Aust. J. Biol. Sci., 1982, **35**, 441–55

Purification, Morphology, and Progesterone Production and Content of Three Cell Types Isolated from the Corpus Luteum of the Sheep

R. J. Rodgers and J. D. O'Shea

Department of Veterinary Preclinical Sciences, University of Melbourne, Parkville, Vic. 3052.

Abstract

A method is presented for the isolation and purification of three cell types, endothelial cells, small luteal cells and large luteal cells, from the ovine corpus luteum. The method involves enzymatic dispersion of luteal tissue followed by centrifugation of separated cells on a Ficoll gradient. The three purified cell types and others, particularly fibrocytes and smooth muscle cells, that were removed during purification, were identified by their morphology. The cell yield, the cellular composition and cellular progesterone content of each fraction from the Ficoll gradient were measured. The endothelial cell fractions were relatively free of contamination by other cell types and had negligible progesterone. Fractions of small luteal cells and those of large luteal cells contained endothelial cells but were relatively free of other cell types. Large luteal cells contained significantly more progesterone, produced more progesterone when incubated in culture, but were less responsive to luteinizing hormone than small luteal cells.

Introduction

It is well established that in many species of mammal, including the sheep, the corpus luteum contains two distinct types of luteal cell (Mossman and Duke 1973). Available evidence supports the idea that the larger type of luteal cell is derived from the follicular epithelium, and the small type, described in some detail for the sheep by O'Shea *et al.* (1979), is probably of thecal origin (O'Shea *et al.* 1980). Very little is known of the functions of each cell type in the ovine corpus luteum, although it is likely, from recent evidence in other species (Lemon and Loir 1977; Koos 1978; Ursely and Leymarie 1979) and the morphology of these cell types, that both are steroidogenic. Investigation of the individual functions of the different cell types and of any functional interactions between them, would be aided if these cells could be separated and isolated in relatively pure populations. Some success in separation of cell types has been reported in the sow (Lemon and Loir 1977) and cow (Koos 1978; Ursely and Leymarie 1979) but previous studies involving separation in the ewe (Jordan *et al.* 1978) have resulted in combined populations of large ('granulosa lutein') and small ('thecal lutein') cells.

This paper reports a technique for the dissociation of luteal tissue and separation of three distinct cell types, endothelial cells, small luteal cells and large luteal cells, from the ovine corpus luteum. Data are presented on the identification, estimation of yield and cell sizes, progesterone content and *in vitro* production by these cell types.

Materials and Methods

Tissues

Ovaries for enzymatic dissociation studies were obtained at an abattoir from cycling ewes within 15 min of death, and placed in ice-cold Krebs Ringer bicarbonate solution (KR) containing antibiotics (penicillin 40 u ml^{-1}, streptomycin 40 u ml^{-1} and Fungizone 1 $\mu g\ ml^{-1}$), 1·25 mM calcium chloride and 11 mM glucose. Mature functional corpora lutea were selected on the basis of size and colour, and their functional status confirmed by subsequent histological examination and assay of tissue progesterone content. Corpora lutea were included only if histological examination confirmed their mature structure, with no evidence of regressionary changes (Deane *et al.* 1966; O'Shea *et al.* 1977) and if their progesterone content per milligram of tissue was >25 ng.

Seven additional ovaries containing corpora lutea were fixed for electron microscopy immediately after their removal. The ovarian artery was cannulated close to the ovary, and 5 ml of KR was first perfused over a period of ≃5 min via a hypodermic syringe using gentle manual pressure. Then 10 ml of 3·0% glutaraldehyde was perfused (≃10 min) in the same manner at room temperature. Whole ovaries were then immersed in 3·0% glutaraldehyde for return to the laboratory.

Dispersion of Luteal Cells

Collagenase digestion

Corpora lutea were dissociated in collagenase (*Clostridium histolyticum* collagenase; EC 3.4.24.3) using a method based on that of Simmons *et al.* (1976). Initially, corpora lutea were dissected from an ovary and sliced with a Staddie Riggs hand microtome. Slices of tissue were first incubated in Dulbecco modified Eagle's medium (DMEM) (10 ml g^{-1}; Commonwealth Serum Laboratories, Melbourne, Vic.) in a shaking water bath at 32°C under a 95% O_2:5% CO_2 atmosphere. After 10 min, the medium was replaced with collagenase (400 units ml^{-1}, type 1A; Sigma Chemical Co., St Louis, Missouri, U.S.A.) in DMEM. The incubation was continued for 40 min and the medium replaced with fresh collagenase medium before a further 2 h incubation. On completion of the incubation, the tissue was dispersed further by pipetting with a Pasteur pipette, the cell suspension was filtered through cloth (square holes, 150 μm), and the eluate collected and washed four times in DMEM.

Trypsin digestion

The filtered cells from the collagenase digest were resuspended in 0·3% trypsin (1:250; Grand Island Biological Co., U.S.A.) in Ca-free 1 mM EGTA,* 25 mM HEPES-buffered KR (10 ml g^{-1} of original tissue) and incubated at 37°C for 45 min. The incubation was terminated by the addition of soybean trypsin inhibitor (final concentration 1 mg ml^{-1}; type 1-S, Sigma) and deoxyribonuclease (final concentration 50 $\mu g\ ml^{-1}$; DN-25, Sigma) in KR. The cells were then resuspended in DMEM containing trypsin inhibitor and deoxyribonuclease before further dispersal of cells by syringing the cell suspension through fine-gauge needles (23–27 gauge).

In developing the above procedures, several methods for tissue dissociation were studied in pilot experiments. These included variations on collagenase digestion; use of Ca-free and Ca- and Mg-free media; EGTA and EDTA treatments; various trypsin concentrations and incubation times; and hyaluronoglucosaminidase (Hyaluronidase, type II; Sigma), protease (Dispase; Boehringer Mannheim, West Germany) and lignocaine treatments.

Separation of Cell Types

The cell suspension was halved and each half layered onto a stepped Ficoll 400 (Pharmacia Fine Chemicals, Sweden) gradient. The gradient was made of 5-ml layers of 3·33, 5·00, 6·67, 7·50, 8·34, 10·00 and 16·77% (w/w) Ficoll in a round-bottomed siliconized glass centrifuge tube (i.d. 24 mm). Cells were centrifuged through the gradient (1200 rpm, g_{av} = 150) for 2·5 min and fractions, from the centre of one layer to the centre of the next, were collected. The cells in each fraction were washed twice and resuspended in DMEM.

* Abbreviations used: EGTA, ethyleneglycol-bis-(β-aminoethylether) *N*,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Cell Counting

An aliquot (75 μl) of the trypsin-digested cells and of each Ficoll fraction for each corpus luteum was counted in a haemocytometer after dilution with trypan blue (Phillips 1973). Viability was assessed by trypan blue exclusion and all viable nucleated cells were classified according to mean diameter into subgroups using the marked divisions of an eye-piece micrometer (1 division = 3·88 μm).

Morphology

For light microscopy of tissues, material was fixed in Bouin's fluid and embedded in paraffin; sections were stained with haematoxylin and eosin. To examine free cells by light microscopy, cells were centrifuged onto glass slides in a cytocentrifuge (Cytospin, Shadon Southern Products Ltd, Cheshire, England), and stained with a modified Wright's stain (Diff-Quik, Harleko, New Jersey, U.S.A.). For electron microscopy, dispersed cells, material retained by the filter cloth, and small blocks of perfused–fixed tissue were fixed in 3% glutaraldehyde in 0·1 M sodium cacodylate buffer, pH 7·3 (2 h for dispersed cells and filter-retained material, and 1 h for blocks cut from perfused–fixed corpora lutea). Fixed dispersed cells were centrifuged at 1500 *g* to form a pellet before further processing. Fixed material was rinsed in cacodylate buffer and post-fixed in 1% osmium tetroxide in distilled water for 2 h at room temperature. Blocks were stained for 30 min in 0·5% aqueous uranyl acetate, dehydrated in alcohol and embedded in araldite (Durcupan; Fluka, Switzerland). Sections were stained with saturated uranyl acetate in methanol, and in lead citrate, and examined in a Philips EM300 electron microscope. Thick sections (1 μm) for light microscopy were stained in 0·5% (w/v) Azure II in 1% (w/v) borax solution.

Progesterone Assay

Progesterone was measured by radioimmunoassay (Hossain *et al.* 1979). For tissue progesterone estimates, a weighed piece of tissue ($\simeq$10 mg) was homogenized in 5 ml of ethanol (Art 983; Merck, West Germany) for 5 min with a Teflon homogenizer. Tissue fragments were removed by centrifugation and the supernatant taken for assay. Cellular progesterone was extracted with 2 ml of hexane from a diluted aliquot of incubation medium in which the cells had been previously sonicated. Assay blanks for hexane and ethanol were below the sensitivity of the assay (5 pg per tube). The within-assay coefficient of variation was 9·0% at 0·95 ng ml^{-1} and the between-assay coefficient of variation was 13·4% at 0·85 ng ml^{-1} (15 assays). Mean recovery of progesterone was 89·6%.

Incubation of Cells

Aliquots (1·0 ml) of small and large luteal cells in DMEM supplemented with 10% foetal calf serum were incubated in a 24-well plate (Flow Laboratories Inc., Virginia, U.S.A.) for 12 h at 37°C in a humidified atmosphere of 5% CO_2 in air, with and without ovine luteinizing hormone (100 ng ml^{-1}, oLH, batch #21; National Institute of Arthritis, Metabolism and Digestive Diseases; biopotency 2·5 times NIH-LH-SI) medium. Each determination was carried out in triplicate and progesterone levels were determined after the cells had been sonicated in the incubation medium.

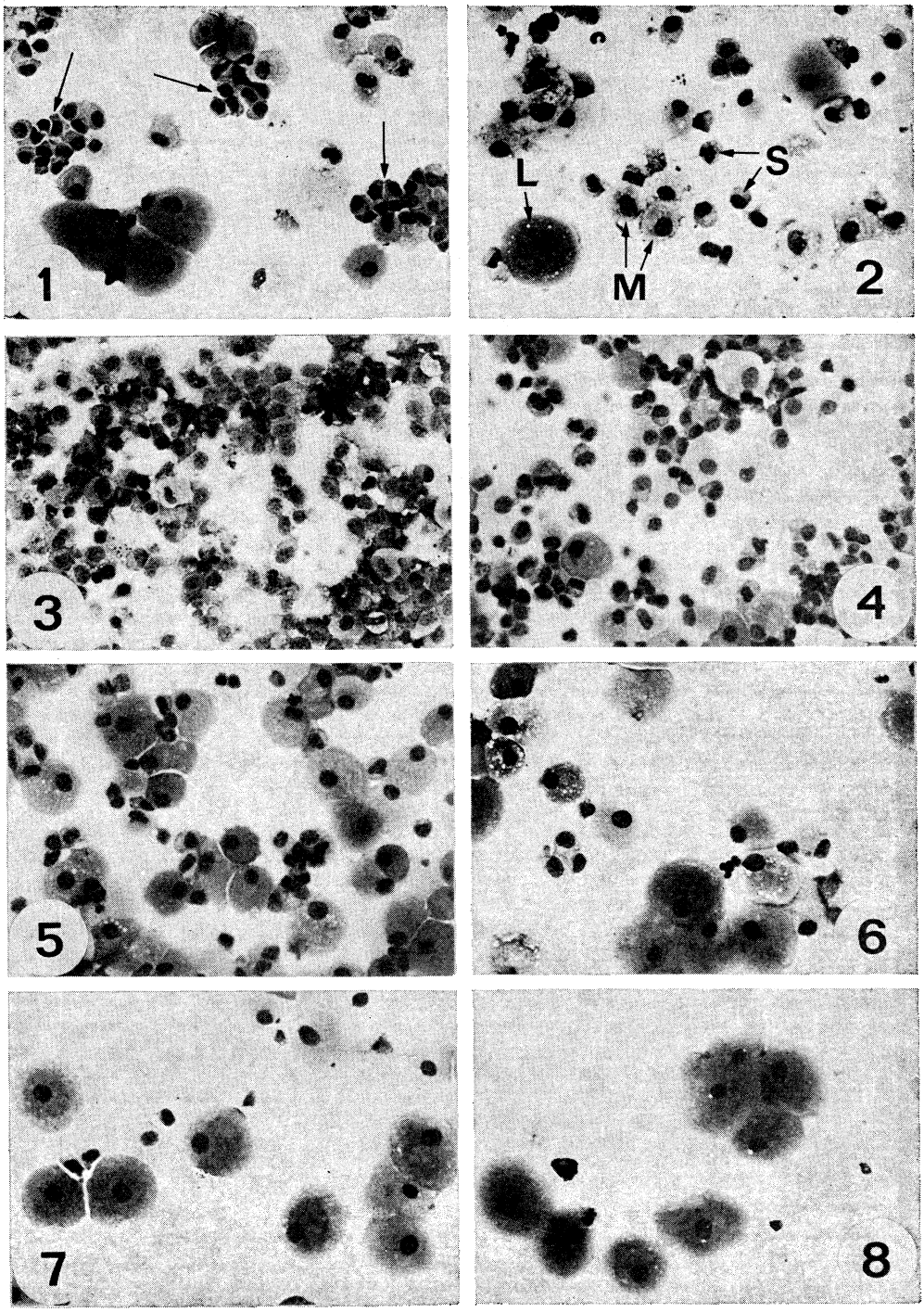
Statistical Analysis

Analysis of cellular progesterone content was carried out using Student's *t*-test. Computation of the standard error of the mean for all percentages was carried out using the arcsine transformation.

Results

Categorization and Identification of Cell Types

Three methods were used for the categorization and identification of isolated cells from dissociated luteal tissue and from Ficoll fractions. Since data from these methods form the basis for terminology and methods of cell classification used throughout this paper, a brief consideration of cell identification with each method, and of correlation between the findings of the three methods, is presented first.


1
L
S
M
2
3
4
5
6
7
8

Examination of living cells in suspension

Cells placed in a haemocytometer chamber in trypan blue were categorized according to mean diameter into subgroups using the marked divisions of an eyepiece micrometer, and subsequently pooled into three broad size classes: small (<3·5 micrometer units), medium (3·5–5 units) and large (>5 units). Although a continuum of cell sizes existed between the small and medium cell classes, a distinct 'break' usually appeared to exist between the sizes of cells in the medium and in the large cell classes. Due to the method of size measurement used, in which cells over 4 units in diameter were measured only to the nearest unit, this 'break' was not able to be quantitated accurately, but approached 1 unit, i.e. at 3–4 μm.

Examination of stained cells after cytocentrifugation

The three categories of cell recognized by size in haemocytometer preparations were again clearly identifiable (see Figs 1 and 2). Small cells showed a high nuclear to cytoplasmic ratio and possessed a generally homogeneous basophilic cytoplasm. Medium cells had a lower nuclear to cytoplasmic ratio and basophilic cytoplasm that was often somewhat vesiculated. The large cells had the lowest nuclear to cytoplasmic ratio. Their cytoplasm usually showed two distinct zones: immediately surrounding the nucleus was a zone with both basophilic and acidophilic staining properties, and a thin band around the periphery of the cell was faintly basophilic.

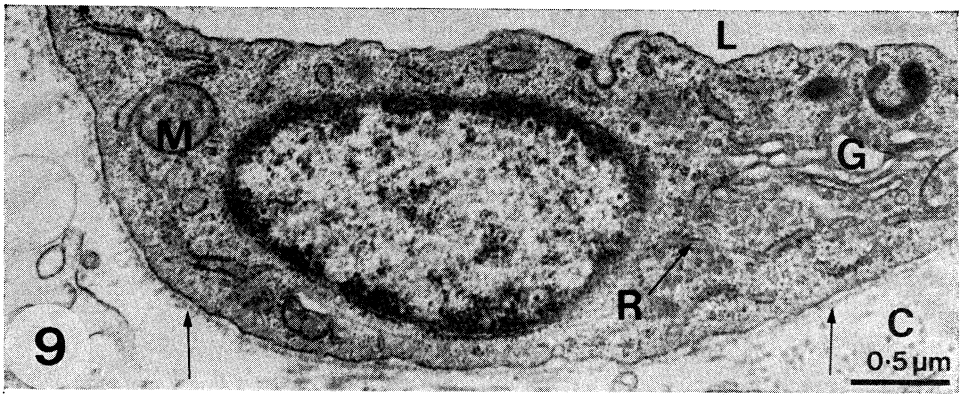
Examination of plastic-embedded cells by light and electron microscopy

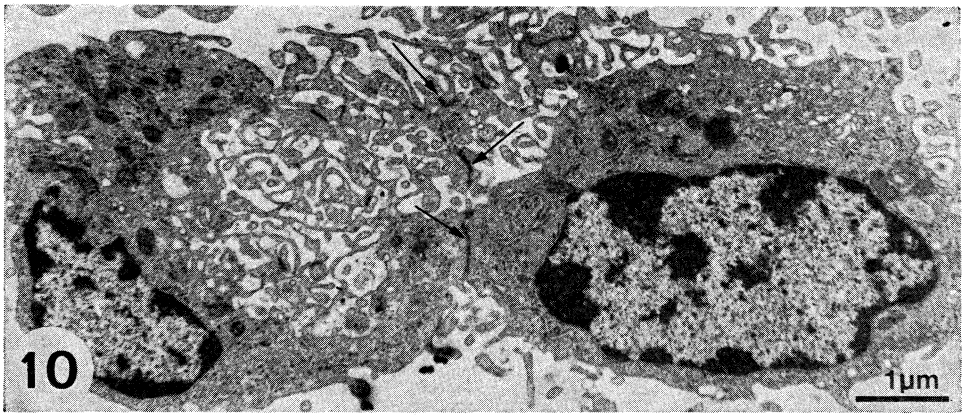
The three broad categories of cells described above were again recognizable, and identification of these cells was possible by comparison with sections of whole luteal tissue, on the basis of criteria described in detail elsewhere (O'Shea *et al.* 1979). However, these cell pellets were not suitable for estimating the number of different cell types because cell distribution within the pellet was uneven, presumably as a result of the effects of centrifugation in the preparation of pellets.

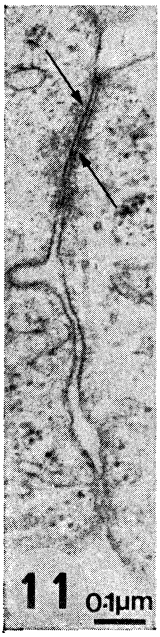
By electron microscopy it was clear that at least the great majority of the small cells were endothelial cells and pericytes, which were not individually distinguishable after dissociation. Identification of these free cells as endothelial was based primarily on their close similarity to unequivocal endothelial cells still attached to one another by adherens-type junctions in typical capillary formations in collagenase preparations (Fig. 9). Such small cells, referred to hereafter as endothelial cells, possessed large eccentrically placed nuclei, often irregular in outline, surrounded by a rim of cytoplasm in which organelles were sparsely distributed (Fig. 12). Mitochondria were small,

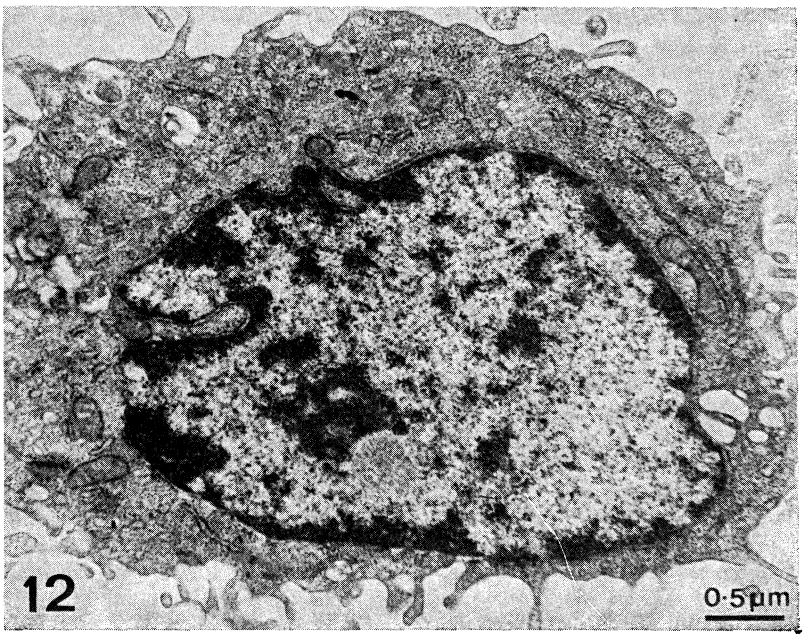
---

Figs 1–8. Cytocentrifuge preparations of collagenase-digested (*1*) and trypsin-digested (*2*) luteal tissue, and Ficoll fractions A–F (*3–8*, respectively). Endothelial cell clumps (arrows) are evident in Fig. 1, but these have broken up following trypsin digestion (*2*). Cells of large (*L*), medium (*M*) and small (*S*) size are seen in Figs 1 and 2, and the separation achieved on the Ficoll gradient is shown in Figs 3–8. Small cells are most concentrated in fraction A (*3*), medium-sized cells in fraction C (*5*), and large cells in fractions E and F (*7* and *8*). In this particular preparation, the cells have not moved down the gradient quite as far as they do on average, and there are significant numbers of medium-sized cells in fraction B (*4*) and of large cells in fraction D (*6*). Diff-Quik stain. Each figure is magnified 180 times.


L
M
G
R
C
9
0·5 µm


10
1µm


11
0·1µm


12
0·5µm

generally oval in outline, and possessed lamellar cristae. Their endoplasmic reticulum was predominantly granular, and free ribosomes were numerous.

Medium cells (Fig. 13) were structurally similar to the small luteal cells of intact luteal tissue and are hereafter referred to as small luteal cells. Their cytoplasm contained moderate numbers of mitochondria, in which cristae of both lamellar and tubular forms were present, and large quantities of tubular endoplasmic reticulum to which some ribosomes were attached (Fig. 14). Free ribosomes and lipid droplets were also present in variable numbers. Occasional nuclei of these cells contained inclusions filled with cytoplasmic material (Fig. 15). Cells of fibroblastic morphology, containing large amounts of rough endoplasmic reticulum in their cytoplasm, were seen infrequently among this population of cells.

Large cells (Fig. 16) were structurally similar to the large luteal cells of intact luteal tissue and are hereafter referred to as large luteal cells. They differed from small luteal cells in containing far larger numbers of mitochondria, whose cristae were predominantly tubular, in the presence of focal aggregates of parallel, flattened cisternae of rough endoplasmic reticulum, and in possessing fewer attached ribosomes associated with their abundant tubular endoplasmic reticulum. No cytoplasmic inclusions were seen within their nuclei, and their cell surfaces frequently showed many interdigitating cytoplasmic processes (Fig. 16, inset). Although membrane-bound granules of $\simeq 0{\cdot}2\ \mu m$ diameter were numerous in the large luteal cells in intact luteal tissue, granules in this size range were infrequently seen in dispersed cells.

Dispersion of Luteal Cells

Collagenase digestion

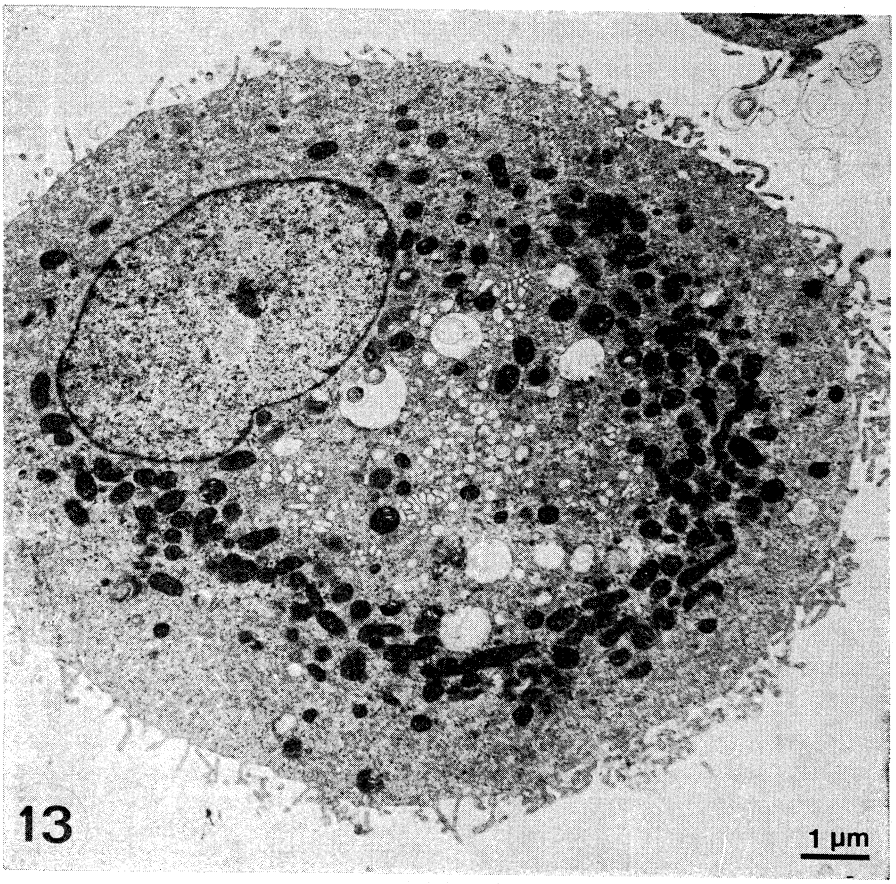
An example of collagenase-digested tissue after filtration is shown in Fig. 1. As seen in cytocentrifuge preparations, dispersion of the cells was incomplete even after prolonged collagenase treatment or collagenase renewal in the incubation medium. Many endothelial cells remained attached to each other in clumps (Fig. 1) and with electron microscopy (Fig. 10) it could be seen that although the basal lamina, which is conspicuous in capillaries of whole luteal tissue (Fig. 9), had been removed by the enzyme, the endothelial cells still remained attached to one another by many adherens-type junctions (Figs 10 and 11). As a result of clumps of endothelial cells persisting after collagenase treatment, it was not possible to obtain pure populations of cells using methods of separation depending on differential cell size.

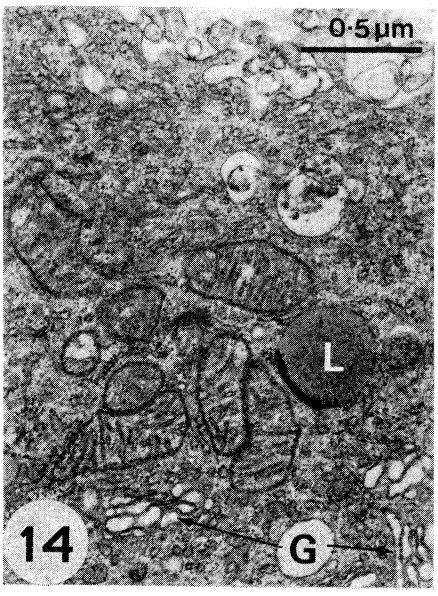
Fig. 9. Endothelial cell from a capillary in undissociated luteal tissue. Note the sparse cytoplasmic organelles, including mitochondria (*M*), Golgi complex (*G*) and rough endoplasmic reticulum (*R*). The basal lamina (arrows) and some scattered collagen microfibrils (*C*) are seen on the outer surface, and part of the lumen (*L*) is present at the top of the figure.

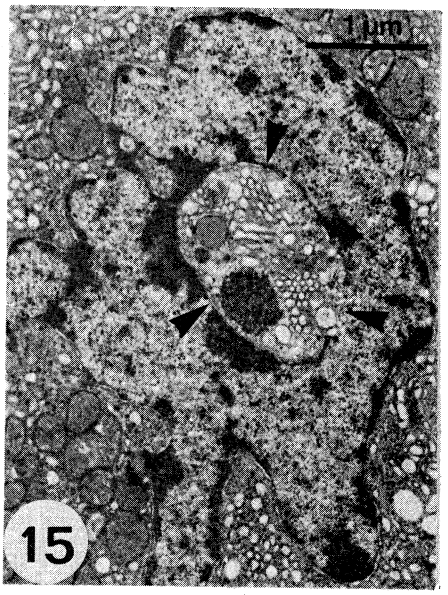
Fig. 10. Two endothelial cells from collagenase-dissociated luteal tissue, still attached to one another by many adherens-type junctions (arrows). No basal lamina nor pericapillary collagen is present.

Fig. 11. Detail of an interendothelial cell junction from collagenase-dissociated luteal tissue. A characteristic intact adherens-type junction (arrows) is present.

Fig. 12. Isolated endothelial cell from trypsin-digested luteal tissue. Note the similarity in nuclear and cytoplasmic structure to the cells in Figs 9 and 10.


13
1 µm


0·5 µm
L
14
G


1 µm
15

Electron microscopic examination of material unable to pass through the filter revealed that larger blood vessels with associated smooth muscle cells, and many fibrocytes in association with some remaining masses of undigested collagen fibres, were selectively removed by filtration. This could have accounted, at least in part, for the relative absence of smooth muscle cells and fibrocytes among the filtered, collagenase-dispersed cells.

Trypsin digestion

Trypsin treatment of collagenase-dispersed cells in calcium-free medium disrupted the adherens-type junctions and produced a suspension composed largely of single cells (Fig. 2). Other tissue-dispersion treatments used were unsuccessful in producing complete dispersion. The cells were fragile when both calcium and magnesium were absent from the medium. As used in these studies, hyaluronoglucosaminidase proved to be toxic and protease was ineffective.

The yield of cells following trypsin treatment was $78 \cdot 3 \pm 11 \cdot 6$ (s.e.m.) $\times 10^6$ viable cells per gram of tissue or $40 \cdot 5 \pm 7 \cdot 5$ (s.e.m.) $\times 10^6$ viable cells per corpus luteum ($n = 13$). Mean viability of these cells was 84% (s.e.m. 81·9–86·1%).

Differential Counts of Dispersed Cells and Cell Fractions

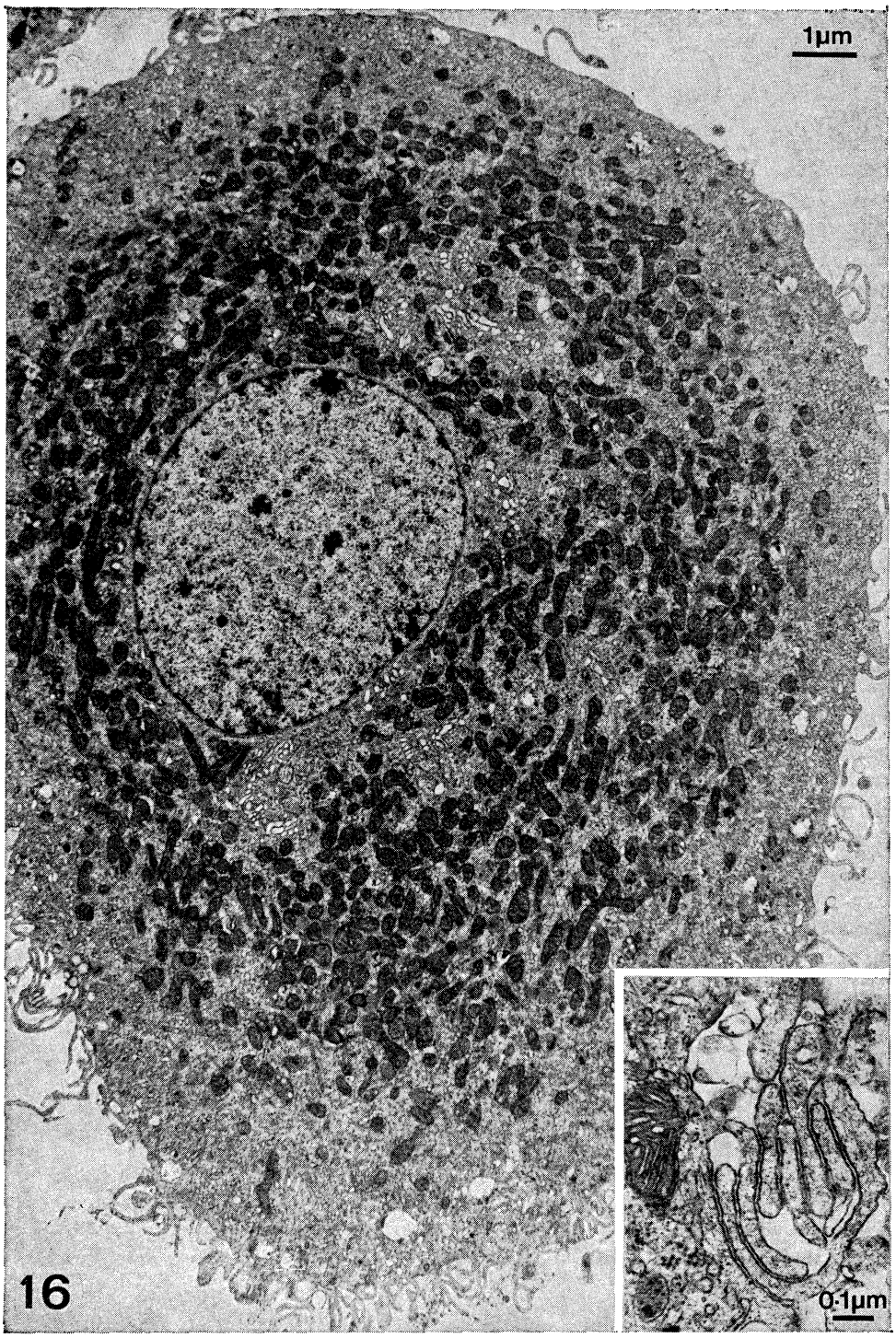
The results of cell counting for trypsin-dispersed cells and for six Ficoll fractions (very few cells were present above the middle of the 3·33% Ficoll layer and this fraction was therefore excluded from further consideration) are shown in Table 1, and a series of Ficoll fractions after cytocentrifugation are shown in Figs 3–8. Ficoll fractions A and B (Table 1) were rich in cells and relatively pure in endothelial cells. Ficoll fractions C and D contained larger numbers of small luteal cells although some endothelial cells were always present. Fractions E and F were rich in large luteal cells, contained very few small luteal cells, and always contained small numbers of endothelial cells. The degree of purity of these fractions varied somewhat from preparation to preparation and was less if the tissue dispersion was incomplete.

The proportion of the total cell volume made up of different cell types was calculated for some Ficoll fractions by using the results in Table 1 and assuming the cells to be spherical with mean diameters of 11·9, 15·6 and 23·6 μm for endothelial, small luteal and large luteal cells, respectively. It was calculated that 57·5 and 54·4% of the total cell volume were of the small luteal type in fractions C and D, and 78·2 and 94·4% were of the large luteal cell type in fractions E and F, respectively.

Fig. 13. Isolated medium-sized cell showing the characteristic features of a small luteal cell. Mitochondria are only moderately numerous, the endoplasmic reticulum is predominantly tubular in form, and the cell surface bears many microvillous processes.

Fig. 14. Detail from the surface region of an isolated small luteal cell. Note the tubular endoplasmic reticulum with some associated ribosomes, and mitochondria with predominantly lamellar cristae. Golgi complexes (*G*) and a single lipid droplet (*L*) are also present.

Fig. 15. Nucleus from an isolated small luteal cell showing a characteristic nuclear inclusion (arrows) containing cytoplasmic material.


1μm
16
0·1μm

Progesterone Content of Cells

The progesterone content per cell is shown in Table 2. Fractions A and B, rich in endothelial cells, contained significantly ($P < 0{\cdot}05$) lower amounts of progesterone than either fractions rich in small luteal cells (C and D) or those rich in large luteal cells (E and F). Fractions with small luteal cells had significantly lower progesterone content than fractions with large luteal cells. Better estimates of the progesterone content of each cell type (Table 2) can be made by making allowances for the proportions of each cell type in each Ficoll fraction (Table 1). As fraction A was composed of 99·5% endothelial cells with cellular progesterone content of 0·4 ng per 10^6

Table 1. Mean viable nucleated cell yield, and the proportion of cells in each of three classes based on cell size in trypsin-digested luteal tissue and in six Ficoll fractions

Cells were isolated from seven corpora lutea. Mean percentage of cells for each class in each sample is given, followed in parentheses by range from mean minus s.e.m. to mean plus s.e.m.

Sample	Ficoll content of each fraction (% w/w)	$10^{-6} \times$ Yield of viable nucleated cells per corpus luteum	Percentage of cells in each class[A] for each sample		
			Small	Medium	Large
Trypsin digest	—	38·7	88·4 (86·7–89·8)	6·6 (5·7–7·6)	5·0 (4·3–5·9)
Ficoll fraction					
A	3·33–5·00	6·17	99·5 (99·1–99·8)	0·5 (0·2–0·9)	0
B	5·00–6·67	3·52	96·0 (94·3–97·3)	3·6 (2·3–5·2)	0·4 (0·2–0·7)
C	6·67–7·50	1·61	62·0 (51·1–72·2)	37·8 (26·2–50·0)	0·2 (0·1–0·4)
D	7·50–8·34	0·85	51·3 (36·9–65·6)	44·4 (30·6–58·5)	4·3 (1·7–7·7)
E	8·34–10·00	0·57	53·7 (46·2–61·3)	10·7 (6·2–16·3)	35·6 (28·4–43·2)
F	10·00–16·77	0·61	25·0 (14·0–37·9)	3·6 (1·9–5·8)	71·4 (56·2–84·2)

[A] Small, endothelial cell; medium, small luteal cell; large, large luteal cell.

cells, it can be assumed that endothelial cells in all other Ficoll fractions had the same progesterone content. The remaining cells in fractions B, C and D must, therefore, have contributed 7·4, 7·0 and 7·2 ng per 10^6 cells, respectively, in order to attain the measured level of progesterone in these fractions. Using 7·2 ng per 10^6 cells as an estimate of small luteal cell progesterone content and allowing for endothelial cells, estimates for large luteal cells were 36·6 and 63·2 ng per 10^6 cells in fractions E and F, respectively. Expressing these estimates of progesterone content per unit volume of cell, endothelial cells contained $4{\cdot}5 \times 10^{-19}$ g μm^{-3}, small luteal cells 36×10^{-19} g μm^{-3} and large luteal cells 53×10^{-19} and 92×10^{-19} g μm^{-3}.

Fig. 16. Isolated large luteal cell. Mitochondria are abundant in the perinuclear cytoplasm, but sparse in the outer regions of the cell. The cell surface shows many microvillous and lamelliform processes (detail inset). Note that the dense cytoplasmic granules (=0·2 μm) normally numerous in functional large luteal cells are barely evident.

Progesterone Production (in vitro)

Progesterone production by both small and large luteal cells *in vitro* is shown in Table 3. Both cell types were capable of synthesizing progesterone and responding to luteinizing hormone by increasing progesterone production. Large luteal cells had a much higher basal rate of progesterone production than did small luteal cells, however, they were much less responsive to a luteinizing hormone stimulus (129% increase in progesterone production) than were small luteal cells (383%).

Table 2. Mean ±s.e.m. cellular progesterone content of trypsin-digested luteal tissue and of six Ficoll fractions, and calculated progesterone content for three cell types

Cells from seven corpora lutea were isolated. Estimates of progesterone content for each cell type were calculated by using progesterone content per total No. of cells and the proportions of each cell type in each fraction (see Results for further details). Two estimates were obtained for large luteal cells

Sample or cell type	Cellular progesterone content (ng per 10^6 total cells)
Trypsin digest	49·8±7·0
Ficoll fraction	
A	0·39±0·06
B	0·69±0·15
C	2·88±0·95
D	3·71±1·03
E	14·01±3·56
F	45·40±13·95
Endothelial cell	0·4
Small luteal cell	7·2
Large luteal cell	36·6, 63·2

Table 3. Progesterone levels at two time intervals during incubation of small and large luteal cells from four corpora lutea (*1–4*), with and without luteinizing hormone in the incubation medium

Progesterone values are means of triplicate determinations. The cells were sonicated in their incubation medium before assay. — Not determined

Time (h)	LH concn (ng ml^{-1})	Progesterone level (ng per 10^5 cells)							
		Small luteal cells				Large luteal cells			
		1	2	3	4	1	2	3	4
0	0	20·4	14·4	16·6	34·2	43·1	—	278·1	102·7
12	0	408·6	244·6	205·9	357·5	1433·6	—	2406·0	607·6
12	100	1574·1	1137·1	724·9	903·8	1634·5	—	2830·2	876·0

Discussion

Using the combination of methods described here, it has been possible to obtain useful separation of the major cell types of the ovine corpus luteum and to make observations on their morphology and progesterone content. In order to purify any population of cells by differential sedimentation, it is essential to completely

disperse the cells, as it is the diameter of a cell or a clump of cells that determines its sedimentation velocity. Collagenase digestion of ovine corpus luteum according to the method of Simmons *et al.* (1976) did not produce complete dispersion of cells, primarily because clumps of endothelial cells remained. Although collagenase was able to separate capillaries from other luteal elements, and to remove the capillary basal laminae, endothelial cells remained firmly attached to one another, often in large groups, by numerous adherens-type junctions. Purification of cell populations dispersed using only collagenase, therefore, proved futile, as large cell populations were always contaminated with the similar-sized clumps of smaller cells. To obtain complete dispersion, it was necessary to break the adherens junctions between endothelial cells.

Borysenko and Revel (1973) found that adherens junctions were of two classes: those able to be broken by trypsin and those broken by EDTA in high concentrations. In the present studies, trypsin in calcium-free medium with EGTA was found to be necessary to completely disperse the cells. The duration and concentrations of trypsin treatment were chosen as minimal to give complete dispersion of cells. It is nonetheless recognized that there are likely to be some disadvantages in using trypsin in a calcium-free medium. Firstly, trypsin not only attacks adherens junctions but it can also attack cell membranes that contain gonadotrophin receptors. Secondly, calcium fluxes are important in LH-stimulated progesterone release (Higuchi *et al.* 1976) and incubation in a calcium-free medium is likely to cause 'leaching out' of intracellular calcium. In rat granulosa cells, 3–4% of intracellular calcium leached out in 30 min, with calcium status restored within 2 h of resuspension in complete medium (Batten and Anderson 1981). In spite of these potential disadvantages of the use of trypsin, viability of luteal cells after trypsin dispersion was found to be relatively high and comparable to that obtained by Simmons *et al.* (1976) using collagenase alone.

Although stained cytocentrifuge preparations and electron microscopy proved valuable in the identification of dissociated cells, neither method was useful for quantitation, as cell distribution in these preparations was not uniform. However, electron microscopy was necessary for positive identification of the various cell types.

Purification of cell populations on a Ficoll gradient proved to be both quicker and easier than sedimentation at unit gravity as used for purification of porcine and bovine luteal cells (Lemon and Loir 1977; Koos 1978; Ursely and Leymarie 1979). As it is quick, the cells are rapidly returned to oxygenated media, eliminating the need to chill the cells during the separation process. The purity of the cell populations provided by this method was adequate for study of the individual functions of the various types of luteal cells *in vitro*. Fractions of small and large luteal cells were almost free of cross-contamination, although both contained some endothelial cells. As an almost pure population of endothelial cells was available, measurements could be undertaken on this population during *in vitro* studies to assess their contribution in any particular function.

It is not known whether the proportions of cells of different types obtained by collagenase/trypsin digestion in this study reflect accurately the proportions of the same cells in the original luteal tissue. Only very limited data (O'Shea *et al.* 1979) are currently available on the proportions of different cell types in ovine luteal tissue. These data do, however, suggest higher relative numbers of large and small luteal cells, and lower numbers of endothelial cells and pericytes, than were found here

in dissociated tissue. The possibility of differential rates of cell loss during the dissociation process, therefore, cannot be excluded. Clearly, there were also substantial losses of cells during the Ficoll fractionation, with preferentially higher losses of the larger cell types, which appeared to be due at least in part to adherence between free cells and the sides of the centrifuge tube.

It is difficult to compare directly the purity and even the identity of these populations with those obtained from bovine and porcine corpora lutea, as little detail of cell identity and purity obtained in these other species has been published. With bovine corpora lutea, Koos (1978) observed three populations of cells, the larger two probably being comparable to small and large luteal cells (Koos and Hansel 1980). The smallest cells (8–11 μm in diameter and comprising 60% of the total cells) whose 'origin and function remain in doubt' were probably endothelial cells as judged from present observations in the sheep. Bovine populations of large luteal cells were contaminated with small luteal cells held together in clumps by 'extensively interdigitating microvilli' (Koos and Hansel 1980). Cytoplasmic processes are also present on ovine small luteal cells, and more extensively on large luteal cells (O'Shea *et al.* 1979) but these did not hold cells together after cell dispersion in this study. Ursely and Leymarie (1978) also reported problems of cross-contamination in bovine luteal cells but kept purity high by rejecting fractions with mixed populations. With porcine luteal cells (Lemon and Loir 1977), many of the clumps apparently did not rise as the sedimentation chamber was filled, leading to a reduction in yield. Purity was not estimated. Finally, comparisons with previous work in the sheep are also difficult because of incomplete data on cell identity. Endothelial cells, which were the most numerous cell type (88%) in the present study, were not identified in the collagenase-dissociated tissues studied by Simmons *et al.* (1976). Possibly the cells that 'predominated in the final cell preparation' and were identified as 'possibly fibroblasts' by these workes may have included endothelial cells and pericytes.

The progesterone content of cells after trypsin–EGTA treatment was higher than for the cells in any Ficoll fraction. The most plausable explanation for this is that during trypsin–EGTA treatment, the cells continued to produce progesterone but were unable to release it as this process requires extracellular calcium (Higuchi *et al.* 1976). Incubation in media containing 2 mM EGTA has been reported to decrease progesterone secretion by 80% in dispersed ovine luteal cells but not to block the production and accumulation within the cell of granules (Schmitz *et al.* 1978) postulated to contain progesterone. Thus, cells collected immediately after trypsin–EGTA treatment would be expected to contain more progesterone than those returned to calcium-rich media for separation on Ficoll gradients.

The progesterone content per cell was tenfold higher in large luteal cells than in small luteal cells, which in turn was higher than in endothelial cells. Progesterone content per unit volume of cell was greatest in large luteal cells, intermediate in small luteal cells, and lowest in endothelial cells. However, these results should be tempered by the possibility that synthesis and release of progesterone may have occurred during the purification procedures and so not accurately reflect *in vivo* levels.

In culture, basal progesterone production by large luteal cells was greater than that of small luteal cells but small luteal cells were considerably more responsive to luteinizing hormone, at the dose used, than were large luteal cells. These findings are in agreement with those for porcine (Lemon and Loir, 1977) and bovine (Koos 1978; Ursely and Leymarie 1979) luteal cells. These findings suggest that small luteal cells,

being more plentiful in corpora lutea than are large cells, may contribute significantly to luteal progesterone production. Further work will attempt to examine other functions of both small and large luteal cells and to investigate potential interactions between the two cell types.

Acknowledgments

We would like to thank Dr J. K. Findlay for his helpful suggestions, Dr P. Bartlett for his advice on tissue dissociation, and Miss A. Veenstra for her technical assistance in electron microscopy. We are also grateful to Dr R. I. Cox for the provision of antisera to progesterone, to United States National Institute of Arthritis, Metabolism and Digestive Diseases for purified ovine luteinizing hormone, and to the Melbourne University Veterinary Research Fund for financial support.

References

Batten, B. E., and Anderson, E. (1981). Effects of Ca^{+2} and Mg^{+2} deprivation on cell shape in cultured granulosa cells. *Am. J. Anat.* **161**, 101–14.

Borysenko, J. Z., and Revel, J. P. (1973). Experimental manipulation of desmosome structure. *Am. J. Anat.* **137**, 403–22.

Deane, H. W., Hay, M. F., Moor, R. M., Rowson, L. E. A., and Short, R. V. (1966). The corpus luteum of the sheep: relationships between morphology and function during the oestrous cycle. *Acta Endocrinol.* **51**, 245–63.

Higuchi, T., Kaneko, A., Abel, J. H., Jr, and Niswender, G. D. (1976). Relationship between membrane potential and progesterone release in ovine corpora lutea. *Endocrinol*ogy. **99**,1023–32.

Hossain, M. T., Lee, C. S., Clarke, I. J., and O'Shea, J. D. (1979). Ovarian and luteal blood flow, and peripheral plasma progesterone levels in cycling guinea pigs. *J. Reprod. Fertil.* **57**, 167–74.

Jordan, A. W., III., Caffrey, J. L., and Niswender, G. D. (1978). Catecholamine-induced stimulation of progesterone and adenosine 3′,5′-monophosphate production by dispersed ovine luteal cells. *Endocrinol*ogy. **103**, 385–92.

Koos, R. D. (1978). Progesterone synthesis by bovine corpus luteum cells separated by unit gravity sedimentation. *Biol. Reprod.* **18**, Suppl. 1, 78A.

Koos, R. D., and Hansel, W. (1980). Fine structure and steroidogenic properties of large and small bovine luteal cells. *Biol. Reprod.* **22**, Suppl. 1, 62A.

Lemon, M., and Loir, M. (1977). Steroid release *in vitro* by two luteal cell types in the corpus luteum of the pregnant sow. *J. Endocrinol.* **72**, 351–9.

Mossman, H. W., and Duke, K. L. (1973). 'Comparative Morphology of the Mammalian Ovary.' pp. 209–20. (The University of Wisconsin Press: Madison, Wisconsin.)

O'Shea, J. D., Cran, D. G., and Hay, M. F. (1979). The small luteal cell of the sheep. *J. Anat.* **128**, 239–51.

O'Shea, J. D., Cran, D. G., and Hay, M. F. (1980). Fate of the theca interna following ovulation in the ewe. *Cell Tissue Res.* **210**, 305–19.

O'Shea, J. D., Nightingale, M. G., and Chamley, W. A. (1977). Changes in small blood vessels during cyclical luteal regression in sheep. *Biol. Reprod.* **17**, 162–77.

Phillips, H. J. (1973). Dye exclusion tests for cell viability. In 'Tissue Culture: Methods and Applications'. (Eds J. Kruse and J. Patterson.) pp.406-8. (Academic Press: London.)

Schmitz, M. H. J., Abel, J. H., Jr, Chen, T. T., McClellan, M. C., Sawyer, H. R., and Niswender, G. D. (1978). The role of calcium in progesterone synthesis in ovarian granulosa lutein cells. *J. Cell. Biol.* **79**, 371a.

Simmons, K. R., Caffrey, J. L., Phillips, J. L., Abel, J. H., Jr, and Niswender, G. D. (1976). A simple method for preparing suspensions of luteal cells. *Proc. Soc. Exp. Biol. Med.* **152**, 366–71.

Ursely, J., and Leymarie, P. (1979). Varying response to luteinizing hormone of two luteal cell types isolated from bovine corpus luteum. *J. Endocrinol.* **83**, 303–10.

Manuscript received 23 December 1981, accepted 28 April 1982