OBSERVATIONS ON THE HISTOLOGY AND ULTRASTRUCTURE OF THE PARS DISTALIS OF THE RABBIT HYPOPHYSIS IN ORGAN CULTURE

A Thesis

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ABSTRACT

The histology and ultrastructure of the pars distalis of the rabbit hypophysis was studied after different periods of organ culture, and the best technique for the maintenance of the maximum proportion of the explant was assessed by comparing cultures grown in different conditions. Explants in air with a medium buffered with N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid (HEPES), not previously used in organ culture, proved more satisfactory than explants in carbogen with bicarbonate-buffered 199, and cultures were maintained for more than 3 weeks. Material from young animals survived better than from old.

The survival of cells was assessed on the basis of their cytological integrity when explants were examined by light microscopy after specific staining and by electron microscopy; DNA and RNA fluorescence with acridine orange was a valuable indicator. Also, cell multiplication was identified by direct observation of mitosis, by the application of the colchicine technique and by autoradiography.

During culture, prolactin cells showed physiological signs of secretion (demonstrated by combined culture with mammary gland) and morphological signs of an increase of secretory activity. Morphological signs of reduced secretory activity appeared in the presence of hypothalamic tissue

(combined culture) or extract. Somatotrophs and gonadotrophs showed signs of low-level secretory activity in solitary pars distalis culture and of increased activity in combined culture with hypothalamus. Secretory granules, and amorphous material judged to be secretory, appeared in intercellular and perivascular spaces during culture.

Somatotrophs became relatively much less numerous than prolactin cells as culture proceeded and gonadotrophs became very rare, but combined culture with hypothalamus appeared to favour somatotrophs and gonadotrophs.

Thyrotrophs disappeared soon after 3 days of culture. Agranular parenchymal cells increased in proportion during culture and some (the stellate cells) became macrophage-like.

Non-parenchymal cells, including fibroblasts, persisted during culture.

Cell modifications during culture included the development of a peripheral epithelioid layer, the appearance of numerous microvilli, microfibrils, coated or smooth vesicles, lytic bodies and desmosomes, and nuclei and nucleoli became enlarged, and intranuclear rods became more common. Cells often became more electron dense during long-term culture. Identifiable granules were retained by many cells throughout culture.

Intranuclear rodlets (fibrous or membranous structures) were identified in uncultured and cultured material; these have not been previously described in pituitary cells.

CONTENTS

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INTRODUCTION

Loeb, in the late nineteenth century, was the first to maintain cells, of blood and some other tissues, outside the body in a viable condition, and thus can be said to have taken the first step in Tissue Culture. Ross G. Harrison in the early part of the twentieth century demonstrated for the first time that nerve fibres grow out from nerve cells in vitro, and he may be called the inventor of tissue culture. Other notable workers were Carrel, Burrows, W.H. and M.R. Lewis and Ebeling. Tissue culture may be subdivided into tissue culture proper, cell culture, and organ culture.

In organ culture, cell multiplication is of subsidiary interest but embryological development or the maintenance of normal organisation and physiological functions is usually the chief object and the outward growth and migration of de-differentiated cells is suppressed.

Strangeways and his team and Maximow made the first real steps in organ culture, working with embryonic organs.

The early work is fully reviewed in "Cells and Tissues in Culture" (Willmer, 1965).

Various methods have been used for the culture of mature organs and described by Fell and Robison (1929), Schaberg and De Groot (1958), Trowell (1959), MacDougall (1964), Coupland and MacDougall (1966), MacDougall and Coupland (1967),

Biswas et al. (1967), Tapp (1967), Mayne et al. (1968), Lucas (1969), Feldman and Lebovitz (1970).

Pituitaries, either entire or as fragments, of the rat and some other animals, have been cultured by a number of workers using different techniques, media and gases. The object of most of the work was to assay the hormones released and formed during culture, and relatively little attention has been paid to the histology and ultrastructure of the cultured explant (Meites et al., 1961; Martinovitch et al., 1962; Nicoll and Meites, 1962a, b; Danon et al., 1963; Solomon and McKenzie, 1966; Deighton and Meyer, 1969; Macleod et al., 1969; Arimura et al., 1969; Krulich and McCann, 1969).

Some workers have studied the histology and ultrastructure of the cultured pituitaries of some animals, e.g. Gaillard (1953), Pasteels (1962, 1963), Trowell (1959) and Mastro et al. (1969) studied rat pituitary, Petrovic (1963a, b) the pituitary of hamster, guinea pig and rat, Tixier Vidal and Gourdji (1965) the pituitary of duck, and Sage (1966) the pituitary of fish, but little or no work has been done on the culture of pars distalis of the adult rabbit except from a purely physiological point of view (Nicoll and Meites, 1962b).

There are several publications on the culture of pituitary in conditions in which the outgrowth of sheets of tissue occurs, notably by Kasahara (1936), Gaillard (1939), Pasteels and Mulnard (1961), Pasteels (1963), Jensen et al. (1964), Emmart

and Mossakowski (1967) and Gyévai et al. (1969).

Organ cultures have been made of the anterior pituitary of the embryonic rat (Chen, 1954), of the young rabbit pituitary (Gaillard, 1942) and of the anterior pituitary of the human foetus (Pasteels, 1963; Jensen et al., 1964). There is very little published work on the organ culture of the pituitary of the rabbit with regard to histology and none with regard to ultrastructure.

In the present work observations have been made on the histology and ultrastructure of the pars distalis of the rabbit hypophysis in organ culture for periods of up to 24 days. A medium buffered with N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid, known in commerce as HEPES, was found to be advantageous (Fisk and Pathak, 1969).

Mitosis in the cultured material has been identified by autoradiography, colchicine-treatment and direct observation. Viability assessments were based on direct observation of standard preparation and DNA- and RNA-fluorescence with acridine orange.

Combined cultures demonstrated the secretion of prolactin during culture.

Preliminary experiments on dehydrogenase activity (formazan technique described by Howe and Thody, 1967) were not successful and were not continued since they had been intended to give an estimate of viability, and simpler methods proved satisfactory.

MATERIALS AND METHODS

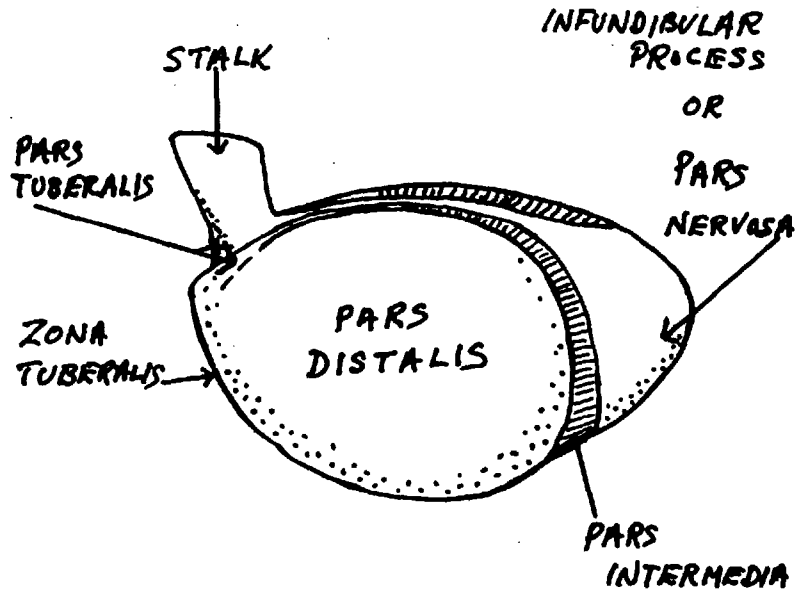
A. Animals

16 mature male rabbits aged from 6 months to 1 year, 25 mature virgin female rabbits aged from 6 months to 1 year, 9 young sexually immature male rabbits aged from 12 hrs to 5 weeks, 11 young sexually immature female rabbits aged from 12 hrs to 5 weeks were used. Rats were used for preliminary work. Aseptic precautions were taken. Adult rabbits were killed by air embolism, and the young animals by decapitation.

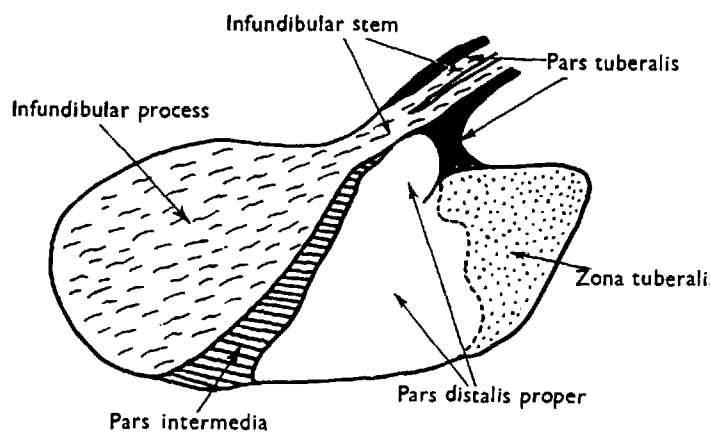
In the rabbit, unlike the rat, the pituitary gland is almost surrounded by bone. The pituitary (see Text-figs. 1 and 2) was removed as quickly and gently as possible and put in culture medium. The pars distalis was separated from the gland and, subsequently, lateral pieces were cut off, avoiding the zona tuberalis and they were subdivided into smaller pieces, usually in the order of 2 x 2 x 1 mm. Slicing was done by hand, using stainless-steel safety-razor blades, the tissue being supported on a piece of rubber and sliced by shearing cuts and the blade being held vertically with the cutting edge at such an angle that the minimum pressure was applied.

B. Media

Various types of culture media were used.



Text-fig. 1. Pituitary gland of rabbit (side view, slightly tilted).



Text-fig. 2. Pituitary gland of rabbit (sagittal section)

(1) Difco, TC medium 199, 10X.

(a) Basic medium

10 ml. TC medium 199, 10X.

2.2 ml. sodium bicarbonate solution (10%)

1.0 ml. Penicillin-streptomycin solution
(Penicillin G_1 Potassium 10,000 units;
Streptomycin sulphate 10,000 mcg. per ml.)

Sterile triple-distilled water added to make 100 ml.

Note:- This was first made up with the recommended amount of bicarbonate solution. Subsequently, after preliminary experiments, the medium containing 2.2 ml. of bicarbonate (10%) in 100 ml was used (MacDougall and Coupland, 1967). Later still, 1.5 ml of sodium bicarbonate solution (10%) was found to be satisfactory.

(b) 9 ml basic medium: 1 ml dehydrated human citrated plasma reconstituted in distilled water.

(c) 9 ml basic medium: 1 ml dehydrated human citrated plasma reconstituted in 1% calcium chloride solution.

(d) 9 ml basic medium: 1 ml rabbit serum (when possible from the same rabbit).

(2) Glaxo, TC medium 199, 10X.

(a) TC medium 199, made up to the standard formula.

(b) 10 ml TC medium 199, 10X
2 ml insulin (80 I.U. per ml)
33 ml glucose solution (5% solution)
Sterile triple-distilled water added to make 100 ml.

(c) 10 ml TC medium 199, 10X
2 ml insulin (80 I.U. per ml)
33 ml glucose solution (5% solution)
16 ml denatured mouse serum
Sterile triple-distilled water added to make 100 ml.

Note:- TC medium 199 Glaxo contains penicillin and sodium bicarbonate (0.088 gms of bicarbonate in 100 ml).

(3) Difco, TC medium 199, 10X

(a) 10 ml TC medium 199, 10X
2.2 ml bicarbonate solution (10%)
1.0 ml penicillin-streptomycin solution
2.0 ml insulin (80 I.U. per ml)
33.0 ml glucose solution (5%)
Sterile triple-distilled water added to make 100 ml.
Later 16 ml. rabbit serum added. This is subsequently

called complete medium and was used in most of the experiments.

(b) 9 complete medium: 1 embryo extract.

(c) 58 ml complete medium containing extract of hypothalamus of 3 rabbits.

Note:- The hypothalamus extract was prepared by macceration in sand and centrifugation.

(d) 10 mg of thiouracyl added to 116 ml of complete medium.

(4) HEPES-buffered medium (Williamson & Cox, 1968).

(a) 100 ml 199
$NaHCO_3$, 13 mM
HEPES, 14 mM
(Plain medium)

(b) 1.6 gm glucose, 2 ml insulin (80 I.U. per ml) and 16 ml rabbit serum added to 100 ml of medium (a): Complete HEPES-buffered medium.

(c) HEPES-buffered medium (plain) with added corticosterone and insulin.

C. Gas phase

(1) 95% oxygen with 5% carbon dioxide (Carbogen). This gas mixture was used in most of the experiments.

(2) Air with 5% carbon dioxide; used mainly with circulating medium.

(3) Material cultured in closed system in air contained in a sealed box.

Carbogen was led into the incubator in which it passed through two humidifiers, containing sterile distilled water and penicillin, before reaching the culture. The actual rate of flow of gas proved to be immaterial within wide limits, in most experiments between 80 - 100 ml. per minute was completely satisfactory.

D. Methods of culture

1. Petri dish method with carbogen (used in all the experiments except when otherwise stated).

Cultures were made in sterile disposable plastic petri dishes (Falcon Plastic Co.) 53 mm in diameter and 12 mm deep containing a sterilised platform of minimesh stainless steel 20 mm x 15 mm and about 3 mm high covered with lens paper. Aseptic precautions were taken.

The petri dish contained about 10 ml. of the medium when

the surface of the medium was just touching the lens paper and about 11 ml. for deeper cultures.

Usually 2 to 4 pieces of the pars distalis were placed on the lens paper in one dish. 4 to 6 petri dishes were placed in a perspex chamber 9" x 6" x 6" with two shelves, and an inlet and outlet for the gas which flowed at 200 to 300 ml per minute. Subsequently, polythene bags standing on foam plastic were found to be preferable to the conventional chamber. 2 to 4 petri dishes on a tray were placed in the polythene bag and a rubber tube carrying the gas from the humidifiers was tied into the mouth of the bag so that the bag inflated when the gas flowed at about 80 to 100 ml. per minute.

The petri dishes were covered, but in preliminary experiments a few petri dishes were left open or half covered. The temperature of the incubator was kept at about 36°C (96.5°F).

There was always some condensation, presumably an outcome of the glass-house effect, inside the lid of the petri dish. This might alter slightly the concentration of the medium, and efforts were made to avoid the possibility of a drop of condensed water falling on the explant. Material was cultured for from 1 to 24 days. Generally the medium was renewed after 10 days and in one or two experiments the medium was not renewed during the three or more weeks of culture.

2. Petri dish method with air (closed system)

In this method, HEPES-buffered medium (Williamson & Cox, 1968) was used. Pieces of pars distalis were cultured in petri dishes in an air-tight plastic box with a dish containing cotton wool and sterile water. The material was cultured for up to 24 days.

In some experiments the medium was renewed after 10 days and in others the material was left for 24 days without renewing the medium. HEPES is stable and not metabolised and maintains pH for much longer than do metabolisable buffers.

3. Circulating medium

New's apparatus (New, 1967) was used for this method (Silica & Scientific Glass Apparatus, Cambridge). One or two pieces of pars distalis were placed on a minimesh steel platform in the culture chamber through which gassed medium circulated. The rate of circulation was kept to a minimum, about 5 to 7 ml. of the medium per minute carried by about 50 ml. gas.

E. Techniques to demonstrate multiplication of cells

(1) Colchicine

Pieces of pars distalis were cultured for 3 days. Colchicine solution was then added to the medium in the petri dish and the material was incubated for a further 3 to 5 hours

and then fixed in Baker's formol-calcium-cadmium.

Various dilutions of colchicine were used; from 1 µg to 50 µg per 10 ml of culture medium were effective without evident toxicity.

(2) Autoradiography

Pieces of pars distalis were cultured for 1, 3 and 7 days. H^3-thymidine solution to give 0.0375 µC per ml. of medium was added and the material was incubated for one hour and then suitably fixed and autoradiographs were prepared (Messier and Leblond, 1957).

Developed autoradiographs were usually stained with aquatic Mayer's haemalum to demonstrate nuclei.

PAS/orange G (with nuclear stain) was also tried on some labelled sections, and a modified technique by which sections were left in orange G for from 1 to 5 minutes and, after differentiating in water, not blotted (which produces distortions of the emulsion) but left in absolute alcohol for from 2 to 3 minutes, was quite successful.

Brookes' stain proved completely unsuited to coated sections.

(3) Direct observation

Cells in mitosis were observed in routine cultures and, in

some instances, the section was given a second, different, stain to make possible an exact identification of the cell type.

F. Combined cultures with hypothalamus

Pieces of pars distalis were cultured with pieces of hypothalamus from the same rabbit for up to 14 days (medium renewed every third or fourth day).

G. Demonstration of prolactin

Small explants of mammary glands of 11- to 13-day pregnant C3H mice were cultured in HEPES-buffered medium with 5 µg/ml insulin and 1 µg/ml corticosterone (Mayne et al., 1968) in a closed system for 48 hours in contact with explants of pars distalis of virgin female rabbit which had previously been cultured for either 4 or 7 days. Explants of mammary gland were cultured with fresh pars nervosa of male rabbit or alone to act as controls.

Note: Corticosterone solution was prepared by adding 1 ml. of ethanol to 500 µg of corticosterone and then adding 4 ml. of distilled water. Sections were cut at 5 µ and stained with haematoxylin and eosin.

H. Light microscopy

Material for light microscopy was fixed in Baker's formol-calcium-cadmium fluid. Paraffin sections at 5 μ were cut and stained:

(1) Haematoxylin and eosin.

(2) Pyronin and methyl green for the demonstration of RNA.

(3) PAS/orange G (with nuclear stain) to stain acidophil cells orange and mucoid cells magenta (Pearse, 1949).

(4) Brookes' stain, which is thought to differentiate the acidophil cells into red-stained prolactin cells and yellow somatotroph cells (Brookes, 1968). A slight modification of Brookes' method was used:-

Stains: 1. Carmoisine L, Michrome No. 102.

2. Orange G, Michrome No. 411.

3. Wool green S, Michrome No. 162.

All stains used were of recent purchase (Edward Gurr, London).

Solutions required:

A. 1% Carmoisine L in 1% acetic acid.

B. 2% Phosphotungstic acid in 95% V/V ethanol.

C. Solution B saturated with 0.5% orange G.

D. 0.5% Wool green S in 0.5% acetic acid.

E. 10% Cupric sulphate, aqueous.

F. Celestin blue.

G. Mayer's haemalum.

Staining method:

1. Bring sections to distilled water.
2. Incubate in 10% aqueous solution of cupric sulphate for 1 hour at 44°C.
3. Wash in running tap water for 20 minutes, transfer to distilled water.
4. Stain nuclei with celestin blue for 1 minute, rinse in distilled water.
5. Stain with haemalum for 1 minute, wash in running tap water for 15 to 20 minutes.
6. Stain in solution A for 15 minutes or more.
7. Rinse well in distilled water followed by 70% alcohol, until red stained cells stand out against a pale background.
8. Stain in solution C for 5 to 7 minutes, the orange G displaces the carmoisine from somatotroph cells. Rinse in distilled water.
9. Return to solution A until the carmoisine has reinforced the staining of prolactin cells without staining the somatotroph cells. This is checked at intervals by microscopy, after rinsing in distilled water.
10. Rinse in distilled water.

11. Stain in solution D for 2 to 3 minutes.
12. Rinse well in distilled water.
13. Place in 1% acetic acid for 2 to 4 minutes to remove excess of wool green.
14. Rinse in absolute alcohol, re-examine to see if green is removed from yellow cells.
15. Transfer to fresh absolute alcohol.
16. Transfer to absolute alcohol + xylene mixture (1:3).
17. Clear in xylene and mount.

Results:

Somatotroph cells:	Pale yellow or dark yellow.
Prolactin cells:	Red or purple or orange, depending on the number of granules present.
Mucoid cells:	Bluish green or dark blue.
Chromophobe cells:	Pale green.
Nuclei:	Bluish red.

I. Fluorescence microscopy

A Gillett and Sibert conference photomicroscope with iodine-quartz lamp was used in this technique, with primary and secondary filters. The primary filter absorbed nearly all visible light, allowing blue-violet light (5,000 Å) to pass through the specimen stained with acridine orange (Armstrong, 1956). The secondary filter absorbed light of smaller wave

length than 5,000 Å which might mask the fluorescence light emitted by the tissues.

Staining method:

1. Bring sections to distilled water.
2. Stain in 0.05% acridine orange in McIlvain's buffer for 5 min. (pH 2.6).
3. Differentiate in McIlvain's buffer for 2½ min. (approx.).
4. Quick rinse in distilled water and blot.
5. Transfer to absolute alcohol + xylene mixture for ½ min.
6. Clear in xylene and mount in D.P.X.

Results:

1. Viable cells: Nuclei fluoresce yellowish-green (because of DNA content) and cytoplasm yellowish-orange (because of RNA content), some cells more intensely orange than others.
2. Pyonotic region: Nuclei fluoresce greenish-yellow and cytoplasm anything from weak greenish-yellow to virtually nothing.
3. Necrotic region: The whole area is dull dark green with occasional spots of yellow fluorescence, presumably from persistent DNA.

Controls pretreated with ribonuclease and subsequently stained did not show any fluorescence in the cytoplasm; those pretreated with distilled water and then stained showed normal fluorescence in the cytoplasm.

J. Electron microscopy

Material for electron microscopy (usually from an explant that was also examined by light microscopy) was prefixed in 6.25% glutaraldehyde in Sorenson buffer at pH 7.4 for 1 to 2 hours and stored in Sorenson buffer. Small pieces were then fixed in Palade's buffered osmium tetroxide (Palade, 1952) for 1 hour at 4^{o}C.

The tissue was then dehydrated in ethanol and embedded in Araldite. Ultrathin sections were cut on a Huxley microtome (Cambridge Instrument Co.), using glass knives, and mounted on copper grids. The sections were stained with uranyl acetate and, usually, lead hydroxide. Later, lead citrate was used instead of lead hydroxide in order to reduce the tendency to precipitate.

In addition, thick (0.5 μ $\pm$) sections were cut for light microscopy and stained with Azure II (5% Azure II in saturated borax solution).

Most of the electronmicrographs were taken on an A.E.I.6B instrument.

RESULTS

I. Nomenclature

The terminology applied to the different cell types in the present work is based either on presumed functional activity (as, e.g. Salazar, 1963) or on general acidic or basic staining reactions or on form. Greek letters were used by Herlant (1964) based on Romeis' system (1940), and Young et al. (1965) used numerals. Many writers have designated cells by initial letters of the hormones presumed to be formed by them.

The following types of cells have been identified in cultured pars distalis of the rabbit (Pl. 8, fig. 1):-

Granular cells

Acidophil	Prolactin (LTH cells; ε -cells; cell type 1). maximum diameter of secretory granule is 800-900 mμ (Pl. 5).
	Somatotroph (STH cells; α-cells; type 2). maximum diameter of secretory granule is 450-500 mμ (Pl. 6).
Basiphil or Mucoid	Gonadotroph (FSH and LH cells; β and γ cells; type 3). Maximum diameter of secretory granule is 300 mμ.
	Thyrotrophs (TSH cells; δ cells; type 4). Maximum diameter of secretory granule is 170 mμ.

Agranular cells or chromophobes

Parenchymal cells

(a) Stellate cells (Type 5) (Pl. 7).

(b) Other agranular parenchymal cells. A chromophobe of light microscopy may show a few granules by electron microscopy and represent a stage of some type of granular cell.

In electron microscopy, only cells devoid of granules, or cells considered to be identical with normally agranular cells but which contain very few granules, are regarded as agranular here; these agranular cells are the stellate cells of Salazar (1963) and Schechter (1969) and the type 5 cells of Young et al. (1965).

Perivascular cells (Pl. 16, fig. 1).

In addition to these cells there are pavement epithelial cells of capillaries, and the red blood corpuscles; wandering white corpuscles might also be encountered.

II. General Results

From 2 to 4 explants of the pars distalis of the hypophysis were taken from each adult rabbit and the approximate size of each piece was recorded.

Preliminary experiments were made with a number of adult rats to assess different techniques and media. Subsequently cultures (up to 24 days) in the more favourable media gave large areas of surviving cells. There were no instances of bacterial infection (tested periodically) and only a few instances of fungus infection (entirely restricted to long-term cultures in unchanged medium). It was impracticable to check for virus or mycoplasma.

Viability was assessed on the basis of the appearance of the nucleus and cytoplasm and the response of the cell to specific staining reactions, particularly the acridine orange technique that demonstrates the DNA and RNA content (Pl. 1, figs. 4 and 5) and the pyronin/methyl green technique. In electron microscopy, cells were judged to be viable if they showed most of the normal organelles without serious artefacts. Particular attention was paid to the ribosomes, endoplasmic reticulum, mitochondria, centrioles and nuclear membrane (with nucleopores) as and when appropriate, all these organelles persist throughout culture.

Pycnotic cells, in light microscopy, had small dark nuclei in vacuolated or dark shrunken cytoplasm that did not react typically to the stains. Little could be distinguished in necrotic areas except cell debris made up chiefly of masses of granular debris that stained with acid dyes

(Pl. 3, fig. 2). Degenerating secretion granules, occasionally very distorted nuclei and some plasma membranes were evident in electron microscopy (Pl. 4, figs. 2 and 3).

The acridine orange technique demonstrated the absence of RNA from pycnotic and necrotic cells and the absence of both RNA and DNA from the necrotic cells.

The absence of RNA was confirmed by the pyronin methyl green technique. Generally, there was no central necrosis in small explants cultured for a short period (3 days), but usually a few cells with pycnotic nuclei occurred near the exposed (upper) surface.

After longer culture, pycnotic nuclei near the exposed surface were more numerous and finally the outermost part of the explant became necrotic.

In large explants, the more central part quickly became necrotic and, as with small explants, the upper surface became necrotic leaving a middle zone of viable cells (Pl. 1, fig. 2).

The extent to which the explant was submerged in medium appeared to affect the survival of cells; this became evident only after prolonged culture. The best results were obtained with half submerged explants (deep medium), but it was difficult to keep parts of the explants viable for 21 days in basic or complete medium. All cultures showed a tendency to develop a few small cavities in peripheral regions. In

explants cultured in HEPES-buffered media the peripheral region remained viable, spongy cavities and epithelioid cells developed with microvilli over free surfaces and, in general, more cells were viable for longer periods than in standard media with carbogen (Pl. 1, fig. 3 and Pl. 3, fig. 3).

Perivascular spaces and intercellular spaces became larger, with a more PAS-positive content, basement membranes became distorted but persisted and capillaries became shrunken in long-term cultures.

In general the acidophil cells enlarged slightly and their nucleoli became more pronounced; those of the more peripheral region of the cultured material had fewer granules than the deeper lying cells. Granules tended to become more coarse as culture progressed, particularly in prolactin cells. Though Brookes' stain differentiated between prolactin cells and somatotroph cells in most of the cultured material (Pl. 2, figs. 3 and 4), in some instances of long-term culture it was difficult to distinguish between these two types of cells.

The basiphil cells of cultured explants and controls did not show so marked and consistent colouring with PAS as is characteristic of these cells when fixed by perfusion (Allanson et al., 1957).

In light microscopy, the granularity of the cells could be

judged only by the intensity of the staining reaction, though in some instances, where there were few large granules, the separate granules of prolactin cells could be distinguished.

Chromophobic cells were numerous in cultured material and there was an apparent increase in the number of such cells in long-term cultures but, by light microscopy, it was impossible to determine whether they were agranular or had very few granules.

III. Detailed Results (Light microscopy)

A. Petri dish methods

1. Mature virgin female rabbits

(a) Explants cultured in complete medium (Difco 199) with carbogen.

Explants cultured for 3 days (6 cultures, 5 successful) showed very little central pycnosis (at most one-tenth of the volume in those which were not more than 2 mm. thick) and epithelioid tissue with microvilli developed around the material, particularly at the exposed surface and sides (Pl. 1, fig. 1). The tissue appeared to resemble the uncultured material except for the presence of a few cavities near the surface and a number of cells near the exposed surface with pycnotic nuclei.

Material stained with PAS/orange G showed many orange-stained acidophil cells and a few basiphil cells (PAS-positive).

Material stained by Brookes' method showed a number of

red or orange prolactin cells with coarse granules; some were lightly granulated and some more heavily. There was a similar number of yellow-stained, finely granulated somatotrophs and very few bluish-green stained basiphil cells (Pl. 2, figs. 3 and 4). All the healthy cells showed abundant RNA.

In 6-day culture (5 cultures, 4 successful), small pieces of material showed little central necrosis, but there were more pyonotic cells towards the exposed side with epithelioid tissue. Brookes' stain showed that the numerous acidophil cells were predominantly prolactin cells with moderate granulation, and somatotrophs were less abundant. A few basiphil cells were present.

In 9-day culture of fairly large material approximately 3 x 2 x 2 mm. (one culture, one successful) the material was still bordered in places by epithelioid tissue. There was widespread necrosis in the peripheral material towards the exposed surface of the explant and a central pyonotic area. The lower and lateral parts of the material seemed quite healthy; there was some peripheral cavitation. There were numerous acidophils, the few basiphils were difficult to demonstrate by either technique.

In 12-day culture (2 cultures, 1 successful) of a large explant in deep medium, about a quarter of the cells survived; these were in the lower part of the explant. The necrotic

area towards the exposed side showed autolytic changes in the outermost region, where nuclei were absent. The region below that showed many pycnotic cells and some living acidophils. Below this in the region of good survival, there were many acidophils and some basiphils. The lowermost part of the explant showed some cavitation and the cells appeared to be less healthy than in shorter-term cultures.

There were many prolactin cells in the region of good survival, most with a full complement of granules, some with only a few granules. Somatotrophs were also numerous in this region (but rather less so than prolactin cells), they were usually heavily granulated.

In 14-day culture (4 cultures, 2 large explants successful, one in shallow medium one in deep) there was a large necrotic region (approximately one quarter of the whole) towards the exposed side of the explant. A layer of about one eighth of the explant contained normal-looking cells and scattered cells with pycnotic nuclei (Pl. 1, fig. 2). In this region prolactin cells predominated over the somatotroph cells, they contained few secretory granules. A few basiphil cells were present. There was a central pycnotic region and below this a region with living cells.

In 21-day culture, no satisfactory results were obtained.

(b) Explants cultured in basic medium with carbogen.

Explants cultured for up to 6 days (3 cultures, 2 successful), looked quite healthy with many prolactin cells and somatotrophs, basiphil cells were more numerous than in cultures in complete medium. In most of the cells, the cytoplasm was more opaque and nuclei stained more darkly than in complete medium.

(c) Explants (large, in deep medium) cultured in a closed system in air for 21 days in HEPES-buffered medium, with added glucose, insulin and serum (2 cultures, 2 successful).

With one explant, the medium was changed once after 10 days, the other was left in unchanged medium. The 2 cultures were very similar, but that with which the medium was changed was somewhat better. Survival of cells was good and, in contrast to cultures in other media, no necrosis occurred at the exposed side and there was a thick outer zone of surviving cells (about a quarter of the explant). There was a central pycnotic area containing some normal-looking cells.

The explant was covered with epithelioid cells. The cells of the upper part of the explant seemed to be fairly well granulated while those of the lower part appeared to have fewer granules but were still living. Perivascular spaces

were distinct in the viable parts of the explant.

The cells of the viable zone were either acidophils, which varied in the extent of their granulation, or chromophobes, and no mucoid cells could be identified (Pl. 3, fig. 1). Although differential staining is less conclusive with long-term cultures, there appeared to be more prolactin cells than somatotrophs.

(d) Explants cultured in HEPES-buffered plain medium for 21 days (2 cultures, 2 successful).

With one of the explants the medium was changed after 10 days. As before, this had little effect (Pl. 1, fig. 3). The absence of additives led to no well-marked differences except for the presence of persistent mucoid cells in the explant. Mucoid cells were much more numerous than in cultures in media with additives.

(e) Explants cultured for 24 days (1 cultured, 1 successful) in HEPES-buffered plain medium.

Large explant, medium was not changed. The results were similar to those of the 21-day culture in plain medium (Pl. 1, fig. 4).

Epithelioid tissue with microvilli occurred at the free

surfaces. Generally the epithelioid cells were large, irregular and agranular but, occasionally, granulated prolactin cells lined the cavities (Pl. 3, fig. 3).

2. Mature male rabbits.

Explants cultured in complete medium (Difco 199) with carbogen for 3 and 6 days (13 cultures, 9 successful).

In light microscopy, the results were essentially the same as were obtained with material from mature females.

3. Sexually immature female rabbits.

Explants cultured for 3 and 6 days in complete medium (Difco 199) with carbogen (5 cultures, 4 successful).

In the uncultured control material, there were numerous heavily granulated acidophils and many basiphils. Brookes' stain did not reveal any prolactin cells.

The material survived culture conditions much more readily than did the adult explants, pycnotic and necrotic areas were similarly arranged. An epithelioid covering developed more rapidly and completely. When cultured material was stained with Brookes' stain, numerous heavily or moderately granulated prolactin cells were demonstrated (in contrast to control material) as well as somatotrophs. There was some cavitation in the viable parts of explants.

Otherwise, the results were similar to those obtained with adult material.

4. Sexually immature male rabbits.

Explants cultured in complete medium (Difco 199) in carbogen for 3 and 6 days (8 cultures, 7 successful).

Light microscopy did not reveal any substantial difference between the pars distalis of immature males and females, either in the control material or in the products of culture.

B. Circulating medium

1. Immature male rabbits.

Explants cultured for 3 days in fast-flowing medium (2 cultures, 2 successful).

Large explants were used and there was some pycnosis in the middle of the material (about one-eighth). There was no peripheral pycnosis but some cavitation, particularly near the periphery. The explant was surrounded by epithelioid tissue with microvilli. Histologically, explants resembled those cultured in petri dishes. The cells appeared to be normal and had large nuclei and nucleoli.

2. Mature males and females.

(8 cultures, 4 successful).

Poor results were obtained in fast-slowing medium.

In 3-day cultures in slow-flowing medium (5-7 ml. medium per minute) there was a central zone of surviving cells (about a quarter of the explant), the outer zone was pyonotic with some necrosis.

The experiments were repeated using air with 5% CO_2 instead of carbogen but the results were equally unsatisfactory. More prolonged cultures were proportionately more unsatisfactory.

C. Multiplication of cells

1. Colchicine method

Pars distalis of the adult female rabbit cultured in complete medium with carbogen for 3 days (4 cultures, 4 successful) showed, after treatment with colchicine, a number of cells in mitosis. Some of these cells were chromophobic but there were also acidophils and a few basiphils (Pl. 2, figs. 3 and 4).

Brookes' stain demonstrated a few prolactin cells in mitosis. Most of the cells that showed mitosis occurred near the periphery of the material and contained fewer secretory granules when compared with the neighbouring mature cells, but some of the dividing cells had a full complement of granules.

2. Autoradiography

Pars distalis of adult males and females was cultured in complete medium with carbogen for 1, 3 and 7 days (6 cultures, 6 successful). After treatment with H^3 thymidine for 1 hour, sectioning and application and development of the emulsion, silver deposits associated with a number of nuclei indicated synthesis of DNA (Pl. 2, fig. 5).

After staining with PAS/orange G, most of the labelled cells proved to be acidophils, some were chromophobes, a few were mucoid cells and a few were fibroblast-like cells. One or two fibroblast-like cells appeared to show labelling throughout the cytoplasm.

3. Direct observation

In some routine 3-day cultures of adult male and female pars distalis, a few acidophils in prophase, metaphase or telophase were observed (Pl. 2, figs. 1 and 2).

D. Combined cultures of pars distalis and hypothalamus

(4 cultures, 3 successful)

The explants from virgin female rabbits were cultured for up to 14 days with hypothalamus. Cultured material appeared to show more mucoid cells and somatotrophs than comparable

simple cultures, and prolactin cells tended to be more heavily granulated and probably fewer in number.

E. Pars distalis cultured with hypothalamic extract

(2 cultures, 1 successful)

A 7-day culture gave similar results to those of the combined cultures.

F. Prolactin secretion

The uncultured mammary gland in section showed duots and small alveoli, alveolar cells were generally without vacuoles and the lumen of alveoli was very small and contained no secretion.

(1) Explanted mammary gland cultured for 48 hrs. in HEPES buffered medium without added hormones was similar to the uncultured material, except for slightly larger lumena.

(2) Explants of mammary gland were cultured for 48 hrs. in combination with pieces of pars distalis which had previously been cultured for 4 days. Corticosterone and insulin were added to the medium.

The explant of mammary gland showed large alveolar lumena with secretion, and alveolar cells showed vacuoles. Explants of mammary gland cultured for 48 hrs. with 7 day cultured pars distalis were similar to those cultured with 4 day explants (Pl. 3, fig. 4).

Explants of mammary gland cultured with pars nervosa of male rabbit for 48 hrs. in medium containing insulin and corticosterone showed very little secretion in the alveolar lumena and alveolar cells were generally not vacuolated.

IV. Detailed Results (electron microscopy)

The identification of the types of parenchymal cells was based on the size of their granules and, to some extent, on cytoplasmic features in accordance with Young et al. (1965) (Pl. 4, fig. 1).

1. Mature virgin female rabbits.

(a) Explants cultured in complete medium (Difco 199) with carbogen.

3-day cultures.

In explants cultured for 3 days, the degree of granulation was similar to that of controls (Pl. 8, figs. 1 and 2).

Most of the prolactin cells, which were relatively few as compared to somatotrophs, showed an abundant opaque granulation; a few had a more sparse granulation restricted to the periphery of the cell. Rough-surfaced endoplasmic reticulum (r.e.r.) was well developed and in places slightly vesiculated and in some cells showed a nebenkern formation. Golgi areas were

particularly well developed, often with immature secretory granules in cisternae. Intercellular spaces were opaque in some explants and there were instances of intercellular bridges.

Many somatotrophs were present, most had abundant electron-opaque granules, some had only a few granules arranged near the periphery. The r.e.r. was well developed, occasionally vesiculated and usually arranged in parallel arrays, and Golgi areas were well developed.

The nuclear membrane was characteristically indented in both types of acidophil.

Gonadotrophs were rare, had few rather pale granules, well developed r.e.r. and Golgi areas and many free ribosomes.

The rare, usually degenerating, thyrotrophs had few rather pale, peripherally arranged granules (Pl. 8, figs. 1 and 2); some still retained r.e.r.

Stellate cells were recognisable and showed the characteristic cell process and intracellular fibrils (85 - 150 Å thick).

Perivascular spaces were well developed, in some explants, intercellular spaces and perivascular spaces were dark (Pl. 8, fig. 2), the latter particularly towards the marginal parenchymal cells where there was evidence of accumulation of

dark material and deformed granules in the region of the basement membrane (itself invisible because of the opacity of the perivascular spaces). A few distinct and not deformed granules (without the granule membrane) were situated in perivascular spaces.

6-day cultures.

In 6-day cultures, prolactin cells were numerous, most were only moderately granulated and some had fewer peripherally arranged granules. R.E.R. was abundant and usually slightly vesiculated, and there were free ribosomes. Golgi areas were fairly well developed, and there were instances of small and large irregular granules in Golgi cisternae and there were numerous small, probably smooth surfaced vesicles (Pl. 9, figs. 1 and 2).

Microfibrils occurred in the cytoplasm of some of the prolactin cells, and microtubules (250 - 270 Å in diameter) were more evident in some of the weakly granulated cells (Pl. 9, fig. 2). Nuclei tended to be more irregular and indented.

Somatotrophs were about as numerous as prolactin cells, that is, relatively less numerous than in 3-day cultures. They were well or moderately granulated and the granules were sometimes arranged near the periphery, and some cells had dark

as well as pale granules. The somatotrophs generally had a well defined r.e.r. and a large Golgi area sometimes with secretory granules, and some cells contained microfibrils and microtubules.

There were instances of intercellular bridges between neighbouring acidophils (Pl. 9, fig. 2) and one instance of an intranuclear rodlet (400 Å thick made of units about 100 Å thick).

Multivesicular bodies occurred in some somatotrophs and in one cell a multivesicular body was combined with a secretory granule.

Very few gonadotrophs could be found; they had few peripherally arranged secretory granules.

No thyrotrophs could be identified.

Dark stellate cells (and a few light stellate cells) with indented nuclei occurred in the parenchyma; their plasma membranes tended to be folded, the folds interlocking with folds of neighbouring granular cells, sometimes forming large masses (Pl. 9, fig. 1).

Occasionally, in stellate cells, one or two dark granular inclusions occurred (in one instance measuring 1.25 x 1 μ). The dark agranular cells (thought to be of the stellate type) contained small vesicles, many free ribosomes and some coated

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HEPES-buffered Medium for Organ Culture

ZWITTERIONIC buffers have already found wide application in biochemical research. Williamson and Cox[1] have demonstrated the advantages for virological research of a synthetic medium for tissue culture consisting of "medium 199" modified by the addition of N-2-hydroxyethylpiperazine-N′-ethane-sulphonic acid (HEPES). Shipman[2] has shown that media buffered with HEPES are suitable for the culture of various types of cell. The advantages are that the medium does not need to be changed at frequent intervals for a long term culture, and the culture can be kept in a sealed, air-containing chamber.

This medium proved to be valuable for our investigation into the organ culture (Trowell's method) of the pars distalis of the adenohypophysis of the sexually mature rabbit. Numerous cultures of adenohypophysis have been made in a variety of media, most of which were 'Difco' medium 199 with or without additives, in a gas phase of 95 per cent O_2 with 5 per cent CO_2. In long term cultures, the explant became necrotic both at the periphery and internally, with viable cells between these two areas (Fig. 1*A*). Viability was assessed by light and electron microscopy. The survival of cells during three weeks of culture in plain medium 199 was poor.

Survival in medium 199 containing 13 mM $NaHCO_3$ and 14 mM HEPES, cultured in air, was much better during three weeks than in the plain medium without further additives. The pattern of survival (Fig. 1*B*) was markedly different from that of cultures in bicarbonate-buffered media. The zone of surviving cells was peripheral; survival was best at the upper surface and margins and poorer at the lower surface. There was an internal pycnotic zone and some necrosis. This suggests that a gas phase of 95 per cent O_2, 5 per cent CO_2, is toxic in the long term. Allison[3] has pointed out that a high partial pressure of oxygen is damaging to lysosomes and causes the release of lysosomal enzymes. We are investigating the effect of culture in 199 with HEPES in air with different quantities of added oxygen.

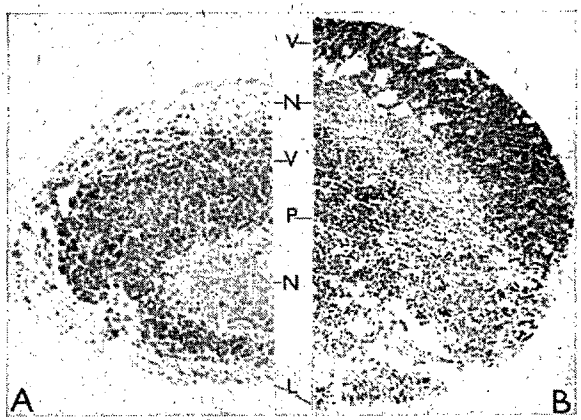

Fig. 1. *A*, 14-day culture of pars distalis of mature rabbit in medium 199 (with additives) buffered with $NaHCO_3$; in 95 per cent O_2, 5 per cent CO_2 (× 60). *B*, 21-day culture of pars distalis of mature rabbit in medium 199 (with no additives) buffered with HEPES; in air (× 60). L, Lower surface; N, necrotic; P, pycnotic; V, viable.

These buffers (particularly HEPES), which are virtually non-metabolizable and interfere little with the biochemical activities of cells and organs, are likely to have wide application in organ culture.

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vesicles (Pl. 7, fig. 3).

The intercellular spaces of the parenchyma were electron opaque in places. The perivascular spaces were well defined and were often dark.

Several instances of apparently unaltered secretion granules in perivascular spaces were observed but the granules were insufficiently numerous to hazard any guess as to their nature, though some were of an order of size that would suggest prolactin or somatotrophin granules.

12- and 14-day cultures.

In 12-day cultures there appeared, as in the material for light microscopy, to have been an increase in the proportion of prolactin cells present (Pl. 10, figs. 1 and 2).

The prolactin cells had increased in size and some cells had many granules, some a moderate number and some few. Example of irregular-shaped granules were found and an instance of a possible fusion of two granules. The granules were larger and a number of granules of 1 μ diameter were observed. A few instances of pale granules occurred and one example of an intranuclear granule (Pl. 10, fig. 2). The cells appeared to be active, they had a large nucleus and nucleolus, well developed Golgi area with some vesicles (light and dark) and large and small granules in cisternae (Pl. 5, fig. 3). The r.e.r. was vesiculated and free ribosomes, forming

polyribosomes, were present. Lytic bodies, microfibrils and microtubules occurred in some prolactin cells.

Many granules were close to the plasma membrane and there were instances of the apparent fusion of granule membrane and plasma membrane. Intercellular spaces were dark.

Somatotrophs were less numerous than prolactin cells, some were moderately granulated and some had few, peripherally arranged granules. In some cells the endoplasmic reticulum was vesiculated and cannalicular. Nuclei tended to be irregular and some contained intranuclear rods. Microfibrils were present but microtubules were not very evident.

A few cells with few small granules were judged to be gonadotrophs; no thyrotrophs were found.

Stellate cells were quite numerous and showed pseudopodial outgrowths, peripheral pinocytotic vesicles and many small vesicles particularly in the Golgi area. A stellate cell had a pseudopod containing a few granules, multivesicular bodies and phagosomes.

There were perivascular cells with dark cytoplasm within recognisable perivascular spaces which, in most regions, were dark so that basement membranes were difficult or impossible to identify. The collagen of the perivascular spaces was abundant and distinct and there were instances of dark and pale granules within perivascular spaces and also

dark granules with a pale halo.

In some regions the perivascular spaces were less dark and much-folded basement membranes were evident.

14-day cultures resembled the 12-day cultures. Prolactin cells were relatively more numerous, they showed signs of considerable secretory activity and were generally lightly granulated. Granules were generally dark, sometimes pale, sometimes dark with a pale halo. Cells showed numerous small Golgi vesicles and sometimes multivesicular bodies. Pinocytotic vesicles were fairly numerous, and cells showed microtubules, in one instance in association with the centriolar apparatus. Somatotrophs also showed signs of secretory activity, gonadotrophs were still recognisable and in the nucleus of one there appeared to be several granules.

As in 12-day cultures, but perhaps more strikingly, the cells considered to be those formerly called stellate had acquired in 14-day culture a number of the characteristics of macrophages. The nucleus was characteristically large and indented with a well-marked, sometimes spirally-arranged, nucleolus and in one instance a rodlet. The r.e.r. was mainly in short stretches with dark contents but there were relatively few ribosomes. Golgi areas were well developed, usually with

numerous vesicles often with rather opaque contents, free ribosomes and polyribosomes were present. Coated vesicles occurred near the plasma membrane and also internally, there were occasional phagosomes and other lytic bodies, and the cells contained microfibrils and microtubules. Mitochondria appeared to be of two types, the normal-appearing pale form and also some much thinner forms which were almost completely electron-opaque. These macrophage-like cells had a very irregular outline resulting in interlocking villous folds where the cell was in contact with another cell, and large processes evidently similar to ruffled membranes where the cell was within a cavity of the explant containing necrotic debris.

Intercellular and perivascular spaces usually contained opaque material.

(b) Explants cultured in basic medium with carbogen.

In 6-day cultures in plain medium (Difco 199) the explants appeared to contain rather fewer prolactin cells and rather more gonadotrophs than in complete medium. Otherwise there was no essential difference in the degree of granulation of cells or in the organelles, intranuclear rodlets were found; intercellular and perivascular spaces were not opaque.

(c) Explants cultured in closed system in air for 21 and 24 days in HEPES-buffered medium.

All the successful 21-day cultures were in HEPES-buffered media, material for electron-microscopy from the explants in complete medium was not entirely satisfactory as it did not show the outermost layer of the explant and it was thus impossible to determine the exact regions studied. A number of sections of the material in plain medium passed through the outermost epithelioid layer and comparison with light microscopical findings were possible.

In plain medium the cells were generally well granulated (Pl. 11, fig. 1). The substance of the cells was often more electron-opaque than in the shorter-term cultures. There were some areas of degeneration between viable cells, and some cavities, of the approximate size of a cell, which were surrounded by groups of healthy granular or agranular cells but without any border of microvilli. There were also some larger cavities (as seen in light-microscopy), which were lined by microvilli borne on the bordering agranular cells (with an occasional cell containing granular bodies). The peripheral epithelioid cells were similar to the cells lining the cavities (Pl. 11, fig. 1).

Prolactin cells were relatively numerous. They contained dark granules and those that had fewer granules had them

arranged peripherally, granules were sometimes of irregular shape. Nuclei were often large and irregular, sometimes with intranuclear rods, and there were instances of intranuclear rodlets (c.f. Pl. 14, fig. 5). The r.e.r. had lost the typical appearance and, when present, was in short stretches, coated with ribosomes, and had moderately opaque contents (c.f. Pl. 5, figs. 4 and 5). Golgi areas were well developed, sometimes containing immature granules (Pl. 13, fig. 2), and had numerous small dark and light vesicles. Centrioles, pinocytotic vesicles, many thin dark mitochondria, microtubules, clusters of microfibrils, pseudopodial outgrowths and villous folds interlocking with similar folds of neighbouring cells were characteristic. Pseudopodial outgrowths of prolactin cells towards a perivascular space or a degenerating cell were observed (Pl. 11, fig. 1).

The organelles of the somatotrophs resembled those of the prolactin cells. One cell had a pseudopodial process, containing granules, that projected into a perivascular space. Since the space contained some free granules in the neighbourhood of the process, it seems possible that the cell-process was extruding granules into it.

The gonadotrophs, which were more numerous than was expected in view of the results of shorter-term cultures, had either few, peripherally arranged granules or a moderate

granulation throughout the cytoplasm. The cytoplasm was perhaps less opaque than that of the prolactin cells and somatotrophs. The r.e.r. was in small stretches containing moderate opaque contents. Golgi areas were well developed with small light and dark vesicles. Mitochondria were present; they were small and not very numerous.

Stellate cells showed again the characteristics of macrophages. Golgi areas were well developed with numerous vesicles and there were free ribosomes, many thin dark mitochondria and instances of small stretches of r.e.r. The cells showed microtubules, and had microfibrils in various forms, arranged like a ball of wool or spirally coiled or in long thin stretches (c.f. Pl. 14, figs. 2 to 4). Large dense bodies and phagosomes occurred in some cells and peripheral coated vesicles. There appeared to be more stellate cells in explants in which the medium was not changed at intervals during incubation.

The agranular cells of the epithelioid layer at the exposed surface of the explant appeared to be of two types, paler cells with smooth microvilli and darker cells with rough microvilli (Pl. 11, fig. 1). Instances were seen of agranular cells, or very sparsely granulated cells, that were associated with a large number of pale granular bodies or clusters of pale granular bodies which were impossible to allocate with certainty either to a cell or to an

intercellular space.

Perivascular spaces with collagen fibres remained well defined and the basement membranes of parenchymal cells were often detected.

Dark and pale granules (somatotroph-size or smaller) were seen fairly often in the perivascular spaces (Pl. 16, fig. 2). Perivascular spaces were not dark and sometimes contained cytoplasmic masses with granules. Intercellular spaces were not dark.

Capillary endothelial cells were still recognisable (Pl. 15, fig. 3), with very much indented but apparently viable nuclei and numerous small vesicles (microvesicles) in the cytoplasm. Vestiges of red blood corpuscles were also found.

There were some macrophage-like perivascular cells (Pl. 16, fig. 1).

21-day cultures in complete medium resembled those in plain medium except for the following differences. Prolactin cells and somatotrophs appeared to be more active and sometimes had lamellar r.e.r. as well as well developed Golgi areas. Gonadotrophs appeared to be less numerous. Perivascular spaces contained some granules and opaque material, and intercellular spaces were opaque.

In 24-day culture in plain HEPES-buffered medium, the

outer viable part of the explant was more spongy and the cavities were larger and most of them were lined by microvilli (Pl. 12, fig. 1). Intercellular spaces were also larger, and often contained masses of interlocked villous folds.

Most of the cells had rather electron opaque cytoplasm, there appeared to be more instances of bridges between neighbouring cells.

The granular cells, most of which appeared to be prolactin cells, were arranged singly or in groups surrounded by the more numerous agranular cells. Their nuclei were generally indented, sometimes with intranuclear rods. Golgi areas were perhaps rather less well developed than in 21-day cultures as were the microtubules. Microfibrils were however more numerous and sometimes arranged around the nucleus with fibrils associated with the nuclear membrane (Pl. 14, fig. 3). Lytic bodies of the primary lysosome, dense body and autophagosome types occurred in some of the acidophil-type cells, as did coated vesicles and pinocytotic vesicles. Some lipid droplets were also present. Macrophage-like cells were more numerous in the parenchyma (Pl. 12, fig. 2).

Fibroblast-like cells were more evident in this culture and their structure more closely resembled the typical fibroblast. They occurred among the parenchymal cells as well as in perivascular spaces. They were pale, had a large

spherical nucleus and well marked nucleolus (Pl. 12, fig. 1), rough endoplasmic reticulum in the form of small or large vesicles with pale contents, a well developed Golgi area with small vesicles, and many microfibrils (often in bunches). When a fibroblast-like cell was at the surface of the explant, the free surface of the cell usually bore microvilli and the adjacent plasma membrane bore many small vesicles at its free surface. One fibroblast showed two centrioles (Pl. 12, fig. 2).

Desmosomes which in short-term cultures were not seen very frequently, were more numerous and were found associated with most of the types of parenchymal cells (Pl. 17, fig. 1).

Neither intercellular spaces nor perivascular spaces were electron-opaque.

Otherwise the 24-day culture resembled the comparable 21-day culture.

2. Mature male rabbits.

3-day culture, complete medium (carbogen).

The survival pattern and general findings were very similar to those observed with explants from female rabbits. There were numerous heavily or moderately granulated prolactin cells.

Some gonadotrophs with moderate granulation were identifiable, though much less common than in controls they

appeared to be more numerous than in explants from female rabbits. Controls showed more gonadotrophs in the male than in the female (as described by Foster and Cameron, 1964).

The nuclei of some of the agranular cells had very well defined rounded reticulate nucleoli and in one instance an intranuclear rodlet.

The epithelioid coat of the explant contained some dark irregular agranular cells with smooth microvilli and dark inclusions judged to be lipid droplets.

Intercellular and perivascular spaces were dark.

3. Immature male rabbits.

3- and 6-day cultures, complete medium (carbogen).

In 3-day cultures, the explant showed some well granulated prolactin cells (uncultered control material revealed only a single probable prolactin cell), numerous somatotrophs, a few gonadotrophs but no thyrotrophs. There were some agranular parenchymal cells. The explants were covered by agranular epithelioid cells with microvilli.

The prolactin cells and somatotrophs showed the signs of secretory activity, but the gonatrophs (which were less numerous than in controls) did not appear to be very active. Among the agranular cells there were a few with a regular outline that resembled granular cells, the rest were cells.

mostly dark, with very many microvilli, villous processes and pseudopodial processes that tended to envelop neighbouring granular cells. The general appearance of these 3-day cultures resembles that of much longer-term cultures of adult material in having large cavities in the outer part of the explant and large intercellular spaces. The cells with many processes resembled the stellate cells of such long-term cultures. Also, again as in longer-term cultures of adult material, desmosomes were relatively numerous in these 3-day cultures. Intercellular and perivascular spaces were not dark except in regions where the intercellular spaces were not enlarged where they had an opaque content. Fibroblast-like cells in the perivascular spaces, which are not seen very often in adult material or in short-term cultures, appeared to be fairly common in controls of young material and short-term cultures.

After 6 days of culture, explants contained more prolactin cells than somatotrophs, gonadotrophs were rare, thyrotrophs were not found and agranular parenchymal cells were more numerous. The pattern of survival resembled that of cultures of adult material, the sections that passed through viable cells did not show the large intercellular spaces and cavities characteristic of 3-day cultures. The granules in prolactin cells tended to be larger, and larger than the characteristic size. Intercellular and perivascular spaces

were dark in many places.

4. The effect of hypothalamus on cultures of pars distalis of mature rabbits.

Two combined cultures of pars distalis of female rabbit with hypothalamus (7 days and 14 days), two cultures of pars distalis in medium containing hypothalamus extract (7 days and 14 days, the 14-day culture failed) were investigated by electron microscopy. As with the similar material investigated by light microscopy, prolactin cells appeared to be less numerous than in ordinary cultures but well granulated; somatotrophs were numerous, some well granulated some only sparsely; gonadotrophs were moderately granulated and more numerous than in ordinary cultures. Both somatotrophs and gonadotrophs showed cytological evidence of secretory activity in having a well developed r.e.r. and normally developed Golgi area while the prolactin cells showed little r.e.r. and the Golgi areas were not prominent. Intercellular and perivascular spaces were not dark.

Pars distalis of male rabbit in combined culture with hypothalamus gave similar results (Pl. 18, fig. 1).

DISCUSSION

I. Techniques

The methods of culture used in the present work were chosen after reviwing previous work on the culture of the pituitary and other endocrine glands. Chen (1954) used the watch glass method of Fell and Robison with a floating lens paper (semi synthetic medium); Schaberg and De Groot (1958) used a solid medium.

Trowell (1959) used a metal bridge with lens paper in a petri dish with medium T8 (synthetic) in carbogen. MacDougall and Coupland (1967) used a metal bridge with lens paper and Parker's medium 199 with 20% serum in oxygen at different pressures (1, 2, 3 atmosphere) with sufficient CO_2 for the required pH. Sage (1966) used Eagle's medium 199 with additives (semi synthetic medium). Deighton and Meyer (1969) used the petri dish method with medium 199 in carbogen. Emmart and Mossakowski (1967) used a collagen clot for tissue culture. Krulich and McCann (1969) incubated pituitary in flasks in Krebs-Ringer bicarbonate solution with carbogen. Martinovitch *et al.* (1962) used the watch glass technique. Meites *et al.* (1961) used medium 199 with air or carbogen. Macleod *et al.* (1969) used medium 199 with carbogen. Petrovic (1963a) used a rolling tube method and also a method in which

organ fragments were kept in suspension by bubbled oxygen. Tixier-Vidal and Gourdji (1965) used a method with gelose and a hanging-drop method with medium 199 or a semi synthetic medium.

The basic method of Trowell (1959) seemed to be the most promising for the present work, bearing in mind the unavoidably limited means available. Preliminary experiments to investigate the method of culture on a floating raft of gauze coated with silicone (Chen, 1954) suggested that this method would produce more failed cultures (through the sinking of the raft or the failure of the explant to be in adequate contact with the culture medium) than that of Trowell. A method by which it was hoped to culture explants submerged in circulating oxygenated medium in New's apparatus (New, 1967), devised for the culture of mammalian embryos, produced poor results except with material from very young animals. This was not surprising in view of the almost unanimous view that the disturbance of the explant or the medium during culture is harmful. Only Petrovic (1963a) has described fairly good results from culturing pituitary fragments in moving liquid.

The present investigation into the changes that occur during the organ culture of the pars distalis of the mature virgin female rabbit was made in order to identify the best medium, gas phase and general technique necessary to keep the maximum number of cells viable for the longest time. The

structural changes that occurred during 24 days of culture were investigated by specific staining techniques for light microscopy and by electron microscopy. Some male rabbits and some immature male and female rabbits were also used.

Liberation of prolactin during culture was demonstrated.

Humidified carbogen was used in most of the experiments but, despite all precautions, there was some evaporation and condensation of the medium and consequently the possibility of an effect on the material.

Light microscopy of explants cultured in carbogen showed that the periphery of the explant contained some pycnotic or necrotic cells; attempts were made to minimise the evaporation and condensation of the medium (which was presumably an outcome of the glass-house effect) and to prevent any desiccation by increasing as much as possible the level of the medium, which, in early experiments, was kept at a minimum so as least to interfere with the diffusion of oxygen (Trowell, 1959; MacDougall and Coupland, 1967; Biswas et al., 1967). Increasing the level of the medium and submerging the explant partially in the medium reduced the peripheral necrosis to some extent in 6- to 9-day cultures.

In spite of all such precautions, the material showed a thick layer of peripheral necrosis (particularly at the upper,

free side of the explant) in 14-day culture in Difco 199 at 1 atmosphere carbogen, with or without additives (Pl. 1, fig. 2), and it was difficult to keep the material viable for 21 days, hence the toxic effect of oxygen, or perhaps carbogen, on the peripheral part of the explant (gas-organ interface) was considered, as suggested by Fainstat (1966) and Allison (1968).

Medium 199 buffered with HEPES (a non-metabolisable buffer) devised by Williamson and Cox (1968) for virological investigations with tissue cultures, does not need the presence of added CO_2 and cultures can be maintained in air. Shipman (1969) has also shown that media buffered with HEPES are suitable for various types of tissue culture.

199 buffered with HEPES proved to be advantageous. The medium did not need to be changed at frequent intervals for long-term culture, and the culture could be kept in a sealed, air-containing chamber.

As HEPES is more stable and non-metabolizable, the pH of the medium remained suitable during 24 days of culture without the medium being changed or the culture disturbed and survival was much better than in medium buffered with bicarbonate + CO_2. Evidently, by this time, living cells were probably relying to some extent on the remains of dead cells for nutrients, as the breakdown of some of the ingredients of 199 probably occurs during 24 days in an incubator. The

antibiotics present also lose their effect. The pattern of survival was markedly different from that of cultures in bicarbonate-buffered media in carbogen. There was no peripheral necrosis but there was an internal pycnotic zone with some necrosis (Pl. 1, figs. 3 and 4). This, in conjunction with the findings when explants are cultured in carbogen (Pl. 1, fig. 2) suggests that carbogen is toxic in the long term. An experiment intended to throw light on this problem was not entirely conclusive. Two explants of pars distalis were cultured in complete medium (not HEPES-buffered) in air with 5% CO_2 for 3 days. Peripheral pycnosis was probably rather less than in carbogen. Allison (1968) has pointed out that a high partial pressure of oxygen favours the release of lysosomal enzymes. Fainstat (1966) has suggested that a gas phase containing more than 90% O_2 is toxic to nearly all the cells in ovarian explants in organ culture, death of the cells occurring near the gas-organ interface and healthy cells near the centre. Smart (1963) suggested that epithelia with little intercellular space are more sensitive to changes in the environment.

The need of more O_2 than that present in air has been suggested by many workers (Trowell, 1959; MacDougall and Coupland, 1967; Biswas et al., 1967; Lucas, 1969); in their work there is no reference to peripheral necrosis or pycnosis.

The central pycnosis and necrosis observed in most of the explants cultured in medium buffered with bicarbonate + CO_2 or medium buffered with HEPES may be caused by deficiency of oxygen in the centre of the explant (Trowell, 1959, 1961). MacDougall (1964), MacDougall and Coupland (1967) and Lucas (1969) described central necrosis in their explants. It is, of course, possible (Trowell, 1961, in discussion with Murray) that toxic substances that do not readily diffuse, such as lactic acid, are produced and accumulate in the central region.

Some workers have used hyperbaric gas (oxygen with enough CO_2 to give the correct pH) for the culture of mature organs. In these experiments the extent of the central necrosis was reduced in some tissues. The experiments also indicated that different organs or parts of organs differ in their oxygen requirements in culture (MacDougall and Coupland, 1967; Biswas et al., 1967).

Difco 199 with additives, if buffered with bicarbonate and CO_2, appeared to give better survival than the plain medium, but this difference was much less noticeable with the HEPES-buffered medium.

A greater proportion of bicarbonate (0.15-0.22%) than that recommended commercially (0.035%) was found to be more favourable with bicarbonate-buffered Difco 199. This gave an

original pH of from 7.4 to 7.8. Probably because of the metabolic destruction of bicarbonate, the medium became more acidic during culture and had to be changed every 3 or 4 days. Dr. B. Athreya (personal communication) found that the pituitary (of the rat) survived better in a more alkaline medium (pH 7.6-7.8). HEPES-buffered medium showed little change in pH during culture.

II. Gross and histologic changes in the cultured pars distalis

The explant tends to flatten during culture otherwise there is little visible change. In a few instances the explant became translucent at the periphery because of marked peripheral necrosis. The blood capillaries shrank, even in short-term cultures, and the intercellular and perivascular spaces became progressively larger, with more PAS-positive material.

The peripheral region of the explant develops some intercellular cavitation which increases during prolonged culture. The cavities are probably of two types, one caused by cell death (Pl. 10, fig. 1), the other caused by movement of cells providing larger surface areas with microvilli (Pl. 12, fig. 1). When the periphery of an explant consists of living cells, the outermost layer assumes to some extent the appearance of an epithelium and generally develops

microvilli. The cells of the epithelioid tissue were generally chromophobic and stained more or less strongly by the PAS technique, as did the microvilli. Sometimes a few acidophil cells with prolactin-sized granular bodies occurred among the epithelioid tissue (Pl. 11, fig. 1). The peripheral cavities, which may be as large as several cells, are lined by epithelioid cells, sometimes with microvilli.

Cavities were comparatively more numerous in cultures in circulating medium. This may be related to the fact that fast flowing medium causes more damage in the outer part of the material than does a slower flow.

It was concluded that circulating medium is unsuitable for the culture of mature pars distalis (Trowell, 1961).

Two types of microvilli were found on epithelioid cells: smooth (usually associated with pale agranular cells) and rough with fuzzy coat (usually associated with dark agranular cells) (Pl. 11, fig. 1). This fuzziness of the microvilli and PAS-positive nature of the cells and microvilli may be associated with the formation of mucopolysaccharides by the dark cells. Hoyes (1967, 1968) has demonstrated the presence of acid mucopolysaccharide on the surface of fuzzy microvilli of human foetal epidermis.

III. Characteristic modifications of parenchymal cells during culture

Prolactin cells

In uncultured control material of the virgin female rabbit, the acidophil cells consist of many somatotrophs and few prolactin cells (Salazar, 1963; Allanson et al., 1966). In young immature animals (male and female) prolactin cells are very rare indeed. In the adult male there are relatively fewer prolactin cells than in the virgin female (Allanson et al., 1966). Salazar (1963) failed to find any prolactin cells in the male.

During culture of mature virgin female material, there is an indubitable relative increase in the number of prolactin cells, and the cells enlarge and the enlarged nuclei show prominent nucleoli. Similarly material from immature rabbits or adult males shows numerous prolactin cells after a period of culture.

In 3-day cultures, most of the prolactin cells showed normal granulation and r.e.r., and Golgi areas were well developed and sometimes showed secretory granules in the cisternae, and sometimes deformed granular material. This, and the appearance of electron opaque material in the spaces in the organ (Pl. 8, figs. 1 and 2), suggests that the cells are actively synthesizing and discharging their secretion.

After 6 days of culture, prolactin cells are more numerous and their granulation becomes more sparse and tends to be arranged at the periphery of the cell. Material cultured in plain medium appeared to have slightly fewer prolactin cells.

Signs of intense secretory activity (synthesis and discharge), such as well developed r.e.r. and enlarged Golgi areas (Foster, 1961) were seen at this stage. Small and large irregular granules were observed in Golgi cisternae (Pl. 13, fig. 1), and the cells contained numerous mitochondria. Continuity of the plasma membranes between some cells - probably prolactin and somatotroph - was seen (Pl. 9, figs. 1 and 2).

At 6 days the spaces of the organ contained more opaque material than in the 3-day cultures and sometimes secretion granules.

The 6-day cultures appeared to be very active in synthesis and discharge of secretion.

After 12 and 14 days of culture, prolactin cells are more numerous still. The maximum dimension of the granules tended to increase to 900 mμ (Pl. 5, fig. 3), with a few granules of up to 1μ and granules were sometimes irregular. Most of the cells had only few, peripherally arranged granules (Pl. 10, figs. 1 and 2). Several instances of the fusion of the granule-membrane with the plasma membrane could be identified with some measure of conviction, and also some granule-sized cavities.

Allanson et al. (1966) and Young et al. (1967) have reported an increase in the number of prolactin cells during pregnancy, and their increased secretory activity (synthesis and discharge) during lactation in the rabbit with the appearance of enlarged and irregular granules. Explants from the anterior pituitary of the human after a period of culture (Pasteels, 1962, 1963) are described as consisting entirely of hypertrophied prolactin cells, whilst cultures from rats contain very few of the other types of granular cells after 2-3 weeks of culture (Pasteels and Mulnard, 1961). Petrovic (1963a, b) has reported that during organ culture of the anterior pituitary of various mammals the prolactin cells are the most active, and that granules disappear after 4 or 5 days, but that granular cells reappear if the explant is transplanted into a host.

In the long-term cultures, many granules still retained their electron-opacity but pale granules also occurred. Pale granules have been observed at various stages of culture, but their significance is not understood. The difference in the opacity of granules cannot be explained on the basis of their maturity, since dark granules occur in the Golgi cisternae as well as near the plasma membrane and sometimes in the spaces of the explant. The existence of pale granules in the pituitary has been reported (Sano, 1962; Barer and

Lederis, 1966; Couch et al., 1969); they are usually regarded as being in a state ready for discharge.

There were two instances of intranuclear granules (one or two granules) of prolactin size (Pl. 10, fig. 2). The r.e.r. of the prolactin cells of 12- and 14-day cultures was well developed and was slightly vesiculated (though not as markedly so as in short-term cultures). Golgi areas were particularly well developed and associated with numerous small light and dark vesicles, and sometimes with secretion granules in the cisternae (Pl. 5, fig. 3). These signs indicate intense secretory activity and recall the characteristics of the prolactin cells of the lactating rabbit described by Allanson et al. (1966) and Young et al. (1967).

Peripheral coated vesicles, microtubules, microfibrils and lytic bodies (including multivesicular bodies) were observed at this stage, especially in 14-day cultures. One prolactin cell bore a cilium (Pl. 17, fig. 2).

When the material was cultured in HEPES-buffered medium, without additives, for 21 and 24 days, prolactin cells were more numerous than other types of cells (Pl. 11, fig. 1). Most of them were heavily granulated and the cytoplasm was usually dark, even in the few sparsely granulated cells. This darkness of the cytoplasm appeared to be caused by an abundance of free single- and poly-ribosomes.

In the 21-day cultures the Golgi area was well developed

and showed numerous vesicles and some granules in cisternae (Pl. 13, fig. 2). In 24-day cultures it was slightly less conspicuous but still showed many vesicles. The r.e.r., when present, was in the form of short stretches with dark contents (Pl. 5, fig. 5), a condition regarded as a first stage in the synthesis of hormone (Pasteels, 1963, describing the cells in the lactating rat). Moreover, nuclei were large, indented, sometimes with rods, and mitochondria were numerous, dark and thin (Pl. 11, fig. 1).

The spaces of the explant did not contain opaque material as in other cultures. In these cultures prolactin cells are probably not actively discharging secretory granules, but are perhaps synthesizing or resting.

Emmart and Mossakowski (1967) have shown (by applying a fluorescent-antibody technique to tissue cultures of fish pituitary) that most of the cells of cultured pars distalis are prolactin cells and that they possess an inherent ability to elaborate the hormone in vitro. Pasteels (1963) has pointed out that the prolactin cells which become degranulated during the first few days of culture acquire a granulation after 1 or 2 weeks.

Gyévai et al. (1969) have reported that acidophils live longer than basiphils during culture. Microfibrils, sometimes perinuclear (Pl. 14, fig. 3), in prolactin cells

become more numerous during culture, and the formation of villous folds in intercellular spaces, which occurred in short-term cultures, became much more evident as culture proceeded.

There were numerous coated vesicles throughout the cytoplasm and also near the plasma membrane, and most of the prolactin cells in long-term cultures (21- and 24-day cultures) showed pseudopodia, structures which might be related to movement or, like the microvilli which are very numerous in most of the cells, to absorption.

Many of the characteristics of prolactin cells in long-term cultures resemble those of the prolactin cells of a human pituitary tumour described by Peake et al. (1969), except that the cultured cells showed microfibrils which were reported to be absent in the prolactin cells of the tumour.

As prolactin cells are more or less degranulated during a phase of the culture (6, 12, 14 days) and acquire granulation at later stages it can be suggested that there is morphological evidence that these cells synthesize, form secretory granules and release them during culture and that a stimulation by the hypothalamus is not required for the synthesis.

This agrees with the generally held view (Everett, 1954, 1956; Meites et al., 1963; Pasteels, 1963; Talwalker et al., 1963) that the hypothalamus influences prolactin cells only

by inhibiting discharge and synthesis. The resemblance of autografts of rats pituitary (Rennels, 1962), in their cytology and physiology, to the tissue *in vitro* also favours this view, as both are more or less completely sheltered from an inhibiting hypothalamic influence.

Convey and Reece (1969), working on the rat, are the only investigators who postulate that a stimulation from the hypothalamus is necessary for the synthesis of prolactin. They found that 2 hours of suckling produced an 85.5% reduction in prolactin content, which, *in vivo*, is completely replaced during the subsequent 8 hours. They were unable to demonstrate that any restoration occurred when suckling-stimulated anterior pituitary was cultured in a flask for 2 hours *in vitro* and from this they deduced that stimulation by the hypothalamus is necessary for synthesis.

The cultures of pars distalis of female rabbit with hypothalamic tissue or extract showed fewer prolactin cells and increased numbers of somatotrophs and gonadotrophs, as compared to the material cultured without hypothalamus. The prolactin cells were moderately (or well) granulated and showed signs of reduced secretory activity, while somatotrophs and gonadotrophs showed signs of secretory activity. All this again favours the view of several workers (Pasteels, 1963; Meites *et al.*, 1963; Talwalker *et al.*, 1963) that

hypothalamus inhibits the release and synthesis of prolactin.

Pasteels (1963) has also shown an increase in number of somatotrophs and gonadotrophs, while prolactin cells remained unmodified, during the culture of rat and human pituitary with hypothalamic extract. He suggests the possibility of the transformation of prolactin cells into somatotrophs and gonadotrophs.

Mitoses in prolactin cells during culture were observed in colchicine-treated material and in a few routine cultures (Pl. 2, figs. 1 to 4), the cells containing a good complement of secretory granules. More frequently, mitoses of chromophobic cells were evident, some of these cells might be degranulated prolactin cells. Emmart and Mossakowski (1967) have stated that cultured pituitary cells undergo degranulation and become chromophobic during mitotic activity.

Pasteels (1963) stated that prolactin cells in tissue and organ culture showed mitotic activity. Allanson et al. (1969) have reported that granulated prolactin cells show mitosis during pregnancy and lactation; they say that when cell proliferation is most active, most of the dividing cells are degranulated.

Autoradiography has also shown labelling of the nuclei of some cells that could subsequently (by differential staining) be shown to be acidophils and chromophobes

(Pl. 2, fig. 5) or, in a few instances, basiphils. Brookes' stain was not effective with emulsion treated sections; consequently it was impossible to determine which type of acidophil was labelled. Mastro *et al.* (1969) are also of the opinion that anterior pituitary cells (rat) retain their differentiated state *in vitro*. They found labelling on acidophil cells during culture, though most labelled cells were chromophobes and a few basiphils.

It is concluded that, *in vitro*, prolactin cells from mature virgin female rabbits, adult males and immature animals hypertrophy, accumulate granules, synthesize as well as discharge their secretion and undergo mitosis, still retaining their differentiated state, for over 3 weeks. The hypothesis that prolactin continues to be synthesised and released during culture is further supported by the results of the combined cultures of pars distalis with mammary gland. However, it must be borne in mind that Rivera (1964) found that the mammary gland of the C3H mouse (used in the combined cultures) is caused to secrete milk by somatotrophin even in the absence of prolactin.

The mammotrophic activity of prolactin released during culture by the pars distalis of all classes of tetrapods has been shown (Nicoll *et al.*, 1966). Pasteels *et al.* (1963) are of the opinion that with human material, only prolactin is

secreted during culture and they do not agree that in the human, somatotrophin and prolactin are identical.

Somatotrophs

In the mature virgin female rabbit the pars distalis, before culture (Pl. 4, fig. 1), contains many more somatotrophs than prolactin cells (Salazar, 1963; Allanson et al., 1966).

During the first 6 days of culture, somatotrophs showed no marked change in number or cytological features, and there were morphological signs of their secretory activity (both synthesis and discharge) (Pl. 6, figs. 1 and 2).

Like the prolactin cells, the number of granules in a cell tended to be smaller after a period of culture. Mitoses were identified in a few somatotrophs after 3 days of culture.

By the 12-14th day of culture, a reduction (as compared with prolactin cells) in the number of somatotrophs occurred and signs of marked secretory activity were generally absent.

In 21- and 24-day cultures, somatotrophs were still less numerous and the endoplasmic reticulum tended to be reduced; apart from this the cells showed other features similar to those of prolactin cells, such as thin dark mitochondria, darkening of the cytoplasm, microfibrils, microtubules, pinocytotic vesicles and lytic bodies.

Similar results were obtained with material from adult

male and young immature male and female rabbits in respect to somatotrophs.

The somatotrophs thus showed evidence of reduced activity as culture progressed and there was a gradual relative reduction in their number but not a total disappearance.

During culture the somatotrophs of the rat and human become less active, their cytoplasm shrinks, they become less numerous and, in the human they disappear completely (Pasteels, 1962, 1963). There is a corresponding reduction in the liberation of somatotrophin. Because of the cessation of the hypothalamic inhibition, the cultured pars distalis is in conditions resembling those in a lactating rabbit. The reported increase of somatotrophs in lactating rabbits (Allanson et al., 1966) does not conflict with the observation recorded here that somatotrophs show signs of decreased activity and became less numerous in organ culture, since in cultures in the presence of hypothalamic extract, they remained rather more numerous and showed more signs of activity (Pl. 18, fig. 1). Moreover, castration or thyroidectomy (again conditions with resemblances to conditions of culture) reduces the functional activity of somatotrophs of rats (Farquhar and Rinehart, 1954a, b).

Rennels (1962) and De Virgiliis et al. (1968) have deduced that the secretory activity of somatotrophs is dependent to

some extent on hypothalamic stimulation and similar findings have also been recorded (Pasteels, 1963; Krulich and McCann, 1969; Couch et al., 1969). Their persistence during culture has been reported in explants from various types of mammal (Chen, 1954; Martinovitch, 1961).

Since it has been reported (Pasteels, 1963) that (with human material) prolactin cells may change into somatotrophs after the addition of hypothalamic extract to the culture medium, it must be stated that the results reported here would not contradict the reverse possibility of the conversion of somatotrophs into prolactin cells in the absence of any hypothalamic influence. The results however do not necessarily suggest that this does occur, and considerable evidence would have to be advanced to demonstrate so surprising a phenomenon.

Gonadotrophs

Many gonadotrophs are present in the uncultured control pars distalis of mature virgin female rabbit (Allanson et al., 1959; Salazar, 1963), they are much more numerous than prolactin cells (Pl. 4, fig. 1).

There was a considerable reduction in the number of gonadotrophs during the first 6 days of culture in complete medium, and the granulation of the cells became peripheral and sparse (Pl. 8, fig. 1). Very few examples of mitosis were

found.

Further reduction occurred in 12- and 14-day cultures and the cells showed signs of reduced secretory activity (e.g. reduced r.e.r.) and sometimes vacuoles were present in the cytoplasm. Tapp (1967), however, regards the vacuolisation of mucus-secreting cells as a normal feature of short-term organ culture.

After 21 days of culture in complete medium, very few gonadotrophs were seen, but the cells showed more normal features and there were occasional microfibrils and some small vesicles in the Golgi area. A similar reduction in gonadotrophs was seen with material from immature male and female rabbits and also adult male rabbits. Material cultured in plain medium for up to 24 days showed more gonadotrophs than with complete medium.

The pars distalis, in vitro, is removed from the influence of the gonads, and gonadotrophs are in conditions that may be comparable to those of the gonadotrophs of castrated animals, and it may be suggested that the results are similar to those of Foster and Cameron (1964), who reported a pronounced reduction in the number of mucoid cells in the pars distalis of the male rabbit after castration.

Essentially similar results with organ cultures to those described here have been obtained by Pasteels (1963) and

Petrovic (1963a) with material from rats, and by Sage (1966) with fish pituitary. These results with cultured material would be explicable as the result of the absence of a stimulus from the gonads, either directly or indirectly through the hypothalamus. Equally they would be explicable on the basis of the absence of a direct stimulation from the hypothalamus in the conditions of culture.

Farquhar and Rinehart (1954a) however, have described an increase in number and activity of gonadotrophs after castration of the rat.

The hypothesis of a direct stimulation of gonadotrophs by the hypothalamus is supported by the results of the experiments on pars distalis cultured with hypothalamus or extracts of it (Pl. 18, fig. 1). In these, the explants, after a period of culture, contained more gonadotrophs, and these showed more signs of activity, than did explants that were deprived of the influence of the hypothalamus. Pasteels (1963) has reported a re-appearance and increase in number of mucoid cells (probably gonadotrophs) in human pituitary in cultures to which hypothalamic extract was added.

Deighton and Meyer (1969) have stated that hypothalamus from the adult female rat is capable of stimulating synthesis of ovulating hormone in organ-cultures of anterior pituitary of 25-day old female rat.

Thyrotrophs

Thyrotrophs were numerous in uncultured controls (Pl. 4, fig. 1) but they soon ceased to be identifiable during culture and even in 3-day cultures the few thyrotrophs present showed degenerative features and very few secretory granules, which were pale and arranged peripherally (Pl. 8, figs. 1 and 2).

Presumably, during culture, these cells discharge their secretion and finally degenerate or become agranular, an outcome which is not surprising in view of the absence of any influence from the thyroid or hypothalamus.

Pasteels (1963) suggests that the thyrotrophs soon disappear in the cultured pituitary of rat and human. Petrovic (1963a) found that mammalian pituitary, during organ culture, showed thyrotrophs with very few secretory granules and reduced r.e.r. Sage (1966) reports that during the culture of fish pituitary, TSH-producing cells become more active after 6 days and Petrovic (1963a) believes that thyrotrophs persist in an agranular form during the culture of a variety of mammalian pituitary explants.

Degranulation and stimulation of thyrotrophs after thyroidectomy has been reported a number of times (Cardell, 1963; Farquhar and Rinehart, 1954b; Herlant, 1964).

In the present work, material that was cultured with

hypothalamus for 7 days showed no identifiable thyrotrophs.

Agranular parenchymal cells

Probably, most of the cells of cultured explants that are designated as chromophobes by light microscopy show a few granules by electron microscopy.

Apart from the cells here designated as stellate cells (following the nomenclature of Salazar, 1963), there seems no reason to suppose that other agranular parenchymal cells are anything more than a phase of granular cells (Pl. 9, fig. 1). They have the cytological characteristics of granular cells and usually all intermediate stages from agranular to heavily granulated cells may be detected. The different phases of granulation of granular cells has already been discussed. Cells in an agranular phase may develop microfibrils during culture.

Stellate cells

The existence of a type of agranular parenchymal stellate cell with many processes that tend to embrace and envelope neighbouring granular cells has been recognized by several authors (Salazar, 1963; Young et al., 1965; Scheohter, 1969). This type of cell often has an elongated nucleus, strands of r.e.r., free ribosomes (Young et al., 1965),

microfibrils (Salazar, 1963; Schechter, 1969, in the rabbit; Cardell, 1969, in the salamander, but not seen by Young et al.), and dense bodies and other inclusions (Schechter, 1969).

Stellate cells occasionally show one or two granules (Cardell, 1969), and are often associated with other cells by means of desmosomes (Schechter, 1969) and are sometimes described as abutting on perivascular spaces (Salazar, 1963; Cardell, 1969).

Most of these features have been identified in uncultured control material in the present work. These cells may be comparable with the follicular cells of the rat described by Farquhar (1957) and Rennels (1964), and the cells with stellate processes described by Rinehart and Farquhar (1955).

During culture the stellate cells (Pl. 7, figs. 2 to 5) change into a macrophage-like form and become relatively more numerous. During the first week of culture, when they are clearly distinguishable from the perivascular cells, they enlarge and begin to acquire the features of a macrophage, later the cells characteristically show pinocytotic vesicles and phagocytotic vacuoles, lytic bodies, lipid droplets and occasional microtubules and the microfibrils often became arranged in bunches and desmosomes are more frequently found. In general appearance, the cells usually became more opaque probably because of increased free ribosomes (Pl. 12, fig. 2).

Sometimes the cells show short stretches of r.e.r. with rather opaque contents. The pseudopodial processes of the cells became larger and more complex and, whenever associated with an enlarged intercellular space, tend to have microvilli and extensive villous folds resembling ruffled membranes (Abercrombie and Ambrose, 1958).

The macrophage-like cells take a large part in providing the epithelioid cells of surfaces and cavities of long-term cultures (Pl. 11, fig. 1), and resemble the epithelioid cells developed from monocytes in long-term culture (Sutton and Weiss, 1966).

Occasionally a pseudopodium appears to invade the granular cytoplasm of a neighbouring cell; this appearance has also been described by Rinehart and Farquhar (1955) with regard to perisinusoidal cells. The fact that, in long-term cultures, the cells believed to originate from the stellate cells often contain a few granules may result from a phagocytotic ingestion of granules rather than from synthesis.

Experimental animals were given injections of indian ink or colloidal gold in an attempt to identify phagocytic cells in the pars distalis and to follow their development during culture. These experiments all failed and no ingested particles could be identified in control or cultured material. Young et al. (1965) found that occasionally, after repeated

injections, perivascular histiocytes take up indian ink or trypan blue and, very rarely, type 5 cells (stellate cells) also appear to do so.

It cannot be doubted that the macrophage-like cells are phagocytic; they are often found among necrotic debris, sometimes surrounding it as small groups. Occasionally rounded forms occur (Pl. 12, fig. 2), probably gorged with debris like the lung macrophages described by Karrer (1958). Their failure to ingest indian ink may be because they are too distant from the blood in the pars distalis. It is, of course, possible that some macrophages of the cultured pituitary may be derived from blood cells (Sutton and Weiss, 1966; Cohn et al., 1966).

IV. Features of the cells of pars distalis and changes during culture

Intranuclear rods

Indentations of the nuclei were a common feature of the parenchymal cells of pars distalis during culture (Pl. 5, fig. 3 and Pl. 11, fig. 2). With increased time, deep finger-shaped invaginations of the nuclear membranes, or intranuclear rods, were observed in granular cells. They were straight or curved, and bounded by the double nuclear membrane, with distinct nucleopores. These intranuclear rods

appear in the enlarged nuclei of enlarged cells during culture, and give, of course, an enlarged area to the nucleus.

The occurrence of marked indentations of the nuclei of type 1 (prolactin) cells of pars distalis in pregnant female rabbits, has been reported by Young et al. (1967) and in cultured prolactin cells by Emmart and Mossakowski (1967).

Colonnier (1965) has described intranuclear rods in the neurons of the visual cortex and supports the view that they facilitate greater nucleocytoplasmic interactions.

The numerous nucleopores associated with intranuclear rods may be for the transfer of RNA (Kurosumi, 1961). Thus the occurrence of intranuclear rods may point to enhanced physiological activity.

Intranuclear rodlet

A compact intranuclear bundle of parallel elements has been observed in granular and agranular cells during culture (Pl. 14, fig. 5). In control uncultured material a similar structure has been observed in one cell (a thyrotroph).

Each element of the bundle measures about 100 Å, resembling a tonofilament. Sometimes the elements have the appearance of a tube measuring about 250 Å in diameter, resembling microtubules. Whether these elements are fibrils or tubules is not clear.

The dimensions also resemble those of spindle fibres and the meiotic midbody (Odot and Renninger, 1960), though the general appearance differs considerably from that typical of this type of organelle.

The straightness of the rodlet reduces the possibility that they are made up of microfibrils or microtubules; it seems possible that they are fine folds of the inner nuclear membrane.

Riley and Seal (1969) have reported the presence of intranuclear fibres in the keratinocyte nuclei of the epidermis of the ear of guinea pigs that had received 4-hydroxyanisole, and pointed out that either these fibres (said to resemble tonofilaments) are synthesized in situ or they originate outside the nucleus and enter it during an abnormal mitosis (comparing them with known instances of intranuclear mitochondria etc.).

Siegesmund et al. (1964) and Magalhães (1967) have described essentially similar rodlets in certain nerve cells. Siegesmund et al. (1964) ruled out the possibility of the rodlet being a result of virus infection.

Dahl (1970) considered bundles of fibres in the nucleus of pancreatic exocrine cells to be without any pathological significance.

Intercellular bridges (continuity of plasma membranes)

Intercellular bridges (Pl. 9, fig. 2) between similar or different types of cells (e.g. prolactin to prolactin or prolactin to somatotroph or agranular cell) have been observed. In some instances secretory granules have been seen in the bridge.

Rose (1960) has shown evidence of an exchange of cytoplasmic components between associated cells in culture.

Sutton and Weiss (1966) have described the disappearance of the plasma membranes between epithelioid macrophages during culture and the subsequent formation of multinucleate giant cells. If, as has been suggested by some authorities, pituitary parenchymal cells can change from one granular type to another (Severinghaus, 1937), it is possible that an exchange of material through such bridges might be a part of the mechanism.

Desmosomes

Desmosomes were rarely observed in control material and they were rare in short-term cultures. Young et al. (1965) have reported desmosomes associated with agranular (Type 5) cells of the rabbit and Salazar (1963) has also reported rare desmosomes in the rabbit.

In long-term cultures (e.g. 21 and 24 days) desmosomes were quite commonly seen on agranular cells; most of these cells

had the appearance of epithelioid cells or fibroblasts (Pl. 17, fig. 1). In material from young, immature animals, desmosomes were seen in 3-day cultures; a desmosome was observed associating a granular, probably a prolactin, cell and an agranular cell.

The cells of long-term cultures of adult material resemble, with regard to desmosomes, those of the immature rabbit after 3 days culture. Prolactin cells with well formed desmosomes have been reported in human pituitary tumours (Peake et al., 1969).

It has been suggested (Smith et al., 1969) that desmosomes maintain a cellular relationship sufficiently close to facilitate transfer.

Mitochondria

There is not much change in the morphology or number of the mitochondria except perhaps a slight increase in number during culture, particularly in prolactin cells and somatotrophs, up to 14 days. They retain a normal appearance except in a few instances where they had irregular swellings, probably artefacts after fixation rather than results of degenerative changes (Malhotra, 1968) because the cells with swollen mitochondria were quite healthy in other respects (Pl. 10, fig. 1).

In 21- and 24-day cultures, there is an increase in the number of mitochondria in all types of viable cell, granular and agranular; the mitochondria become filamentous (and occasionally branched) and dark, the cristae appearing as lighter lines against a dark background (Pl. 11, fig. 1). Consequently it seems that mitochondria multiply during culture. However, these cultures were maintained in air, and it is possible that the changes were related to the lower oxygen tension.

In these long-term cultures, the mitochondria are often in the neighbourhood of microfibrils in the granular or agranular cells. As these fibrils are probably associated with cell movement or synthesis of sedentary protein, it is not surprising that they should be close to mitochondria. Sutton and Weiss (1966) have also reported cytoplasmic filaments in a topographical relationship with mitochondria. Miller (1953) has also suggested that an increase of mitochondria is not related to release or discharge of hormone but rather to synthesis of hormone or precursor.

Lever (1955) has suggested that dense mitochondria change into lipid. Farquhar and Rinehart (1954a) have said that in the cells which are resuming functional activity and acquiring granules, mitochondria become dark, osmiophilic and appear to be of uniform density.

Microtubules

In the uncultured material, microtubules were observed in all types of granular cells of the pars distalis but they are rare or absent in agranular cells.

During culture there was no very notable change in the occurrence of microtubules that could be related to the period of culture, and they were observed in all types of cells, but less commonly in agranular cells.

Branson (1968) has suggested that the microtubules in the cells of the stratum granulosum of the epidermis of the newborn rat are connected with the elongating or flattening movements of the cells. Although, undoubtedly, cells change shape during culture, this is not associated with any evident change in the number or arrangement of microtubules.

Microtubules were rather more numerous in some of the cells discharging secretion (peripherally arranged granules, large Golgi etc.) and showing signs of active synthesis (Pl. 14, fig. 1).

This may suggest that microtubules have some connection with intracellular transport. Rodriguez et al. (1968) have described dense-core microtubules and suggested their possible function in the transport of newly synthesized material.

Microfibrils

In the uncultured control pars distalis, a few agranular cells (Pl. 7, fig. 1) and no granular cells showed microfibrils; there is no recorded observation of microfibrils in the granular cells of uncultured pars distalis.

During culture, microfibrils appeared in all types of parenchymal cell, and with increased duration of culture there was an increase in the occurrence of microfibrils. The fibres were arranged as a network or entangled to form a ball-like structure, or a small dense bundle (in agranular cells in long-term culture) or scattered in the cytoplasm (Pl. 14, figs. 2 to 4). Some agranular epithelioid cells had microfibrils more concentrated near the plasma membrane.

As suggested by Franks et al. (1969) and De Petris et al. (1962), these fibres may well be associated with cell movement as they become more evident during culture (when a certain amount of cell migration must be occurring) and are more numerous in the agranular epithelioid cells.

A perinuclear arrangement of these fibres in some granular cells, in long-term cultures (Pl. 14, fig. 3), in which r.e.r. has disappeared suggest that they may have some association with the disappearing ergastoplasm. A close association between free ribosomes and scattered microfibrils was also

observed. Microfibrils were abundant in the fibroblast-like cells that appeared during long-term culture (Pl. 12, fig. 2).

Young et al. (1967) have pointed out that sedentary protein synthesized by free ribosomes (Palade, 1958) may take the form of microfibrils.

Sutton and Weiss (1966) suggest a transport role for filaments which provide a surface for the movement of colloids; they also suggest a relation to movement.

Cilia

Electron micrographs of cultured material showed three examples of prolactin cells, one fibroblast and one probable gonadotroph each with a cilium. In one instance, the cilium emerged from the cell into the intercellular space (Pl. 17, fig. 2). The prolactin cells with cilia showed signs of considerable secretory activity, and in this resembled those described by Barnes (1961) and Lever (1962) in the pituitaries of mouse and rat respectively.

Cilia were not found in the electron micrographs of uncultured control material but Salazar (1963) has described them as occurring normally in the cells of the pars distalis of the rabbit, and Young et al. (1965) have reported their occurrence in the zona tuberalis of the rabbit.

Fibroblasts bearing a cilium (9 + 0) have been reported by

Wheatley (1969) to occur in tissue cultures of embryonic mammalian fibroblasts.

No suitable transverse section of a cilium was found in the electron micrographs and it was impossible to classify those occurring in these cultures.

Dingemans (1969) has stated that in the cultured adenohypophysis of the mouse, prolactin cells undergoing mitosis are devoid of cilia.

Lytic bodies

In cultured material, especially long-term, as compared with controls, multivesicular bodies, dense bodies (Pl. 14, fig. 3) and autophagic vacuoles were more commonly seen in the granular parenchymal cells, particularly prolactin cells. Similarly, macrophage-like cells characteristically showed phagocytotic vacuoles (Pl. 12, fig. 2).

Golgi areas were very well developed at 14 days of culture but were usually less so in long-term cultures (e.g. 24 days). Small vesicles both light and coated, were numerous in the Golgi areas. These findings accord with the view that Golgi vesicles represent the primary lysosomes (Friend and Farquhar, 1967) or proto-lysosomes (Gordon et al., 1965) which eventually participate in the formation of other types of lytic body, e.g. multivesicular bodies.

Smith and Farquhar (1966) observed an increase in the number of lytic bodies in the prolactin cells of post-lactating rats, and concluded that the increased lysosomes provide a regulatory mechanism to take care of over production of secretory granules. It seems unlikely that this would provide the explanation of the lytic bodies in the prolactin cells of the cultured rabbit pars distalis, which are more likely to indicate degenerative changes caused by culture conditions. De Virgiliis et al. (1968) suggested that when the secretory activity of the somatotroph returns to a normal rate after stimulation, the increased Golgi membranes are no longer necessary and the excess is segregated into multivesicular bodies, but agreed with Smith and Farquhar (1966) as to the probable regulatory role of multivesicular bodies.

Coated vesicles

Coated vesicles, which in uncultured material occur in small numbers in all types of cell in the pituitary, appear to become progressively more numerous during culture (Pl. 14, fig. 4). They are seen near the cell membrane or away from it, and found in all types of parenchymal cell but are perhaps more numerous in cells that show signs of physiological activity. They are also characteristic of the perivascular

cells (Pl. 16, fig. 1).

The peripheral vesicles have the appearance of being coated on both sides of the membrane (presumably derived from the plasma membrane), and are similar to those described by Bowers (1964) in the pericardial cells of an Aphid, when he suggested that coated vesicles are involved in the selective uptake of protein by the cell from the environment.

If coated vesicles are indicative of protein absorption (presumably pinocytotic), their presence shows that most cells in cultures, particularly long-term cultures, are actively engaged in absorption from their environment.

In material that was treated with H^3-thymidine during culture, some labelling was seen in the cytoplasm of certain branched elongated cells that resemble the perivascular cell (which are difficult to identify by light microscopy). It seems possible that this labelling may be caused by pinocytosed thymidine. As the cells were exposed to labelling for only an hour, the explanation given for a similar phenomenon in nervous tissue (Hopewell and Wright, 1970), that the cytoplasmic labelling is the result of cell death and phagocytosis, is very unlikely to apply to the instance noted here.

In addition to the coated vesicles near the periphery of the cell there are coated vesicles in the Golgi region (Pl. 17, fig. 2). These may be primary lysosomes (Friend and Farquhar, 1967), but there is no evident structural difference

between the coated vesicles of the Golgi region and those seen elsewhere.

Some dense-walled bowl-shaped depressions in the plasma membrane (often seen in long-term culture) have been regarded as an early stage in the development of a coated vesicle; however, similar structures in cultured monocytes are illustrated by Sutton and Weiss (1966) who suggest that they are lysosomes in the act of discharge.

Dark cells

A few dark cells, granular or agranular, were seen in early cultures and (Pl. 9, fig. 2), with increased duration of culture, there appeared to be an increase in the number of dark cells. The darkness of the cell does not depend on the secretion granules; in 21- and 24-day cultures most of the cells were dark (Pl. 12, figs. 1 and 2).

This darkness may be caused by an abundance of free ribosomes in the dark cells. The cells do not generally show signs of active discharge of secretion and it appears that they are in a stage of resting or restoring material for secretion; The presence of numerous coated vesicles, both deep in the cytoplasm and near the cell membrane, may support this view.

Lever (1955) refers to light, dark and intermediate cells in the adrenal cortex; they may represent different stages in the functional mechanism, and may become transformed one into another.

Young et al. (1967) have also referred to occasional dark cells, thought to be prolactin cells, in the adenohypophysis of the lactating rabbit.

Nebenkern and multilamellate bodies

An arrangement of r.e.r. in concentric whorls known as a nebenkern is generally regarded as indicative of intense synthetic activity. Instances of their occurrence in prolactin cells of rats after stimulation with oestrogen and during lactation (Hymer et al., 1961) and in rabbits during lactation (Young et al., 1967) have been recorded.

Similar formations were seen in prolactin cells during culture, especially the short-term cultures. In longer-term cultures nebenkern-like formations were observed (Pl. 13, fig. 3) with a markedly reduced ribosome content and narrower channels in the endoplasmic reticulum (similar structures were seen by Petrovic, 1963a) and these seemed to represent a transition from the nebenkern to an organelle made up of very closely packed fine whorls devoid of ribosomes and very similar to the multilamellate body described by Norman (1969) in

secretory cells of a fly after the release of secretion.

In the long-term cultures, some instances of multilamellate bodies devoid of ribosomes were seen (Pl. 13, fig. 4).

It seems possible that the nebenkern of an actively synthesizing cell may change into a multilamellate body (at least, during long-term culture). Moreover, a relation seems possible between this organelle and the origin of microfibrils which are frequently numerous in the neighbourhood of multilamellate bodies in long-term cultures (Pl. 13, fig. 4).

Pale granules

In light microscopy, explants became generally more PAS-positive as culture proceeded. Most of the PAS-positive reaction seems to be in perivascular and intercellular spaces and the normal diffuse PAS-positive reaction of the cytoplasm of cells becomes rather more marked. It appears that mucus is secreted by some cells, particularly the agranular type, after a period of culture.

In electron microscopy, cells that contained clusters of granular bodies with contents that range from very pale to quite opaque, occurred in a number of cultures, more commonly in long-term cultures. Some of these structures resembled, to

some extent, clusters of mucous granules. The significance of these pale granules is not clear. They may represent new-formed secretion in a state that is less dense than normal; they may represent granules that are undergoing dissolution or they may represent a new secretion of mucus. This last explanation would be similar to that advanced by Pasteels (1963) who found that human prolactin cells could, in culture transform into mucus-secretory cells (after the addition of hypothalamic extract). However, many of these structures have a resemblance to published illustrations of lytic bodies.

It has been shown by Berenson et al. (1958), Grossfeld (1959) and Daniel et al. (1961) that fibroblasts are a source of mucopolysaccharide during culture.

Rinehart and Farquhar (1955) have suggested that the mucopolysaccharides of the ground substance of perisinusoidal spaces is secreted by perisinusoidal cells.

Undoubtedly, fibroblasts and macrophages became relatively more numerous during culture and it is possible that the general enhancement of the PAS-positive reaction during culture reflects their secretion of mucus.

V. Material cultured with thiouracil

Only 6-day cultures were studied by electron microscopy. In these, as in cultures in ordinary media thyrotrophs were

not identifiable. Examples were seen of modifications in somatotrophs that were essentially similar to those described by Foster et al. (1969) and involved the dilation of the r.e.r. but no evidence of any dense secretion in the cisternae.

There would be little reason to expect thiouracil to have any effect on thyrotrophs since, in the animal, its effect on the pituitary thyrotrophs is an outcome of its direct effect on the thyroid.

VI. Intercellular spaces; perivascular spaces and the contained cells

A system of spaces permeates the pars distalis. Between the parenchymal cells there are intercellular spaces; these are separated from a space that surrounds the capillaries by the basement membrane of the parenchymal cells. The space surrounding the capillaries is called the perivascular space (perisinusoidal space, pericapillary space). It contains collagen fibres and is described as being filled with an amorphous ground substance (Young et al., 1965). The perivascular space is limited internally by the basement membrane of the capillary epithelial cells (Pl. 15, fig. 1).

The three-dimensional structure of the pars distalis has not been worked out, consequently the relationships of the numerous stretches of perivascular space is not known, nor is it known whether the parenchyma is basically one cell thick or

more than one cell. There is some confusion of terminology in the various descriptions of the pars distalis that probably springs from this. Some authors do not seem to have observed the basement membranes.

The capillary cells and the contained red blood corpuscles persist for a time during culture (Pl. 15, fig. 2). The capillary cells with their basement membrane may be recognisable after three weeks with the cells and lumen shrunken (Pl. 15, fig. 3). Bousquet and Meunier (1962) have reported the disappearance of the last traces of capillaries after 5 days of culture of rat pituitary.

Cells resembling histiocytes are generally recognised to occur in the perivascular spaces and this was confirmed in the present work. Cells considered to be fibroblasts were seen with pale cytoplasm and an indistinct plasma membrane in the perivascular spaces of control material. There is no other reference to the presence of fibroblasts in the pituitary.

During culture, as already noted, there is an increase of PAS-positive material, and sometimes dense electron-opaque material and more or less deformed secretion granules occur in the perivascular spaces (and intercellular spaces). Also, the perivascular spaces become dilated and collagen appears to become more abundant and basement membranes often become

very folded and sometimes unrecognisable.

The histiocytes become more macrophage-like with dense irregular nuclei, opaque cytoplasm, thin dark mitochondria, phagocytotic vacuoles and small vesicles (Pl. 16, fig. 1). The cells develop long pseudopodia which, in one or two instances, do appear, as described by Rinehart and Farquhar (1955), to enter granular cells in a region where, in the culture, the basement membrane appears to be lacking.

Large intercellular spaces (Pl. 17, fig. 1) with microvilli and villous folds occur in long-term cultures (24 days) and also in some short term cultures of young immature rabbits. The formation of these spaces appears to be the outcome of cell movements and would increase the absorptive surface of the explant. Their earlier appearance in young material probably reflects the greater viability of young cells that has been noted in other contexts. That these spaces are not the mere result of localised cell death is demonstrated by this young material in which cell death is rare.

VII. Discharge of secretory granules

The discharge of secretory granules from cells has been described by a number of workers; some report that secretory granules lose their individuality inside the cytoplasm of the

cell or in the inpocketings of the cell, and their substance is given out in an amorphous form (Sano, 1962).

Others (Pasteels, 1963; Couch et al., 1969) describe the intact granule leaving the cell and passing into the perivascular space, no longer with a granule membrane. They do not refer to the basement membrane which is ordinarily present (and appears to be visible in the electron micrograph published by Couch et al., 1969) which would prevent the extrusion of a granule into the perivascular space proper. De Virgilis et al. (1968) in their abstract, also refer to granules passing into the perivascular space. In their text however, they describe the granule passing into the "extracellular space" which the illustrations make clear is the space between the plasma membrane of the granular cell and its basement membrane.

Girod et al. (1965), in their illustration, show the extrusion of a granule into intercellular space from a prolactin cell of the hedgehog. Although organ culture would not seem to be a means of investigating granule extrusion, certain observations seem relevant. It is likely - and the findings support this - that material discharged from the granular cells will tend to accumulate more in the cultured organ than it would in vivo in an organ with circulating blood. In several cultures, recognisable granules, devoid of granule membrane, together with deformed granules and amorphous opaque material

occurred in intercellular spaces as well as in the space between parenchyma cells and basement membrane (Pl. 8, fig. 2). In these cultures there was evidence too of amorphous opaque material that had passed through basement membranes into the perivascular spaces.

This may indicate that granules per se pass out of the cell and lose their form, presumably by dissolving in tissue fluid in the intercellular space, and some evidence could be seen in the cultures of a mechanism of discharge by the fusion of granule membrane and plasma membrane as described by several authors.

Several authors have suggested that mature granules are partially dissolved before liberation. The existence of dark granules in the intercellular space appears to contradict this suggestion, but throws no light on the significance of the pale granules which occurred in many cultures.

In cultured material there are undoubted instances of secretory granules in the perivascular space (Pl. 16, fig. 2), sometimes free granules devoid of a membrane and sometimes granules within cytoplasm. The existence of granule-containing cytoplasm in the perivascular space (i.e. not separated from it by a basement membrane) may support the contention of Rinehart and Farquhar (1955) that parenchymal cells put out protuberances containing granules through the basement membrane into the perivascular space. Their electron micrographs

(and probably one by De Virgiliis et al., 1968) also provide some support for this view. Salazar and Peterson (1964) also describe cytoplasmic processes of parenchymal cells entering the intercellular space and there liberating granules either with or without a membrane, and also the existence of membrane-bound granules in the perivascular space.

The view that macrophage-like cells withdraw granules from parenchymal cells by means of pseudopodial extensions is advanced by Rinehart and Farquhar (1955).

The existence of macrophage-like cells containing granules, both in the parenchyma and perivascular space, in cultured material may support this view of Rinehart and Farquhar (1955) or the granulation of the cells may be the outcome of the phagocytosis of debris or extruded granules by these cells.

It is possible, of course, that the granular cytoplasm and free granules in the perivascular spaces seen in cultured material may be there because of the abnormal condition of culture and possible disruptions of basement membranes. The wide differences in the findings concerning granule extrusion may, of course, arise from different methods of killing, fixing and preparing material for electron microscopy that produce different artefacts.

SUMMARY AND CONCLUSIONS

1. Explants cultured for 24 days show areas of viable cells (about a quarter of the explant). Viability was assessed on the basis of general cytological features; DNA- and RNA-fluorescence with acridine orange; RNA reaction with pyronin, and the occurrence of mitosis.
2. Cultures in air with HEPES-buffered medium 199 gave better results than cultures in carbogen with bicarbonate-buffered 199; it seems probable that the high proportion of oxygen in carbogen is harmful.
3. Large explants survive better than small in long-term cultures.
4. Explants from young immature rabbits survive better than those from mature animals.
5. Epithelioid tissue with microvilli develops during culture at the exposed surfaces of the explant.
6. Cavities (generally lined by microvilli), which increase in number and size with the duration of culture, develop in the viable region of explants.
7. Perivascular spaces and intercellular spaces increase in size with prolonged culture and show a stronger PAS-positive reaction. Collagen fibres in perivascular spaces appear to increase in number.

8. An increase in the number of PAS-positive droplets in intercellular spaces occurs during culture.
9. Granular cells become lightly granulated during culture. This is more evident from 6 to 12 days of culture but later at 21 to 24 days they may become more heavily granulated.
10. From 3 to 14 days, prolactin cells are most active in synthesizing and releasing their secretion, at 21 and 24 days they appear to be more active in synthesis than release, or to be quiescent.
11. Prolactin cells, which are few in uncultured controls, become the most common type of granular cell in long-term cultures, and their secretory granules may increase in size.
12. Somatotrophs are more numerous than prolactin cells in uncultured material and in short-term cultures but less numerous in long-term cultures. In short-term cultures they show signs of secretory activity which diminish during further culture.
13. Gonadotrophs decrease in number during culture and show reduced secretory activity, though in 21 and 24 days cultures they were much more numerous than was expected.
14. Thyrotrophs disappear soon after 3 days of culture.
15. Agranular cells increase in number and some, the stellate cells, tend to become macrophage-like and electron dense.

16. Fibroblast-like cells were occasionally seen in perivascular spaces, but in some long-term cultures they were also seen among the parenchymal cells.

17. The cytoplasm of most of the cells becomes electron dense during prolonged culture.

18. Small light and dark Golgi vesicles, lytic bodies, pinocytotic vesicles, and desmosomes, become more numerous during culture. Microfibrils appear in granular cells and become more numerous in agranular cells during culture. An increase in the number of microfibrils, in long-term cultures, may be related to the presence of multilamellate bodies (which, themselves, may arise from the nebenkern of the actively secreting cell).

19. With prolonged culture, the nuclei of acidophil cells, particularly prolactin cells, become larger and indented with intranuclear rods, and nucleoli become more evident. These nuclear changes probably indicate an increased secretory activity.

20. Occasional intranuclear rodlets were seen in all types of parenchymal cell; they have not previously been recorded in the pars distalis.

21. In long-term cultures, mitochondria increase in number and become filamentous and electron dense.

22. More acidophils than basiphils were undergoing mitosis in 3-day cultured material. A similar number of chromophobes showed mitosis.

23. Secretory granules, sometimes deformed always without membranes, were observed lying freely in intercellular and perivascular spaces of cultured material. In many cultures, intercellular and perivascular spaces contained amorphous electron-dense material. This was interpreted as discharged secretion that had accumulated.

24. Explants cultured in plain medium do not differ much from those cultured in media with serum, though gonadotrophs are more numerous in explants in plain medium.

25. Combined cultures (in a suitable medium) of mammary gland (from mice) with previously cultured pars distalis, show stimulated secretion in the mammary gland and demonstrate that pars distalis, previously cultured for up to 7 days, is still synthesizing and liberating prolactin.

26. With hypothalamic extract or tissue, cultured explants show fewer prolactin cells and an increase in the number of somatotrophs and gonadotrophs, and these show signs of increased secretory activity.

27. Circulating medium was not satisfactory for culturing adult rabbit pars distalis though material from young rabbits gave good results.

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PLATES

All the figures are of pars distalis of mature virgin female rabbit cultured in complete Difco 199, except where otherwise stated.

PLATE 1

PLATE 1. Stained sections of various cultures, viable regions indicated by arrows.

Fig. 1. 3 days. x 70.

Fig. 2. 14 days. x 30.

Fig. 3. 21 days; plain HEPES-buffered medium. x 30.

Fig. 4. 24 days; plain HEPES-buffered medium, acridine orange stain. x 50.

Fig. 5. Same as Fig. 4, viable region. x 560.

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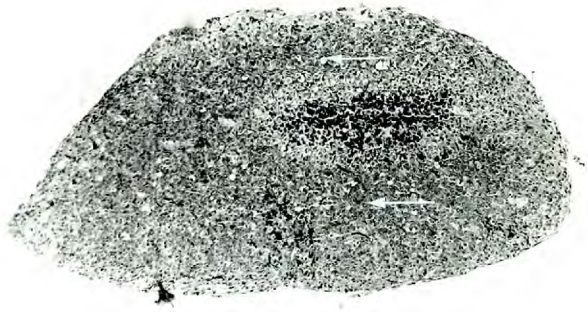

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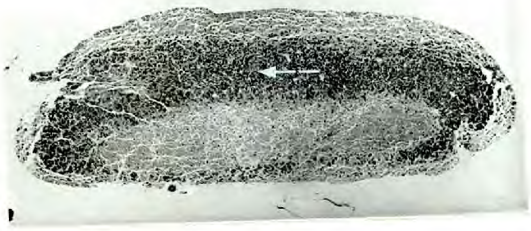

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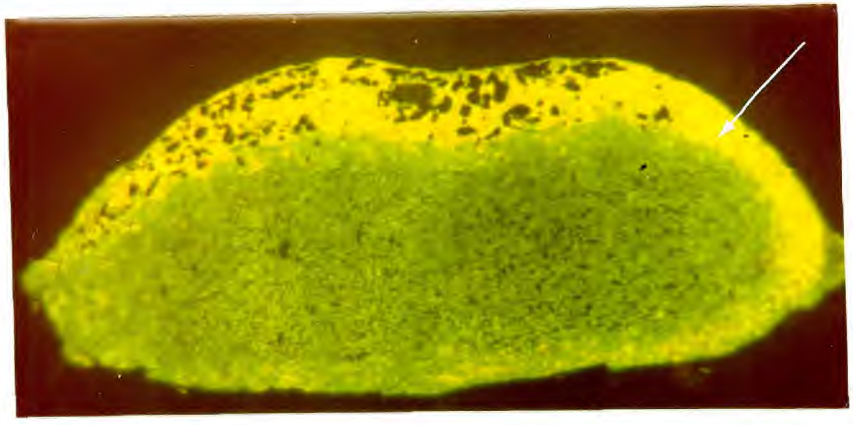

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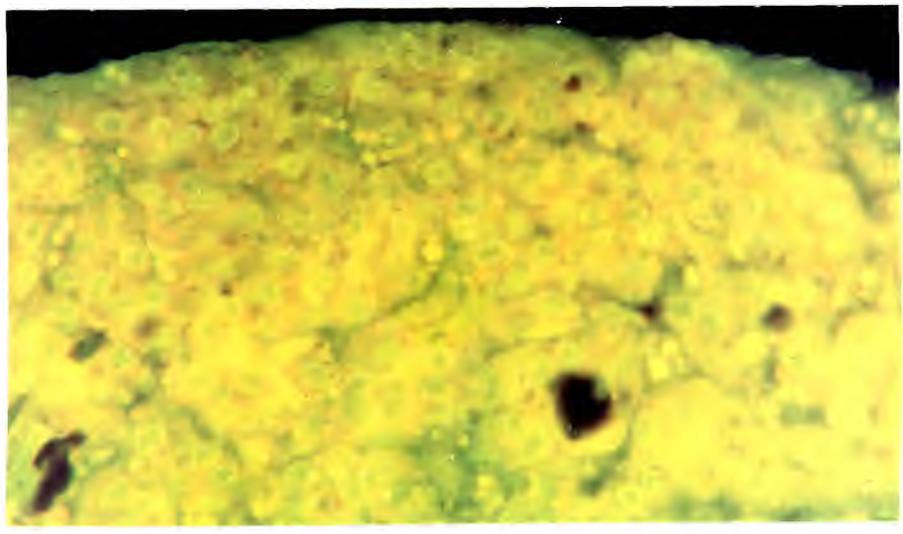


PLATE 2

PLATE 2. Stained sections of various cultures. Aspects of cell division indicated by arrows.

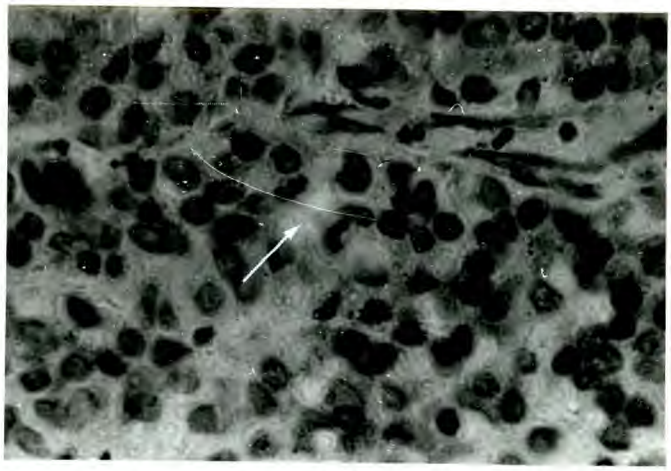
Fig. 1. 3 days; acidophil in telophase. x 800.

Fig. 2. 3 days; acidophil in metaphase. PAS/orange G stain. x 560.

Fig. 3. 3 days; colchicine treated. Mucoid cell in mitosis. Prolactin cells (reddish orange), somatotrophs (yellow), mucoid cells (bluish green) and chromophobes (pale green). Brookes' stain. x 560.

Fig. 4. 3 days; colchicine treated. Prolactin cell in mitosis. Brookes' stain. x 560.

Fig. 5. 1 day; H^3-thymidine-labelled nuclei (2 chromophobes, 1 acidophil). x 880.



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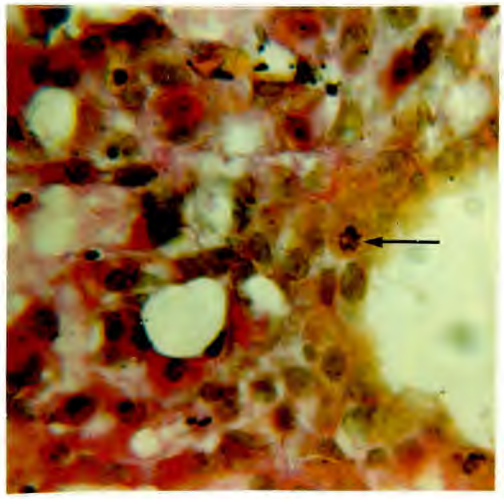

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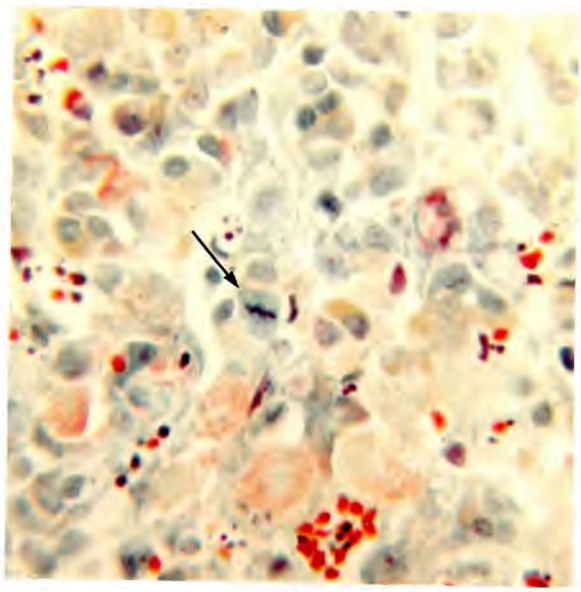

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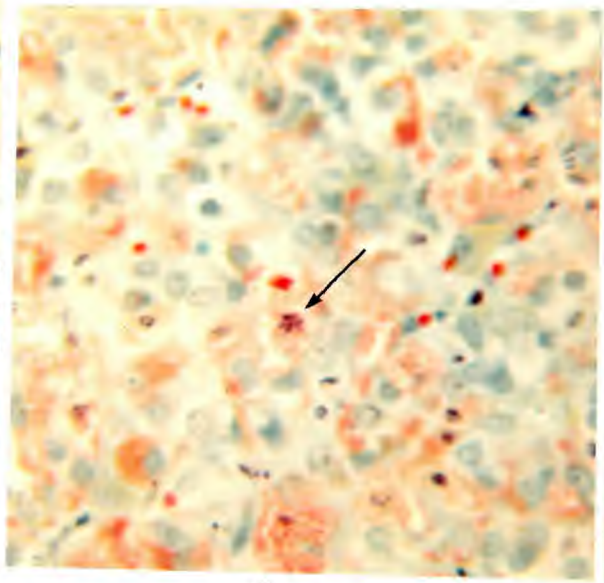

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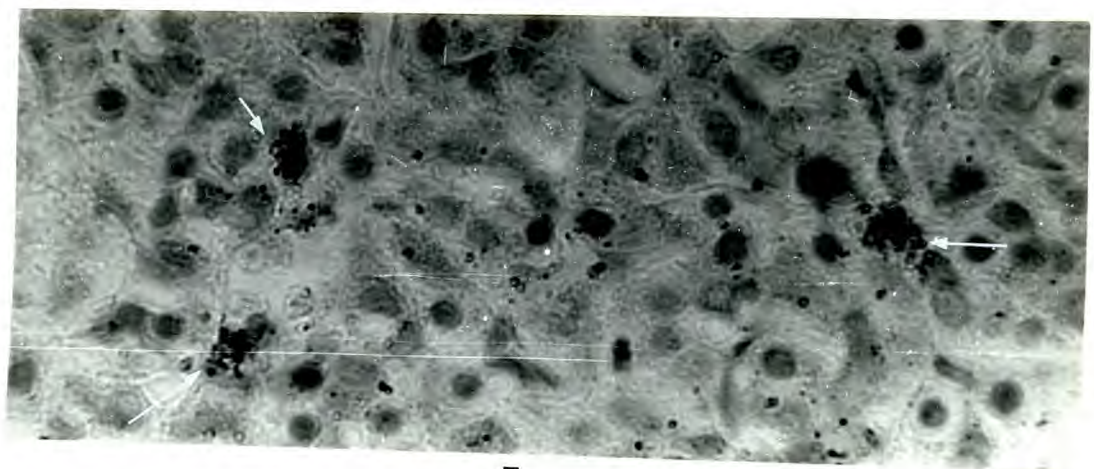

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PLATE 3

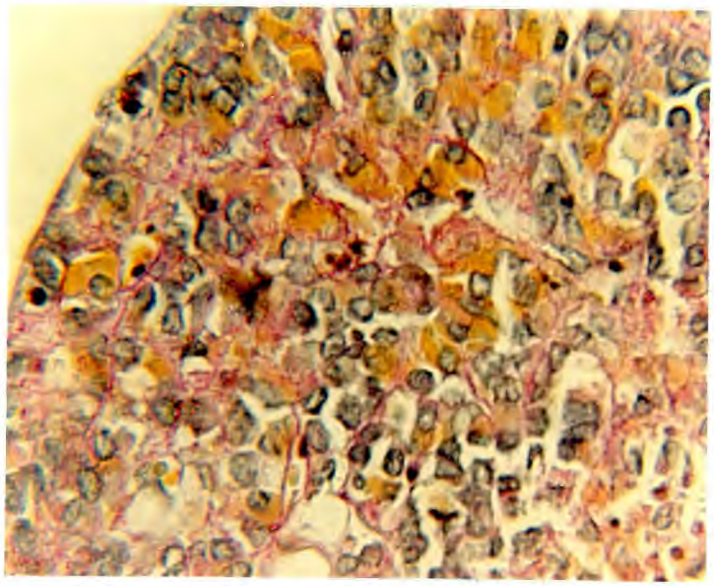
PLATE 3. Stained sections

Fig. 1. 21 days; complete HEPES-buffered medium, viable region showing acidophil cells (orange) and chromophobes (pale pink or unstained cytoplasm), PAS/orange G stain. x 560.

Fig. 2. 21 days; plain HEPES-buffered medium. Internal region, most cells pycnotic or necrotic. PAS/orange G stain. x 560.

Fig. 3. 24 days; plain HEPES-buffered medium. Viable region with epithelioid coat (arrow). Prolactin cells (red) and chromophobes (pale green). Brookes' stain. x 560.

Fig. 4. Mammary gland after combined culture with 7-day cultured pars distalis. Lumen with secretion. x 400.



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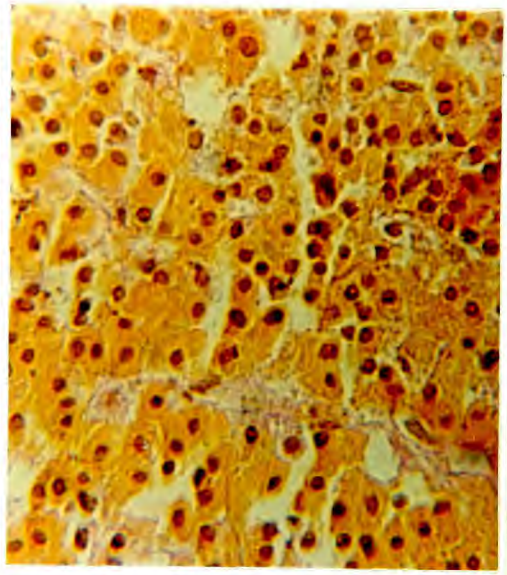

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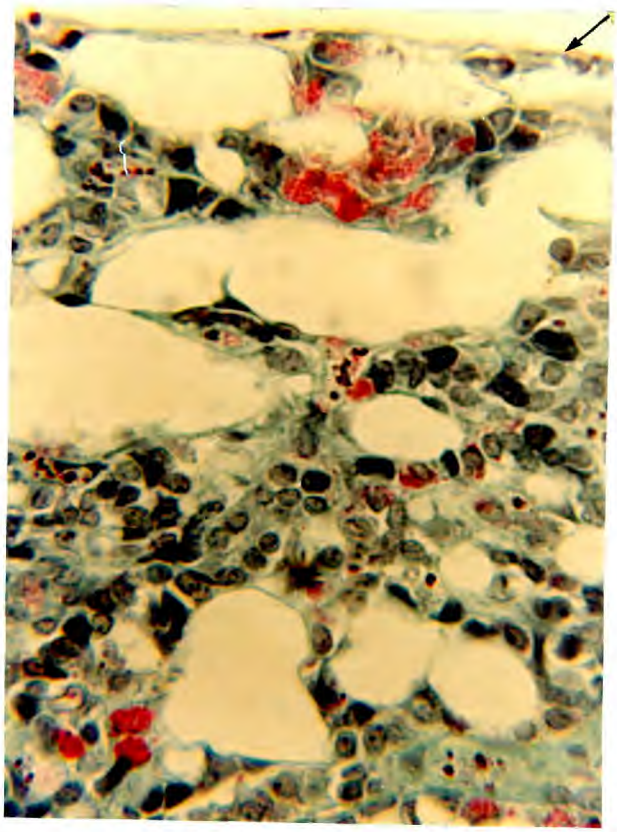

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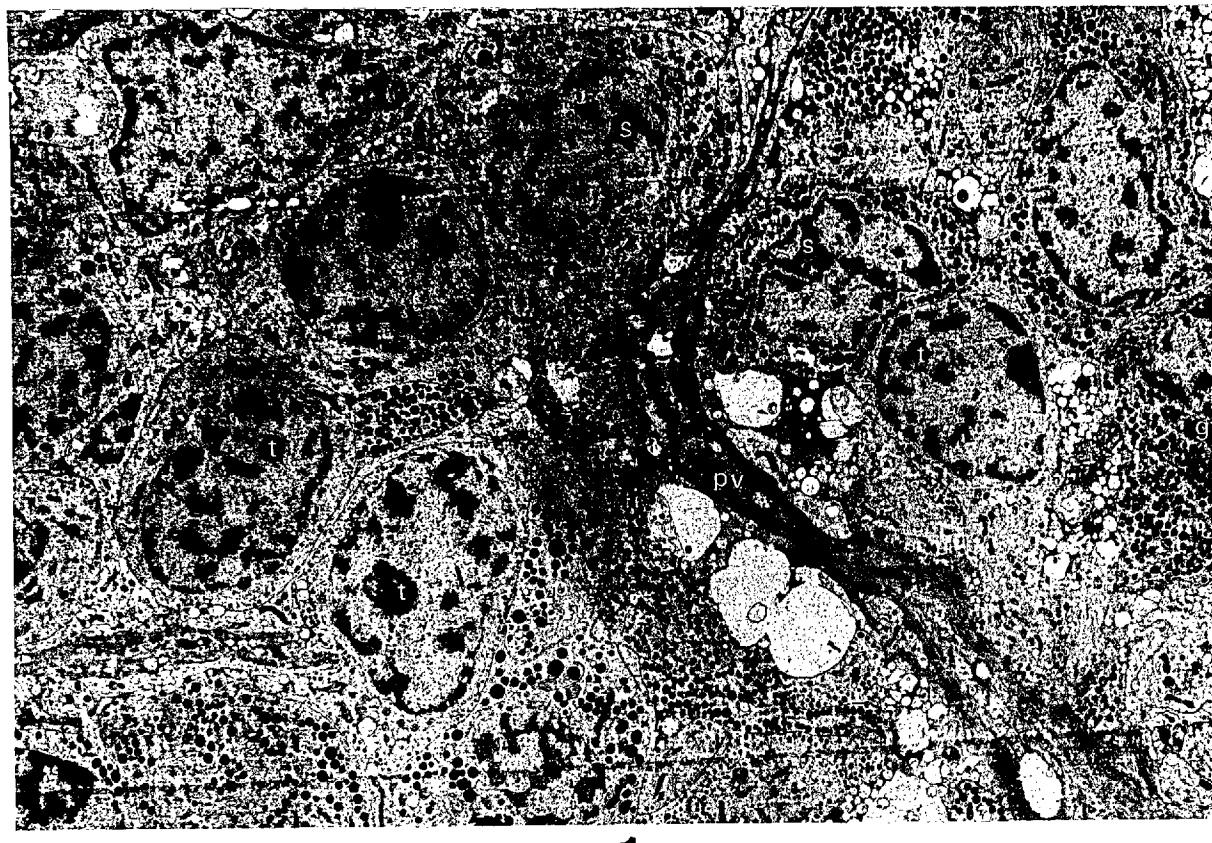
PLATE 4

PLATE 4. Electron micrographs of pars distalis.

Fig. 1. Uncultured, control material, prolactin (p), somatotrophs (s), gonadotroph (g), thyrotroph (t), stellate cell (st) and perivascular space (pv). x 4,500.

Fig. 2. A pycnotic cell, surrounded by viable cells. x 6,000.

Fig. 3. Necrotic material with perivascular space (pv). x 3,000.



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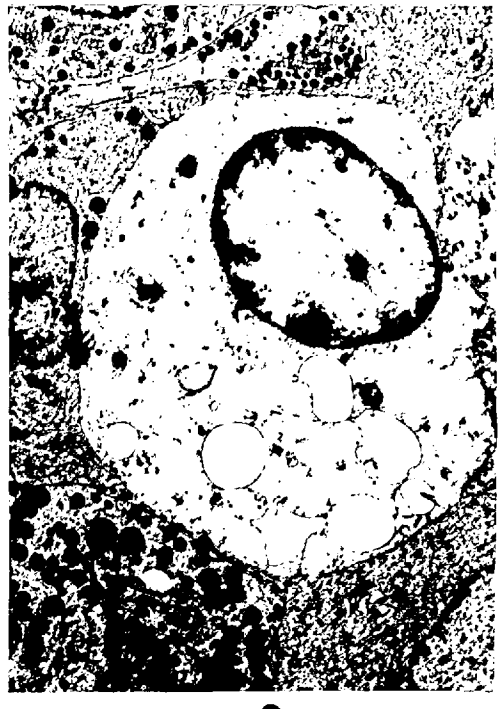

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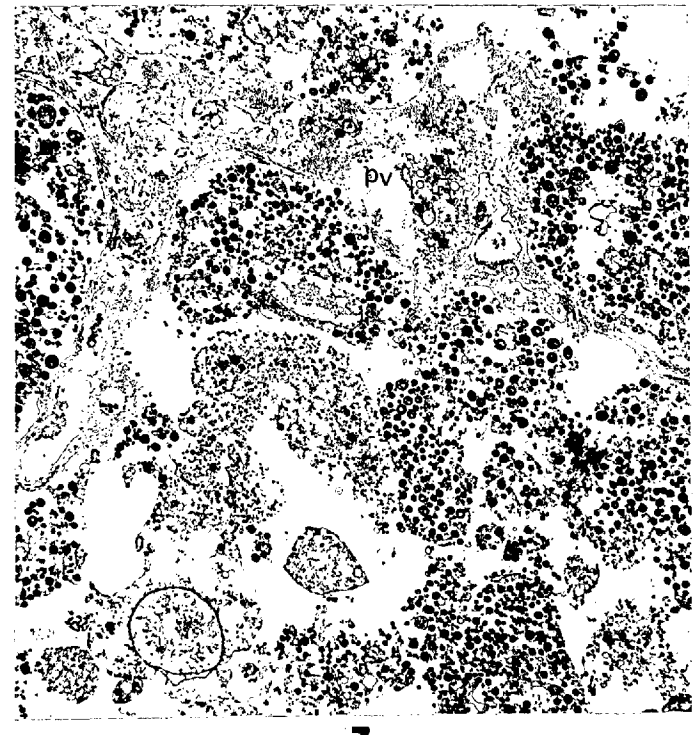

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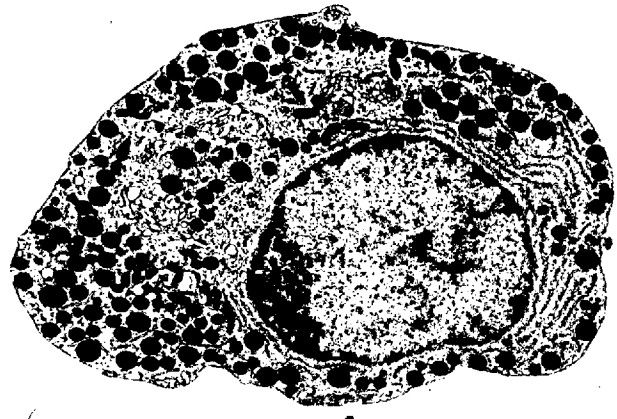
PLATE 5

PLATE 5. Electron micrographs of prolactin cells after 3 days culture (Fig. 1), 6 days culture (Fig. 2), 12 days culture (Fig. 3), 21 days culture (Fig. 4) and 24 days culture (Fig. 5). x 6,000.



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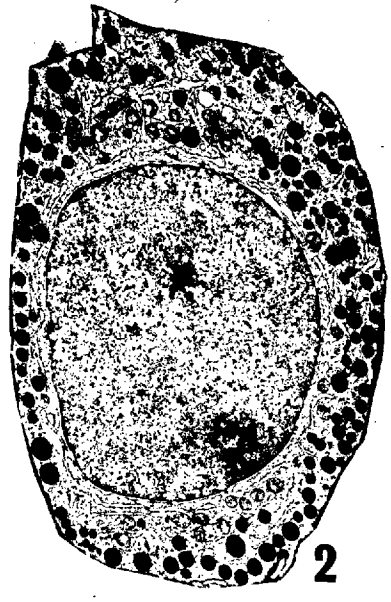

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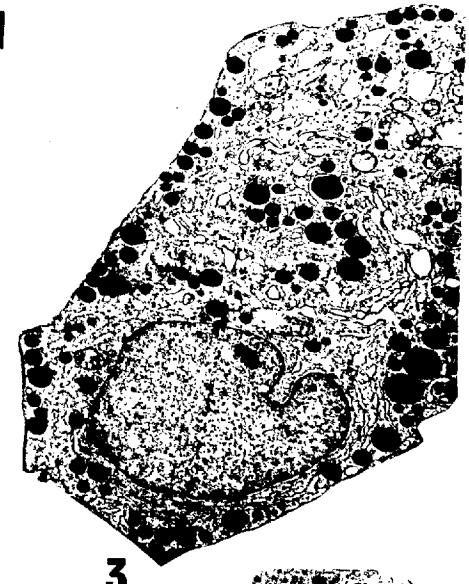

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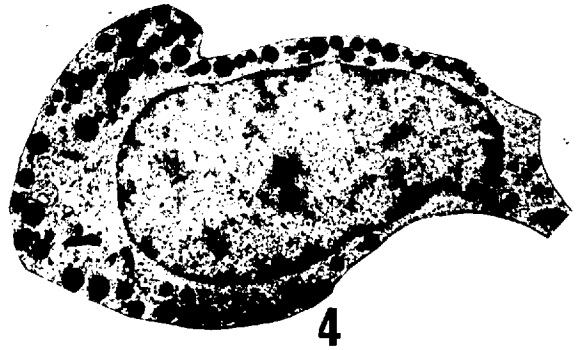

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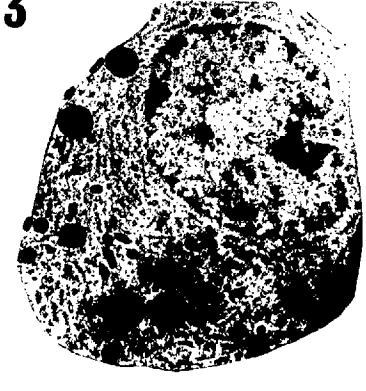

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PLATE 6

PLATE 6. Electron micrographs of somatotroph cells after 3 days culture (Fig. 1), 6 days culture (Fig. 2), 12 days culture (Fig. 3), 21 days culture (Fig. 4) and 24 days culture (Fig. 5). x 6,000.

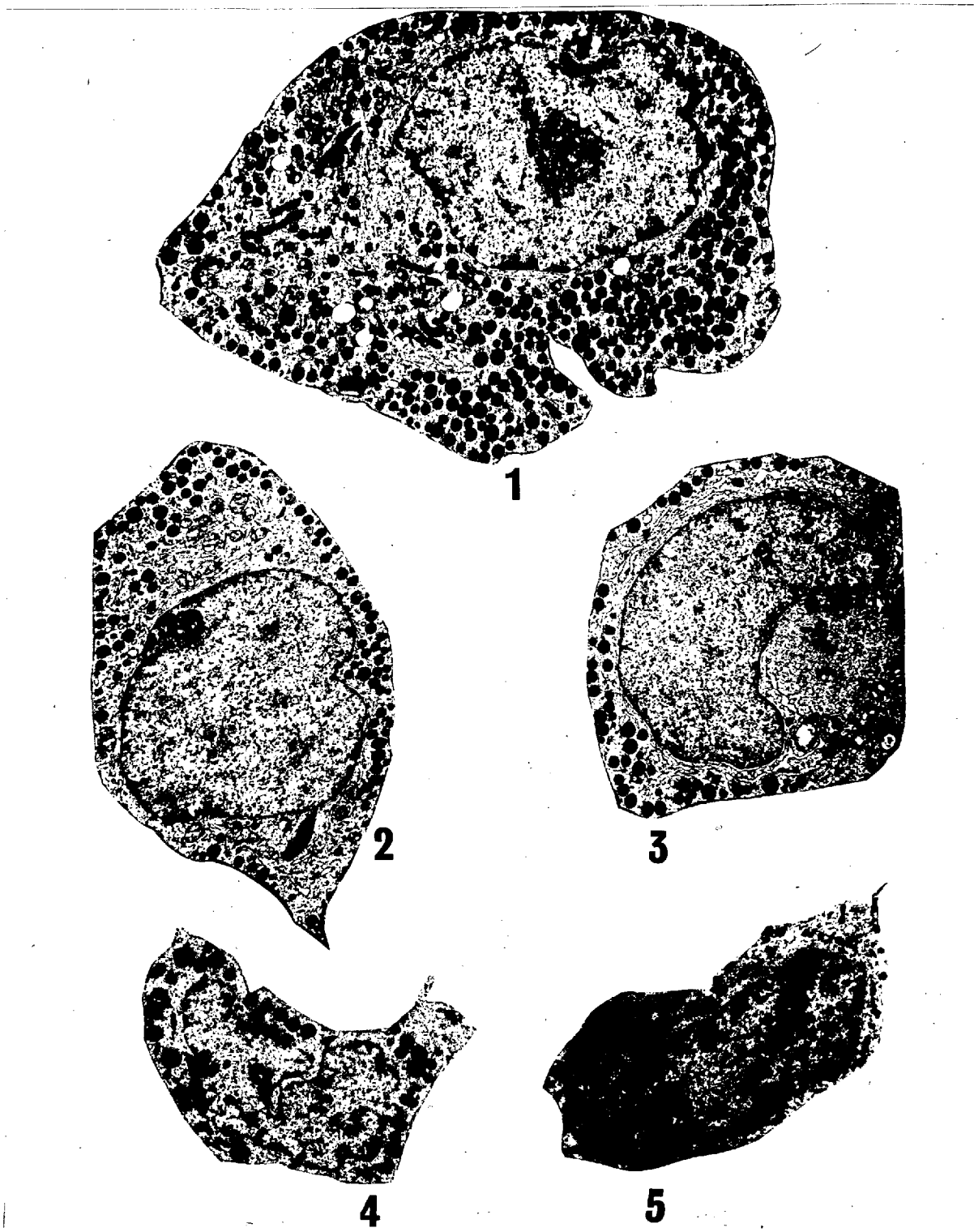
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PLATE 7

PLATE 7. Electron micrographs of stellate cells. Uncultured control (Fig. 1) and after 3 days culture (Fig. 2), 6 days culture (Fig. 3), 21 days culture (Fig. 4) and 24 days culture (Fig. 5). x 6,000.

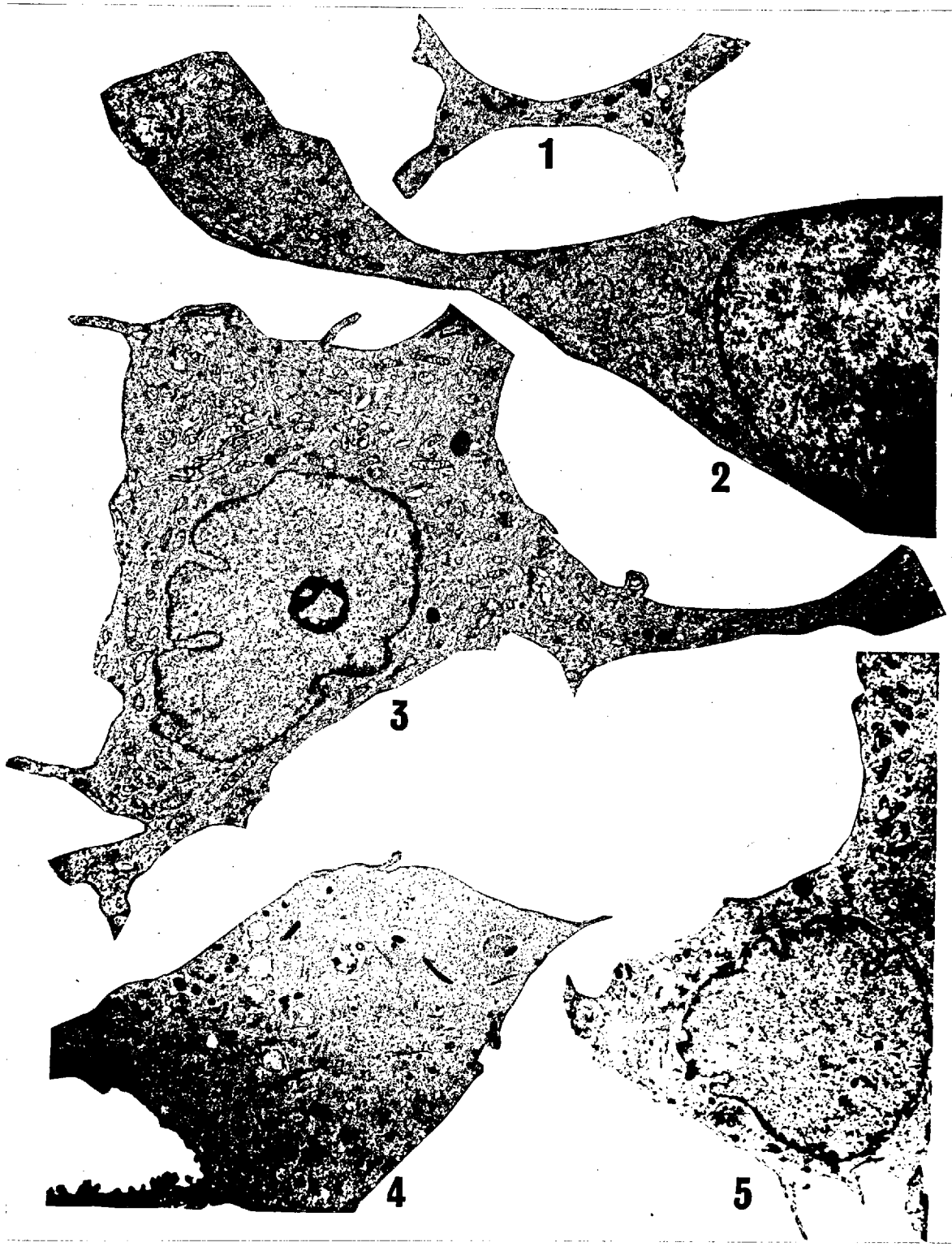
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PLATE 8

PLATE 8. Electron micrographs of a 3-day culture. Prolactin (p), somatotroph (s), gonadotroph (g), degenerating thyrotroph (t) and stellate (st) cells. Arrows indicate intercellular spaces.

Fig. 1. Some granular cells are moderately granulated and some lightly. Well developed r.e.r. and Golgi areas in acidophils. Thyrotroph probably degenerating. x 6,000.

Fig. 2. Intercellular spaces contain electron opaque material and some deformed granules (arrows), erythrocytes (rbc). x 6,000.

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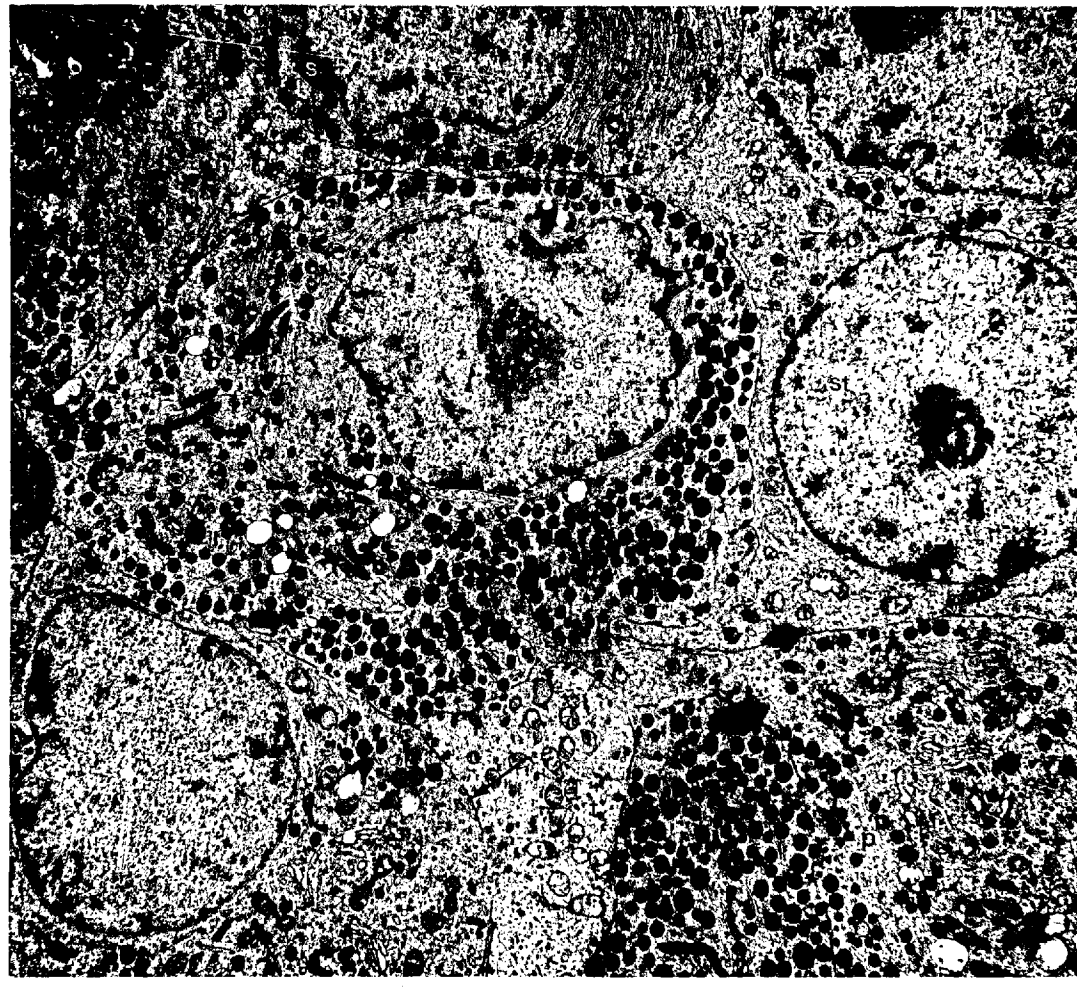

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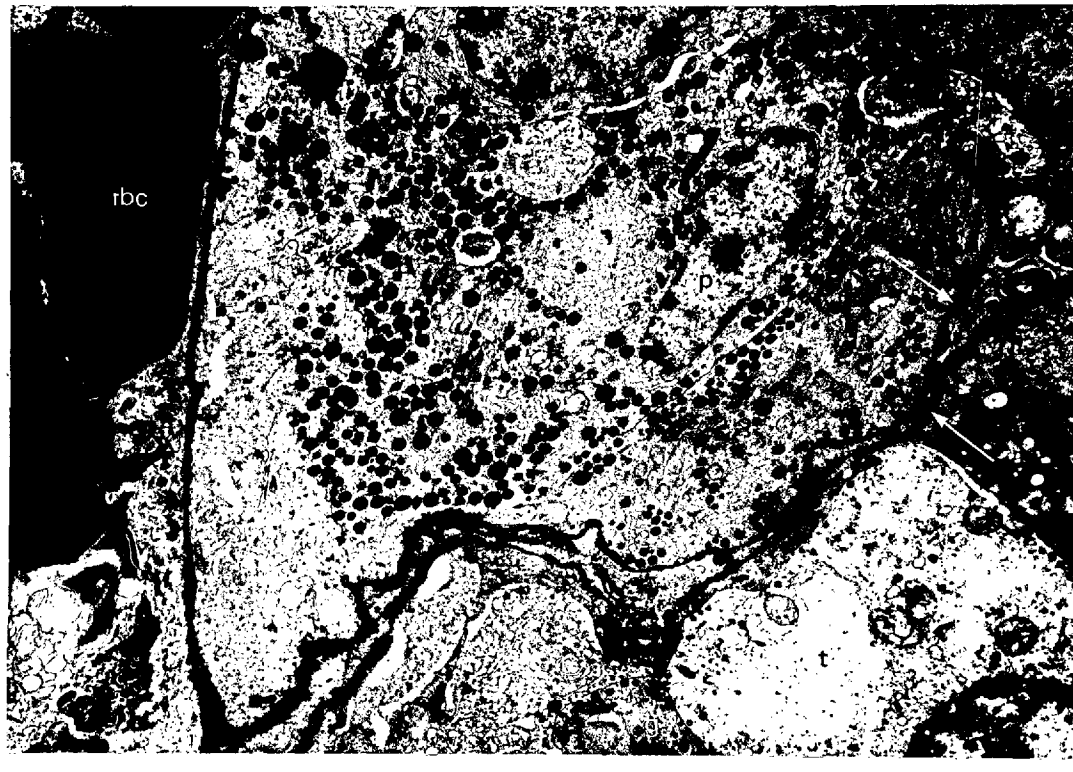

PLATE 9

PLATE 9. Electron micrographs of a 6-day culture. Prolactin (p) and somatotroph (s) cells. Dark intercellular spaces indicated by arrows, stellate cells (st), agranular cells (ag).

Fig. 1. Granular and agranular cells, some with well developed r.e.r. and Golgi areas. x 6,000.

Fig. 2. Cells with peripheral light granulation. Intercellular bridges (b) between a somatotroph and a prolactin cell. A central cell has dark cytoplasm. Microtubules and microfibrils in both types of granular cell. x 6,000.

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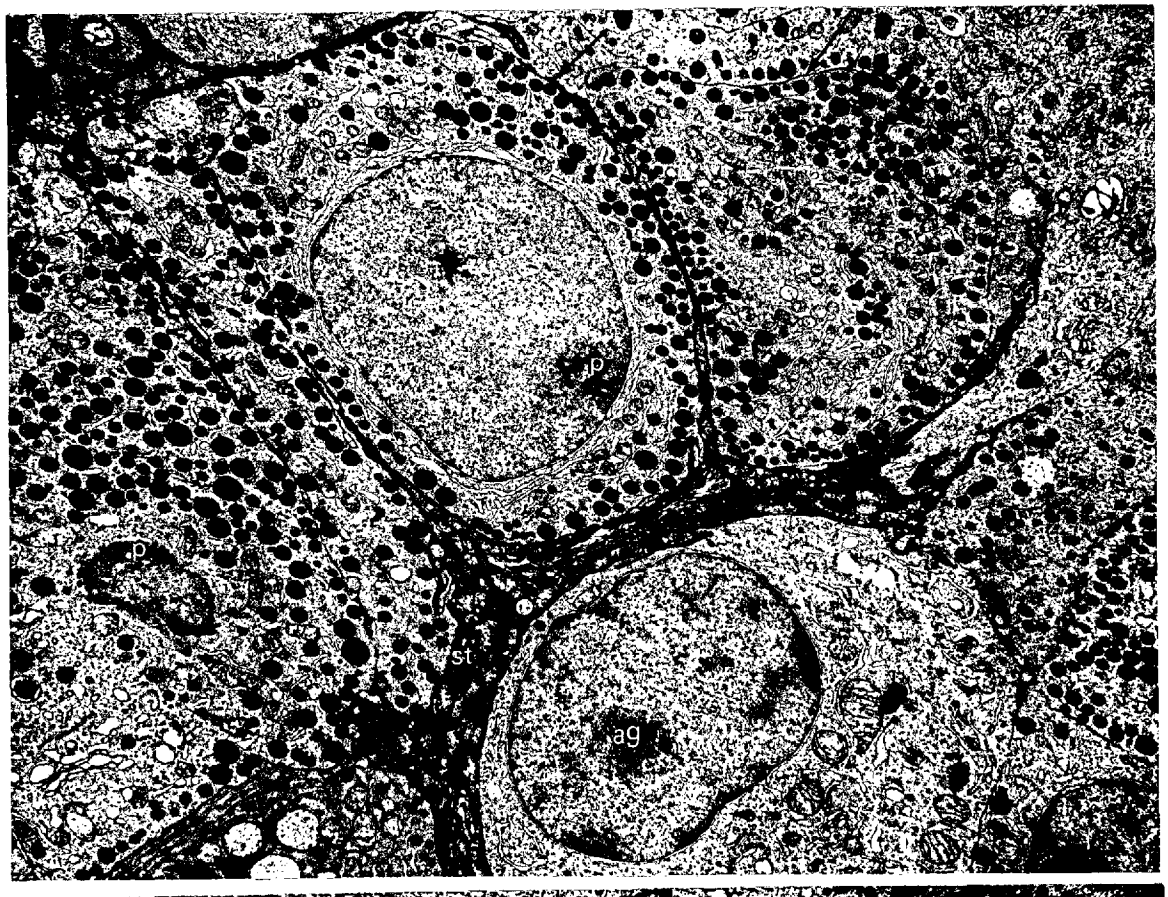

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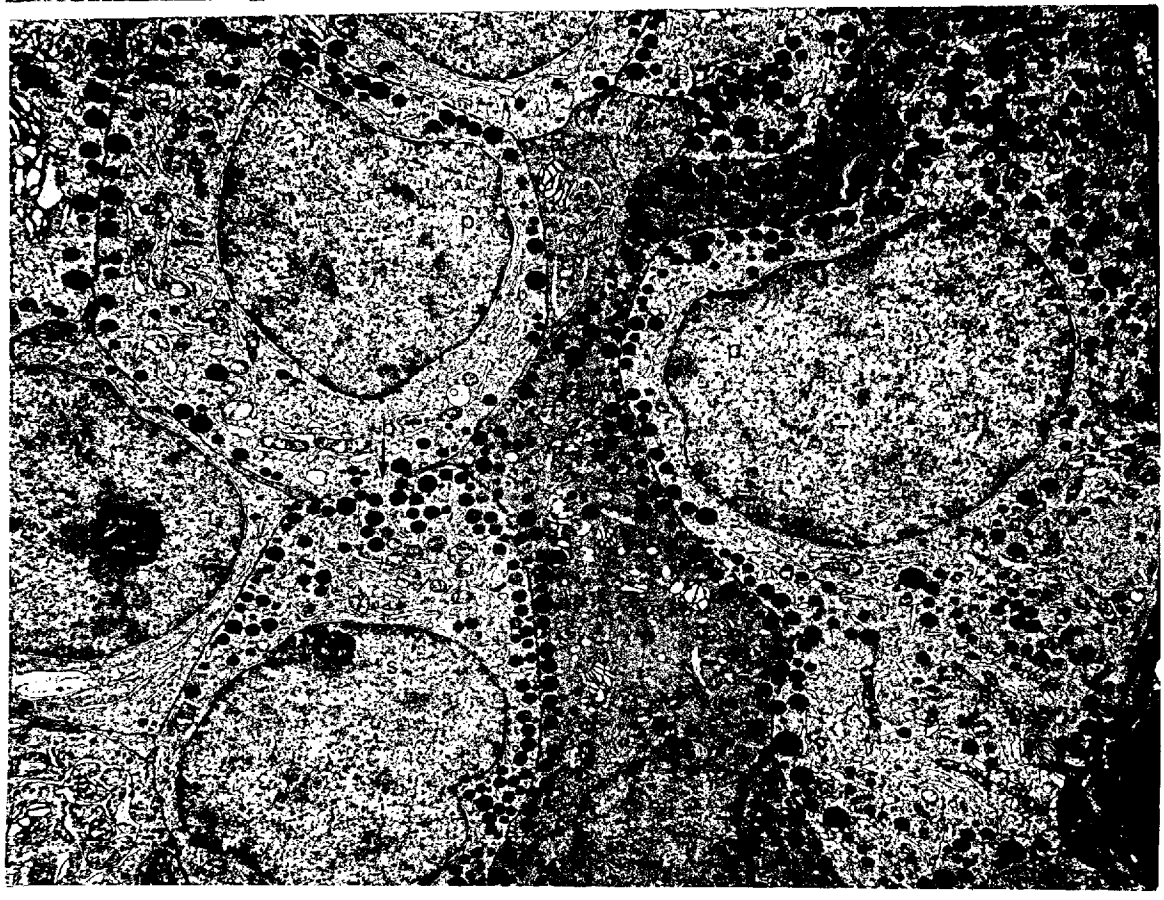


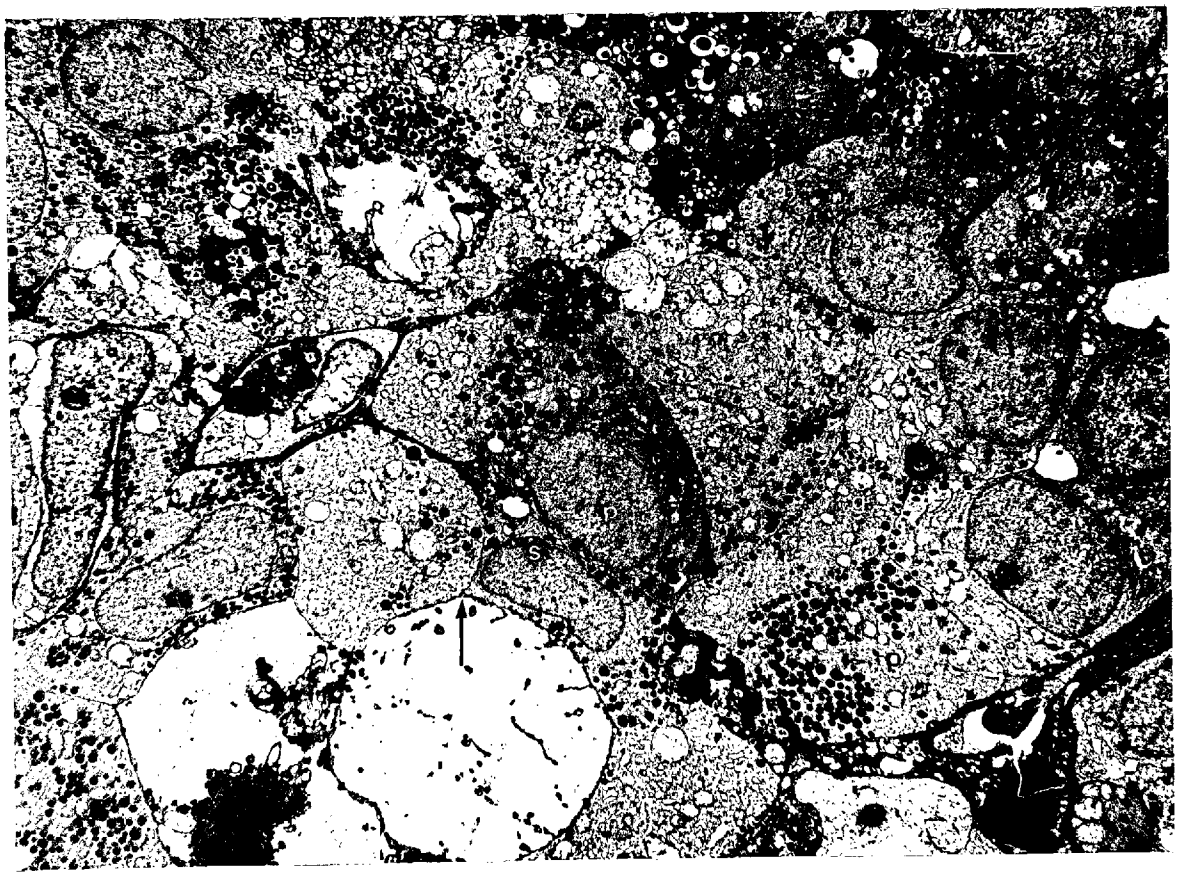
PLATE 10

PLATE 10. Electron micrographs of a 12-day culture. Prolactin (p), somatotrophs (s) and gonadotroph (g) cells. All intercellular spaces are dark. Inflated mitochondria probably fixation artefact.

Fig. 1. Prolactin cells more numerous than somatotrophs. Some cells have light peripheral granulation. Nuclei indented. A cavity of about the size of a cell (arrow). x 3,000.

Fig. 2. Prolactin cells with well developed Golgi areas and slightly vesiculated r.e.r. Centriole (arrow), intranuclear granule in neighbouring cell. x 6,000.

1



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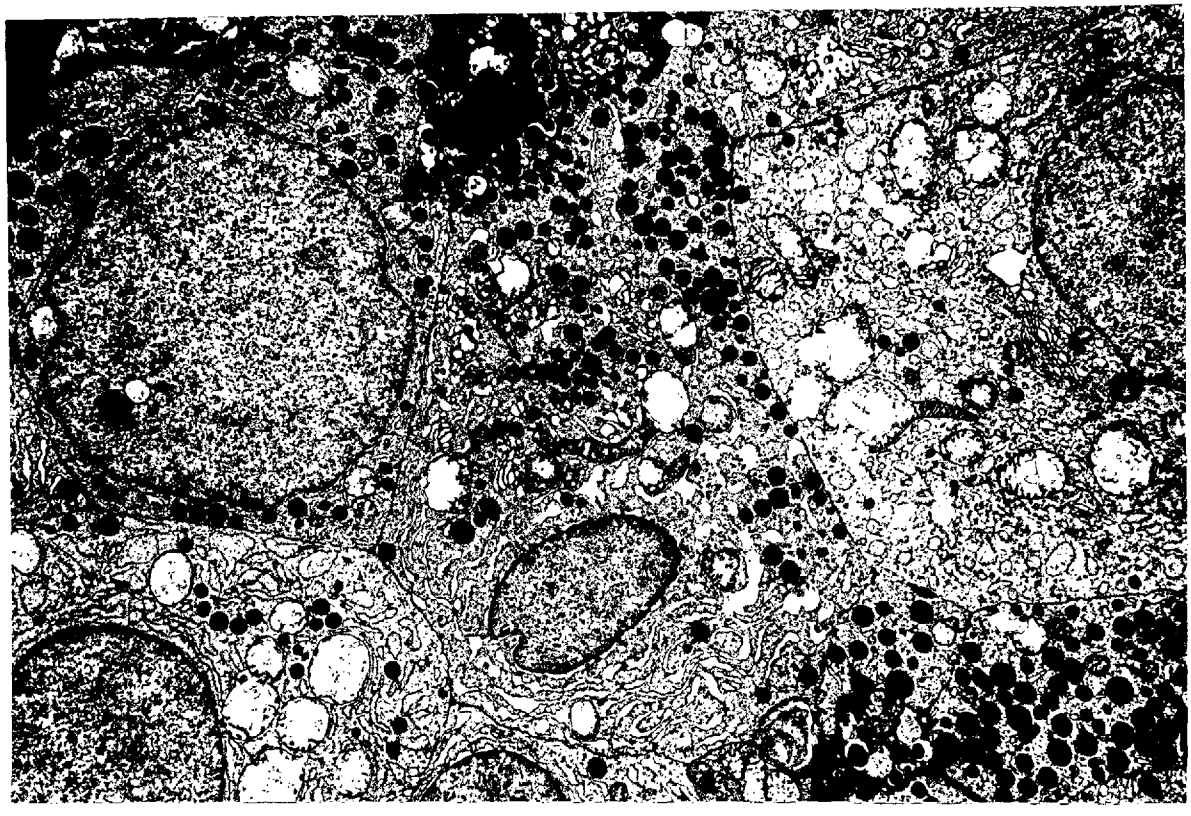
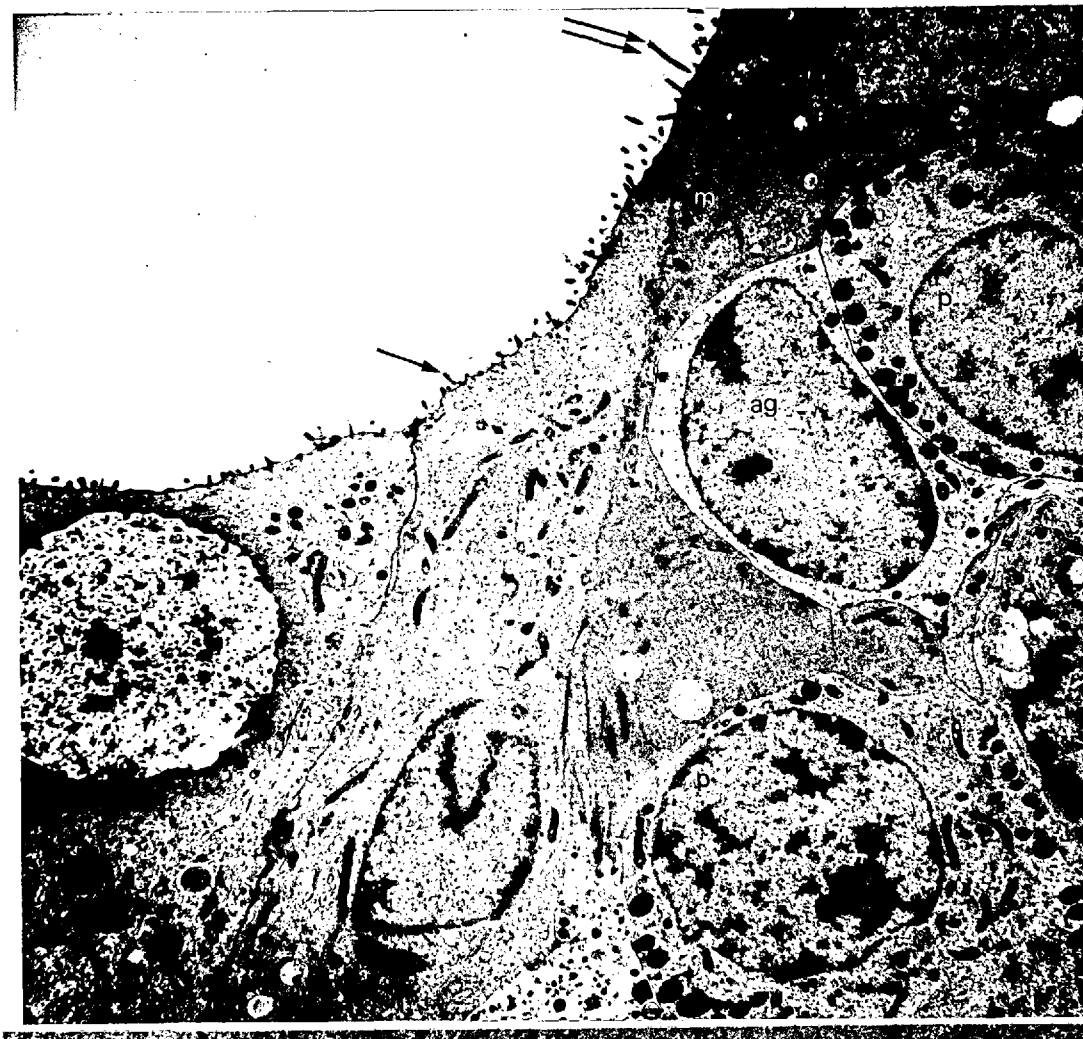


PLATE 11

PLATE 11. Electron micrographs of 21-day cultures.

Fig. 1. Prolactin cells (p), a dark irregular stellate cell (st), and an agranular cell (ag). Free surface of explant shows smooth (arrow) and fuzzy microvilli (double arrow). Dark thin mitochondria (m) in all types of cell. Plain HEPES-buffered medium. x 6,000.

Fig. 2. Agranular parenchymal cells. Intranuclear rod (arrow), lytic body (l), lipid droplets (ld). x 6,000.



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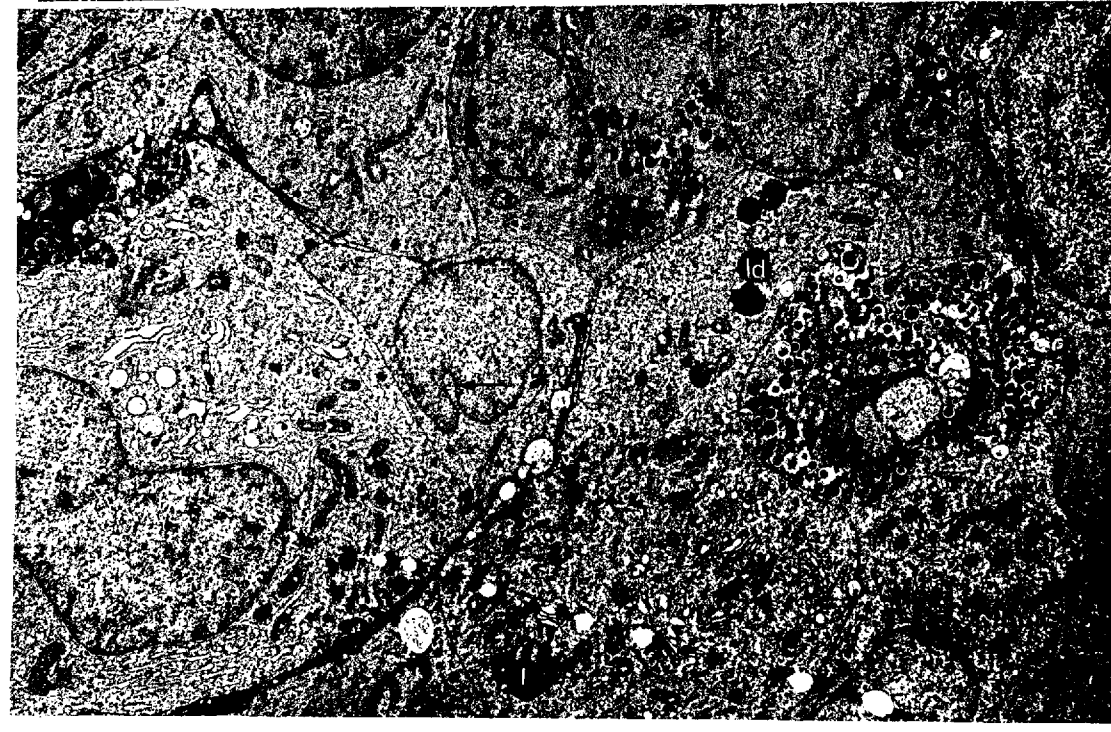

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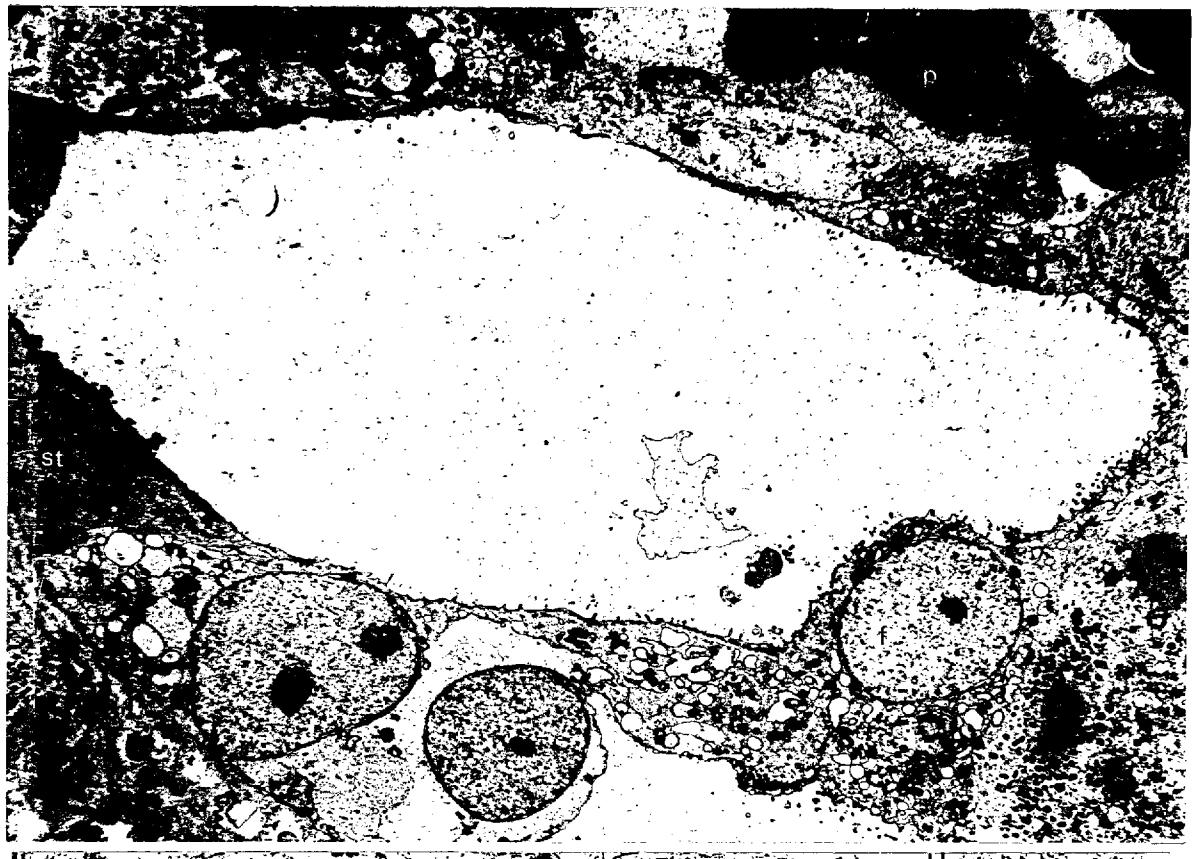
PLATE 12

PLATE 12. Electron micrographs of a 24-day culture. Plain HEPES-buffered medium.

Fig. 1. Viable region with spaces lined by epithelioid cells with microvilli. Some fibroblast-like cells (f) with dilated r.e.r. and stellate cells (st). Dark prolactin cells (p). x 3,000.

Fig. 2. Granular cell (acidophil), probably somatotroph (s) and a macrophage-like cell (mp) with phagocytotic vacuoles (arrow) and a ruffled membrane (double arrow). A fibroblast (f) shows microfibrils and two centrioles. x 10,000.



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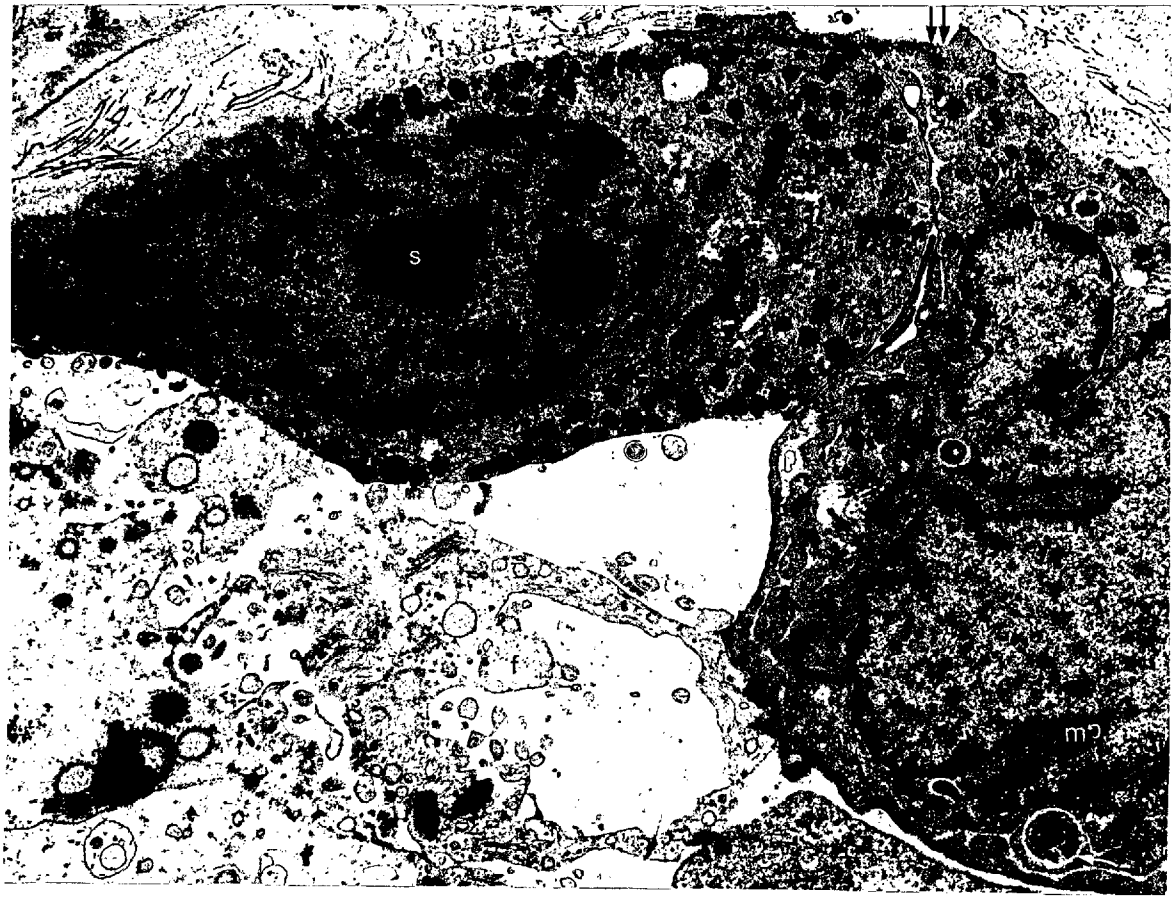

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PLATE 13

PLATE 13. Electron micrographs of cultures to show special features.

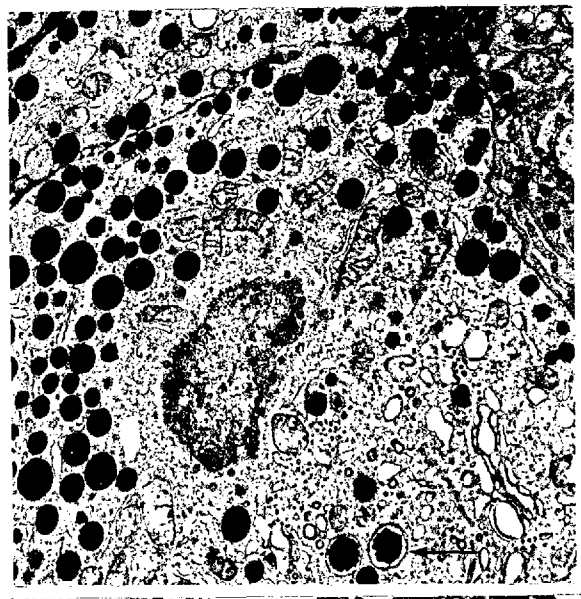
Fig. 1. A portion of a prolactin cell, 6-day culture. Granules in Golgi cisternae (arrow). x 9,000.

Fig. 2. A portion of a prolactin cell, 21-day culture. Granules in Golgi cisternae (arrow). Plain HEPES-buffered medium. X 9,000.

Fig. 3. A portion of a cell with Nebenkern-like formations, 14-day culture. Coated vesicles (arrow). x 20,000.

Fig. 4. Multilamellate body in an agranular cell and bundles of microfibrils (arrow), 21-day culture. Plain HEPES-buffered medium. x 20,000.

1



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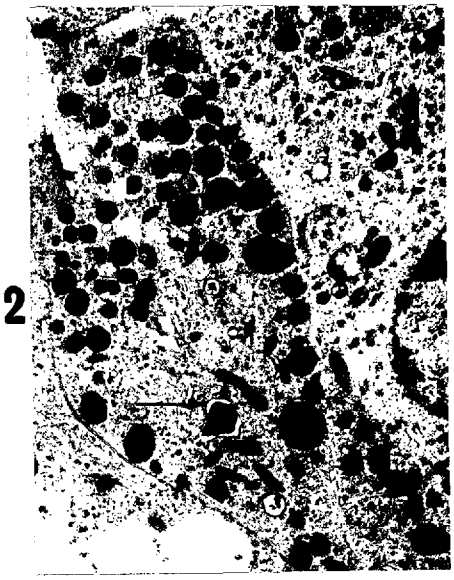

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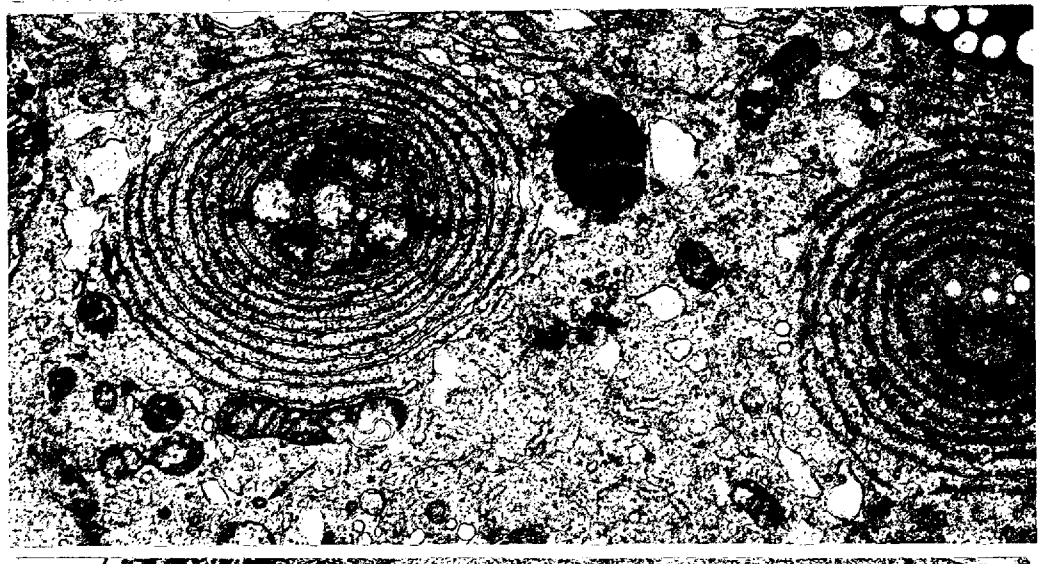

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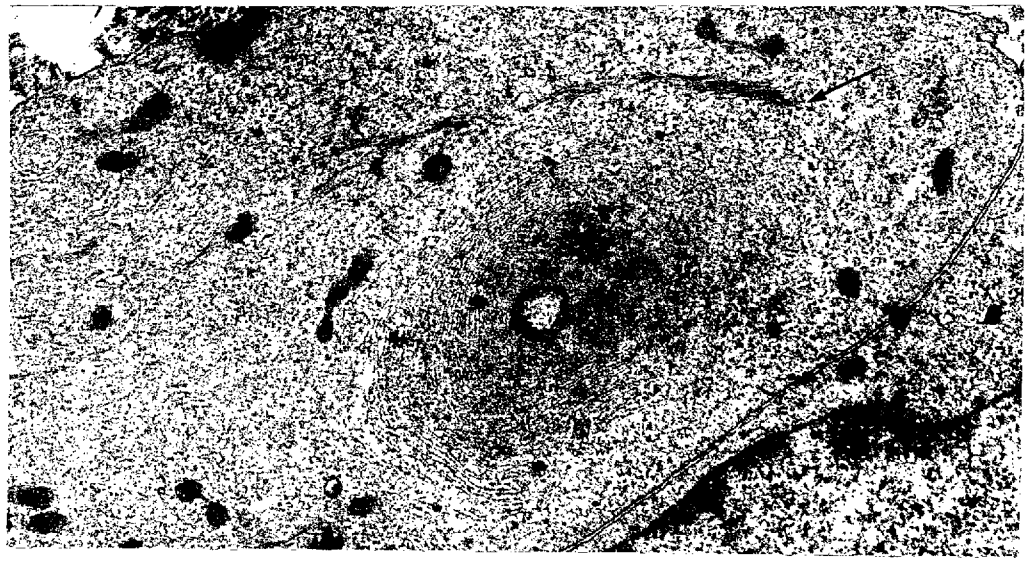


PLATE 14

PLATE 14. Electron micrographs of cultures to show special features.

Fig. 1. A portion of a prolactin cell with microtubules (arrow), 6-day culture. x 12,000.

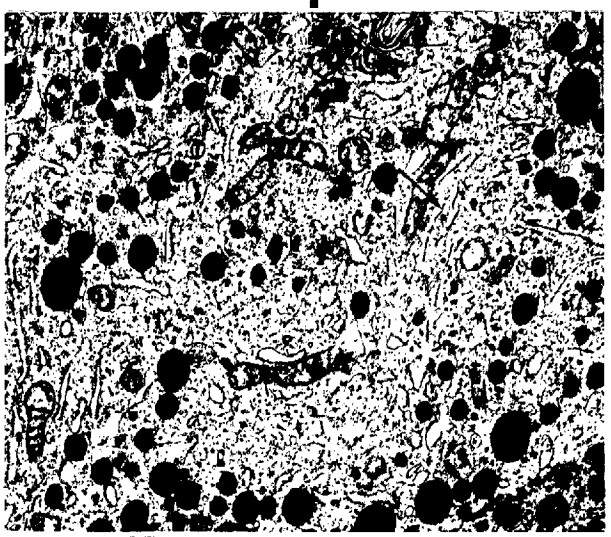
Fig. 2. A portion of a prolactin cell with ball of microfibrils (arrow), 6-day culture. x 12,000.

Fig. 3. A portion of acidophil cell with perinuclear microfibrils (arrow) and lytic body (l), 24-day culture. Plain HEPES-buffered medium. x 12,000.

Fig. 4. A portion of a stellate cell with bundles of microfibrils (arrows). The cell shows peripheral coated vesicles, villous folds, desmosomes, and mitochondria (m), 21-day culture. Plain HEPES-buffered medium. x 12,000.

Fig. 5. Granular cell (probably prolactin) with intranuclear rodlet (arrow), 21-day culture. Complete HEPES-buffered medium. x 12,000.

1



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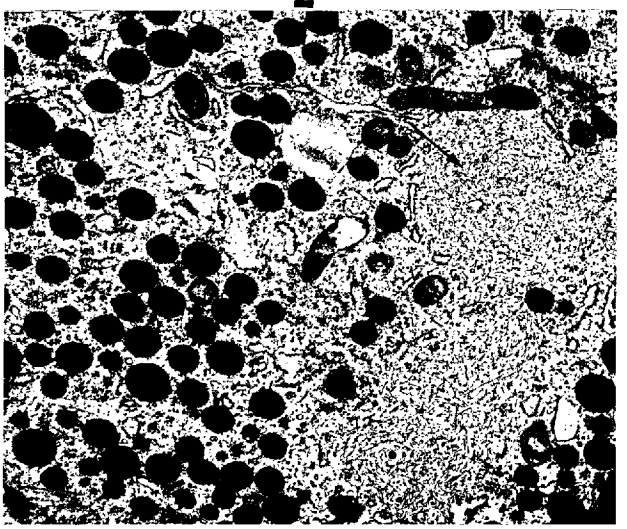

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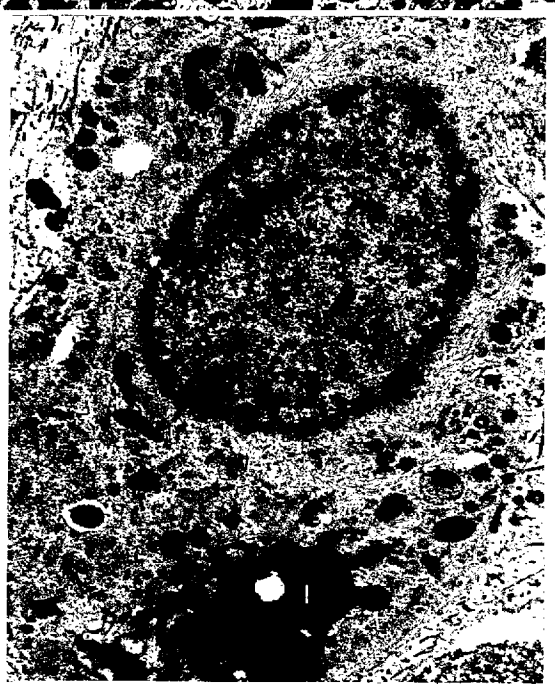

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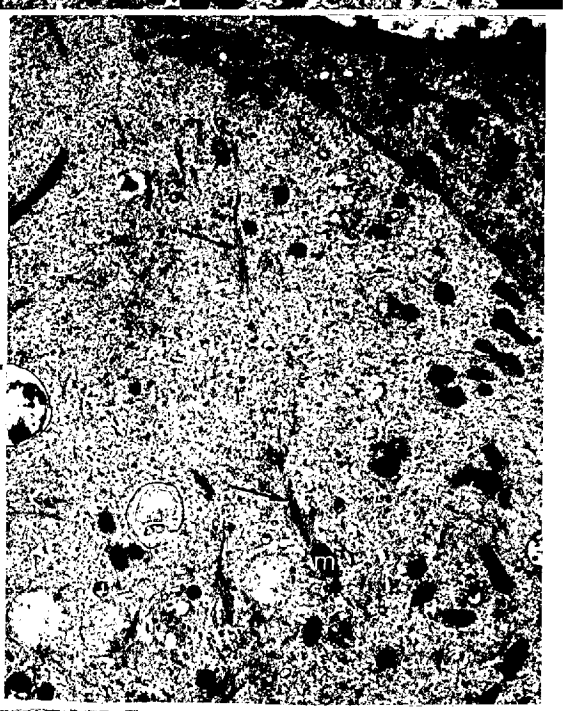

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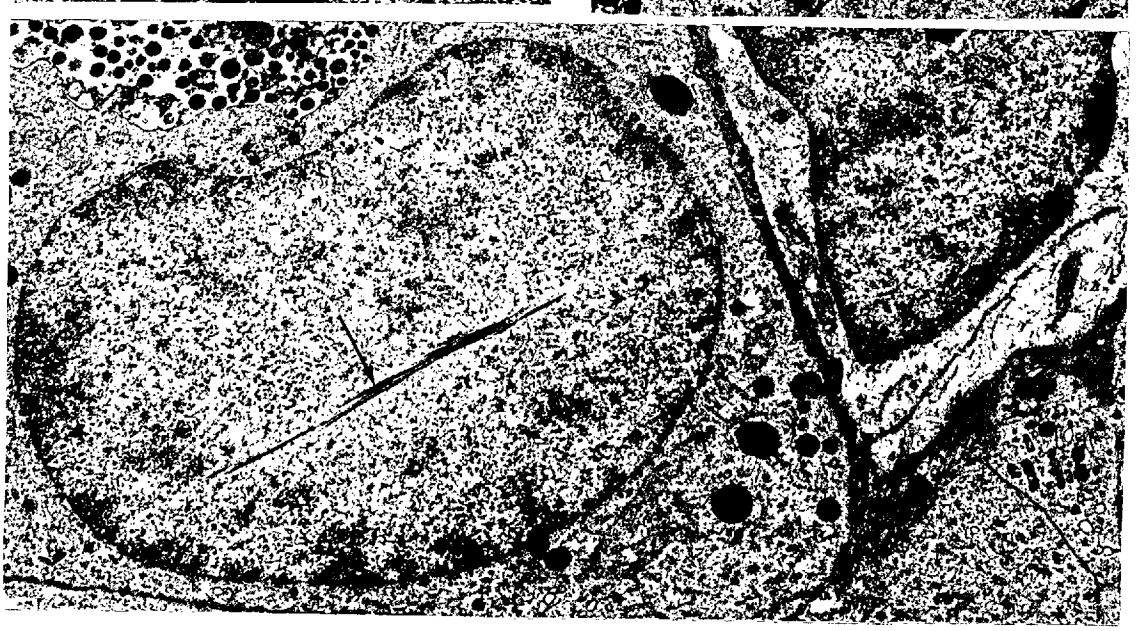


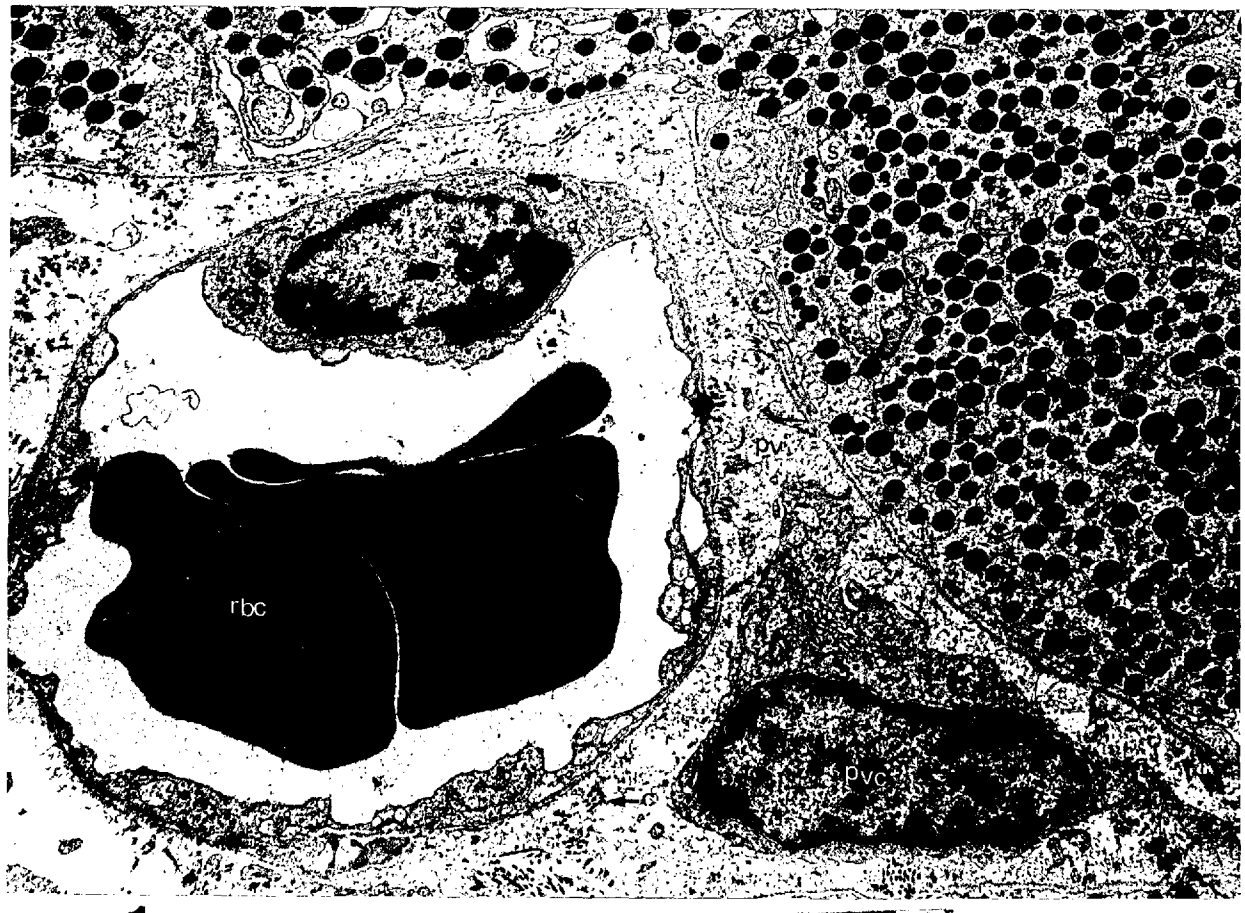
PLATE 15

PLATE 15. Electron micrographs of control and cultured pars distalis showing capillaries and perivascular spaces.

Fig. 1. Uncultured, control material from immature male rabbit. Large capillary with erythrocytes (rbc). Capillary epithelial basement membrane and parenchymal basement membrane (arrows) limiting the perivascular space (pv). A perivascular cell (pvc) and collagen fibres (c) in perivascular space. Somatotroph (s). x 9,000.

Fig. 2. Capillary after 6 days culture, showing capillary epithelial cell and surrounding perivascular space limited by basement membranes (arrows). Erythrocytes (rbc). x 9,000.

Fig. 3. Distorted capillary after 21 days culture, showing lumen and epithelial cell. Collagen fibres (c) in perivascular space. Plain HEPES-buffered medium. x 9,000.



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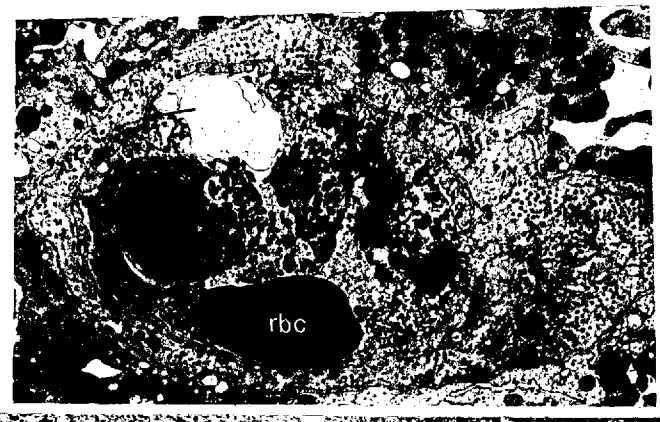

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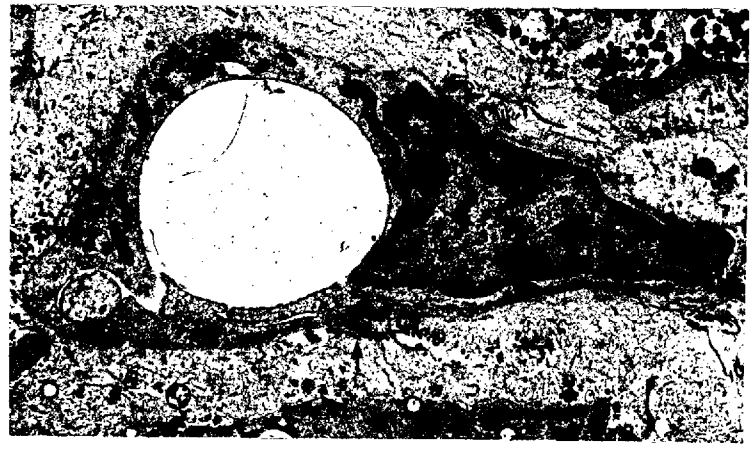

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PLATE 16

PLATE 16. Electron micrographs of cultures in plain HEPES-buffered medium.

Fig. 1. Perivascular cell (pvc) with centriole (arrow), coated vesicles, a long pseudopodial process and dark irregular nucleus. Also an agranular parenchymal cell (ag) with r.e.r., numerous small vesicles, peripheral coated vesicles and dark thin mitochondria (m), 21-day culture. x 6,000

Fig. 2. Perivascular space containing secretory granules devoid of limiting membrane (arrow) and surrounded by parenchymal cells, 21-day culture. x 12,000.

Fig. 3. A portion of a fibroblast-like cell with dilated r.e.r. (arrow), microfibrils (mf) and microvilli (mv), 24-day culture. x 9,000.

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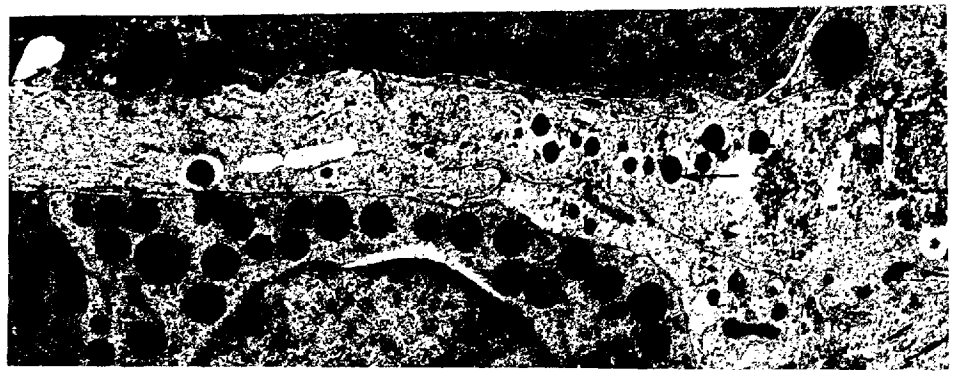

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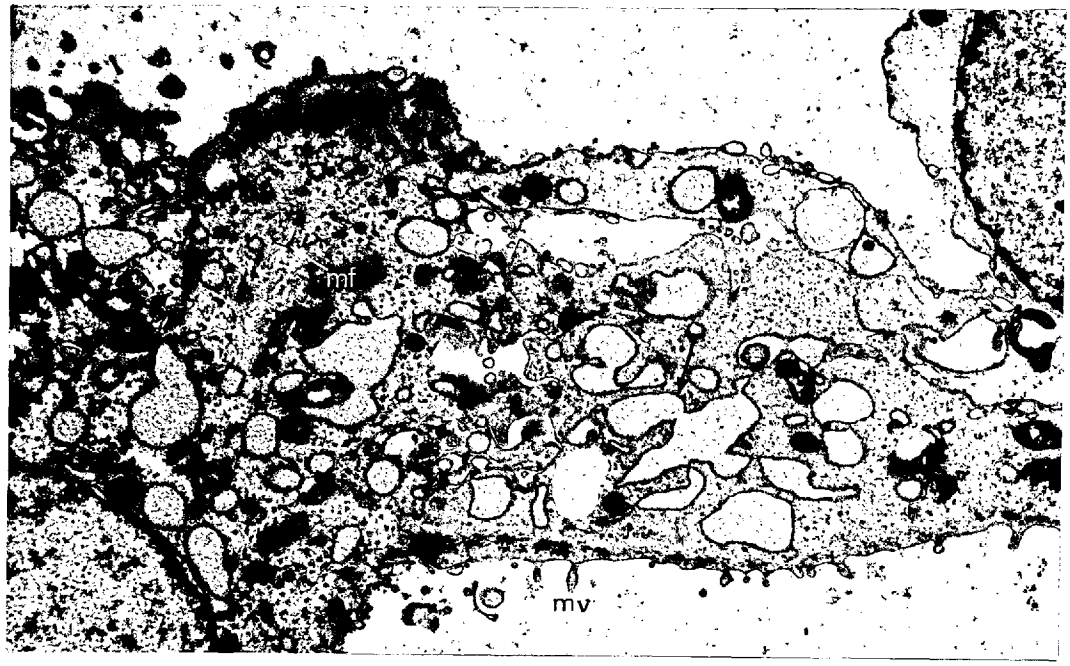


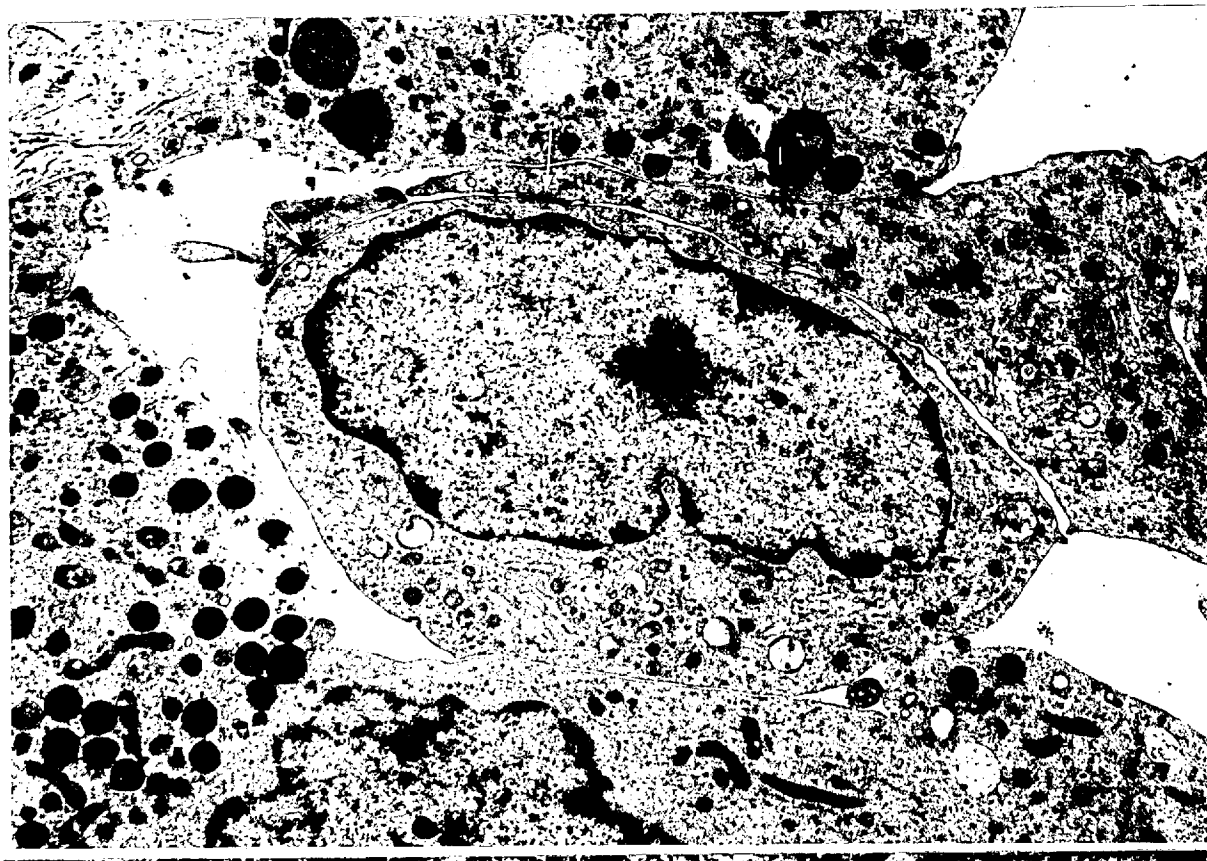
PLATE 17

PLATE 17. Electron micrographs of cultures to show desmosomes, cilia and lytic bodies.

Fig. 1. Parenchymal cells showing desmosomes (arrows) and lytic body (l). Large intercellular spaces. 24-day culture, plain HEPES buffered medium. x 12,000.

Fig. 2. Portion of a prolactin cell with cilium (arrow), well developed r.e.r. and Golgi areas with many small vesicles, some coated. 14-day culture. x 18,000.

1



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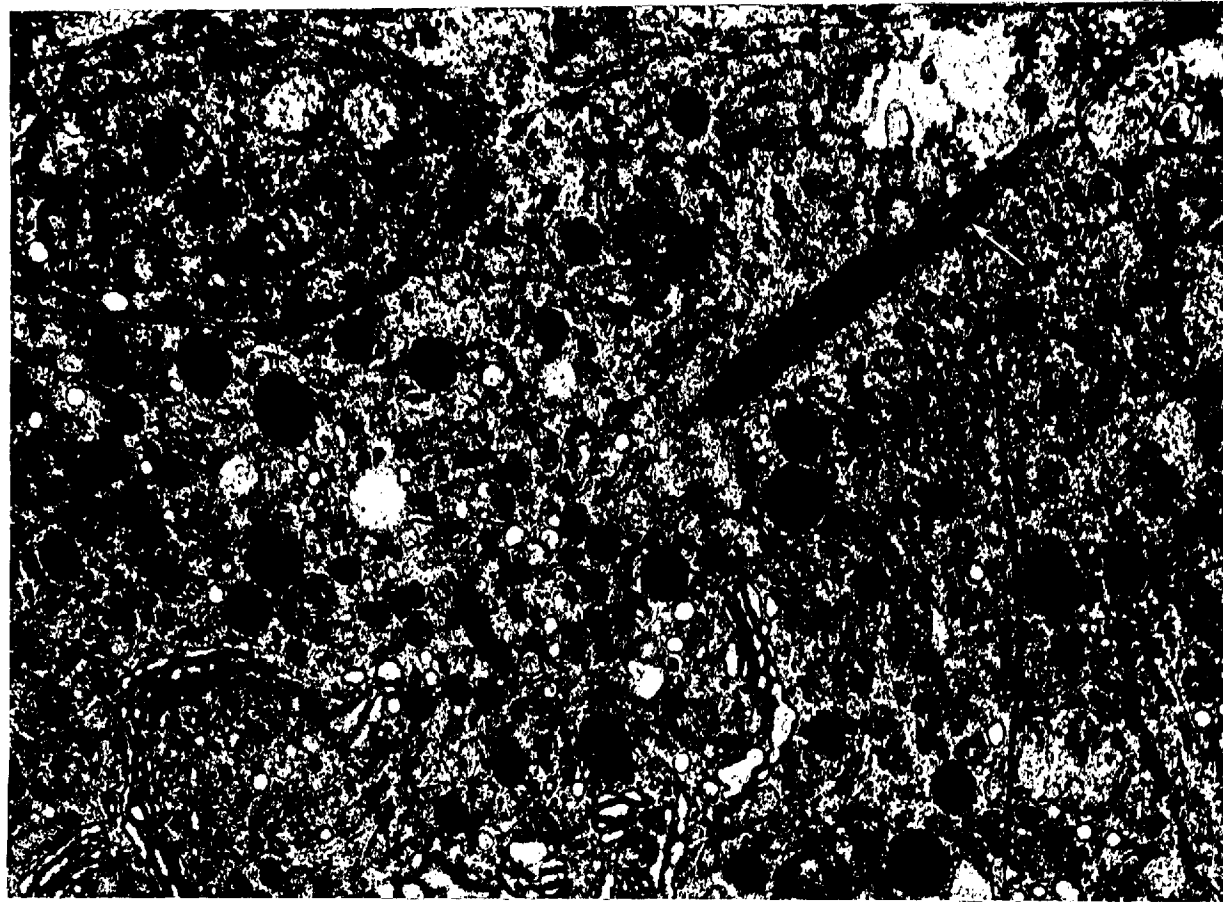

PLATE 18

PLATE 18. Electron micrograph of 14-day culture, adult male rabbit pars distalis cultured with hypothalamus.

Fig. 1. Somatotrophs (s) and gonadotrophs (g) show signs of active secretion. The few prolactin cells (p) are well granulated but show no indication of enhanced synthetic activity. Perivascular space (pv). x 3,000.

1