyte maturation and ovulation and also to prepare other reproductive organs for sperm transport and implantation. The follicle is a highly organised unit: in early stages it consists of the oocyte and granulosa cells, later a thecal layer develops from ovarian stroma. The cell types of the follicle are in intimate association and complex interactions occur. The ovarian compartments can be studied in isolation but communication between them is probably essential for integrated follicular function and development.

The ovary of an adult mouse contains thousands of follicles at various stages of development. Follicles fall into three main groups: 1) a non-growing pool of small (primordial) follicles in which the oocyte and pregranulosa cells are quiescent, 2) the small, growing, preantral follicles marked by oocyte growth and an increase in granulosa cell numbers and layers (in later stages the thecal layer differentiates and the zona pellucida forms), and 3) the antral follicles which contain a fully grown oocyte with granulosa cells continuing to multiply. Follicular fluid appears forming the antrum (Peters et al 1979). Each day primordial follicles move from the non-growing pool and enter a growth phase. In the mouse this can last for 19 days. The largest proportion of the time taken for a follicle to reach maturity is spent in the preantral phase yet the majority of follicular research concentrates on the antral phase. The hormonal control and role of the large ovarian follicle, particularly the production of ovarian steroids has been extensively investigated. Larger

follicles can be dissected out and studied in vitro. The granulosa cells can be harvested and studied by tissue culture methods. The development and function of the smaller follicles has received much less attention and remains a largely open question.

Follicles are recruited from the non-growing pool and progress to the antral stage with a few reaching ovulation. The majority of follicles are lost through atresia. Little is known of the factors initiating and controlling growth. Many factors might be important: classical endocrine mechanisms, paracrine and autocrine mechanisms, growth factors, stromal-epithelial interactions, are all of interest.

The pituitary hormones follicle stimulating hormone (FSH) and luteinising hormone (LH), are important recruiting antral follicles for ovulation but their role in the development of smaller follicles remains uncertain. Baker and Scrimgeour (1980) indicated they might be necessary for "priming" follicular growth in fetuses and neonates. The ovaries of normal and anencephalic human fetuses were investigated. Normal fetuses between 34 weeks and term contained growing follicles including some antral follicles, none were found in the ovaries of anencephalic fetuses of a similar age. In addition the administration of PMSG (pregnant mare serum gonadotrophin) to mice between birth and five days old increased the growing follicles (Peters 1979). FSH had a similar effect on neonatal mouse ovaries in organ culture (Neal & Baker 1973). As preantral growth continues after hypophysectomy the gonadotrophins are not required for all stages of follicle development (Jones & Krohn 1961, Faddy et al 1976, Edwards et al 1977,). Once initiated follicle growth continues irrespective of the animals endocrine status. Follicles are recruited from the non-growing pool throughout the oestrus cycle, during pregnancy and after hypophysectomy. The role of classical hormones in controlling the initiation and growth of small follicles remains uncertain. When antisera specific to FSH or LH was injected into mice the number of small antral follicles decreased (Eshkol & Lunenfeld 1975). It is not known if this effect is due to a decrease in the number of follicles entering the growing phase or a decrease in growth rate. Gonadotrophin releasing hormone (GnRH)-deficient hypogonadal mice have low plasma and pituitary levels of gonadotrophins. Halpin et al (1986) compared the follicle dynamics of hypogonadal and normal

mice. Their results suggest that the gonadotrophins may regulate the initiation of growth and influence the rate of loss through atresia. They suggest that intraovarian mechanisms may control the number of large follicles formed.

The role of paracrine and autocrine mechanisms in controlling ovarian function has recently been recognised (reviewed by Hillier 1985 and Hsueh 1986). Ovarian steroids (androgens, oestrogens and progesterone), and peptides (GnRH, insulin, insulin-like growth factor-1 (IGF-1), vasoactive intestinal peptide (VIP)) may all have paracrine functions. Growth factors may also be involved. There is evidence that epidermal growth factor (EGF) can affect oocyte maturation and may inhibit the action of FSH on granulosa cells (Hsueh et al 1981). Stromal-epithelial interactions have been shown to be important in the morphogenesis of many reproductive tissues (reviewed by Cunha et al 1983). The morphogenic effect of hormones such as the steroids might be reliant on interactions between the stromal and epithelial components of a tissue. For example when the simple, columnar uterine epithelium of neonatal mice was combined with vaginal mesenchyme the uterine cells differentiated into a squamous vaginal-type epithelium (Cunha et al 1983).

Isolated follicle preparations maintained under the controllable conditions of tissue culture present a potentially powerful technique for investigating the complex intraovarian mechanisms which may regulate the development and growth of small follicles.

ESTABLISHING A METHOD FOR ISOLATING AND CULTURING FOLLICLES

There are three possible approaches to establishing an *in vitro* system for investigating the intraovarian mechanisms controlling early follicle development. Organ culture, the maintenance of complete ovaries or ovarian fragments *in vitro*, is one approach which has provided valuable insights into follicular development (eg Ryle 1972). This approach retains follicular structure but does not permit the interactions between theca and stromal cells to be investigated. As the tissue is relatively large there may also be diffusional limitations on follicular growth. The opposite approach is to use monolayer cultures of mixed or single ovarian cell types after mechanical and/or enzymatic disaggregation of the ovary. Granulosa cells aspirated from

antral follicles are often cultured in this way. The technique is limited due to possible de-differentiation of the cells in monolayers and the generally unphysiological conditions. The third approach is a limited disaggregation which releases the follicle unit from surrounding tissues but keeps it intact. The intact follicles can then be cultured and studied *in vitro*. This is the approach utilised in this project. To develop the method two major problems had to be addressed. Firstly a method was needed to free follicle from the ovarian stroma with minimal disruption of the follicular unit and secondly the culture system had to maintained the normal follicular shape.

A number of workers have attempted to produce isolated ovarian follicles. Grob (1964) used pronase to produce isolated follicles, these were then grown *in vitro* (Grob 1971) or *in vivo* (Grob 1969). Nekola and Malbandov (1971) also used pronase to produce a suspension of follicles, broken follicles, cell clumps and fully dispersed cells. Nicosia et al (1975) used collagenase to isolate rabbit ovarian follicles. Nicosia and co-workers compared pronase and collagenase and found the former tended to degrade the follicle unit. Eppig (1977) also used collagenase to disaggregate mouse ovaries. As enzymes can damage cells, particularly cell surface receptors, initial attempts to free follicles were mechanical. Ovaries were teased apart under the dissecting microscope using fine needles. By this method some isolated follicle were produced but many appeared damaged. Numbers were low and many follicles remained in small clusters which were very difficult to reduce further. The method was very time consuming for the small numbers produced. Attention then turned to enzymatic methods. Collagenase was chosen as pronase (and trypsin) are know to cause some damage to cells. This was an important consideration in follicles required for development studies. Varying levels of collagenase and incubation times were assessed. A concentration of 1.5mg/ml and an incubation time of 30 minutes was finally adopted. This consistently produced large numbers of free follicles.

Several problems remained. The isolated follicles had a tendency to adhere to the glassware, leading to large losses. Siliconizing the glassware and the addition of DNase to the enzyme solution reduced this problem. It also proved difficult to obtain samples containing relatively even numbers of follicles. The follicles are relatively large and quickly settle, simply shaking the

vessel is not sufficient as the largest follicles settle almost immediately. Some form of constant agitation was required. Shaking devices were tested to overcome this problem. These however used rotational movements which caused the follicles to concentrate towards the centre of the test-tube. This meant that the first few samples withdrawn contained higher numbers (and larger follicles) than later samples. After several fruitless attempts to produce an even suspension of follicles it was decided to take advantage of the effects of settling and rotation. The final suspension of follicle was transferred to a watchglass gently rotated to produce a concentrated area of follicles in the centre of the glass. Once settled these follicles can be viewed under the dissecting microscope and approximately even numbers aspirated using a Gilson pipette. These apparently minor technical problems actually took some time to solve.

The final requirement was for a culture system which allowed the follicular elements to retain their normal 3-dimensional shape and attachments. When cultured directly on a plastic substrate follicles soon attach and are disrupted as the granulosa cells begin to migrate over the surface. It had originally been planned to use a roller culture system similar to those used for embryo cultures, to overcome the problem of attachment. However an investigation of the literature suggested another approach. A paper by Chambard et al (1981) drew attention to the use of collagen gel. The authors cultured thyroid epithelial cells on, or embedded in collagen gel. When embedded in the gel the thyroid cells formed follicle-like structures with the correct epithelial cell polarity ie with the apical pole of the cells being orientated to the interior of the follicular lumen. Preformed monolayers of these cells could be converted to follicles by a collagen gel overlay. Collagen gels were investigated further. Gel solution was produced from rat tail tendons. There were problems in embedding the follicles in the gel and the gel solution had to be handled with care as it could rapidly polymerise once the pH and ionic composition was adjusted but eventually the technique reported in the next section was devised. A novel approach to the isolation and culturing of mouse ovarian follicle had been achieved.

MATERIALS AND METHODS

ANIMALS

8-11 day old mice were used. The mice were F₁ females of matings between inbred strains of CBA males and C57B1/6 females. Breeding pairs were obtained from Bantam & King. F₁ hybrids were chosen for hybrid vigour and to maximise genetic uniformity.

MEDIA

All preparatory steps were carried out in HEPES-buffered media M199 (Gibco) and follicles were cultured in bicarbonate-buffered M199. Both media were supplemented with gentamicin (Sigma), amphotericin B (Sigma), glutamine (Flow Laboratories) and sodium pyruvate (BDH). The culture media also contained 10% donor calf serum (Flow). The serum was heat-inactivated at 55°C for 35 minutes. Preparation media had a osmolality of 300 mOsm Kg⁻¹ as measured by a Wescor vapour pressure osmometer. pH was 7.4. The culture media had an osmolality of 290 mOsm Kg⁻¹, pH was maintained in the physiological range by gassing with 5% CO₂.

COLLAGEN GEL SOLUTION

Collagen gel was extracted from rats tail tendons after the methods of Ehrmann & Gey (1956) and Chambard et al (1981). The tails were sterilised overnight in 70% alcohol. Beginning at the tip, the tail was gripped between two pairs of artery forceps and fractured into small sections. Each section with its attached strands of tendon was pulled free before making the next fracture. The tendons were collected into 70% alcohol, then rinsed in sterile distilled water. 1g of tendon was added to 100ml of 1:1000 acetic acid and stirred at 4°C for 48 hours. The solution was centrifuged at 4-5000 revs on a MSE centrifuge for 1 hour. The solution was kept at 4°C until needed. Immediately before use, 200µl of serum and 200µl X10 Medium 199 were added, pH was adjusted by added 500mM NaOH, between 120-140 µl was required.

FOLLICLE ISOLATION

All steps were carried out using cold preparation media. 24 mice were used in a typical experiment. The animals were killed by decapitation and the ovaries

were removed and placed in a watchglass containing media. All subsequent steps were carried out in a laminar flow hood. The ovaries were cleared, transferred to a fresh watchglass and bisected. The bisected ovaries were incubated at 37°C in media containing 1.5mg/ml of collagenase (Sigma type 1) and 40 units/ml of DNase I (Sigma). After 30 minutes the ovaries were centrifuged to pellet the tissue and the collagenase solution was removed. The pellet was resuspended in 10ml of media and centrifugation repeated. This step was repeated to ensure that all the enzyme solution had been removed. The follicles were then resuspended in 3ml of media and transferred to a watchglass.

Under a dissecting microscope follicles were isolated by repeated pipetting of the bisected ovaries using Gilson pipettes. As the fragments decreased in size pipette tips of progressively smaller diameter were used. To minimise mechanical trauma from repeated pipetting freed follicles were harvested frequently. The follicles were collected using a 200µl pipette tip and filtered through a 125µm nylon mesh (Nybolt ASTM 120-125) to remove any lumps. The filter was flushed to remove any trapped follicles. When pipetting no longer freed follicles the remaining fragments were discarded. The follicles were pelleted by gentle centrifugation, the supernatant removed and the follicle pellet resuspended in 3ml of media.

The follicles were transferred to a watchglass and gently pipetted to break up any clumps. Gentle rotation concentrated the follicles in the centre of the watchglass. 20µl samples were pipetted from the central concentration into the wells of a Terasaki plate (Flow Laboratories). The rotation was repeated until 16 samples had been obtained. A tissue was used to remove excess fluid from the wells and 10µl of the collagen gels solution was added and pipetted once to mix with follicles. The follicles were incubated at 37°C until the gel set (2-3min). 20µl of gel solution was pipetted into another set of wells and the 10µl gels transferred into these wells. The double gels were allowed to set. This double gelling is necessary because follicles can be lost as the collagen gel contracts during culture and processing for histology. The set gels were transferred to the wells of a tissue culture plate.

CULTURE CONDITIONS

0.5ml of culture medium per gel was used and 4-5 gels were cultured in each well. The gels were incubated at 37°C in 5% CO₂. The media was changed at 24hr then every third day.

MICROSCOPY AND HISTOLOGY

Follicles were monitored during culture using inverted phase microscopy. At the termination of culture the gels were fixed overnight in aqueous Bouins fluid, embedded in paraffin wax and sectioned at 5µm. Sections were stained with haematoxylin and eosin and mounted with DPX.

CLASSIFICATION OF FOLLICLES

Follicles were observed under x400 magnification and classified according to the system of Mandl and Zuckerman (1951):

- Stage I Oocyte surrounded by a single layer of squamous granulosa cells.
- Stage II Growing oocyte surrounded by a single layer of cuboidal granulosa cells
- Stage III Growing oocyte surrounded by two layers of cuboidal granulosa cells
- Stage IV Growing oocyte surrounded by three layers of cuboidal granulosa cells
- Stage V Oocyte surrounded by four or more layers of cuboidal granulosa cells but no antrum.
- Stage VIa Antrum forming.
- Stage VIb Oocyte surrounded by cumulus mass and a fully formed antrum.

Figure 1 illustrates this classification system.

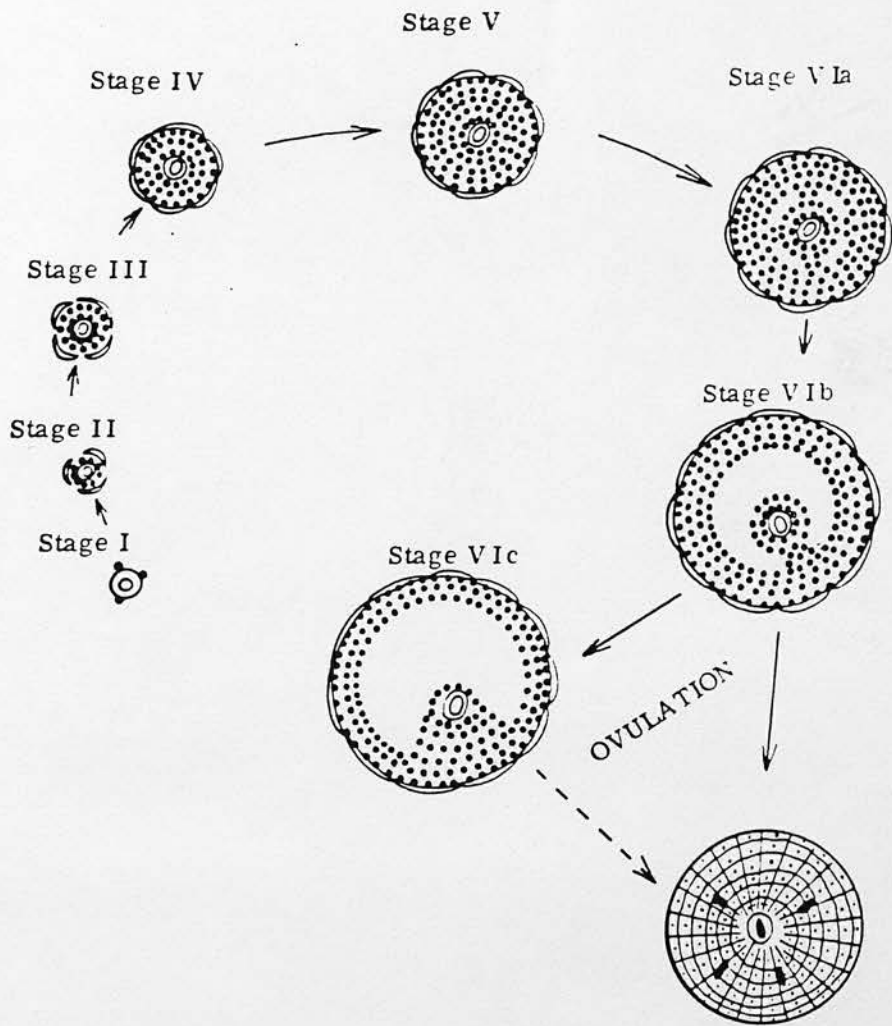


Figure 1 Follicle classification (Mandl & Zuckerman 1951).

EXPERIMENTS TO ESTABLISH THE PATTERN OF FOLLICLE GROWTH IN COLLAGEN GEL CULTURES

Follicles were isolated as described above and the 16 gels obtained were cultured with four gels per well. For each experiment/dissagregation 4 gels from one well were allowed to equilibrate in the incubator for one hour and then harvested for histology. These '0' hour gels were used to obtain information on the numbers and distribution of follicles in the gels at the beginning of the culture period. At set points over the culture period the 4 gels in a well were harvested. This allowed the pattern of growth over a 14 day culture period to be investigated. The results from nine disaggregations are reported, all the donor calf serum used was from the same batch (Flow Laboratories). Differential follicle counts using the above classification system were carried out on all gels. In addition follicles were classed as disrupted (this included free oocytes and any follicles with a disrupted granulosa layer) and intact (granulosa layer(s) intact). The quality of the intact follicles was also assessed. Follicles with more than slight retraction of the oocyte from the granulosa layers, with nuclear contraction, with obvious separation of the granulosa layers, or with more pyknotic cells than cell layers were classed as intact but damaged.

Every section was counted in the 0 hour gels and gels from 6 or more days in culture. Every third section was counted for gels from 1 to 5 days in culture. Follicles were counted if the nucleolus was present. Nucleolar diameters were measured for Stage I, II and III follicles in 0 hour gels. The nucleolar diameters of 22 follicles at each stage were measured using a image shearing micromasurement system (Vickers Instruments, York). Measurements were made under x100 oil immersion magnification. The measurements obtained were used to produce a correction factor to compensate for overcounting. Correction factors were obtained using the equation (Abercrombie 1946):

$$\frac{\text{Section thickness}}{\text{Section thickness} + \text{nucleolar diameter}}$$

Total numbers of follicles were calculated by multiplying the number counted by 3 (the sampling frequency) and the correction factor. As very few Stage V follicles were identified in the 0 hour gels the Stage VI correction factor was used. Stage 1 (primordial) follicles while present in the gels were not counted. Free oocytes and disrupted follicles were treated as Stage III follicles.

AUTORADIOGRAPHY EXPERIMENTS

Tritiated methionine (Amersham International, specific activity 85Ci/mmol) was used as a marker for protein metabolism. Incubation with the isotope was carried out in the 3 hours before the culture was terminated. The culture medium was removed from the wells and the gels washed twice with methionine-free medium 199 (Gibco). 0.25ml per gel of $\frac{1}{4}$ - strength methionine media containing ^3H -Methionine (5 $\mu\text{Ci/ml}$) was added. The follicles were returned to the incubator and cultured for three hours. At the end of the culture period the radioactive medium was removed and the gels washed twice with normal culture medium. The gels were then fixed in Bouins fluid and treated as above. Sections were brought down to water through an alcohol gradient and coated with Kodak K2 Nuclear Emulsion. After exposure at 4°C in light-tight boxes the slides were developed in Kodak D19 developer and fixed with Iford Hypam Fixer. Initially sections were stained with methyl green-pyronin and assessed using an image analysis system ($\mu\text{Magiscan}$, Joyce-Loebl). Later sections were mounted unstained, follicles located and classified under phase contrast microscopy, then grains counted using an oil immersion lens..

MONOLAYER CULTURES

Follicles were isolated as above but the final follicle pellet was resuspended in culture medium. Follicles were pipetted directly into the wells of a culture plate. The culture medium and conditions used were the same as for the gel cultures.

TRANSPLANTATION EXPERIMENTS

Follicles were isolated as before and placed into gel. After the gel had been cultured for one hour to ensure complete setting it was transferred under the kidney capsule of host animals (adult F₁ hybrids). The animals had been ovariectomised at least three weeks before use. All operations were carried out

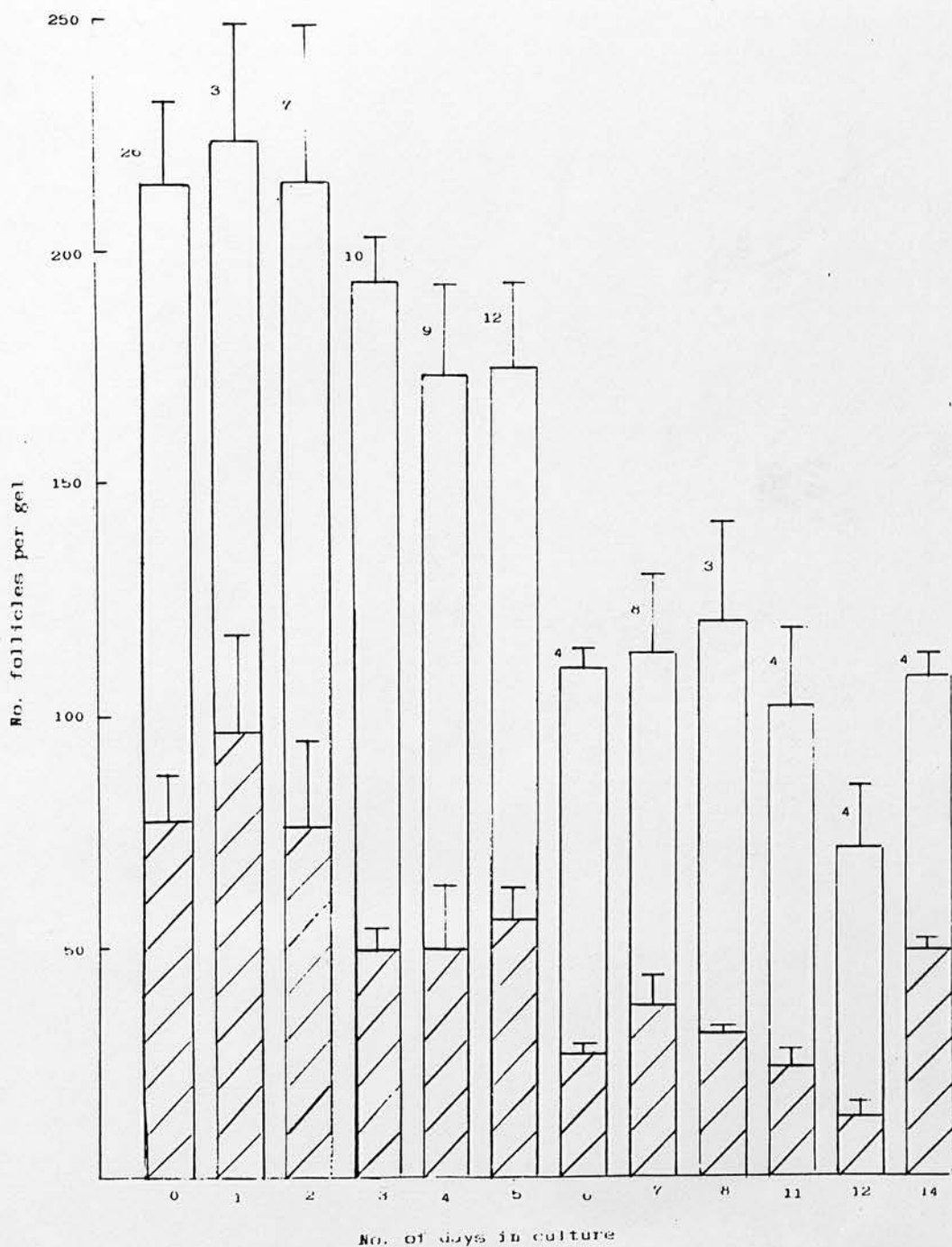


Figure 2 Number of follicles per gel (including free oocytes) over the culture period. The hatched area represents the number of disrupted follicles per gel. Values given are mean + s.e.m. Sample size (n) is shown to the left of the bars.

under intraperitoneal Avertin anaesthesia (6.03g/Kg body wt). Vaginal smears were taken before and after the follicular transplants had been inserted.

ORGAN CULTURES

Ovaries were obtained from F₁ hybrids of several ages. The cleared ovaries were bisected and set in collagen gel. They were then cultured as above for 4-8 days.

RESULTS

EXPERIMENTS TO ESTABLISH THE PATTERN OF FOLLICLE GROWTH IN COLLAGEN GEL CULTURES

Observation of the gels by phase contrast microscopy indicated that the follicles retained their shape with the oocyte maintaining a central position. Numerous processes were observed growing outwards from the follicles. As the cultures progressed the collagen gels tended to shrink making viewing more difficult. Figure 2 summarises the mean values of the total number of follicles in a gel over the course of the culture. The mean number at 0 hours was 214 (s.e.m. = 18.5). The range was from 63 to 445. About 36% of the follicles were obviously disrupted and of those classed as intact 20% had some histological evidence of damage. An average of around 200 follicles per gel had been aimed for. Figure 3 show the distribution of follicles of each stage over the course of the 14 days in culture. The most marked change is the increase in numbers of stage IV and stage V follicles. Up to and including 6 days in culture about 90% of the follicles are stage II and III. At seven days this drops to 70% and by 11 and 12 days to 50%. On the final day in culture only 32% of the follicles were stage II and III, the remaining 68% were stages IV and V. Stage V follicles were very rare in the 0 hour gels but represented 27% of the intact follicles by day 12. Figures 4a and b are sections of gels cultured for 6 days, figure 5 is a section of intact ovary from a 10 day old F₁ hybrid. It can be seen that many follicles have maintained an appearance identical with those in the intact ovary.

AUTORADIOGRAPHY EXPERIMENTS

The initial experiments described in the methods section produced encouraging results. Granulosa cells were heavily, and oocytes more lightly, labelled.

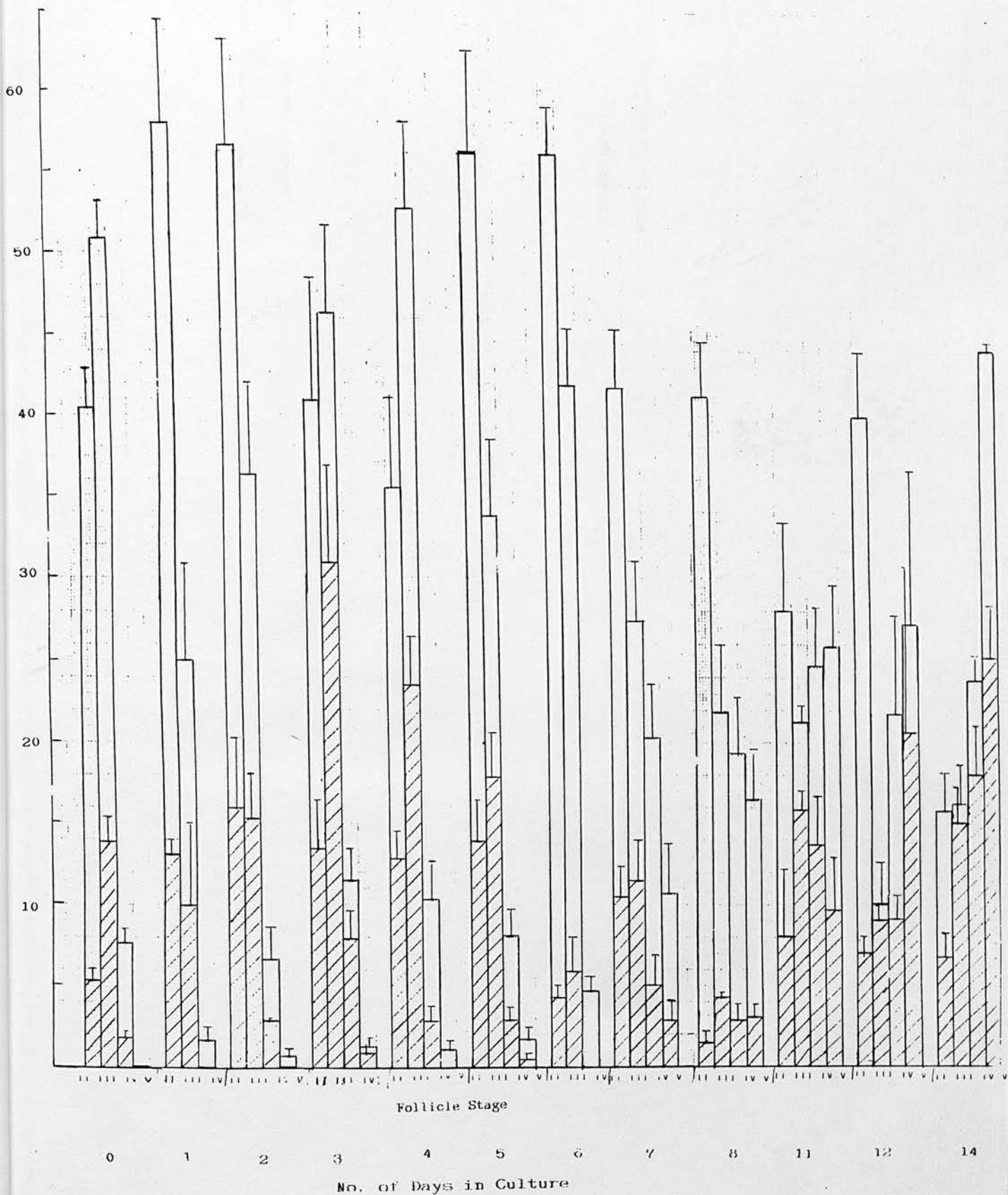


Figure 3 Distribution of follicle stages as a % of the total number of intact follicles per gel. Hatched area represents the % of intact follicles with histological evidence of damage. Values given are mean plus s.e.m.

However when viewed using μ Magiscan image analysis system the MGP stain used was found to interfere with reproducible grain counting. Unstained sections were then tried and these appeared to be overexposed - grains were large and tended to coalesce. Experiments are under way to establish the required exposure time and the optimum level of tritiated methionine to give more discrete grains which will permit quantitative results to be obtained.

MONOLAYER CULTURES

Follicles cultured directly on a plastic substrate quickly became adherent and began to spread. Within 2-3 days most of the follicles had been disrupted as the granulosa cells spread out over the surface as shown by figure 6. Oocytes were shed into the culture media and were removed when the cultures were fed.

TRANSPLANTATION EXPERIMENTS

These experiments were undertaken to provide an *in vivo* assessment of follicular function. The gels are robust and can be implanted quite easily. Initial attempts were made before the follicle isolation procedure had been perfected. These attempts were encouraging - the transplanted gels stayed in place and the ovarian graft survived. A later experiment using follicles from older mice showed that the follicles could thrive in these conditions - 12 days after the operation antral follicles were seen in the grafts, the grafts were also infiltrated by blood vessels. These results indicate that the isolation procedure does not irreversibly damage the follicles. It is also hoped to transplant gels after a period in culture to assess their function in more physiological conditions.

ORGAN CULTURES

The method of embedding ovarian fragments in collagen gel appears to work well. Follicles as assessed by inverted phase microscopy and limited histological sections continue to grow. The collagen gel provides an alternative to culturing on microfilters or steel mesh grids as has been done in the past. Only pilot experiments have been carried out but the technique does look promising.

Figure 4a&b Follicles after 6 days in gel culture (x160).

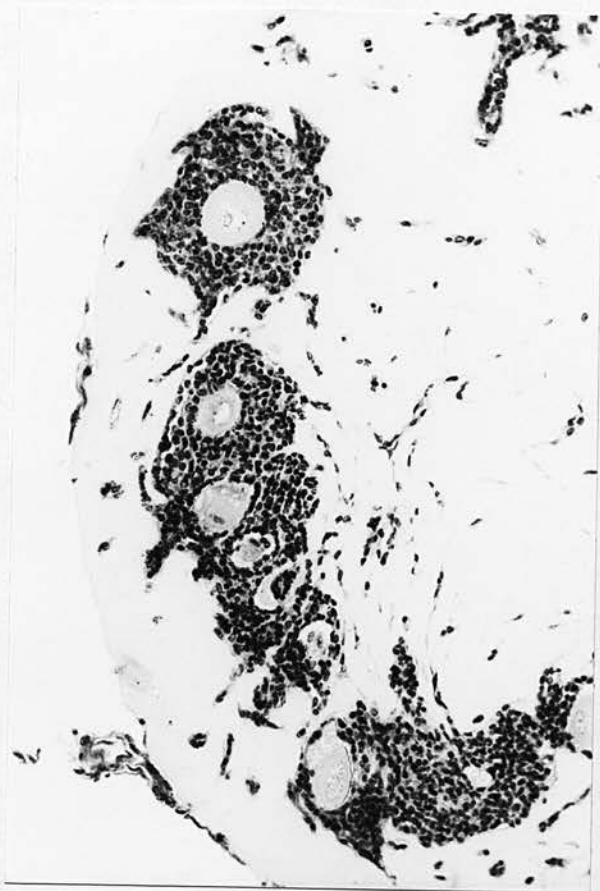


Figure 5 Section of the ovary of a 10-day old F₁ hybrid mouse (x160).

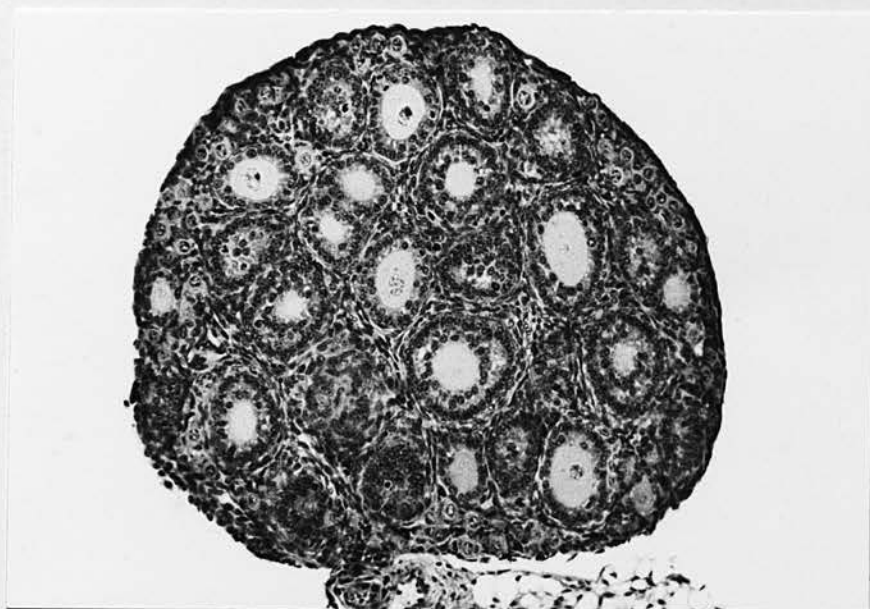
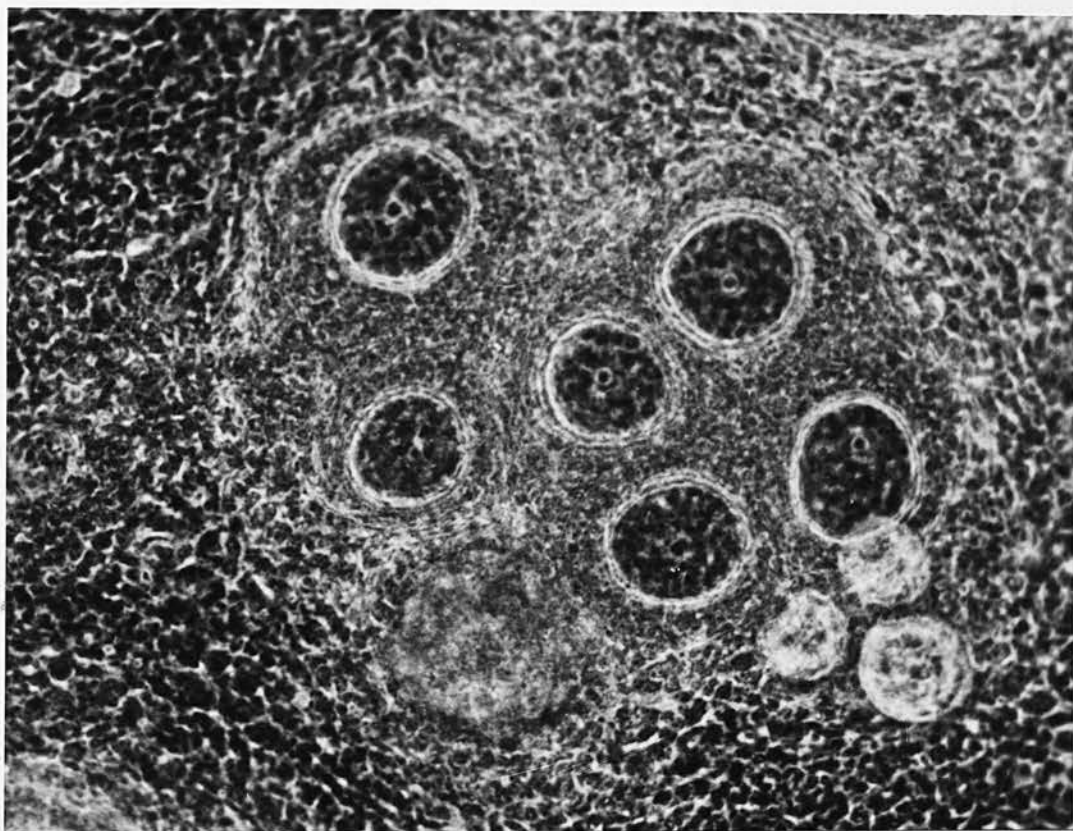


Figure 6 Monolayer culture of isolated follicles after 48 hrs.



DISCUSSION

The main achievement of the work reported has been the development of a new technique which permits mouse ovarian follicles to be isolated and grown *in vitro* for at least 2 weeks. The available results are incomplete and further experiments are in progress. Large numbers of isolated follicles can be produced. If 16 gels are formed from 48 ovaries a mean of about 200 hundred follicles per gel is achieved. However the range is wider than desired. The gels are usually cultured in groups of four, so there are approximately 800 follicle per culture well. The collagen gel provides a matrix for more normal growth but does not inhibit the diffusion. Roy and Greenwald (1985) have developed a similar method using collagenase and pronase to isolate the follicles. They have only used freshly isolated follicles for receptor analysis (Roy et al 1987). The ability to transfer cultured follicles under the kidney capsule for *in vivo* assessment is another advantage.

Ten-day old mice were chosen because they contain low numbers of stage V follicles. The multilaminar follicles seen after 6 days in culture must be growing from small or even primordial follicles. While intact small follicles often appeared identical to their *in vivo* counterparts the multilaminar follicles were often less healthy looking and pyknotic cells were uncommon. These follicles had reached a stage where they might require additional support to continue growing. Media supplemented with donor calf serum was chosen to represent basal conditions as it contains little gonadotrophin or sex steroids but can be expected to contain non-specific growth factors. This media may not be appropriate to support growth in later stages. One major long-term aim is to produce a fully defined media. Experiments comparing donor and fetal calf serum are in progress. Experiments using media supplemented with gonadotrophins and/or oestrogen are planned. Other hormones or growth factors could easily be tested in this system. Histology will allow gross morphological changes such as an increase in granulosa cell layers to be identified and it is hoped that autoradiography will permit more subtle changes in protein metabolism to be investigated. Granulosa or oocyte specific effects should be discernible.

Another approach being considered is using autoradiography to investigate the effects of hormones on growth by looking at the levels of ODC (ornithine decarboxylase) in the cultures. ODC is the rate-limiting enzyme in the metabolism of the polyamines. Its levels rise rapidly in growing tissue and it provides a sensitive, early indicator of cell growth and differentiation (Snyder & Russel 1970). Recently a tritiated DMFO (DL-difluoromethylornithine), an irreversible inhibitor of ODC, has become available. This could be used to localise and quantify ODC in the cultures. This approach has been utilised by Lowkvist et al (1987) to study ODC localization in chick embryo during organogenesis and by Holinka and Gurpide (1985) in human endometrial cells.

Biochemical studies of the spent media could provide information on the production of hormones or other substances by the follicle. Measurements of lactate production are planned. However interpretation of these results will be complicated by the limitations of the procedure as it stands. The follicle suspension contains a variety of cells - follicle units, damaged follicles and isolated granulosa cells, stromal cells, ovarian surface epithelium, and other cell types. A method of producing a purified follicle suspension is being sought. It would be useful to separate follicles into stages and look for stage-specific effects or secretions. This can be done using calibrated pipettes (Roy & Greenwald 1985) but a method which would allow large numbers to be conveniently handled is required.

The culture system can be used to investigate a number of other problems. Density-dependent effects can be studied by altering the numbers of follicles in a gel or the number of gels cultured together. The gel approach also allows elegant recombination experiments to investigate mesenchymal-epithelial interactions which may be specific to thecal and granulosa cells. Co-cultures of follicles in gel and preformed stromal monolayers would allow the role of diffusible messengers between the cell types to be assessed. Culturing follicles in spent media from stromal cultures would provide similar information. Stroma from the ovary and other tissue could be used. Alternatively follicles could be combined with the chosen stroma before the gelling stage to investigate the role of stroma-granulosa contact in follicular development. If pure follicle suspensions can be achieved this would be a very fruitful area of study.

Collagen is a major component of the extracellular matrix *in vivo* and has been used extensively as a substrate in tissue culture. It can markedly affect growth and differentiation (reviewed by Yang & Nandi 1983). Substrate can influence cell shape, orientation and interactions with neighbouring cells, responsiveness to hormones or other mitogens and secretion of cell products. Mammary epithelium for instance proliferates better on collagen than glass or plastic substrates and secretes more milk proteins (Yang & Nandi 1983). Thyroid epithelium (Chambard et al 1981) and mammary epithelium (eg Shannon & Pitelka 1981) achieve a more tissue-like configuration when provided with a collagen substrate. Other extracellular matrix components can be added to the collagen gel (eg glycoaminoglycans) to make it more physiological.

The system developed has potential for investigating many aspects of the development of small ovarian follicles. Improvement and development of the system is required but preliminary results are encouraging. It would be particularly exciting if the system could be utilised to investigate two of the major transition points in follicle development: the recruitment of quiescent primordial follicles into the growing pool and antrum formation.

REFERENCES

- ABERCROMBIE M (1946) Estimation of nuclear populations from microtome sections. *Anat. Rec.* 94:239-247
- BAKER T G, SCRIMGEOUR J B (1980) Development of the gonad in normal and anencephalic human fetuses. *J. Reprod. Fert.* 60:193-199
- CHAMBARD M et al (1981) Influence of collagen gel on the orientation of epithelial cell polarity: Follicle formation from isolated thyroid cells and from preformed monolayers. *J. Cell. Biol.* 91:157-166
- CUNHA G R et al (1983) Hormone-induced morphogenesis and growth: Role of mesenchymal-epithelial interactions. *Rec. Prog. Horm. Res.* 39:559-595
- EDWARDS R G et al (1977) Normal and abnormal follicular growth in mouse, rat and human ovaries. *J. Reprod. Fert.* 51:237-263
- EHRMANN R L, GEY G O (1956) The growth of cells on a transparent gel of reconstituted rat-tail collagen. *J. Natl. Can. Inst.* 16:1375-1403
- EPPIG J (1977) Mouse oocyte development *in vitro* with various culture systems. *Develop. Biol.* 60:371-388

- ESHKOL A, LUNENFELD B (1975) Use of antisera to gonadotrophins in reproduction research. In Immunization with hormones in reproductive research ed. E Nieschlag, North-Holland Publishing Company, Amsterdam p55-81
- FADDY M et al (1976) An analytical model for ovarian follicle dynamics. J. Exp. Zool. 193:173-186
- GROB H S (1964) Enzymatic dissection of the mammalian ovary. Science 146:73-74
- GROB H S (1969) Growth and endocrine function of isolated ovarian follicles cultivated *in vivo*
- GROB H S (1971) Monolayer culture of ovarian follicular elements derived from isolated mouse follicles. Biol. Reprod. 5:207-213
- HALPIN D M G et al (1986) Effects of gonadotrophin deficiency on follicular development in hypogonadal (hpg) mice. J. Reprod. Fert. 78:119-125
- HILLIER S G (1985) Sex steroid metabolism and follicular development in the ovary. Oxford Rev. Reprod. Biol. 7:168-222
- HOLINKA C F, GURPIDE E (1985) Ornithine decarboxylase activity in human endometrium and endometrial cancer cells. In Vitro & Dev. Biol. 21:697-706
- HSEUH A J W (1986) Paracrine mechanisms involved in granulosa cell differentiation. Clinics in Endocrin. Metabol. 15:117-134
- HSEUH et al (1981) Inhibition of ovarian and testicular steroidogenesis by epidermal growth factor. Endocrinology 108:2002-2004
- JONES E C, KROHN P L (1961) The effect of hypophysectomy on age changes in the ovaries of mice. J. Endocrin. 21:497-509
- LOWKVIST B et al (1987) Localization of ornithine decarboxylase in the chick embryo during organogenesis. Cell Tissue Res. 247:75-84
- MANDL A M, ZUCKERMAN S (1951) The relation of age to numbers of oocytes. J. Endocrin. 7:190-193
- NEAL P, BAKER T G (1973) Response of mouse ovaries *in vivo* and *in organ culture* to pregnant mare serum gonadotrophin and human chorionic gonadotrophin 1 Examination of critical time intervals. J. Reprod. Fert. 33:399-404
- NEKOLA M V, NALBANDOV A V (1971) morphological changes of rat follicular cells as influenced by oocytes. Biol. Reprod. 4:154-160
- NICOSIA S V et al (1975) Rabbit ovarian follicles 1. Isolation technique and characterization at different stages of development. Biol. Reprod. 13:423-447

- PETERS H (1979) Some aspects of early follicular development in mammals. In ovarian follicular development and function. ed A R Midgley and W A Sawyer, New York, Plenum p1-15
- PETERS H et al (1979) Follicular growth in fetal and prepubertal ovaries of humans and other primates. Clinics in Endocrin. Metabol. 7:469-484
- ROY S K, GREENWALD G S (1985) An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects. Biol. Reprod. 32:203-215
- ROY S K et al (1987) Radioreceptor and autoradiographic analysis of FSH, hCG and prolactin binding sites in primary to antral hamster follicles during the periovulatory period. J. Reprod. Fert. 79:307-313
- RYLE M (1972) The growth *in vitro* of mouse ovarian follicles of different sizes in response to purified gonadotrophins. J. Reprod. Fert. 30:395-405
- SHANNON J M, PITELKA D R (1981) The influence of cell shape on the induction of functional differentiation in mouse mammary cells *in vitro*. *In vitro* 17:1016-1028
- SNYDER S H, RUSSELL D H (1970) Polyamine synthesis in rapidly growing tissues. Fed. Proc. 29:1575
- YANG J, NANDI S (1983) Growth of cultured cells using collagen as a substrate. Int. Rev. Cytol. 81:249-285