# ELECTRON MICROSCOPIC OBSERVATIONS ON CERTAIN SECRETORY EPITHELIAL CELLS OF THE GASTRO-INTESTINAL TRACT IN THE MOUSE.

VOL. I : TEXT.



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PREFACE.

This investigation was undertaken while on the staff of the Anatomy Department of Glasgow University, and I am much indebted to Professor G. M. Wyburn for constant advice, guidance and encouragement.

The earlier and major part of the work was carried out in the Department of Chemistry, and I am grateful to Dr. I. M. Dawson, Senior Lecturer in Electron Microscopy, for the generous advice and facilities extended to me during this period. Latterly, the study was completed within the Anatomy Department.

I am particularly indebted to Mr. Hamilton S. Johnston for expert technical assistance, and am grateful to Miss Vida Henning for three excellent drawings (Figs. 15, 32 & 33).

"there are smicroscopes of different kinds .....
on the other hand some insert in a certain tiny tube very tiny glass spheres whose diameter does not exceed the diameter of very small pearls, namely of this size , a novelty lately invented and for this purpose extremely well adapted: if on the surface of this sphere you apply the foot of a flea between the eye and the lamp, you will see a thing marvellous to behold, its upper leg in the grim likeness of a horses foot: but the hair thereon will remind you of an immense beam, or rather how extremely wonderful it is that in so small a sphere such excessively large objects can be represented".

Trans. fr. Kirch, 1664.

"Goe on Dioptrick Artist still goe on,
May your glass eyes even Vie perfection
With natures noblest sense .....
Nay then you pretty sprits' and fairy elves
That hover in ye air look to your selves.
For with such prying Spectacles as these,
We shall see you in yr owne essences."

Powers, 1661.

## GENERAL INTRODUCTION.

The epithelium of the gastro-intestinal tract has been intensively studied by many investigators drawn from a variety of disciplines, and therefore much is known of its structure and function, but our knowledge is still far from complete.

Morphological investigations have in the past been limited by the resolution of the light microscope, but within the last decade the position has altered with the advent of the electron microscope. The latter has become a powerful and reliable research instrument, capable of resolving structure down to macromolecular levels.

The present thesis, which is in five parts, is an electron microscopic study of certain epithelial cells in the gastro-intestinal tract of the mouse. It begins with a general account of the electron microscopical techniques used in the examination of biological tissues, as these techniques have been, and still are, undergoing rapid development. The second part describes the fine structure of the Paneth cell, the function of which is quite unknown. The gastric parietal cell, which secretes

the hydrochloric acid of the gastric juice, is the subject of the remainder of the thesis. Thus the third part is devoted to this cell in the starving animal. In the next, the structural changes induced by pilocarpine nitrate are examined. Finally, light microscopists have demonstrated a Golgi complex within the parietal cell. Surprisingly, several electron microscopic studies have failed to confirm its existence, and therefore the fifth part attempts to discover the underlying reason for this discrepancy.

This thesis is based on the following published work:

HALLY, A.D. (1958). The fine structure of the Paneth cell. J. Anat., Lond., 92, 268-278.

_____ (1959). The fine structure of the gastric parietal cell in the mouse. J. Anat., Lond., 93, 217-224.

_____ (1960). The secretory changes produced by pilocarpine in the gastric parietal cells of the mouse. In The Ultrastructure of Cells. London: Edward Arnold & Co.

_____ (1960). Electron microscopy of the unusual Golgi apparatus within the gastric parietal cell. J. Anat., Lond., 94, 425-432.

# TABLE OF CONTENTS.

# P A R T  I.

## ELECTRON MICROSCOPICAL TECHNIQUES.

## ELECTRON MICROSCOPICAL TECHNIQUES.

### INTRODUCTION.

The electron microscope, which was a rarity ten years ago, is still in its infancy; a stage characterised by rapid growth and development. Consequently, the ancillary techniques used for the preparation of biological specimens have been continuously adapted and altered in an endeavour to utilise fully the increasing resolving power of electron microscopes.

The steady improvement in such processes as fixation, embedding, and microtomy - resulting from progressive modification of the conventional methods previously used in light microscopy - has from time to time been accelerated by the introduction of radically new techniques.

Better microscopes necessitated improved ancillary techniques, and similarly an advance in a particular method often creates a need for development in another. For example, it may be impossible to exploit the potential of a new microtome without an improvement in the existing cutting edges of knives. In the following account the evolution of each technique is described separately for the sake of simplicity, but in reality all are closely interdependent.

The main stages in preparing biological specimens for electron microscopy are fixation, washing and dehydration, embedding, and microtomy, which are described in turn. Next, certain aspects of microscopy are discussed, including the design, operation, and maintenance of the electron microscope, and the preparation of micrographs. Finally, the problems arising during the interpretation of electron micrographs are outlined.

## FIXATION.

When electron microscopy was first applied to biological materials, it was found that several of the fixatives which were satisfactory for light microscopy produced gross artefacts at the electron microscopical level. Porter, Claude, and Fullam (1945) concluded that for tissue culture cells, the best fixative was osmium tetroxide vapour. This confirmed the earlier findings of Strangeways and Canti (1927), who had shown that tissue cultured cells examined by dark-ground illumination were minimally altered by a 2% solution of osmium tetroxide, whereas other fixatives cause marked cellular changes, such as shrinkage, coagulation of the cytoplasm and nucleoplasm, alteration in the shapes of mitochondria, and a fusion or solution of fat globules.

When improved embedding methods and microtomes made it possible to study tissue sections by electron microscopy, disappointing results were obtained with osmium tetroxide solution. Palade (1952) made a major advance when he examined the action of osmium tetroxide and, using neutral red as the indicator, found that acidification of the tissues occurred just prior to fixation. Accordingly, he tested the effect of various buffering solutions, and demonstrated that a 1% osmium tetroxide solution buffered to pH 7.4 with veronal acetate gave optimal results - i.e. fixation comparable to that obtained with osmium tetroxide vapour on tissue culture cells. Palade's fixative quickly became established, and in a modified form remains to this day the most widely used fixative in electron microscopy. A subsequent modification was to render it isotonic with plasma: Palade himself did not consider it always necessary, but believed that the addition of sucrose "improved fixation in certain cells with a watery cytoplasm", such as fibroblasts and leucocytes. Rhodin (1954) and Zetterqvist (1956) emphasised the need for isotonicity to minimise swelling or shrinkage of such organelles as the mitochondria, and used sodium chloride or calcium chloride to raise the tonicity. However, these studies were all based on the final appearance of

the cells, and thus included volume changes produced by subsequent dehydration and embedding. Indeed, it is a matter of common observation that when tissues are immersed in isotonic buffered osmium tetroxide solution they undergo an increase in volume, and thus there is no intrinsic merit in isotonicity.

Bahr et al. (1957) made a quantitative study of the volume changes occurring in tissues during fixation in buffered isotonic osmium tetroxide, dehydration through alcohols, and embedding in methacrylate. A rapid 30% increase occurred during fixation, but this was approximately corrected by the 22% shrinkage during dehydration, and the further contraction of about 8% caused by polymerization in methacrylate. Thus, the only advantage of exact tonicity of the buffered osmium tetroxide solution is that it happens to produce a degree of swelling which is subsequently corrected during dehydration and embedding in methacrylate. However, this concertina-like action is far from ideal, as it is liable to produce distortion within the tissues. Bahr's results also imply that absolute linear measurements of cell organelles must be treated with some suspicion, although as linear dimensions vary only as the cube root of the volume changes, the errors are considerably smaller.

Post-mortem change is another problem of fixation which merits consideration. In addition to the time interval between death and the immersion of the specimen in fixative, there is a further period which elapses before the fixative reaches the deeper parts of the specimen. Quantitative studies of the rates of penetration of fixatives into tissues are sparse, but Underhill (1932) found that a 1% solution of osmium tetroxide penetrated 270μ into the liver within 15 min. Medawar (1941), who was interested in the relative rather than the absolute rates of penetration of different fixatives, used a readily controllable albumin-gelatine gel as the test specimen, and concluded that 1% osmium tetroxide penetrated 170 microns within 15 minutes. Allowing for the fact that different test specimens were used, these two results are in fairly good agreement, and suggest that in standard tissue blocks 1 $mm^3$. in size, there is probably a delay of over 15 minutes before the more centrally-placed cells are fixed. This delay is of importance, because even with the light microscope mitochondrial changes have been detected within a few minutes of death. In order to study post-mortem changes under the electron microscope, it is essential to choose a tissue where there is no problem in penetration: Zetterqvist (1956)

observed swelling of the mitochondria and vacuolation of the cytoplasm at 5 min. post-mortem, in the epithelium of the small intestine. Rhodin (1954) had earlier stated that well-fixed tissue was found only with 40μ of the surface of the block.

Therefore, with tissue blocks of 1 $mm^3$. size, it is important to discern and discard sections showing post-mortem changes.

Present Method.

(1) The fixative was prepared according to Zetterqvist (1956), and consists of a buffered isotonic solution of 1% osmium tetroxide, made up as follows:-

| | | |
|---|---|---|
| Stock Solution I. | Sodium Acetate | 9.714 gms. |
| | Veronal Sodium | 14.714 gms. |
| | Distilled water to | 500 ml. |
| Stock Solution II. | Sodium Chloride | 40.25 gms. |
| | Potassium Chloride | 2.1 gms. |
| | Calcium Chloride | 0.9 gms. |
| | Distilled water to | 500 ml. |

The final mixture contains:

| | |
|---|---|
| Stock Solution I | 10 ml. |

| | |
|---|---|
| Stock Solution II | 3.4 ml. |
| 2% Osmium tetroxide in distilled water | 25 ml. |
| 0.1 N Hydrochloric acid | 11 ml. |

The pH was checked either by an electric pH meter or Lovibond comparator, and if found to lie outwith the range 7.2 - 7.6, was adjusted to 7.4 by the further addition of either hydrochloric acid or the alkaline veronal-acetate solution I.

(2) The animal was killed and the appropriate tissue rapidly removed, cut with a fresh razor blade into small pieces less than 1 $mm^3$. in size, and placed in fixative within 2 min. of death. The duration of fixation was 1 hr.

WASHING AND DEHYDRATION.

Washing with saline or water is necessary to remove the excess osmium tetroxide which would otherwise be reduced by the action of alcohol during dehydration. Prolonged washing is undesirable, however, as it extracts large amounts of organic material from the tissue (Bahr, 1955).

Dehydration causes considerable shrinkage, which occurs rapidly if the tissue is transferred directly into 100% ethanol after washing. A stepwise dehydration through increasing

concentrations of alcohol produces a more gradual volume change, but the total change is the same (Bahr et al., 1957). Moreover, the diminution in volume occurs in the higher concentrations of alcohol above 70%, and thus there is no need to start the dehydration series with concentrations of less than 70%. Prolonged immersion in alcohols, as in saline, causes loss of organic matter from the tissue, so that dehydration times should be as short as possible.

Present Method.

| | | |
|---|---|---|
| Wash (Isotonic Saline) | | 15 mins. |
| 70% Ethanol | ... | 20 mins. |
| 90% Ethanol | ... | 20 mins. |
| 100% Ethanol | ... | 30 mins. |

During washing and dehydration each fluid is renewed two or three times.

## EMBEDDING

Paraffin proved to be too soft for the ultrathin sectioning in electron microscopy. Celloidin-paraffin methods produced a harder block (Pease & Baker, 1948), but traditional media were superseded by Newman, Borysko and Swerdlow in 1949, when they introduced a method using the well-known plastic "Perspex"

- n-butyl methacrylate. Following fixation, washing, and dehydration through graded alcohols, they immersed the tissue in the liquid monomeric form of n-butyl methacrylate for some hours. Finally, it was embedded in a mixture of n-butyl methacrylate containing the catalyst benzoyl peroxide, and polymerized by heat or ultra-violet light. As polymerization proceeds the liquid methacrylate comes increasingly viscous and sets to a glass-like solid.

Methacrylate embedding quickly became standard practice and is still the most commonly used method. However, increasing experience revealed that frequently tissues were damaged during polymerization, and Borysko (1956) found that many cells became swollen and were destroyed. He concluded that polymerization proceeds unevenly within a tissue, and that as the polymerized methacrylate shrinks in volume, stresses develop which are initially relieved by flow. Later, as the viscosity rises, flow is retarded, and shrinkage of areas of methacrylate tear the tissues apart. Borysko accordingly raised the temperature of polymerization to 60°C. in order that the thermoplastic methacrylate would remain fluid until polymerization was nearer to

completion, and thereby greatly reduced the incidence of polymerization damage.

During examination in the microscope, the electron beam may raise the temperature of the specimen considerably, causing the methacrylate to sublime. * Although sublimation is advantageous in that it improves the contrast of the specimen, it has two disadvantages. First, softening of the plastic may cause distortion of the specimen (Morgan et al., 1956), and second, the sublimed methacrylate contaminates the microscope, which ultimately reduces its performance.

Recently, in an attempt to overcome these difficulties, thermosetting epoxy resins have been introduced in place of methacrylate (Maaløe & Birch-Andersen, 1955; Glauert et al., 1956) as they do not shrink during hardening, nor do they soften or sublime in the electron beam. Thus polymerization damage, distortion, and contamination of the microscope are minimised, but consequently contrast is less satisfactory. Moreover, the high viscosity of these compounds renders impregnation of tissues difficult.

* Methacrylate has a low sublimation point around 33°C.

Present Method.

The method is mainly that of Borysko (1956). Following dehydration through ethanols the tissue is immersed in a 1:1 mixture of ethanol and monomeric n-butyl methacrylate for an hour, followed by three hours in pure monomeric n-butyl methacrylate. Finally, it is embedded in gelatin capsules containing a 10:1 mixture of butyl methacrylate and methyl methacrylate with 1% benzoyl peroxide as a catalyst.

The capsules are polymerized in an oven at 60°C. for 12 - 24 hours.

## SPECIMEN GRIDS AND SUPPORTING FILMS

When ultrathin sections were first examined in the electron microscope they were mounted on metal grids which had a fine mesh of about 200 squares to the inch. Unfortunately, many of these sections were destroyed in the electron beam, so that to provide additional support the grids were coated with a thin layer of some plastic material, such as collodion or "Formvar". This method considerably reduced the wastage rate of sections, but these plastic films are not heat-resistant, and so tend to soften under the electron beam which allows the specimen to "drift". Watson (1956), therefore, introduced carbon films which are heat

resistant, and are considerably thinner than plastic ones. Thus, with carbon films, drift is reduced and contrast improved, but their preparation involves expensive complex equipment, and owing to their brittleness they are easily damaged.

Present Method.

A clean glass jar is filled brimful with distilled water and the surface swept clean with a glass rod. Two drops of a 1.5% solution of collodion dissolved in amyl acetate are placed on the water from a pipette. As the solution spreads rapidly across the surface the amyl acetate volatilizes, leaving a thin film of collodion.

The thickness of the film is judged by its interference colours, and it must be silver and free of wrinkles. Copper grids (Smethurst High-light 200 squares/inch) are dropped gently onto the film and then a sheet of paper lowered on top until it touches the film and surrounding water. The paper, when thoroughly wet, is then lifted up together with the adherent film and grids, and dried off in the oven. The grids are finally removed from the paper and stored in Petri dishes, ready for use.

## ULTRAMICROTOMY.

The electron microscope raised fresh problems in

microtomy, because although sections of 1 - 2μ were satisfactory for the limited resolution of the light microscope, the new microscope, for reasons discussed later (p.24, 55) necessitated sections only a fraction of a micron thick - "ultrathin" sections.

Microtomy of any given tissue depends on the following factors: fixative and embedding medium, microtome, and knife.

Fixative and Embedding Medium.

The choice of fixative is determined largely by factors other than its effect on the cutting properties of tissues, in contrast to the new embedding media which were adopted primarily for their excellent cutting properties. However, routine fixation with osmium tetroxide does not affect the tissues adversely in this respect, although Dalton's fixative - which consists of potassium dichromate in addition to osmium tetroxide - renders tissues more friable and less suitable for microtomy. The post-osmication method used to demonstrate the Golgi apparatus (p. 131 ) has a similar detrimental effect.

Microtomes.

Claude & Fullam (1946) first successfully cut ultrathin sections of about 0.3μ by means of a special high-speed microtome in which the blade rotated at 49,000 r.p.m. The machine

was, however, cumbersome and unreliable, and Pease & Baker (1948) obtained better results by proceeding along more orthodox lines. They simply reduced the minimum rate of advance of a standard Spencer microtome by the insertion of a wedge-shaped block of metal. Subsequently, specially designed microtomes have incorporated a number of improvements, including vibration-free couplings, nylon bearings, and separately mounted motor drives to ensure a uniform speed of the knife on the cutting stroke. An alternative method of advancing the block is that of thermal expansion (Porter & Blum, 1953; Sjöstrand, 1953b). This involves even heating of the rod which holds the specimen, so that the linear expansion causes it to advance towards the knife. There are fewer moving parts in this system, which is incorporated into several of the present-day instruments. Modern ultramicrotomes have a "by-pass" mechanism, whereby the specimen block meets the knife on the cutting stroke, but is deflected away from it on the return stroke; this is important, for the specimen block is advancing throughout each stroke, and therefore on meeting the knife on the return upward stroke it will be distorted (Fig. 1a), and in addition this may cause the freshly-cut section to adhere to the face of the block.

Knives.

Improved cutting edges were required for ultrathin sections, and excellent edges were obtained by careful grinding of razor blades using the finest abrasive powders. While this conventional development with steel knives was taking place, Latta & Hartman (1950) invented a novel "glass knife". This quickly superseded the steel knife, as it was cheap, readily available, and more easily prepared, and it remains the most widely used form of knife today.

A problem encountered with all knives is that of wrinkling and compression of the sections. Hillier & Gettner (1950) fixed a liquid bath on the knife and adjusted the meniscus to reach the cutting edge: each section as it was cut floated on the surface of the liquid (Fig. 2c). Various liquids have been tested, including acetone, alcohols, and dioxane, but all have a low surface tension and a slight solvent action on the embedding material, which tends to soften and hence flatten the sections. Gentle heat also aids this process. A third method of reducing compression is to hold a pledget of cotton wool soaked in a volatile solvent such as chloroform or xylene (Sotelo, 1957; Satir & Peachey, 1958) above the sections as they float on the liquid bath, which

allows the vapour to stretch them. This method is effective, but in the writer's experience is not without risk, as it may damage the sections.

Fernandez-Morán (1956) introduced a specially ground diamond knife which, with its much harder edge, is ideal for cutting hard tissues. Moreover, the edge is more durable and will last for months, whereas glass knives blunt rapidly with use, and have to be prepared each day. However, diamond knives have one grave defect, i.e. their poor "wetting" qualities. Consequently it is difficult to adjust the meniscus to the cutting edge. Moreover, their durability entails the difficult task of keeping them clean over prolonged periods. Fearnhead (1958), seeking a less costly substitute, used a sapphire knife in place of diamond, to cut teeth.

Although glass knives have been in use for many years there is a diversity of opinion about the best form of knife (Fig. 1c). It is generally agreed that the clearance angle should be small, about 5 - 10°. The included angle, however, varies greatly. At one extreme Gelber (1957) recommends an angle of 80 - 90°, whereas Latta & Hartman (1950) used a 45° knife, and Sjöstrand (1956) advises 57°. Such divergent views suggest that

the angle is not critical.

Today, sections less than 400Å (1/20μ) can be obtained with a modern microtome, glass knives, and standard fixatives and embedding medium. Indeed, Huxley (1957) with a microtome of his own design achieved sections of about 100Å in thickness in the course of an excellent investigation into the fine structure of muscle.

Present Method of Microtomy.

1. Preparation of Knives:

Glass knives are prepared mainly according to the method of Latta & Hartmann (1950). Strips of plate glass 12" long, 1" broad, and 3/8" thick, are obtained. Each strip is cleaned thoroughly with a warm solution of detergent, rinsed with water, and then dried carefully with lens tissues. A glass cutter is used to score the surface at an angle of 45° to the long axis, at intervals of 1" along the strip, taking care that the scores do not reach the edges (Fig. 2a). With two pairs of glass pliers, a smoothly increasing force is applied mainly to distract the glass knife from the strip, rather than to bend it (Fig. 2a). A good knife has a slightly concave smooth cutting edge, rising to a "horn" at one end. Near the other end, a stress

line diverges from the cutting edge, and the best part of the knife lies just beyond the point where this line leaves the cutting edge (Fig. 2b). However, only a fraction of these knives are satisfactory, and in order to eliminate some of the remainder, the knife edge is examined under a 60x binocular microscope by reflected light, when it should appear as a thin even bright line. Examination under a 600x reflecting microscope eliminates more unsatisfactory knives, but even then, some of the remainder prove inadequate.

A strip of ½" self-adhesive tape (Minnesota Mining Co. Electrical Tape 33) is then applied to the knife to act as a liquid bath, which is sealed with molten dental wax (Fig. 2c), and the knife is ready for use.

2. Description of Microtome.

The Cooke and Perkins ultramicrotome operates on the thermal expansion principle, and is rubber-mounted on a vibration-free bench (Fig. 3). The glass knife (K) is clamped in an adjustable knife holder (H), on a sliding stage (S), which can be moved transversely, or advanced accurately towards the block by means of the fine micrometer screw feed (M). The specimen block is held in a chuck (C) on the end of the object arm (A). The

motion of the object arm is as follows: rotation of the wheel (W) causes the arm to move within the guide plate, so that it describes a parallelogram, meeting the knife on the descending cutting stroke, but bypassing it on the upward stroke.

An electric coil (E) produces the thermal advance by heating the object arm. The thickness of the sections depends on the rate of heating, which is therefore accurately controlled by a rheostat (R) with ammeter (AM). In spite of a metal shield which covers the heating coil, the advance is readily affected by draughts, but these are minimised by enclosing the entire microtome within a large glass cabinet.

The driving wheel can be turned either manually, or by an electric motor which is mounted separately on the bench. The motor drive ensures a constant speed of the knife, and therefore uniform section thickness.

A 60x binocular microscope and lamp are essential for controlling the process.

3. Procedure for Microtomy:

After the block has been trimmed to a pyramid using a special chuck (Fig. 3a), it is inserted into the chuck on the object arm and orientated at the universal joint (J) so that it

meets the glass knife symmetrically (Fig. 4b).

The glass knife is clamped in the knife holder and adjusted with a clearance angle of about 5 - 8°, and then advanced until it is close to the specimen, using the micrometer screw for the fine adjustment. Now the liquid bath is filled from a Pasteur pipette with 20% ethyl alcohol until the meniscus just reaches the cutting edge of the knife. The motor drive is switched on, and the knife further advanced in 2μ steps, until a section is cut off, and floats in the liquid bath. At this stage the thermal advance is turned on so that more sections are cut. The thickness of the sections is judged by their interference colours, and the thermal advance is gradually reduced until fresh-cut sections appear dull grey. The thermal advance and the motor drive are stopped after about two dozen satisfactory sections are floating on the liquid. Should the sections be compressed or wrinkled at this stage, they are encouraged to expand and flatten by applying gentle heat from an electric bulb for 10 min. Finally, a copper grid, held in jeweller's forceps, is lowered gently until it touches the liquid surface where the sections are floating, and the surface tension causes a drop of liquid to adhere to the grid with the sections (Fig. 4c). The excess fluid

is then removed from the grid with filter paper, and the grid stored ready for use on a glass slide in a Petri dish.

## MICROSCOPY

### The Development of the Electron Microscope.

The resolution of a microscope or any lens system, is its ability to distinguish detail, and is the minimum distance between two neighbouring particles at which they can be seen to be separate. In an ideal lens system Abbe demonstrated that the resolution was directly proportional to the wavelength of the light used, as expressed in the following equation:

$$R = \frac{0.61\lambda}{N.A.}$$

where R = resolution.

$\lambda$ = wavelength of light.

NA = numerical aperture of the lens.

As the wavelength of visible light ranges from 4000 - 8000Å, and the numerical aperture of even oil immersion lenses is only about 1.4, the maximum theoretical resolution of the light microscope is approximately 0.2μ, and this limit has been practically reached.

In 1926, de Broglie calculated that there were waves

associated with a beam of electrons, and that their wavelength was extremely small. The formula for the wavelength of moving electrons is:

$$\lambda = \frac{12.3}{\sqrt{V}} \text{ Å}$$

where V = the accelerating voltage in volts.

At an accelerating voltage of 75 Kv, then

$$\lambda = \frac{12.3\text{Å}}{\sqrt{75,000}} = 0.05\text{Å}$$

Consequently, the wavelength of such an electron beam is several thousand times shorter than that of visible light. The Abbe equation applies to any ideal lens system, and therefore it became theoretically possible to achieve, by using electrons, a resolution far better than that of the light microscope. Electrons, due to their electric charge, can be deflected by magnetic fields and Busch succeeded in focussing and producing a magnified image of a source of electrons. Thus, the path was cleared for the design of an electron microscope, and Ruska built several in the 1930's.

Within the last decade, electron microscopes have become readily available, and today there are several capable of

resolutions better than 10Å, representing a 200-fold improvement on that of the light microscope.

The Main Features of the Electron Microscope.

The following description refers primarily to the Philips EM.75B - a small electron microscope of modern design with a resolution better than 50Å (Fig. 5). The basic optical system of an electron microscope resembles that of the light microscope (Fig. 6). Thus, the source of illumination in the electron microscope gives rise to a powerful beam of electrons which traverse successively (1) a condenser lens which concentrates the beam, (2) the object, (3) the objective lens, which produces a magnified intermediate image of the object, and (4) a projector lens, which further magnifies a portion of the intermediate image and projects the final image onto a fluorescent screen, which in turn makes the image visible to the human eye.

Although the optical systems of the light and electron microscopes are basically similar, the design of the electron microscope is modified by the poor penetrative powers of electrons,* which are unable even to pass through air. Consequently, the microscope column containing the lens system is sealed, and the contained air withdrawn by means of fast vacuum pumps. Moreover,

* This characteristic of the electron beam also necessitates exceptionally thin tissue sections.

the electron beam is focussed by means of 'magnetic' lenses in place of glass ones.

1. The Electron Gun. (Fig. 7a).

The electron microscope column or tube resembles a cathode ray tube. The electron gun consists of a cathode of tungsten wire maintained at a negative potential of 75 Kv. When a current passes through the filament it is heated to around 2,200°C, causing thermionic emission of electrons, which accelerate rapidly towards the anode plate, and pass through an aperture ; in its centre. The Wehnelt cylinder acts like an electrostatic lens, and concentrates the beam near its source.

2. Magnetic Lenses.

Magnetic lenses consist of a hollow cylindrical solenoid enveloped in an iron case. The magnetic field, generated by the electric current passing through the coil, causes the electrons to converge, and thus magnetic lenses are always convergent and never divergent. The magnetic field strength can be augmented by means of "pole-pieces," which are carefully designed pieces of ferrous metal with small central apertures which allow the passage of the beam. Pole-pieces are incorporated into the powerful lenses, with short focal lengths,

which are used as objectives or projectors (Fig. 7b).

The path of electrons through a magnetic lens is helical, unlike that of visible light through a glass lens (Fig. 7b). Moreover, the focal length of a lens can be varied continuously by altering the lens current, and this change in focal length is associated with a rotation of the image.

Provided the various magnetic lenses are aligned carefully with respect to each other, the inevitable slight fluctuations in lens current and high tension voltage produce only a negligible displacement of the image, but malalignment increases the movement, resulting in loss of resolution. Fortunately, alignment is facilitated by this characteristic helical path of the electrons, as usually the centre of rotation of the image produced by varying a particular lens current coincides with the optical axis of that lens.

The objective lens produces the initial enlarged image of the object, and therefore limits the resolution of the microscope. No matter how carefully pole-pieces are constructed, asymmetries in the magnetic field will persist, causing astigmatism of the lens. In order to reduce this residual astigmatism, the EM.75B includes an adjustable stigmator. This consists of

two pairs of iron segments which can be rotated on the pole-piece (Fig. 7a).

The projector lens is used at a constant current in the EM.75B, and the magnification can be varied continuously from 1,200x to 12,000x by raising the upper part of the projector lens mechanically. This method has the advantage that there is no rotation of the image with change in magnification.

Magnetic lenses generally have a depth of field of the order of 1μ, which is large when one considers the depth of resolution of which they are capable, and this is due to the small angular apertures used. The depth of focus is even larger, and for all practical purposes is infinite. Consequently, when the final image is focussed on the fluorescent screen, no further adjustment is necessary for taking photographs, either on plates or film, although they are about 15 cm. apart (Fig. 5).

3. The Vacuum System. (Fig. 8).

A high vacuum ($10^{-5}$ mm. Hg.) is produced in the microscope column by means of a "pre-vacuum" rotary oil pump, and a "high-vacuum" oil diffusion pump, arranged in series (Fig. 8). The pre-vacuum pump achieves a vacuum of $10^{-4}$ mm. Hg., enabling the oil diffusion pump to evacuate the column to the requisite

high vacuum. A Penning gauge indicates the vacuum. This simple system is made possible because of the specially designed oil diffusion pump on the EM.75B. The pump contains only a small quantity of silicone oil, which on meeting large volumes of air cools rapidly and therefore is not oxidised or 'cracked'. In other microscopes, during the preliminary evacuation of the column - when the rotary pumps are withdrawing large amounts of air - provision is made to by-pass the oil diffusion pump(s) to prevent 'cracking' of their oil. This complicates their vacuum systems and increases the likelihood of leaks developing.

Closing the column valve isolates the pumps from the column, which can then be brought to atmospheric pressure by opening the air inlet valve, for such purposes as cleaning, or changing cameras. There is in addition a safety magnetic valve, which is closed when the pumps are on. In the event of power failure, or when the pumps are switched off, it opens, allowing air into the pre-vacuum pump, and this inrush of air closes a second valve which is between the two pumps (Fig. 8). These valves prevent oil from the pre-vacuum pump being sucked along the pipe to the microscope column via the oil diffusion pump.

4. Electronics.

Complex electronic equipment is necessary to attain a high degree of stability on the high tension and the lens currents. Small fluctuations in the high tension voltage cause the filament to emit electrons of various wavelengths, and chromatic aberration results. Further, the focal length of a lens varies with the energy of the electrons, and thus the focussing properties will fluctuate. Finally, the focal length of each lens is inversely proportional to the current flowing through its coils, and so focus will not be maintained unless the lens current is constant.

The EM.75B has an objective lens with an extremely short focal length of 0.8 mm., and a small bore, and both these factors reduce the lens sensitivity to fluctuations in the high tension and lens current. The stabilising equipment is accordingly much simpler than that of other microscopes, and consists merely of magnetic stabilisers.

## ADJUSTMENT AND OPERATION OF THE MICROSCOPE

### Performance Check.

An electron microscope is capable of its maximum resolution only when it is scrupulously clean, in good alignment,

electrically and magnetically stable, and has minimal astigmatism. Consequently, careful maintenance and frequent checks on performance are essential.

(1) Alignment.

The characteristics of the well-aligned EM.75B microscope are as follows:-

(a) Electron beam: Switching on the high tension causes the screen to light up. The 'Wehnelt' image of the unsaturated filament, when focussed by means of the condenser lens, is symmetrical.

(b) Condenser lens: Variation of the condenser lens current towards condenser focus from the over-focussed position increases the intensity of illumination. Moreover, the illumination remains uniform over the screen during this variation. A fault in this particular EM.75B is that there is a considerable shift of illumination close to condenser focus, but this is of no consequence as such high intensities of illumination are not required.

(c) Objective lens: At maximum magnification, small variations of lens current on either side of objective focus cause the specimen image to rotate about the centre of the screen. Moreover, reversal of the current through the coils of the objective

lens results in no movement of the portion of the image lying at the centre of the screen.

(d) Projector lens: Variation of the projector lens current about the working position causes the image to rotate about the centre of the screen.

(e) Apertures: The objective aperture is externally adjusted to the centre of the screen, and incidentally visually checked for cleanliness, but the centration of the projector and condenser apertures depends on good alignment. On increasing the projector current the projector diaphragm should begin to restrict the field of view symmetrically. Malcentration of the condenser diaphragm, if gross, will prevent the screen lighting up during step (a), and if mild, will cause uneven illumination over the screen during step (b).

(2) Stability.

Stability is most simply tested by examining a heat-resistant specimen of carbon black (soot) at maximum magnification.

The edges of particles in an electron beam diffract some of the electrons which results in the appearance of diffraction or Fresnel fringes. These vary according to the focus (Fig. 10). Under-focussed particles are surrounded by a bright white fringe

or 'halo' which enhances the contrast of the edge. At focus, this halo vanishes, and the contrast is poor. At over-focus, a black fringe appears, but the contrast at the edge remains poor.

To check the stability, one edge of a particle is brought exactly to focus by adjusting the superfine objective lens control, the position of which is then recorded. The particle is next slightly overfocussed until a thin overfocus fring appears, and observed for one minute, during which time its appearance should not alter. Finally, it is accurately refocussed, and the position of the superfine control again noted. The two readings should coincide.

This stability check is sensitive to any instability of either the high tension voltage, or of the objective or projector lens currents, which would cause blurring of the Fresnel fringes, and fluctuation of the focus position of the superfine control. Further, contamination in the column, particularly near the electron gun or condenser lens system, or on the objective aperture, may cause either intermittent slight movements of the image or irregular fluctuations in the illumination.

(3) Astigmatism.

A resolution of better than 50Å is attainable with

the EM.75B provided the astigmatism is less than 2μ.

A heat-resistant particle is slightly overfocussed at maximum magnification and then, by altering the superfine objective control, brought back through focus. During this operation the overfocus fringe disappears, but owing to the presence of astigmatism, it does not do so symmetrically over the whole edge of the particle at the same time (Fig. 10). Accordingly, the position of the superfine control is noted when it has completely disappeared. The astigmatism equals the difference between the two readings, and should be less than four click stops on the superfine control, as each one represents a change of 0.5μ in focal length of the objective lens. Should the astigmatism be greater than 2μ, then it is corrected by slowly rotating the stigmator plates on the lower objective pole-shoes, until it reaches a minimum. Usually the astigmatism is checked visually, but if there is any doubt as to its magnitude, then it is measured photographically on a through-focus series (Fig. 10).

Specimen Drift.

In spite of a well-adjusted microscope, a common cause of loss of resolution is slight movement of the specimen during a

photographic exposure. This movement or 'drift' may be due either to instability of the specimen stage - mechanical drift - or to the heating effect of the electron beam causing expansion or contraction of the specimen grid, or softening and flow of the supporting film or specimen itself - thermal drift. Mechanical drift is rare on the EM.75B, where the specimen holder is moved in a horizontal plane during scanning, although it was common in the EM.100 where it moved in a practically vertical plane, and therefore gravity encouraged drift. Thermal drift sometimes does occur, and results in loss of resolution. The slight movement elongates particles in the direction of drift, and similarly, pairs of membranes perpendicular to the drift may be broadened and appear as a single thick membrane (Fig. 10).

Drift can be accurately measured by photographing a specimen repeatedly at known intervals of time, and calculating the displacement per unit time.

$d$ = distance between two successive images in mm.

$t$ = time interval in secs.

$m$ = magnification

then Drift $D = \frac{d \times 10^7}{mt}$ Å/sec.

With a resolution of 50Å then the total drift over the time of exposure should be less than 10Å.

Thermal drift is minimised by using as low an intensity of illumination as possible, but too low an illumination requires long exposures, during which even very slow drift may cause a significant loss of resolution, so that a compromise is required to steer between the Scylla of rapid drift and the Charybdis of excessive exposure.

Other factors responsible for thermal drift are weak supporting films, or poor contact between the specimen grid and the specimen holder so that the heat generated by the beam in the specimen is not conducted away rapidly into the specimen holder.

Calibration of the Electron Microscope.

The indicated magnifications on the instrument are only approximations, and therefore the true magnification must be determined for each individual microscope. A replica of a diffraction grating with 14,400 lines to the inch, is shadowed with palladium metal, and then a series of photographs taken at known indicated magnifications. As the average distance between the lines is known, it is possible to calculate fairly accurately, at low magnifications, the true magnification at each setting,

by measuring across a large number of the lines. But at higher magnifications there are so few lines visible on a plate that such measurement would be inaccurate, as there is no guarantee that a particular space between two adjacent lines is exactly $1/14,400^{th}$ of an inch.

Therefore, first the true magnification is calculated for a particular low indicated magnification, and then the other magnifications are derived from it by measuring the ratios of the magnifications of the series of photographic plates.

A calibration curve is shown (Fig. 9) for the EM.75B at 60 Kv, and at known projector and objective settings, because these also affect the final magnification. For example, separate calibration studies indicate that there is a 1% change in magnification for every one tenth of a unit change on the coarse objective control.

Photography of Biological Specimens.

Accurate focussing is essential for maximum resolution. As already stated, Fresnel fringes appear at the edges of structures viewed near focus, and substantially increase contrast in the underfocussed position. When photographing biological specimens - because of their inherent low contrast - there is a strong

tendency for the microscopist to underfocus, as the improved contrast masks the loss of resolution from the unaided human eye. To overcome this, there is a device known as a 'wobbler' incorporated into the Philips EM.75B. This consists of two pairs of electric coils, which temporarily increase the angular aperture, and considerably reduce the depth of field of the objective lens. Consequently, a slight variation from true focus causes considerable blurring, which makes accurate focussing easy.

The intensity of illumination is controlled by the condenser lens, and is set so that an exposure of 3 - 6 secs. is required, which is long enough to be reproduced accurately, and yet sufficiently brief to make drift unlikely.

## PHOTOGRAPHIC TECHNIQUES.

Light photography necessitated the development of special "sensitised" silver halide emulsions which are sensitive to the entire visible spectrum - "panchromatic" emulsions. The sensitivity of the original unsensitised emulsions includes not only the blue end of the visible spectrum, but electromagnetic waves of shorter wavelengths down to and including the extremely

short waves associated with electrons. These emulsions are known as "blue sensitive", because it is the reaction to visible light which is of general import (Fig. 13a).

Unsensitised emulsions are suitable for electron microscopy, and owing to their insensitivity to orange-red light, safelights of that colour of a convenient high intensity may be used in the dark room.

The characteristics of a photographic emulsion of concern to the electron microscopist are the "speed" or sensitivity to electrons, the contrast, and the grain. Under given processing conditions, the sensitivity and contrast of an emulsion are obtained by plotting the density of the silver image against the logarithm of the "exposure" - the produce of light intensity and the time. The density is a measure of the opacity of the silver image and is defined as follows:-

If a beam of light I falls on an exposed emulsion, a fraction of it is transmitted: $I_t$. The transmission T of an emulsion is the ratio $\frac{I_t}{I}$, i.e. maximum opacity T = zero, when completely transparent T = 1.

Photographic Density, $D = \text{Log } \frac{1}{T}$

e.g. if a silver image transmits 10% of the incident light

then,

$$D = \text{Log}\frac{1}{T} = \text{Log}\frac{1}{\left(\frac{I}{I_t}\right)} = \text{Log}\frac{1}{\left(\frac{10}{100}\right)} = 1.$$

$\therefore$ Photographic Density = 1.

Similarly, Density 2 means 1% of the incident light is transmitted.

When the Density is plotted against the logarithm of the exposure the graph obtained is known as the characteristic curve (Fig. 13b).

If two adjacent portions of the emulsion x and y are given different exposures $E_1$ and $E_2$, then when developed the density of x will be d, and y $d_2$. The ability to distinguish between these will depend on the contrast, i.e. the difference of the densities: $d_2 - d_1$. Thus, the contrast depends for a given difference in exposure on the slope of the characteristic curve. With increasing exposure, the contrast of the emulsion rises over portion AB to reach a maximum at B, remains constant over BC and then declines over CD to a low level. Therefore, for maximum contrast the exposure should fall between $E_1$ and $E_2$, but unfortunately as the contrast increases so does the grain. Grain depends on the size and distribution of the silver halide

crystals in the emulsion, and for fine grain the crystals must be small and evenly distributed. Large irregularly-spaced crystals cause coarse grain, and so obscure detail when the negative is enlarged. Fine grain is necessary in electron microscopy, and therefore usually the exposure is chosen to lie on AB near B, on the so-called 'toe' of the curve. Under-exposure - near A - must be avoided as it results in poor contrast (Fig. 37).

In practice it is possible to compensate for moderate overexposure by reducing the time of development, but it is impossible to obtain adequate contrast from grossly under-exposed negatives.

In printing, the paper is processed according to the manufacturers instructions and the exposure varied until an optimal positive print is obtained.

Photographic Materials and Methods.

(1) 35 mm. Film.

Ilford 5 b 11 fine-grain blue-sensitive recording film was developed in Kodak D.76 developer - a fine grain developer - for 10 min. at 68°F. with intermittent agitation. After a rinse in a 3% acetic acid stop bath, it was fixed in Amfix - a rapid fixer -

for 2 min. and then washed for 30 min. in running water. Finally, it was immersed in 'Photoflow' for ½ min. - a wetting agent which reduces drying marks - and then placed in a drying cabinet at 70°F.

(2) 6 x 6 cm. Plates.

Gevaert Diapositive contrasty plates were developed in G.251 or Kodak D.76 for 4 minutes in a dish, with continuous agitation. Thereafter the processing was similar to that of the film, except that owing to the thinner emulsion on the plates only 15 mins. washing is necessary.

(3) Enlarging.

The film or plate negatives were enlarged in a Kodak Precision Enlarger with the appropriate Ektar objective lens. The lens was focussed at full aperture and then closed one stop for the exposure. The exposure was selected - by trial and error if necessary - to result in an optimal print after developing in D.163 for 1½ - 2 mins. Kodak bromide glossy printing paper was used, and because of the intrinsically low contrast of biological specimens, grade 3 (hard) or even grade 4 (extra hard) was required. Following development the papers were rinsed in an acetic acid stop bath and fixed in Amfix for

1 - 2 mins. After washing in running water for 30 mins. they were immersed in glazing solution and transferred to a flat glazer.

Cleanliness, accurate control of temperatures and times, and care to use fresh solutions, are all necessary for good results.

## THE INTERPRETATION OF ELECTRON MICROGRAPHS.

The complexity of living material and its high water content makes it inevitable that considerable changes or artefacts will be introduced during the drastic procedures involved in the preparation of tissue for electron microscopy.

There is always uncertainty as to how far the microscopic image of a biological specimen corresponds to the in vivo condition, but with light microscopy it is possible to examine fresh living tissue and then study directly any artefacts produced by fixation or subsequent processing (Strangeways & Canti, 1927; Borysko, 1956). With the electron microscope, however, because of its inability to examine living tissues, such direct methods of investigation are not possible.

Several indirect methods of investigation are available

to assess the reliability of osmium tetroxide fixation and current methods of processing for electron microscopy. First, other chemical fixatives can be used, but this method is open to criticism on the grounds that certain artefacts might be common to a number of different chemical fixatives. A second method, which is free of this criticism, is to use physical methods of fixation such as freeze drying. Lastly, it is possible to carry out combined electron microscopical and x-ray diffraction studies on a limited number of highly ordered biological systems, and directly compare the results.

The artefacts in biological specimens fall into two categories (1) the "avoidable", which arise from technical errors, and (2) the "inevitable" which result directly from the technique itself. The latter are of greater importance, as they may be undetected, or even unsuspected.

Avoidable Artefacts.

These artefacts may be introduced during any stage in the preparation and microscopy of biological specimens. Autolytic changes have been recognised under the light microscope within a few minutes of death (Duthie, 1935; Zollinger, 1948) and similarly Palade (1952), Rhodin (1954), and Zetterqvist (1956),

detected changes under the electron microscope within 5 minutes of death. The mitochondria become swollen, the cristae disorganised, and the cytoplasm vacuolated. Sometimes, a single cell may show what are apparently post-mortem changes, although it is surrounded by well-preserved cells, and this may reflect the functional state of the cell at the time of fixation (Fig. 12a).

Prolonged fixation, washing, or dehydration, causes progressive destruction of tissue, and increasing extraction of its constituents such as lipids and proteins (Bahr, 1955; Dallam, 1957). These artefacts, however, being directly dependent on time, are readily controllable, and tissues should not be left overnight in alcohols. Conversely, inadequate washing may be responsible for the random precipitation of particles of reduced osmium tetroxide throughout the specimen.

Borysko (1956) demonstrated that tissue culture cells were sometimes damaged by the process of polymerization in methacrylate. Moderately damaged cells are swollen, the cytoplasmic ground substance is fragmented, and mitochondrial and other membranes are often ruptured (Fig. 12b).

Although the temperature of specimens was known to be raised considerably by the electron beam (Hall, 1954) and

damage had been noted earlier (Porter & Blum, 1953; Williams & Kallman, 1955) the first systematic search for possible artefacts caused during microscopy was that of Morgan et al., (1956), in which it was shown that this thermal effect could cause sublimation and differential flow of the methacrylate and result in patchy destruction and distortion of fine structure.

Artefacts which may be introduced by microtomy include knife-marks caused by irregularities in the knife-edge, compression of the section, and "ripple" in which the section appears striped due to a cyclic variation in thickness (Figs. 36 & 42).

Various artefacts may arise from faulty manipulation or adjustment of the microscope. Provided the microscope is adjusted properly, astigmatism should not be a problem, and the effects of focussing errors and drift have already been described.

Inevitable Artefacts.

Even when all the avoidable artefacts have been eliminated, the problem of the reliability of osmium tetroxide fixation remains. As mentioned previously, it is possible to examine with the light microscope the changes induced in living cells by osmium tetroxide fixation. But although these studies

have been reassuring, they are limited by the resolution of the light microscope. However, x-ray diffraction provides an independent method by which the quality of fixation can be assessed, although it is applicable only to certain highly organised biological systems, such as myelin. Comparative studies of myelin by electron microscopy and x-ray diffraction (Fernandez-Moran & Finean, 1957) reveal that the concentric membranes of lipoprotein discovered by x-rays are apparent under the electron microscope. Further, the spacing of adjacent membranes in the electron microscope is in fairly good agreement with the x-ray diffraction data.

Although osmium tetroxide fixes phospholipids it does not always preserve their morphology. For example, Chou & Meek (1958) have shown that certain phospholipid globules in the neurone of the common leech do not appear under the electron microscope, but their place is taken by several curved parallel membranes. They further showed that the addition of calcium ions to the osmium tetroxide fixative - which are known to stabilise phospholipid (Baker, 1946) - resulted in the appearance of multilaminar spheres with the same diameter as the original

lipid globules present in the living neurones. Thus, the usual osmium tetroxide fixative does not, in this instance, preserve the form of these phospholipid membranes, although it does fix them, and prevents their extraction during subsequent processing.

During fixation, artefacts may also arise from aggregation of dispersed molecules, and Baker (1958) gave a timely warning that there is a tendency to consider that fixatives which give a sharp contrasty image are "good", when there may be no evidence for the assumption that such appearances are more life-like than the aesthetically less pleasing "fuzzy" images. A good example of aggregation is furnished by the finding (Baker & Sjöstrand, 1958; Hanzon et al., 1959), that the microsomal particles of ribonucleic-acid (Palade, 1955a) which are such a prominent cytoplasmic constituent in osmium-fixed material, are not seen in freeze-dried tissue. Freeze-drying is important, because it is a physical method of fixation, in contrast to the other more widely-used chemical ones, such as osmium tetroxide, and therefore is unlikely to cause similar artefacts. Theoretically, freeze-dried tissue is practically free of artefact, except for

those arising from the formation of ice crystals (Gersh, 1958). These studies suggest that in vivo the ribonucleic-acid or RNA molecules are dispersed, but are aggregated and rendered visible by osmium tetroxide. The structure of mitochondria, Golgi apparatus, nuclear and cell membranes, is similar in freeze-dried tissue to that in osmium-fixed tissue, which is strong evidence that both these methods preserve the ultrastructure close to the in vivo state.

In addition to the evidence derived from physical methods like freeze-drying, the general similarity in structure after treatment by a variety of chemical fixatives (Luft, 1956; Freeman & Low, 1956: Morgan et al., 1956) which have been introduced within the last few years, testify to the reliability of osmium tetroxide.

Most fixatives used for electron microscopy contain a heavy metal, because the electron-scattering power, or density of an element, depends directly on its atomic weight, and it was believed that high concentrations of such compounds would give increased density to intracellular structures and thus 'stain' them. Consequently, in osmium-fixed material the term "osmophilic" is used to describe dense membranes and other structures, on

the assumption that such dense areas are the site of high concentrations of reduced osmium tetroxide. This view has been disputed by Ornstein (1957), who on quantitative grounds believed that electron-dense structures were due to high concentrations of biological substances - such as proteins and lipids - rather than to the presence of fixatives. It is true that after fixation with compounds containing only elements of low atomic weights, such as formalin, many organelles within cells appear dense, and membranes are recognisable (Morgan et al., 1956), but generally the contrast is poor. Furthermore, the efficacy of 'electron' stains has been proved by Watson (1958a & b), who by means of a variety of heavy metal compounds considerably enhanced the contrast of osmium-fixed tissues. Finally, Merriam (1958) showed that tissue fixed in osmium tetroxide lost much of its density as seen under the electron microscope, if the reduced osmium oxides in it were re-oxidised or 'bleached' by hydrogen peroxide to the soluble tetroxide, and then removed by washing. Swift & Rasch (1958) carried this work a stage further by 're-staining' such bleached sections with osmium tetroxide which restored the density of the intracellular components.

To sum up, it is clear that the density of given biological components under the electron microscope may be due to either a high concentration of biological substances, or the presence of 'electron stain', or a combination of these factors.

The fact that osmium tetroxide acts as an electron stain raises the fundamental problem: what is the chemical significance of 'osmophilic' membranes, and in particular are they indicative of the presence of protein or lipid? Sjöstrand (1953a) postulated that the unit cell membrane about 90Å thick was protein, but since then improved resolution has revealed that each unit cell membrane consists of two dense layers separated by a light interspace (Rhodin, 1954; Zetterqvist, 1956). Moreover, intracellular membranes such as those of the mitochondria, endoplasmic reticulum, Golgi complex, and the nuclear envelope, have a similar structure (Low, 1956a & b; Robertson, 1959).

Chemical studies do not resolve this problem, as osmium tetroxide reacts in vitro with many groups of biological compounds including both proteins and lipids (Bahr, 1954; Wolman, 1957).

A promising technique (Stoeckinius, 1959; Revel et al., 1958) is to prepare artificial phospholipid membranes, and

then process them in the usual way. Electron microscopically, these protein-free membranes - which appear as a dense membrane 90Å wide composed of two dense lines separated by a lighter interspace - bear a striking resemblance to the natural unit membranes. Unfortunately, a similar type of study with protein yielded the same type of result, and consequently the dilemma remains. Careful comparison of x-ray diffraction and EM data from myelin (Fernandez-Moran & Finean, 1957) suggests that the osmium is deposited at the lipoprotein interface of the unit membranes.

Brachet (1957) expressed deep concern that electron microscopists generally were relying almost exclusively on a single fixative - osmium tetroxide - and although within the last few years an increasing number of different fixatives have been introduced (Luft, 1956; Low & Freeman, 1956) it is still true that the vast majority of biological electron microscopic work involves fixation with osmium tetroxide.

However, increasing attention is being paid to cytochemistry at electron microscopic level and already it is possible to localise more or less accurately succinic dehydrogenase (Barnett & Palade, 1958), alkaline phosphatase (Barnett, 1959),

and nucleic acids (Swift & Rasch, 1958).

## GEOMETRIC ASPECTS OF THE INTERPRETATION OF ELECTRON MICROGRAPHS.

In the early days of section-cutting for electron microscopy, some errors arose from lack of familiarity with the problems involved in interpreting two-dimensional data in terms of the original solid structure. For example, intracellular components which consistently appeared as dense lines in random sections, were thought to be fibrillae (Dalton et al., 1950; Oberling et al., 1953) in spite of the fact that statistically fibrillae would be expected to appear as round or oval dots in the great majority of micrographs, and as lines only in the rare case of their being cut longitudinally (Fig. 11a). Dalton (1951) and later Sjöstrand (1953a) correctly deduced that these components were membranes.

Pairs of membranes, lying relatively close together, are a common feature of many cellular organelles, and each membrane pair appears in micrographs as two dense lines separated by a light interspace. As a result of the great depth of field in electron microscopes, the entire thickness of the section is in

focus, so that images formed from different levels of the specimen will be superimposed in the final image. Let us consider how the appearance of a double membrane will vary according to the plane and thickness of section, resolution of the microscope, and width of the interspace separating the two membranes.

The membranes of mitochondria are double (Fig. 11b), each membrane is ~60Å thick and the interspace ~100Å. Suppose such membranes deviate from the long axis of the beam by an angle $\theta$; in a section 500Å thick, if $\theta$ is zero, the membranes will be sharp and clearly defined (Fig. 11b), but as $\theta$ increases the adjacent edges will approach one another until the interspace approximates to the resolution of the microscope ~50Å, at which stage they will no longer be separated. Beyond this critical angle, the image will increase in width and the edges will be less sharp. With a thicker section of 1,000Å, the critical angle would be smaller, but conversely an improvement in microscopic resolution to 25Å would increase this angle. The angle is a measure of the probability of resolving the double membranes in random sections, and even with the relatively large interspace between mitochondrial double membranes it is so small that in a

single micrograph only a few of the cristae and limiting membranes will be resolved (Fig. 44). Each individual or 'unit' mitochondrial membrane can occasionally be resolved into a triple structure of two dense ~20Å lines with a ~20Å interspace. In order to resolve such a membrane into its component layers the critical angle must be considerably smaller even with a microscopic resolution of 10Å, and thus it is not surprising that only a few instances have been reported (Low, 1956a; Robertson, 1959). Low emphasizes the fact that provided the changes of resolving a certain feature are statistically very poor, then even a few successful demonstrations provide strong evidence that it is a real and constant feature.

The density of membranes which have been sectioned very obliquely - where $\theta$ is large - may be so reduced as to make them invisible, particularly if the surrounding ground substance is fairly dense; and therefore it is not uncommon to find mitochondrial cristae apparently free in the cytoplasm, as the obliquely cut limiting membranes are not visualised (Fig. 44). These appearances may lead to the erroneous assumption that there are gaps in membranes which have in reality been cut obliquely over part of their course: Lever (1956 & 1957) described gaps

in the mitochondrial membranes in the cells of brown fat and adrenal cortex, which were probably of this nature (Napolitano & Fawcett, 1958).

A study of skeletal muscle (Huxley, 1957) provides an excellent illustration of how important the thickness of sections may be in the interpretation of fine structures, due to this superimposition of structures at different levels within each section. Other investigators (Hodge, 1956; Spiro, 1956; Sjöstrand, 1956; Sjöstrand & Anderssen, 1957) agreed that the thin myofilaments found in the I band were continuous with the thicker ones of the A band (Fig. 14) and that the contraction of muscle was associated with shortening of the single myofilaments. Huxley, however,-primarily on the basis of earlier x-ray diffraction and phase contrast studies (Huxley, 1953; Hanzon & Huxley, 1953) - believed that the thin filaments continued into the A band between the thick filaments, and that as contraction occurred the two sets of filaments slid past one another (Fig. 14). With the aid of very thin sections, 200 - 300Å in thickness, he demonstrated clearly thin myofilaments between the thick ones in the A band.

He explained the failure of previous investigators

to find the thin filaments within the A band on the grounds that the normal silver sections used for electron microscopy are over 600Å in thickness, and that with a system of thick and thin filaments in close hexagonal array, only suitably angled sections less than 300Å would contain a single layer of filaments, whereas in 600Å sections the thin filaments would be obscured by the overlying thick ones (Fig. 14).

He thus conclusively proved the existence of two separate types of filament in skeletal muscle.

Slight errors are liable to arise in the measurement of double membranes because, as the deviation angle $\theta$ increases (Fig. 11b), the individual membranes become thicker and less sharp, and the interspace diminishes.

Estimates of the diameters of spherical organelles are liable to be low. Consider random sections of such an organelle bounded by a single membrane ~70Å thick. Increasing eccentricity of the plane of section results in a decrease of diameter, and blurring of the bounding membrane (Fig. 11c).

Suppose the membrane appears fairly sharp in sections over the range $B_1$ to $B_2$, and all other sections are rejected, then the diameters obtained will range from $d_1$ or $d_2$ up to D, the true or equatorial diameter, and therefore an average of the measurements will give a false low result. If a particular type of spherical organelle varies in size, then the results of measurement will be even more unreliable.

In conclusion, great care must be exercised in the interpretation and measurement of fine structures in electron micrographs, in order to avoid serious errors.

# PART II.

## THE FINE STRUCTURE OF THE PANETH CELL

## IN THE STARVING MOUSE.

## INTRODUCTION

### Discovery.

It was Schwalbe (1872) who first discovered - in the small intestines of certain animals - what were later to become known generally as "Paneth" cells. While examining fresh material from rat, mouse, and bat, he noticed a hitherto undetected type of cell in the lining of the Crypts of Lieberkühn*.

This cell was readily distinguished from the other types of cell in the crypt by numerous brilliant granules, which lay in the upper half of the cell, adjacent to the lumen.

Little attention was paid to Schwalbe's discovery, and sixteen years elapsed before Paneth (1888) re-discovered these cells, and described them at some length.

In view of their main distinguishing feature Paneth called them 'Körnchenzellen' or 'granule cells'. The granules were brilliantly refractile in the fresh state, although less so than fat globules, and were larger than Schwalbe had indicated

* "Sofort in die Augen fallen aber 3 bis 4 kleine Haufen dunkler, glänzender Körner, die dicht und das centrale Lumen hernumgruppirt sind, wodurch dann, wie bereits oben erwähnt wurde, ein Bild zu Stande kommt, ähnlich wie es kleiner pancreatischen Drüsen des Darms in frischen Zustande zeigen". Schwalbe (1872). Arch. f. micr. Anat. 8, p.136.

in his original drawing. Sudan black failed to stain them. Paneth found the granules dissolved slowly in ether and alcohol, and rapidly in dilute acids, but resisted alkalis and distilled water. Osmium tetroxide or picric acid were the fixatives of choice, and with certain other fixatives, such as alcohol, it would have been difficult to identify these cells. This emphasis on the difficulties of achieving adequate preservation of the Paneth granules may partly explain the conflicting views of subsequent authors on the species distribution of these cells.

The granules stained with haematoxylin, saffranin, methylene blue, eosin and 'alle Aniline-farben'. Haematoxylin distinguished these cells from the goblet cells of the crypt particularly well: "Haematoxylin färbt den Inhalt der Theka der Becherzellen nach Pikrin saure-Härtung bekanntlich nicht, während die Körnchen in den Lieberkühnschen Krypten sich damit intensiv tingiren". He noted that the granules stained differently from those of the exocrine cells of pancreas.

The granules were occasionally observed within the lumen of the crypt. He further noted that starvation of the animal for 48 hours resulted in an accumulation of granules in the Paneth cell, but that after ingestion of food many cells appeared

empty of granules, and that granules appeared in the lumen of the crypts.

Paneth's comprehensive account established the granule cell as a distinct type of exocrine cell, in which feeding provoked cytological changes.

Distribution.

(1) Species:

The classical paper of Paneth stimulated a constant flow of research, which established the widespread existence of these cells, in a wide variety of vertebrates.

They are particularly common among the mammals, among which they are found in (1) anthropoids: man (Paneth, 1888), macaque (Zipkin, 1904), and the marmoset (Filho, 1959). (2) ungulates: horse, sheep, and ox (Möller, 1899) and the pig (Deimler, 1905; Tang, 1922). (3) carnivores: cat and dog (Kaufmann-Wolf, 1911). (4) rodents: rat and mouse (Schwalbe, 1872) and guinea-pig (Klein, 1906). (5) marsupials: the opossum (Klein, 1906) and finally (6) chiroptera: bat (Schwalbe, 1872).

In lower vertebrates Paneth cells occur sporadically. For example, they have been found in reptiles, such as the lizard

(Nicolas, 1891) and in birds (Clara, 1926), although Nicolas (1891) failed to find them in frog, viper, tortoise and triton, and similarly Filho (1959) was unable to find any within the turtle or boa-constrictor.

(2) Site:

Although generally in higher mammals Paneth cells lie in the small intestine near the fundi of the Crypts of Lieberkühn, Kaufmann-Wolf (1911) claimed that in man, occasionally, a few could be seen on the villi themselves. The opossum is unique among mammals in having intestinal villi rich in Paneth cells (Klein, 1906).

The cells are not confined to the small intestine, because they are found in 12% of normal human appendices (Kerr & Lendrum, 1936). Kaufmann-Wolf (1911) described them in the large bowel of man, but subsequent workers have refuted this claim (Stöhr & Lewis, 1913; Kerr & Lendrum, 1936; Hertzog, 1937). There is little doubt that in pathological states in man they are found frequently in the large bowel* (Schmidt, 1905; Hergzog, 1937),

* Kerr & Lendrum (1936) failed to find any Paneth cells in a series of 25 consecutive cases with pathological lesions of the large bowel. However, other workers have shown only a comparatively low incidence of about 10%, and a negative series of 25 cases is not sufficient to disprove this low rate statistically, if one accepts the usual 5% level of significance.

and the most recent study (Watson & Roy, 1960) has demonstrated a very high incidence rate in ulcerative colitis. Pathological studies of this topic in other animals are very rare, but a carcinoma of the small intestine in a mouse - induced by the carcinogen 20-methyl cholanthrene - was reported to contain an exceptionally large number of Paneth cells (Dunn & Kessel, 1945).

Thus, there is good evidence that local pathological states in man are associated with increased numbers of Paneth cells.

Hamperl (1923) described these cells in the large bowel of certain normal ungulates such as the sheep and goat. Later, Hamperl also showed that in the bat they extended in the large bowel as far as the anus.

(3) Quantitative Studies:

The concentration of Paneth cells is not constant throughout the small intestine. Mols (1930) found more in the jejunum than in the duodenum. According to Filho (1959), the incidence in the mouse increases from the duodenum, reaching a maximum near the beginning of the ileum. Filho alleges that the numbers in the female mouse are 50% greater than in the male, but that this difference is abolished on feeding the animals, as more

cells in the female discharge their granules.

To summarize the distribution, the Paneth cell occurs in a wide variety of vertebrate species, and although in most they are confined to the small intestine, there are numerous important and well-established exceptions. Finally, pathological states within the gastro-intestinal tract tend to be associated with an increased number of Paneth cells.

Morphology.

The general structure of the Paneth cell resembles that of a serozymogenic exocrine cell. Numerous large secretory granules occupy the upper half of the cell.

The position of the Golgi apparatus is controversial. Holmgren (1902) described a widespread system of canals within the cell, but Corti (1926) using a silver method, found that the Golgi apparatus lay above the nucleus. Basophilic filaments - shown by Bensley (1898) to be common to serozymogenic cells - lie below the nucleus (Klein, 1906). The mitochondria are filamentous, and not particularly numerous.

The chemical nature of the granules has been intensively studied since the days of Paneth. There is general agreement

that they are not fatty (Paneth, 1888; Filho, 1959). Hinsche and Anderegg (1938) obtained a positive ninhydrin reaction, indicating the presence of amino-acids. Although they do not stain with mucin stains like mucicarmine (Klein, 1906; Bolande et al., 1954), they are P.A.S. positive (Bolande et al., 1954; Filho, 1959).

Filho (1959) in a recent histochemical study states that the granules are composed of protein and neutral mucopolysaccharides.

Van Genderen and Engel (1938) have shown, by estimating the dipeptidase activity of serial horizontal sections through the mucosa of the rat ileum, that a peak of activity occurs at the level of the fundi of the Crypts of Lieberkühn, where there are Paneth cells. Although this evidence is rather indirect, and cannot be regarded as conclusive, it implies that Paneth cells are concerned with the digestion of protein.

Secretory Cycle.

Nicolas (1891) described the secretory cycle of the Paneth cell. Unfortunately, he used Flemming's fluid, which makes the granules crescentic instead of spheroidal. Paneth (1888) had observed a similar appearance with alcohol and attributed

it to poor fixation, and later Klein (1906) came to the same conclusion.

Mols (1930) gave a detailed description of the secretory cycle in the mouse. Initially, the cell is slender and contains numerous mitochondria. The secretory granules begin to appear by the direct transformation of the mitochondria above the nucleus. At a later stage, the apical half of the cell becomes filled with large granules, which are ultimately discharged into the lumen. Mols noticed that the nucleus becomes irregular in outline during the latter portion of the cycle.

In man, Policard (1920) found no evidence of a direct transformation of mitochondria into secretory granules.

Cytogenesis.

With the solitary exception of Mols (1930), it is generally agreed that the Paneth cell does not undergo mitosis (Paneth, 1888; Klein, 1906; Leblond & Messier, 1958).

Probably these cells develop from principal cells further up the crypt (Paneth, 1888) or from mucous cells (Mols, 1930), but the problem of their origin remains unsettled.

Recent studies of the renewal rate of intestinal epithelium (Leblond & Messier, 1958) have not contributed to this

problem, because they were primarily concerned with the cells of the villus - which have an exceptionally high rate of turnover - and were therefore unlikely to reveal the life-cycle of the Paneth cell which, owing to its protected situation at the base of the crypts, has probably a much slower renewal rate.

Physiology.

The distribution in the various species sheds little light on the possible function of the Paneth cell. Although largely confined to the small intestine in mammals, the definite exceptions like the bat, make it difficult to ascribe any digestive function to these cells which could be common to all the species. Nor can it be related to any particular class of diet, as they occur in herbivora, granivora, insectivora, carnivora, and omnivora. Investigators agree that the ingestion of food causes the cells to discharge their granules (Paneth, 1888; Nicolas, 1891; Klein, 1906; Schmidt, 1905; Mols, 1930), but there is controversy as to whether protein, carbohydrate, or fat is the main stimulus. Miram (1912) found a maximal response after fat, whereas Mols (1930) considered the most potent stimulus to be protein. Filho (1959), after a careful quantitative study, came to the extraordinary conclusion that distilled water and fat were equally effective!

Thus, the information on this aspect of the function is bewildering.

Doubt also exists as to the method by which the stimulus is mediated: Mols (1930) concluded that hormonal and nervous stimuli were ineffectual, in contrast to Cordier (1923), who found that pilocarpine caused discharge of granules.*

With regard to the nature of the secretion there are three main schools of thought. The first, represented by Prenant (1907) and Kostitch (1924), and very recently by Filho (1959), believes it to be a modified mucus. The second regards the Paneth cell as serozymogenic (Paneth, 1888; Klein, 1906; Cordier, 1923). A third view (Bizozzero, 1892), is that it is an immature goblet cell, but this is incompatible with the embryological evidence that in the human and the mouse the appearance of goblet cells precedes that of the Paneth cells.

* The present writer attempted to repeat Cordier's experiments, using freshly prepared pilocarpine nitrate solution, which caused salivation when injected into the animals. Two experiments, each with six animals, were performed, but on examination of light microscopic 5μ sections stained with Heidenhain's iron haematoxylin and Southgate's mucicarmine there was no evidence that the pilocarpine injections had caused the Paneth cells to discharge their granules.

In addition, Klein (1906) considered that the distribution of Paneth cells up to the tips of the intestinal villi in the opossum, was strong evidence against this theory.

The evidence for and against the other views will be considered in the discussion.

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The present investigation with the electron microscope is an attempt to discover first, whether the fine structure resembles that of a typical serozymogenic cell or that of a mucous cell and second, the mechanism of formation of the secretory granules.

## METHODS

Healthy adult stock mice 6 - 9 months old were used. They were fasted for 18 hours prior to killing, during which time they were allowed water ad libitum, and kept in cages with a wire mesh floor to prevent coprophagy. They were killed by a blow on the head, and small portions of jejunum, 8 - 10 cms. from the pylorus, were removed with scissors, cut by a clean razor blade into minute fragments about 1 $mm^3$ in size, and finally dropped into fixative. Next, the jejunum and stomach were

examined to ensure that they were empty. If food was present, then the specimen was discarded.

The fixation and embedding were standard (see pp. 8, 12) in this first investigation, except that (1) the duration of fixation was 4 hours, and (2) the temperature of polymerisation was 45°C.

A Philips electron microscope 100Å fitted with a 50Å objective lens was used for survey micrographs. High resolution pictures were taken on a Siemens Elmskop I at direct magnifications of up to 40,000 times, and enlarged photographically as required.

## OBSERVATIONS

Four types of cell form the lining epithelium of the intestinal crypts of the mouse jejunum - the 'principal' or unspecialised cells, Paneth cells, mucous cells, and intermediate cells - which have some of the features of both the mucous and the Paneth cell.

White blood cells with small dense granules are occasionally interposed between the bases of epithelial cells. No argentaffin cells were seen, as this study was confined mainly to the bases of the crypts, where they are rarely found (Schofield,

1952). A typical cross-section through the base of the crypt (Fig. 16), shows two or three Paneth cells in a ring of about eight principal epithelial cells. The Paneth cell is readily distinguished by its large secretory granules, well-developed endoplasmic reticulum, irregular nucleus, and darker cytoplasm.

The typical mucous cell has light, more closely packed granules (Fig. 31). Finally, 'intermediate' cells - so-called because of their granules, which have features intermediate between Paneth and mucous granules - are also present.

## THE PANETH CELL

The main features of the Paneth cell (Figs. 15 & 16) are the secretory granules, Golgi complex, clusters of dense particles, endoplasmic reticulum, and nucleus. There are microvilli on the apical surface, and mitochondria and vacuole-containing bodies in the cytoplasm.

### The Cell Membrane.

The cell membrane of the Paneth cell, like that of the other epithelial cells, appears as a thin electron dense or osmophilic line, separated from a thin homogeneous basement membrane by a narrow lighter zone. The basement membrane lies between the cell and the sub-epithelial space, which contains

collagen fibres and various cells.

Laterally the cell membrane is separated from that of the neighbouring cell by a narrow interspace. At the level of the nucleus, the cell membrane shows one or more narrow shelf-like folds, while near the free surface there are one or more terminal bars, characterised by adjacent dense cytoplasm and broadening of the interspace (Figs. 15 & 28a).

The Microvilli.

Microvilli project from the free surfaces of the Paneth cells and other epithelial cells of the crypt (Figs. 16 &17 ). The cell membrane, which is single elsewhere, splits at the free surface of the cell to form a triple-layered membrane bounding the microvilli (Fig. 29). The microvilli, which are about 0.4μ in length and 0.1μ in diameter, are rather irregularly spaced (Fig. 28a), and are absent or much reduced where there are subjacent secretory granules (Fig. 30). They number approximately $35/\mu^2$, giving about a four-fold increase of the free surface area. In cross-section, the microvilli contain several circular profiles (Fig. 29) which represent tubular structures running longitudinally, as in longitudinal section only striations are seen. Microvilli form the striated border of the small intestine (Granger & Baker,

1950), so that it is not surprising that a smaller 'striated border' is present on the Paneth and neighbouring crypt cells. The great majority of light microscopists believed that there was no such border on the crypt cells, but Prenant (1907) said "j'ai observé inversement que les cellules de ce fond, et même peut-être les cellules de Paneth sont pourvues à leur face libre d'un pinceau de cils bien distincts et non soudes en un plateau strie". Thus Prenant anticipated the present findings by almost half a century!

The microvilli on the free surface of the Paneth cell and adjacent crypt cells, are similar to those found on a variety of other epithelial cells, such as the absorptive columnar cells of the intestinal villi (Zetterqvist, 1956), distal convoluted tubule cells of kidney (Pease, 1955), gall bladder (Yamada, 1955), mucous cells of the small intestine, and epithelial cells of rat trachea (Rhodin & Dalhamn, 1956). The microvilli of a secretory cell such as the Paneth cell are generally shorter, less frequent, and less regularly spaced than those of an absorptive cell such as the columnar cell of the intestinal villus (cf. Figs. 28a & b). Those of the latter cell, which form the intestinal striated border, increase the free surface area fourteen-fold

(Zetterqvist, 1956), compared with the approximate four-fold increase in the Paneth cell.

The Nucleus.

The outline of the nucleus is irregular (Figs. 16 & 18) in accordance with Mols (1930). This irregularity is due to infoldings, at least some of which are tubular, because frequently one sees in sections nuclei which contain an apparently isolated area of cytoplasm.

The nuclear membrane is double, consisting of two dense lines with an intervening lighter zone. The outer membrane is studded with cytoplasmic particles on its outer surface, and resembles a single membrane of the endoplasmic reticulum. The inner membrane has numerous nuclear particles associated with its inner surface (Fig. 27). Pores can be seen on cross-section (Fig. 27) and annuli on tangential sections. These annuli are not as prominent as those found in other mammalian cells such as the dorsal root ganglion cell (Dawson et al., 1955) or the hepatic parenchymatous cell (Watson, 1955).

Watson (1955) noted connections between the outer nuclear membrane and the endoplasmic reticulum in cells, such as the exocrine cell of pancreas, which have a well developed

endoplasmic reticulum. No connections of this kind were found in the Paneth cell, although they were occasionally seen in the principal cells.

The nucleolus is a dense sponge-like reticulum.

The Granular Endoplasmic Reticulum.

A system of paired membranes, arranged concentrically on the nucleus, fill most of the basal two-thirds of the cell (Figs. 16, 18, & 19). These are the ∝-cyto-membranes of Sjöstrand (1956), or endoplasmic reticulum (Palade, 1956). On section, each membrane pair below the nucleus lies parallel to the basement membrane, and extends upwards around the nucleus; the inner pairs encircling it, while the outer ones continue upwards into the upper third of the cell, and end among the secretory granules. Each membrane has a smooth surface and a surface studded with small opaque particles. The membranes of each pair are continuous with one another at their ends, and so enclose a flattened space lined with the smooth surfaces, while the rough surfaces face the cytoplasmic ground substance (Figs. 19 & 27). The membrane pairs among the secretory granules are disposed at random, are shorter, and appear as circular or elongated profiles (Fig. 23).

The Golgi Complex.

Dalton & Felix (1954) and Sjöstrand & Hanzon (1954) have described the ultrastructure of the Golgi complex, which has subsequently been identified in most cells.

The Golgi complex in the Paneth cell is extensive, and occupies a discoidal space above the nucleus, the plane of the disc being transverse (Figs. 16 & 18). It consists of several smooth membrane pairs, each pair being closed at its ends to form a flattened sac. There is no communication between adjacent membrane pairs. The flattened sacs may be expanded at their margins to form the Golgi vacuoles. Other vacuoles on section have their own limiting membrane and appear to be independent of the membrane pairs. Small Golgi vesicles surround the membranes and vacuoles (Figs. 23 & 24). These vesicles or granules are 450-500Å in diameter. Smaller vesicles about 100Å in diameter are also found.

The Golgi complex of the adjacent principal cells has similar structural elements, but is more compact, smaller, and has relatively more vacuoles and fewer vesicles (cf. Figs. 16 & 22) than that of the Paneth cell.

The Secretory or Paneth Granules

The secretory granules are the most prominent feature of the Paneth cell and fill the upper third of the cell (Figs. 16 & 20). They are dense spherical bodies 0.75-1.5μ in diameter, each lying in a vacuole 1-2μ in diameter. Some of the vacuoles, especially the smaller ones, have a single enclosing membrane (Figs. 23 & 24).

The vacuole is not a constant feature, however, as there are occasional cells where the space surrounding the granule is filled with a moderately osmophilic material (Fig. 17). The appearance of the granule with its surrounding vacuole was the same after fixation in Palade's hypotonic fixative or Dalton's osmium-dichromate solution, and washing with Tyrode's solution instead of distilled water.

Formation of Secretory Granules:

Survey micrographs show that in addition to the large secretory granules in the upper third of the cell, other secretory granules and related vacuoles lie within the Golgi region. These, in contrast to the large granules, are smaller, often irregular in outline, and lie eccentrically within their vacuoles, which have an enclosing membrane (Figs. 18, 23, & 24). Three types can

be distinguished:

(1) In close relation to the Golgi membranes are vacuoles, enclosed by a single membrane which sometimes is partly deficient. These vacuoles contain a cluster of small vesicles at one part of their circumference. The contained vesicles are similar in shape and size to the Golgi vesicles (Fig. 24).

(2) The second type of vacuole resembles the first in having an enclosing membrane which is sometimes partly deficient but contains, in addition to the cluster of vesicles, some of the fine granular substance typical of the large secretory granule. An example is seen in Fig. 20, where the deficiency in the enclosing membrane at the site of the cluster of vesicles allows them to communicate freely with the adjacent Golgi vesicles.

(3) The small secretory granules within the Golgi region have embedded within their finely granular substance vesicles similar to the Golgi vesicles. As in the case of the previous two types of vacuole, the enclosing membrane may be deficient, allowing continuity between the contents of the granule and the adjacent Golgi ground substance and vesicles (Figs. 25 & 26).

Thus it appears that the large secretory granule develops

from a vacuole arising within the Golgi complex. First, the vacuole incorporates Golgi vesicles through a deficiency in its enclosing membrane (Type 1). Secondly, it comes to contain finely granular substance in addition to the cluster of vesicles (Type 2). Thirdly, by further accumulation of granular substance it becomes a small secretory granule, in which vesicles can still be seen embedded in the mass of granular substance (Type 3).

As the small secretory granule increases in size it moves upwards towards the free surface of the cell, comes to lie symmetrically within its vacuole, and loses its enclosing membrane. Before it is extruded into the lumen, the overlying cell membrane is stretched, so that the microvilli disappear (Fig. 30).

Clusters of Dense Particles:

Apart from the typical granule, the Paneth cells have characteristic clusters of very dense small granules arranged irregularly, usually within a vacuole. Each cluster is about the size of a large secretory granule and the average section shows two or three clusters in the upper part of each Paneth cell (Figs. 20 & 23). There is no evidence that they are extruded into the crypt or transformed directly into secretory granules.

The Cytoplasmic RNA Particles:

Small opaque particles about 175Å in diameter are found free in the cytoplasm of the Paneth cell. They are similar to the particles attached to the membranes of the endoplasmic reticulum, and most of the apparently free particles are attached to obliquely-sectioned membranes (Fig. 19). In contrast, there are in the cytoplasm of the neighbouring principal cells (Fig. 22), numerous small groups of free particles.

Palade (1955a) proved that both the free and attached particles contain large amounts of RNA, and constitute the basophilic component of the cytoplasm.

The Mitochondria.

Rod-shaped mitochondria are scattered throughout the basal two-thirds of the cell (Fig. 20). Below the nucleus they lie transversely, but elsewhere those close to the cell membrane are parallel to the long axis of the cell, while the others are dispersed at random. Their ultrastructure is typical of mitochondria in other cells (Fig. 21). Dense granules lie in the mitochondria of the principal cells, but are rare in the Paneth cell (Fig. 18). Weiss (1955) concluded that similar intramitochondrial granules in duodenal epithelial cells were involved in cation transport.

The Vacuole-containing Bodies.

These consist of a vacuole about 0.3 - 0.5μ in diameter, bounded by a single membrane, containing numerous smaller vacuoles 500-800Å in diameter. In the majority of sections, there are one or more in the upper half of the cell, often among the secretory granules (Fig. 20).

Similar structures have been described in the neurone by Palay & Palade (1955), in the gall-bladder epithelium by Yamada (1955), and in the tracheal epithelial cells by Rhodin & Dalhamn (1956), who introduced the term vacuole-containing body.

In the present study they were also found in the neighbouring principal cells. Zetterqvist (1956) described similar structures in the columnar absorbing cell of the intestinal villus, and noted that the smaller ones about 0.3μ, were close to the Golgi complex.

In the Paneth cell, no close relationship exists between the vacuole-containing bodies and the Golgi complex.

The Cytoplasmic Vacuoles.

Vacuoles about 0.1 - 0.2μ in size, with an enclosing membrane, lie in the upper part of the cell close to the free surface (Fig. 28a).

# DISCUSSION.

## The Endoplasmic Reticulum and Cytoplasmic Basophilia.

The granular endoplasmic reticulum of the Paneth cell consists of a highly organised and extensive system of interconnected flattened sacs or 'cisternae', arranged concentrically on the nucleus. This arrangement is strikingly similar to that of other cells which are definitely serozymogenic, such as the exocrine cells of the pancreas (Sjöstrand & Hanzon, 1954; Palade, 1956) and the chief or pepsin-producing cells of the gastric mucosa (cf. Figs. 18 & 36).

What is the significance of a highly-developed granular endoplasmic reticulum? The answer to this question lies in recent studies which have clarified the relationship between the classical cytoplasmic basophilia or ergastoplasm, the 'microsomes' obtained from cell fractionation, and the endoplasmic reticulum revealed by the electron microscope.

(1) Cytoplasmic Basophilia and Ergastoplasm.

Langerhans (1896) noted that after fixation with dilute acetic acid the exocrine cells of pancreas contained basal striations.

Bensley (1898) showed that these striations or basal 'filaments' were characteristic of the serozymogenic cells in gastric, pancreatic, and salivary glands. They were strongly basophilic, and had a marked affinity for dyes which stained nuclear chromatin. Bensley, who believed they were the precursors of the secretory granules, termed them 'prozymogen'.

Garnier (1900) concluded, from a perusal of the previous literature and a study of glands in a variety of mammals, that cytoplasmic basophilic substance or 'ergastoplasm' was a general constituent of cells, but was particularly prominent in specialised cells which had to synthesize absorbed material to form their secretion.*

In 1906, Klein found similar filaments within the Paneth cells.

Thus, the 'ergastoplasm' or filamentous basophilic substance is a constant feature of serozymogenic cells, and of

* l'ergastoplasm ... "il s'agit lâ d'une structure générale de la cellule, mais surtout, apparente dans les unites vivantes chargees plus spécialement d'utiliser les matériaux assimilés pour l'élaboration du material de secretion". Garnier (1900). J. de l'Anat. et Physiol. 36, 22-94.

the Paneth cell. Garnier believed it played an active part in the process of secretion, and described it as a 'veritable organ of the secreting cell'. He found no evidence that it was transformed directly into secretory granules.

The significance of this cytoplasmic basophilic substance was enhanced considerably by Brachet (1941) who, using pyronine G staining, and ribonuclease digestion as a control, showed that cytoplasmic basophilia was due to the presence of ribonucleic acid (RNA). Brachet and his co-workers also demonstrated that cells actively engaged in protein synthesis were rich in RNA. Conversely, cells not noted for protein synthesis were poor in RNA, even if - like muscle cells - they had a high metabolic rate. Independently, Caspersson (1950) arrived at similar conclusions, using the ingenious method of ultraviolet microspectrophotometry. Leslie & Davidson (1951) proved quantitatively that in embryonic tissue, synthesis of RNA <u>preceded</u> that of protein. In this connection, it is of historical interest that Garnier (1900), had observed a half century earlier, that the ergastoplasm became most prominent <u>before</u> the appearance of secretory granules, during the secretory cycle in serozymogenic cells.

Finally, Gale (1955) produced direct evidence of the role of RNA in protein synthesis. He found that after disrupting staphylococci ultrasonically, they became incapable of respiration, but retained their capacity to synthesize protein in the presence of a source of energy and suitable amino-acids. Removal of their nucleic acids from the bacteria produced an inhibition of protein synthesis, which could be reversed subsequently by the addition of staphylococcal RNA and DNA.

The foregoing evidence is proof that RNA is responsible for cytoplasmic basophilia, and plays the central role in protein synthesis.

(2) The Endoplasmic Reticulum and Related Biochemical Studies.

Porter et al., (1945) found a reticulum consisting of interconnected vesicles in the cytoplasm of fibroblasts, which were examined in toto, under the electron microscope. Subsequently this structure has been found in all nucleated cells examined, and is known as the endoplasmic reticulum (ER). It varies greatly from one type of cell to another in disposition, quantity, and morphology, but essentially is a continuous membrane-bound system of vesicles, tubules, or flattened sacs (Palade, 1956). There are two main forms of ER: in one - the

'agranular' or 'smooth' - the bounding membrane is smooth, but in the other - the 'granular' - the membranes are studded with granules 150 - 200Å in diameter on their cytoplasmic surface.

Particles morphologically similar to those attached to the granular ER are found free in the cytoplasm of many cells. Palade (1955a) noticed that in many types of cell a close correlation existed between the distribution of those free or attached particles and that of cytoplasmic basophilia, and therefore, postulated that both types of particle were essentially the same, and consisted of RNA. Microsomes are one of the fractions obtained by homogenising and then ultracentrifuging cells. They are very rich in RNA and actively synthesize protein.

Palade's hypothesis was substantiated by combined biochemical and electron microscopic studies (Palade & Siekevitz, 1956a & b), in which the microsome fraction of both liver and pancreas were found to be fragmented granular ER. In addition, they proved that the RNA of the microsomal fraction was confined to the granules by incubating the microsomes with the enzyme ribonuclease - which dissolved the granules but left the membranes intact.

More recent information on the role of the ER in the elaboration of zymogen in the exocrine cell of the guinea-pig pancreas, comes from further studies by Siekevitz and Palade (1958a & b). They conclude that first, the RNA particles on the ER membranes synthesize the amino-acids into protein, which is then transferred into the cavity of the ER. Subsequently, it reaches the zymogen granules by a route which is at present obscure. In thin sections of pancreas, they observed electron-dense material within the ER during active secretion, which was assumed to be newly-synthesized protein.

In the pigeon pancreas, Douglas & Munro (1959) found pancreatic lipase within the ER vesicles of the microsome fraction, which is additional evidence that the ER is concerned with the early formation of protein secretion in serozymogenic cells.

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Mucous cells are poor in granular ER (Fig. 31), (Rhodin & Dalhamn, 1956; Palay, 1959). On the other hand, the exocrine pancreatic cell contains a highly organised endoplasmic reticulum - which is known from the preceding evidence to be directly involved in the elaboration of protein for the zymogen

granules. The fact that the endoplasmic reticulum of the Paneth cell is morphologically identical with that of the pancreatic cell is strong evidence that it is serozymogenic rather than mucous in nature.

Partly on the basis of ethionine studies, Filho (1959) concluded that the Paneth cell was not serozymogenic. Injections of DL-ethionine - an analogue of the essential amino-acid methionine - cause a necrosing pancreatitis (Goldberg et al., 1950). It is the exocrine cells in the pancreas which are affected, and a diminution in their cytoplasmic basophilia is an early sign of damage. Filho injected mice with DL-ethionine and failed to cause necrosis of the Paneth cells. On the assumption that all serozymogenic cells would react uniformly, he deduced that as the Paneth cell had responded differently from the pancreatic cell, it could not be serozymogenic. But is there proof to support the initial assumption? On the contrary, Alvizouri & Warren (1954) were unable to find any changes within the serozymogenic cells of the salivary glands in animals suffering from an ethionine-induced pancreatitis. In the light of this work, Filho's premise is no longer tenable, and consequently his conclusions are invalid.

The Paneth cell granules resemble those of other serozymogenic cells in their affinity for haematoxylin and neutral gentian, and in their failure to stain with "mucin" stains, such as mucicarmine. They are P.A.S. positive (Bolande et al., 1954; Filho, 1959), but this does not disprove that the cell is serozymogenic as Lillie (1951), in a comprehensive analysis of many tissues from several species, found a marked difference between the P.A.S. reactions of pancreatic exocrine cells and mucous cells, but that in general the reactions of the Paneth and pancreatic cells were similar.

In conclusion, the published evidence that the Paneth cell is a mucous cell is unconvincing, and the present study gives strong morphological support to the view that it is serozymogenic.

The Role of the Golgi Complex in Secretion.

During the first quarter of the present century it was commonly supposed that certain intracellular structures such as the mitochondria or 'ergastoplasm' were directly transformed into secretory granules. Mols (1930), for example, believed that the secretory granules of the Paneth cell arose directly from mitochondria, although Policard (1920) had found

no evidence of such a transformation. In recent years, as the mushroom growth of biochemical knowledge has revealed the astonishing complexity of such organelles as the mitochondria, it has become increasingly apparent that to extrude in the secretion even portions of such highly organised organelles would be extremely wasteful, and therefore it seems on a priori grounds to be an improbable occurrence.

The Golgi apparatus or complex is the organelle which has been linked most consistently with the secretory process, since Nassanov (1924) first stated that the immature granules arose within it. Nassanov used osmium methods of impregnating the Golgi apparatus, after initial fixation in good cytological preservations such as Regaud's or Champy's solutions, and consequently his results were superior to those of the silver impregnation techniques used by earlier investigators. Nassanov examined diverse secretory tissues and found a constant close relationship between the developing secretory granules and the Golgi complex.

Bowen (1928 & 1929) confirmed Nassanov's findings, and pointed out that as the Golgi complex did not diminish during secretion it was unlikely to contribute directly to the secretion.

Recent electron microscopic studies (Dalton & Felix, 1953 & 1954; Sjöstrand & Hanzon, 1954) have established the Golgi complex on a firm morphological basis, whereas with the light microscope identification had depended on its staining reactions.

As described in the observations, the Golgi complex plays an intimate role in the formation of the Paneth secretory granules. Near the apex of the cell the granules are large and enclosed within a vacuole, but the granules in the Golgi complex are considerably smaller, irregular in shape, and often have an incomplete enclosing membrane. Thus, the present study indicates that the small immature granules arise within the Golgi complex, and suggests that the Golgi vesicles contribute directly to these granules.

Sjöstrand & Hanzon (1954) noted a close topographical relationship between the Golgi complex and the secretory granules in the pancreatic cell. The earlier findings of Weiss (1953) that it was the "ergastoplasmic sacs" i.e. the granular ER, which gave origin to the secretory granules, can be discounted on the grounds that at that time the Golgi complex had not yet been

recognised and, therefore, the "ergastoplasmic sacs" may well have been Golgi complex.

During the present investigation it was repeatedly observed that in the goblet cell there was a distinct textural difference between the mucous granules in the Golgi complex and those outwith it (Fig. 31).

Hagenzu & Bernhard (1956) also showed that the Golgi complex was implicated in granule formation in secretory cells, including those of endocrine glands. Moreover, the acrosome of the spermatid arises within the Golgi complex (Burgos & Fawcett, 1955).

Thus accumulating evidence, derived from electron microscopy and biochemical studies of secretory cells, implicates the Golgi complex in the formation of secretory granules, and in the Paneth cell the Golgi vesicles appear to be actually incorporated into the granules.

# PART III.

## THE FINE STRUCTURE OF THE GASTRIC PARIETAL CELL IN THE STARVING MOUSE.

# INTRODUCTION.

## Earlier Observations on the Cytology of the Parietal Cell.

The parietal cell is found in the gastric mucosa of higher vertebrates, including mammals. In amphibia, such as the frog, on the other hand, where no typical parietal cells are present, it is believed that the single type of cell present in the body of the gastric gland secretes both hydrochloric acid and pepsin.

Prior to Heidenhain's (1870) discovery of the parietal cell, these gastric glands of mammals were thought to consist of a single cell type - the 'rennin cell' or 'Labzelle'. Heidenhain showed that there were peripherally placed cells - the (parietal) 'Belegzelle', in addition to the (chief) 'Hauptzelle', which formed the main lining of the gland. He concluded that the chief cell secreted pepsin, and therefore by exclusion the hydrochloric acid was secreted by the parietal cell.

Heidenhain thought that the chief cells formed a continuous layer, and thereby excluded the parietal cells from contact with the lumen, but this was subsequently disproved by Müller (1895), among others, who demonstrated by Golgi's silver bichromate method that these cells communicated with the lumen either

directly in the upper part of the gland, or indirectly via narrow intercellular canaliculi, which passed between the chief cells in the lower part of the gland. Moreover, this method revealed tiny passages - intracellular canaliculi - which entered the cell at the apical surface and branched dichotomously as they passed towards the base of the cell.

Next, the canaliculi were observed in fresh gastric mucosa by means of intravital and supravital methods. (Fitzgerald, 1910; Harvey & Bensley, 1912). Twenty years later, Beams & King (1932) using a neutral red method, stressed that the canaliculi were visible in the fresh state, and finally Moussa & Khattab (1957) found them in the fresh unstained mucosa by phase contrast.

The characteristic oxyphil granules of the parietal cell were regarded as bioblasts (mitochondria) by Altmann (1894), whose pictures showed parietal cells containing numerous spheroidal or short rod-like granules. Subsequent workers have, in the main, supported this conclusion: Lim & Ma (1926) stained them with acid fuchsin. Histochemically, the granules contain phospholipine and a protein moiety (Menzies, 1949). The granules stain with Janus

Green B - long recognised as a specific reaction of mitochondria - and which is related to the presence of oxidative enzymes restricted to the mitochondria (Lazarow & Cooperstein, 1953).

Ma, Lim & Liu (1927) thought the Golgi complex of the parietal cell was merely lipoid within the intracellular canaliculi, but most other workers— including Moussa & Khattab (1957)— believe it to be a separate organelle.

The Function of the Parietal Cell.

Heidenhain (1870) first suggested that the parietal cell secreted acid primarily because there was evidence that the pepsin was produced by the chief cells, and as the secretion of these two substances could occur independently of each other, it was unlikely that a single cell was the source of both. Langley (1880) strengthened this hypothesis by showing that a close relationship existed between the quantity of granules in the chief cells, and the peptic activity of the gastric mucosa.

Various investigators conceived the idea of using indicator dyes such as neutral red supravitally to determine the pH in the gastric tubule. Harvey & Bensley (1912), finding that the dye in the lumen of the body and basal portion of the gastric glands was alkaline and became acid only at the foveolae, concluded

that hydrochloric acid was secreted as a neutral precursor. Intermittent attempts were made following the work of Harvey & Bensley to demonstrate acid within the canaliculi of the parietal cell, and finally Bradford & Davies (1950) succeeded. The actively secreting gastric mucosa in vitro transported a large number of basic dyes and they found the pH of the intracellular canaliculi was at a pH of 1.4. They noted, however, that only some of the tubules contained dye, and within a single tubule only a proportion of the parietal cells were active. They concluded that acid was secreted in the free state into the canaliculi of the parietal cell. However, this was not the final word, because White, Swigart & Rehm (1956) injected indicator dyes intravenously until the gastric juice became intensely coloured, and then rapidly cooled the living mucosa by pouring on liquid nitrogen. Under examination at $-18^{o}C$. the gastric mucosa was colourless. Control observations revealed that indicators in true solution did not appear coloured if viewed in depths corresponding to the lumina of the gastric glands, and further observation proved that the tubules were only stained if the stomach was inactive. They concluded that indicators were valueless in the investigation

of the site of acid formation, and that all previous results based on such methods were invalid!

However, if the use of dyes has been found inadequate, there is still very good indirect evidence that the parietal cell is the source of HCl.

First, the active gastric mucosa concentrates hydrogen ions several million times, and this requires an enormous expenditure of energy. Thus it is not surprising that acid production is reduced by deficiencies of certain members of the Vitamin B complex which are concerned with energy metabolism. Moreover, there is a good correlation between the concentrations of cozymase and nicotinic acid, two members of the B complex, on the one hand, and the number of parietal cells at different levels of the gastric mucosa on the other (Bradford et al., 1948), which implies that they have a high rate of metabolism, and are concerned with HCl formation.

Secondly, during acid secretion the gastric mucosa secretes hydrogen ions into the lumen of the stomach, and equimolar amounts of bicarbonate into the blood. The production of bicarbonate is therefore associated with acid formation. The enzyme carbonic anhydrase - which catalyzes the formation of bicarbonate - was found in large amounts within the parietal cell

by Davenport (1940), which suggested it was the site of HCl formation. Recently, this hypothesis has been substantiated by the finding that powerful inhibitors of this enzyme, such as diamox, reduce acid secretion in animals (Janowitz et al., 1952).

Third, several quantitative studies have established a direct relationship between the numbers of parietal cells present, and the total acid produced by maximal stimulation. For example, Card & Marks, (1960) found that in man the number of parietal cells per unit volume of mucosa - the parietal cell mass - showed a wide variation in different individuals, and was proportional to the total amount of acid produced in each case.

Finally, histamine, which acts directly on the acid-secreting cells of the gastric mucosa (Alivasatos, 1960), produces cytological changes within the parietal cell, such as loss of phospholipid from the mitochondria (Menzies, 1952a & b).

In conclusion, there is abundant evidence obtained by several widely differing methods, that the parietal cell is the source of the hydrochloric acid in gastric juice.

## METHODS.

Twenty healthy adult mice were fasted, but allowed free

access to water, for 24 hr., and then killed by a blow on the head. Small portions of mucosa were removed from the anterior wall of the stomach, and placed in fixative within 2 min., of death, after which the stomach was widely opened to ensure that it was empty. If food was found, the specimen was rejected. Subsequent processing was standard in this study.

Electron microscopy was carried out on a modified Philips EM.100A and a Philips EM.75B electron microscope.

## OBSERVATIONS

In sections through the body of the gland, the chief or zymogenic cells - which secrete pepsinogen - are characterised by numerous secretory granules, and an extensive granular endoplasmic reticulum (Fig. 36). The secretory granules vary in density (Figs. 34 & 36), and the arrangement of the granular endoplasmic reticulum resembles closely that found in other serozymogenic cells, such as the exocrine cell of the pancreas, or the Paneth cell (cf. Figs. 18 & 36). The RNA granules on the membranes of the endoplasmic reticulum are readily seen in Fig. 37.

The parietal cell - characterised by large dense ovoid mitochondria and a system of intracellular canaliculi - is readily

distinguished from the mucous neck cell and the chief cell (Figs. 34 & 35). Often a parietal cell is interposed between two mucous neck cells, so that its apex reaches the lumen of the gland (Fig. 38). Alternatively, a parietal cell which does not reach the lumen of the gland directly, communicates with it via an intercellular canaliculus as in Fig. 35, where such a canaliculus leads from the narrow apex of a parietal cell towards the main lumen. The intercellular canaliculus is a narrow cleft between two adjacent cells, sparsely lined with short microvilli.

The Parietal Cell.

The parietal cell is pyramidal, with a large basal surface next to the basement membrane, and the apex towards the lumen of the gland. It is larger than the other cells of the gastric glands, and has a centrally placed nucleus (Fig. 35).

The large ovoid mitochondria are the dominant feature of the cytoplasm in survey micrographs, and appear to be separated into a peripheral group and a central perinuclear group by a ring-like lighter zone of cytoplasm containing the intracellular canaliculi (Figs. 32 & 38).

Higher magnifications reveal other organelles, including

the cytoplasmic vacuoles of the agranular endoplasmic reticulum, RNA granules, a scanty granular endoplasmic reticulum, and vacuole-containing bodies.

Intracellular Canaliculi.

Intracellular canaliculi - consisting of a branching system of tubular passages within the parietal cell, which open on to its free apical surface - are seen in most sections.

The canaliculi - cut in cross-section - are clearly seen in Fig. 40, where they are more distended than usual. In some sections, they are cut longitudinally throughout the greater part of their course, and in one fortunate section (Fig. 38), two intracellular canaliculi can be seen extending from the basal part of the cell, upwards on both sides of the nucleus to reach the free surface of the cell, where they open directly into the lumen of the gland. In most micrographs the canaliculi appear as a complex system of membranes (Figs. 32 & 38), as their walls are not smooth, but have numerous microvilli projecting into and reducing the lumen (Figs. 33 & 39). The canaliculi are more readily observed in animals which have received histamine injections, where they are moderately distended (Fig. 41).

A continuous cell membrane about 100Å thick, bounds

the microvilli, and has occasionally been resolved into a double membrane resembling that of the microvilli of the intestinal brush border (Zetterqvist, 1956) and the Paneth cell (Fig. 29). An additional membrane which underlies this double membrane, and is apparent in both cross and longitudinal sections of microvilli (Figs. 43 & 44), is a unique structural feature which distinguishes the microvilli in the parietal cell from those of other cells (Rhodin, 1954; Yamada, 1955; Zetterqvist, 1956).

The microvilli of the parietal cell are 0.7μ in length and .09μ in diameter. The number of microvillous projections is about 10 per $\mu^2$ - calculated by measuring the distance between the centres of adjacent microvilli at their bases in longitudinal sections, and assuming that they are hexagonally spaced. Direct measurement of the spacing between cross-sectioned microvilli is unsuitable, as they converge on each other towards the centre of the canaliculus (Fig. 39).

According to the above figures the microvilli increase the secretory surface area about three-fold.

Cell Membrane.

Basally, the cell membrane is related to a typical

basement membrane, and is infolded at infrequent intervals (Fig. 42). These folds are not comparable, however, to the highly complex system found by Pease (1956) in epithelia noted specially for water transport. A good example of such infolding occurs in the nasal gland of the duck (Hally & Scothorne, 1960), which secretes a strongly hypertonic salt solution (Scothorne, 1959).

Mitochondria.

The great size and numbers of the 'oxyntic granules' or mitochondria is the dominant feature of the parietal cell in survey micrographs (Figs. 35 & 38). Mostly ovoid, a few are in the form of short cylinders, and the mean thickness - measured parallel to the cristae - is 0.57μ. This is greater than mitochondria of most other cells: for example, the corresponding figure in the intestinal epithelium is 0.25μ (Zetterqvist, 1956), and is 0.4μ in the renal tubular epithelium (Rhodin, 1954).

Their fine structure is typical of mitochondria in general, with an outer double membrane and internal cristae (Figs. 42 & 44). However, the ground substance of the parietal cell mitochondria is denser than that of mitochondria in other cells (Fig. 35). The cristae show two dense 75Å lines separated by a

40Å interspace, and are unusually well-developed, extending almost the full width of the mitochondria (Fig. 42). Moreover, the cristae are close-packed - about 30 to every micron length of mitochondrion.

Vacuole-containing Bodies.

Micrographs of parietal cells usually contain one vacuole-containing body. This cytoplasmic organelle is less than 0.5μ in diameter, and consists of a spherical vacuole - bounded by a smooth membrane - within which are smaller vacuoles 400 - 600Å in size (Fig. 39).

These inconspicuous organelles were not described in earlier studies of the parietal cell (Sedar, 1955; Challice et al., 1957; Kurosumi et al., 1958), but recently the present findings have been confirmed in several species (Lawn, 1960; Ito & Winchester, 1960; Sedar, 1960).

Cytoplasmic Vacuoles of the Agranular Endoplasmic Reticulum.

Spherical vacuoles ranging from about 0.05 - 0.2μ in diameter, pack the cytoplasm between the mitochondria and the intracellular canaliculi (Figs. 39 & 44). Each vacuole is bounded by a smooth membrane, but although they are often contiguous, they show no evidence of interconnections, and so do not form a reticulum.

A few lie more peripherally among the mitochondria (Fig. 42), but the majority are adjacent to the canaliculi.

Granular Endoplasmic Reticulum and RNA Granules.

The granular endoplasmic reticulum appears as a few elongated profiles representing tubules, randomly scattered throughout the cytoplasm (Fig. 42). RNA particles, about 150-200Å in size, are scattered in small groups of four to seven throughout the cytoplasm (Fig. 42).

Golgi Complex.

The Golgi complex has been identified with the electron microscope in a wide variety of cells, since it was first described by Dalton & Felix (1954) and Sjöstrand & Hanzon (1954) as a system of several smooth parallel membrane pairs, small granules or vesicles, and large vacuoles.

Nothing conforming to this description has been found in the parietal cell, although a spurious resemblance to such a Golgi complex may arise where an intracellular canaliculus is cut transversely, and some of the microvilli - cut longitudinally - appear as smooth double membranes, while others - sectioned transversely - resemble Golgi vesicles.

It has not yet been possible to determine which cytoplasmic inclusion in the parietal cell corresponds to the 'Golgi complex' described under the light microscope, although preliminary work indicates it is neither the mitochondria nor the intracellular canaliculi.

## DISCUSSION.

### The Intracellular Canaliculi, Mitochondria, and their Functional Significance.

The intracellular canaliculi consist of a branching system of microvillus-lined passages within the cell, which open at the free surface of the cell to communicate either directly, or indirectly via an intercellular canaliculus, with the lumen of the gland. Thus their general arrangement conforms to the light microscopical observations of Harvey & Bensley (1912).

What is the significance of this system of canaliculi? The parietal cell is approximately pyramidal, with the apex towards the lumen of the gland, so there is a large basal surface area for the exchange of materials with the underlying capillaries (Figs. 35 & 38). On the other hand, the secretory surface, at the apex of the cell, would be small were it not greatly augmented by the intracellular canaliculi, which can be regarded as invaginations

of the secretory surface. Assuming that the total length of canaliculi within a cell is 50μ— and this is probably an underestimate, as in a single section (Fig. 38) 40μ can be seen— and as the average diameter is about 1μ, this would give an additional surface area of $160\mu^2$ provided the walls of the canaliculus were smooth. The microvilli, however, will further increase this surface by a factor of three, giving a final figure of about $500\mu^2$, which compares favourably with the available secretory surface area of other cells. Finally, the presence of the canaliculi ensures that secretion formed anywhere within the cell has to be transported only a short distance to reach the secretory surface, and so pass into the lumen of the gland.

Therefore, the intracellular canalicular system within the parietal cell enables it to be compact, while retaining extensive basal and secretory surfaces, and ensuring that secretion formed within the cell has ready access to the lumen of the gland —factors which will increase the efficiency of a secretory cell.

A unique feature of the microvillus of the parietal cell reported here, is an additional membrane underlying the bounding cell membrane, but its significance is obscure.

Intracellular canaliculi have been described from time to time in other mammalian cells. Elias (1949) reviewed the earlier claims to have found intracellular biliary canaliculi in the hepatic parenchymatous cells, and demonstrated that in the living organ they do not exist. Fawcett (1955), using the electron microscope, confirmed that the biliary canaliculi were entirely intercellular.

In amphibian liver cells, Dawson (1931) and Pollister independently found intracellular canaliculi, but subsequently Pollister (1932) withdrew his claim on the grounds that it had been based on a misinterpretation of thick sections, in which an intercellular canaliculus was superimposed on the cytoplasm of a cell below, and therefore appeared to be intracellular.

The gastric parietal cell is therefore unique among vertebrate cells in possessing intracellular canaliculi.

A cell which secretes hydrochloric acid, and yet has no intracellular canaliculi, is found in the gastric mucosa of frogs (Sedar, 1960). This 'granular' cell is the only type of cell in the gastric gland, and is therefore believed to have the dual function of secreting both HCl and pepsin. It does not contain canaliculi, although there are complex folds of the apical

or luminal cell membrane which considerably increase the available secretory surface, and may therefore be the analogues of the canaliculi.

Among invertebrates, they are found in certain glands of insects. Beams & King (1932), for example, demonstrated intracellular canaliculi in the salivary glands of various grass-hoppers, and it is of interest that the secretion from these glands is also acid, with a pH of 5.5 (Bodine, 1925).

The pharyngeal glands of the honeybee secrete 'royal jelly', which is acid (pH 4.5-5.0), proteolytic, and rich in vitamins of the B complex. The secretory cells possess intracellular canaliculi (Beams et al., 1959), but they differ in several respects from those of the gastric parietal cell. For example, in the honeybee the canaliculi are smooth-walled, as they lack microvilli. Although this cell and the gastric parietal cell, while differing in function, both possess canaliculi, the major differences in their fine structure suggest that this discrepancy is more apparent than real, and would justify a preliminary classification of intracellular canaliculi into two types: 'microvillous' and 'smooth'.

To sum up, there is no simple relationship between cell

function and the presence of intracellular canaliculi, and possibly the latter serve a more general function such as that of increasing the surface area, which might be necessary in a variety of secretory processes.

Abundant large ovoid mitochondria, with numerous cristae and dense ground substance, are a feature of the parietal cell. There is convincing biochemical evidence that mitochondria are concerned with almost all oxidative processes involving the consumption of oxygen, and recently Green, Lester, & Ziegler (1957) have shown that structurally intact cristae are necessary for oxidative phosphorylation - the energy-producing mechanism of the cell. The number and size of the mitochondria, with their unusually extensive and close-packed cristae, give the parietal cell an exceptionally large total area of cristae. Consequently, the fine structure of the parietal cell implies that in the active state it has a high rate of oxygen consumption.

There are two types of cell with comparable close-packed extensive cristae. The first is the brown fat cell, which is believed to be concerned with neolipogenesis, a process requring very large amounts of energy (Napolitano & Fawcett, 1958). The second is that of cardiac muscle, which is metabolically

very active. Moreover, as the mitochondria are relatively sparse, occupying a small fraction of the cytoplasmic volume - which is taken up largely by myofilaments - the energy demands on each mitochondrion will be correspondingly severe. Thus, these mitochondria have - in common with those in the parietal cell - very high metabolic rates.

Although it is generally agreed that the parietal cell secretes the hydrochloric acid of gastric juice, the exact nature of the metabolic mechanism, and the form in which it is secreted, are unknown. Any theory of the mechanism of hydrochloric acid secretion, however, must consider the efficiency of the process, i.e. the ratio of acid hydrogen ions secreted to oxygen molecules consumed (Davies, 1947). This ratio, according to most works including Davenport (1957), is less than 4. This figure, however, implies that the parietal cell has a greater oxygen consumption rate than that of any other mammalian cell, and as Davies (1957) considers this unlikely, he concludes that any mechanism which entails a ratio of only 4 is untenable.

The fine structure of the parietal cell, however, and in particular the unique system of intracellular canaliculi and very abundant mitochondria, indicates that it is a highly

specialised cell with an unusually high oxygen consumption rate, and so provides morphological support for the views of Davenport (1957).

The endoplasmic reticulum and cytoplasmic vacuoles.

The examination of spread cells, in toto, led to the introduction of the term 'endoplasmic reticulum' to describe a system of interconnected vesicle-like bodies lying in the cytoplasm. Since then it has been identified in every type of cell examined, except in the mature erthrocyte (Palade, 1956). In ultrathin sections, the reticular nature is lost as the likelihood of seeing the interconnecting tubules is much reduced owing to the thinness of the sections, and therefore the endoplasmic reticulum appears to consist of unconnected sacs, tubules, or vesicles, (Palade, 1956). Structurally, the endoplasmic reticulum has two forms: the 'agranular' which is bounded by smooth membranes, and the 'granular' which is bounded by membranes studded on their cytoplasmic surface with RNA granules. The relative volume, shape, and distribution of those two forms of reticulum vary widely in the different types of cell, and Palade (1956) classifies types of endoplasmic reticulum ranging from the highly organised granular reticulum

of the plasma cell, to the mainly smooth agranular reticulum found in such cells as the rat spermatocyte - which he describes 'as characterized by a randomly disposed, predominantly smooth-surfaced reticulum made up primarily of interconnected vesicles and tubules'. He further states that such a reticulum occurs in certain other cells, and quoting the work of Sedar (1955), includes the parietal cell in this group. According to Sedar, the cytoplasmic vacuoles adjacent to the canaliculi constitute the smooth agranular reticulum of the parietal cell, but these vacuoles do not conform to the above description, as they are neither randomly disposed, but are concentrated around the canaliculi, nor are they interconnected - although being close-packed and small - random sections would be expected to show such interconnections if they did exist.

According to the present observatinns the cytoplasmic vacuoles within the gastric parietal cell do not form a true reticulum, and differ in form and distribution from the agranular reticulum of such cells as the rat spermatocyte. They probably do, however, correspond to the agranular reticulum of other cells, as originally suggested by Sedar (1955).

Since this study was completed several papers have appeared which are relevant to the problem of whether or not the vacuoles form a true reticulum. Kurosumi et al., (1958) concluded that in the rat the vacuoles were independent. Lawn (1960) also worked with rats, and agreed that the reticulum was vacuolar, but found occasional intercommunications. There is considerable species variation, because Sedar in the bullfrog (1957) found that the agranular reticulum consisted of interconnected tubules. The agranular reticulum of the human parietal cell is also tubular (Lilliebridge, 1960), and sometimes opens freely at the secretory surface between the bases of the microvilli. This last finding is very important, as it provides a route whereby the contents of the endoplasmic reticulum could be secreted into the lumen of the gland. If secretion is mediated via this route, then what is the function of the microvilli? Either they serve merely as a 'reserve' of cell membrane for use during changes in diameter of the canaliculi, or else they could be involved in the <u>reabsorption</u> of some constituent of the primary secretion, such as water. However, such possibilities are highly speculative, and much further

investigation is required before the role of the endoplasmic reticulum in the production of hydrochloric acid is finally unravelled.

Challice et al., (1957) considered that these cytoplasmic vacuoles might represent cross-sectioned microvilli, but it is unlikely that large numbers of microvilli would be consistently cut transversely, and the unique structure of the microvilli, with the additional underlying membrane, enables the two structures to be readily distinguished (Fig. 43).

# PART IV.

## THE SECRETORY CHANGES PRODUCED BY PILOCARPINE IN THE GASTRIC PARIETAL CELLS OF THE MOUSE.

## INTRODUCTION.

The parietal cells of the mammalian stomach are generally believed to be the source of the hydrochloric acid in gastric juice. The present study of the changes produced in the fine structure of the parietal cell by pilocarpine injections, is a sequel to the preceding study of the resting cell in the starving mouse.

Menzies (1952a & b), who injected rats with histamine and pilocarpine, described light microscopic changes in the mitochondria and intracellular canaliculi of the parietal cells. He further demonstrated that after a single injection these changes affected only the parietal cells near the bases of the gastric glands, whereas repeated injections altered the parietal cells throughout the gland. In view of these findings, it was decided to use repeated injections of pilocarpine nitrate in order to produce a uniform picture, and thus reduce sampling errors.

## METHODS.

Six healthy adult mice aged 6 - 9 months were fasted for 24 hr., during which time they were allowed water ad libitum,

and then given 12 injections of pilocarpine nitrate at ½-hourly intervals. Each injection was 25 mg. of pilocarpine nitrate/kg. body weight, and the strength of the solution was so adjusted that the average mouse (wt. 40 gm.) received 0.25 cc. Control animals were also starved for 24 hr., and then injected with sterile water. Half an hour after the final injection each animal was killed, and small portions of gastric mucosa excised, and placed - less than 2 min. after death - in a fixative solution of buffered isotonic 1% osmium tetroxide. Processing was standard in this investigation.

A Philips EM.100A electron microscope was used initially, and latterly a Philips EM.75B with adjustable stigmator.

OBSERVATIONS ON CONTROL ANIMALS.

The fine structure of the gastric parietal cells in the control animals was the same as in the starved animals already described in the preceding investigation.

OBSERVATIONS ON TREATED ANIMALS.

After the first few injections the mice salivated profusely, and became motionless in a corner of their cage.

In survey micrographs two features distinguish the

treated animals from the controls. First, the intracellular canaliculi are moderately distended, so that the lumen of each one is clearly seen, and second, large vacuole-containing bodies lie scattered among the mitochondria (Figs. 45, 46, & 48).

Intracellular Canaliculi.

The diameters of the canaliculi are usually moderately increased in the treated animal to 2μ-3μ compared with 1μ in the controls (Fig. 45; cf. Figs. 38 & 48). In a small proportion of cells grosser changes occur, the canaliculi occupying most of the cell. Small evaginations protrude from the canalicular wall into the underlying cytoplasm, and their development resembles - on a minute scale - the histogenesis of an alveolar gland. These evaginations, which are lined with microvilli, are so numerous that the cytoplasm separating them from each other is reduced to thin sheets (Fig. 51). Finally, in a few cells the canaliculi are further increased in diameter, and have only an occasional microvillus on their otherwise smooth walls (Fig. 52).

The fine structure of the microvilli is unchanged in the treated animals, and the second membrane underlying the cell membrane which, as previously stated, distinguishes the microvilli

of the parietal cell from those of other cells - is still present (Figs. 50 & 53).

Vacuole-containing bodies.

Large rounded vacuolar bodies, ranging in diameter from 1μ - 3μ (Figs. 46 & 47), lie between the mitochondria. These 'vacuole-containing bodies' (Rhodin & Dalhamn, 1956) are bounded by a single smooth membrane enclosing smaller 500Å vacuoles (Fig. 49). The vacuole-containing bodies in the controls are smaller - under 0.5μ in diameter - and less numerous (cf. Figs. 39 & 48).

Although a micrograph may show up to about 8 vacuole-containing bodies in the treated parietal cell compared with 1 or 2 in the control, there is probably either little or no change in the absolute numbers in each cell following pilocarpine injections, because the chance of a spherical body appearing in a random section is proportional to its diameter, and consequently the observed fourfold increase in diameter would be expected to raise the apparent numbers from about 2 to 8.

Mitochondria.

The abundant mitochondria are more pleomorphic than

in the controls, and Y-shaped forms are not uncommon. The internal structure is unchanged; the cristae are close-packed and extensive, and the ground substance dense (Fig. 50).

Cytoplasmic vacuoles.

In the pilocarpine-treated animals cytoplasmic vacuoles lie closely packed adjacent to the canaliculi, but fewer are seen towards the periphery of the cell, as in the controls. An occasional vacuole appears to communicate directly with a canaliculus (Fig. 49).

Sometimes the number of cytoplasmic vacuoles is much diminished in the treated cells, but this is not a constant finding.

## DISCUSSION.

### Possible Mechanisms underlying the Changes in the Intracellular Canaliculi.

The changes produced in the intracellular canaliculi by pilocarpine - which are presumably related to the accumulation of secretion within the lumen of each canaliculus in the active cell - raise several problems in relation to the general properties of cell membranes. As the canaliculi increase in width the microvilli become more widely spaced over their walls, and in a

few cells virtually disappear. This decrease in the number of microvilli per unit area could occur in several ways, which will be considered in turn.

First, the smooth intervillous membrane between the bases of the microvilli could stretch, the microvilli themselves remaining unaltered. Is it likely that stretching of cell membrane can occur in this way? In a quantitative estimate of the elasticity of the cell membrane Ponder (1948) showed that during haemolysis the red cell membrane underwent little or no increase in area before rupturing. Recent work (Sjöstrand, 1953a; Rhodin, 1954; Geren, 1954; Robertson, 1955 & 1957; Zetterqvist, 1956; Fernandez-Morán & Finean, 1957) has emphasised the basic structural similarity of the cell membrane in a wide variety of cells, and supported the concept of Danielli (1936) that it consists of a bimolecular lipid layer sandwiched between two unimolecular protein layers. Thus it is probable that the elastic properties of the red cell membrane and that of the parietal cell resemble each other, and therefore the latter is unlikely to be capable of stretching. Moreover, stretching of the intervillous portion of the membrane would not account for the virtual absence of microvilli from the most dilated canaliculi.

Second, the canalicular walls could derive additional membrane from the underlying cytoplasmic vacuoles, but fusion of a vacuole with the cell membrane is a rare event as judged by this series of electron micrographs.

Third, the microvilli could shorten and their membrane flow into the intervillous smooth membrane, thereby increasing its area. This is the most likely mechanism, as it explains the virtual disappearance of the microvilli from the most dilated canaliculi. Thus, the microvilli, in addition to increasing the secretory surface area, appear to provide a "mobile reserve" of membrane, which permits the canaliculi to alter in diameter without stretching of their component cell membranes.

Shortly after the completion of this work, Palay demonstrated in the intestinal epithelium, that the cell membrane of the mucous goblet cells has complex folds during the early stages of the secretory cycle, which disappear as the developing mucinogen granules distend the upper part of the cell. He suggests that these reversible folds constitute a reserve of cell membrane which allows the expansion of the cell necessary to accommodate the freshly synthesised mucinogen granules (Palay, 1959). These folds therefore appear to have a function similar to that postulated for the microvilli.

Apart from the problem of the increased width of the canaliculi, their complex outlines in the active cells (Figs. 51 & 53) suggest that this is not merely a passive distension but an active process whereby many small evaginations develop from the walls of the main canaliculi.

Vacuole-containing bodies.

The marked increase in size of the vacuole-containing bodies produced by pilocarpine is most striking. It is not an artefactual swelling, because a single section through one of the enlarged bodies may contain more small vacuoles than would fill a vacuole-containing body from a control animal (cf. Figs. 49 & 39). Moreover, small vacuole-containing bodies are present in the adjacent gastric zymogenic cells in treated animals.

Palay & Palade (1955) first observed this organelle in the neurone, and suggested that the contained vacuoles might be tubular invaginations of the bounding membrane, but subsequent investigations - including the present one - provide no support for this contention. Rhodin & Dalhamn (1956) found it in the various epithelial cells of the tracheal mucosa, and introduced the term 'vacuole-containing body'. Following the original discovery it has been encountered in a large number of diverse cell types including: basophiles (Palade,

1955a); intestinal epithelial cells (Zetterqvist, 1956); regenerating nerve fibres (Estable et al., 1957); capillary endothelium (Gloor, 1958); ciliary epithelium (Holmberg, 1957); Paneth cells (Fig. 20); avian nasal gland (Hally & Scothorne, 1960); and the protozoan Chlamydomonas (Sager & Palade, 1957). The fact that several of these investigators each discovered the vacuole-containing body in more than one type of cell suggests that it is widely distributed in animal cells, but is frequently unrecognised.

Sotelo & Porter (1959) found large numbers of a similar structure in the developing rat ovum, and from a survey of the literature also concluded that it is probably a universal organelle of animal cells.

The chemical nature and function of the vacuole-containing body is obscure, and the enlargement of these organelles in the parietal cell induced by pilocarpine is the first occasion on which they have been shown to undergo functional changes.

Mitochondria.

In a histochemical study Menzies (1952a & b) demonstrated that under the influence of pilocarpine the mitochondria within the parietal cell underwent certain changes. They lost their phospholipine

content, as judged by a failure to react to Baker's acid haematin test (1946), and became enlarged and vacuolated. It is of interest, therefore, that apart from pleomorphism no evidence of mitochondrial change is apparent under the electron microscope, as the cristae and ground substance are unaltered. In particular, the absence of any sign of vacuolation within the mitochondria in the present study suggests that the vacuolation seen by Menzies was an artefact.

Thus the effect of pilocarpine is to produce moderate changes in the canaliculi and vacuole-containing bodies in the great majority of parietal cells, and gross changes in a substantial minority. As little is known of the biochemical mechanism of hydrochloric acid secretion (Davies, 1957), it is impossible to discuss the functional significance of these changes.

## PART V.

## ELECTRON MICROSCOPY OF THE UNUSUAL GOLGI APPARATUS WITHIN THE GASTRIC PARIETAL CELL.

## INTRODUCTION.

In 1954 two groups of electron microscopists (Dalton & Felix, 1954; Sjöstrand & Hanzon, 1954) identified in certain cells a characteristic cytoplasmic organelle at the site known to be occupied by the 'Golgi apparatus' of light microscopy. When first discovered, this organelle was identified as the 'Golgi apparatus', but subsequent evidence - reviewed later in this introduction - and the results of the present study, show that the two are not always identical, and therefore the organelle will be distinguished by the term 'Dalton Complex' (Baker, 1957b). The Dalton complex consists (Fig.22) of (1) several flattened sacs which in section appear as parallel membrane pairs, (2) large vacuoles, and (3) small granules or vesicles, about 500Å in diameter. Since the original discovery, many workers have confirmed the existence of the Dalton complex in a wide variety of cells from both vertebrates and invertebrates (Hagenau & Bernhard, 1956; Rhodin & Dalhamn, 1956; Lacy & Challice, 1956; Lacy, 1957).

In a further electron microscopic study Dalton & Felix (1956), using a modified classical Golgi osmium method, found that post-osmication resulted in the deposition of reduced osmium tetroxide on the Dalton complex in several types of cell. Although the Golgi

method consistently blackened the Dalton complex, the action on its several components was capricious. For example, in the exocrine cells of the pancreas the small vesicles were blackened, whereas in the epithelial cells of the epididymis it was the vacuoles and flattened sacs which were affected.

Lacy & Challice (1956) and Lacy (1957) applied unmodified classical silver and osmium methods to demonstrate the Golgi apparatus. They confirmed the finding of Dalton & Felix (1956) that the established Golgi methods blackened the Dalton complex, but once again the reactions of the individual components were not uniform. For example, in the exocrine cells of pancreas, silver methods blackened the flattened sacs, although as previously mentioned, Dalton & Felix had found that osmium methods blackened the small vesicles. Thus, in a single type of cell silver and osmium methods may give different results.

To sum up, electron microscopy has demonstrated in a wide variety of cells a morphologically distinct organelle - the Dalton complex. Classical osmium and silver methods used for demonstrating the Golgi apparatus consistently blacken the Dalton complex, although their detailed actions on the components of the complex are variable.

Light microscopists are generally agreed that a Golgi apparatus exists within the gastric parietal cell (Kolster, 1913; Beams & King, 1932; Menzies, 1949; Moussa & Khattab, 1957). It was therefore expected, in view of the foregoing evidence from other cells, that electron microscopy would reveal a Dalton complex. However, electron microscopical studies on the gastric parietal cells of the mouse and rat (Challice et al., 1957; Kurosumi et al., 1958; and the present author's study) failed to demonstrate it, and thus posed the question: what organised structure - if any - represents the Golgi apparatus within the parietal cell? The present investigation, using a post-osmication method, is designed to resolve this problem.

## METHODS.

Healthy adult Swiss albino mice, aged 6 - 9 months, were fasted for 48 hr., and then fed. 1 hr. after feeding they were killed by a blow on the head. Small portions of gastric mucosa, 1 $mm.^3$ in size, were then placed in fixative within 2 min. of death. The specimens were fixed in isotonic buffered 1% osmium tetroxide for 1 hr. After fixation, the specimens were washed briefly in distilled water, and divided into three groups. Each group was transferred to an aqueous 2% osmium tetroxide solution, and

incubated at 37°C. for 48, 15, and 3 hr. respectively. Following this post-osmication they were rinsed in distilled water, dehydrated through graded ethanols, and embedded in methacrylate in a routine manner.

Thick 2μ sections were cut for phase-contrast and light microscopy.

## OBSERVATIONS.

### Light Microscopy.

After 48 hr. post-osmication, the parietal cells are easily distinguished from the other cells of the gastric tubule by the intense black granules of reduced osmium tetroxide - constituting the 'Golgi apparatus' - which are scattered throughout their cytoplasm (Fig. 54a).

After 15 hr. post-osmication, some of the parietal cells are heavily impregnated, but the majority show only a few black granules in the cytoplasm which contrast with the grey mitochondria and clear canaliculi (Fig. 54b). A typical supranuclear Golgi apparatus is often present in other cells of the gastric tubule (Fig. 54b).

Thus, depending on the duration of post-osmication the Golgi apparatus of the parietal cell consists of either (1), a few black

granules scattered throughout the cytoplasm, which contrast sharply with the mitochondria and intracellular canaliculi, or (2), numerous black granules practically filling the cytoplasm; in which case the mitochondria are no longer recognisable, although the intracellular canaliculi remain visible (cf. Figs. 54a & b).

Electron Microscopy.

In moderately impregnated parietal cells, the Golgi apparatus consists only of black granules between the mitochondria (Fig. 56), but heavily impregnated cells contain - in addition to the black granules in the cytoplasm - blackened mitochondria (Fig. 55). Thus, prolonged post-osmication leads to deposition of reduced osmium tetroxide within the mitochondria of the parietal cell.

Clearly, in moderately impregnated cells the 'Golgi apparatus' is represented by black granules in the cytoplasm. Let us, therefore, examine the relationship between these black granules and the other organelles of the parietal cell.

(1) The cytoplasmic vacuoles of the agranular endoplasmic reticulum.

The agranular endoplasmic reticulum of the parietal cell consists of numerous cytoplasmic vacuoles which are widespread throughout the cell, but are most concentrated near the intracellular canaliculi.

Following post-osmication these cytoplasmic vacuoles are no longer visible in moderately impregnated cells, but instead the black granules of the Golgi apparatus have a similar distribution within the cytoplasm (cf. Figs. 39 & 56).

In order to ascertain the exact relationship between the black granules and these vacuoles, it is necessary to examine an earlier stage of post-osmication, before the Golgi apparatus becomes visible to the light microscope. After 3 hr. post-osmication small black particles only 50 - 200Å in size lie within the cytoplasmic vacuoles (Figs. 57 & 58). Some particles appear to lie outside the vacuoles, but in a tangential section of a vacuole the obliquely cut membrane bounding the vacuole may not be resolved, and thus the contained particles will appear to lie free in the cytoplasm. Moreover, if free particles do exist, one would expect to find them also in the cytoplasm near the periphery of the cell - where cytoplasmic vacuoles are sparse - but in Fig. 57 there is a conspicuous absence of particles from this region. Thus the great majority, if not all, of these particles lie within the cytoplasmic vacuoles.

Therefore the Golgi apparatus of the parietal cell is the agranular reticulum: rendered visible by the progressive deposition

within its vacuoles of tiny particles of reduced osmium tetroxide.

(2) Vacuole-containing bodies.

These organelles contain black particles, even at an early stage of post-osmication (Figs. 57 & 58), and therefore make a small contribution to the image of the Golgi apparatus.

(3) Lipid globules.

Moderately blackened cytoplasmic inclusions, similar to those described as lipid globules by other authors, are observed within the parietal cell (Fig. 55). They are too small and sparse to contribute significantly to the light microscopic image of the Golgi apparatus.

(4) Mitochondria.

There is a stage in post-osmication when the cytoplasmic vacuoles are blackened and the mitochondria are practically unaffected (Fig. 57), but as osmication proceeds the mitochondria blacken (Fig. 55). The distribution of the black particles within the mitochondria is characteristic, for they are confined to the 'outer mitochondrial space', which is between the outer and inner mitochondrial membranes and extends into each crista. Particles are therefore seen within a crista, and never on the surface facing the mitochondrial ground substance (Fig. 59).

This selective blackening of the mitochondria is pecular to the parietal cell, for the mitochondria in the adjacent zymogenic cells are unblackened (Fig. 55).

## DISCUSSION.

The present study of the gastric parietal cell in the mouse shows that during post-osmication, reduced osmium tetroxide is selectively deposited in the agranular reticulum and the vacuole-containing bodies. As the latter occupy a very much smaller volume of the cytoplasm than the former, almost the entire Golgi apparatus is composed of the agranular endoplasmic reticulum.

However, with heavier impregnation the mitochondria are also blackened in a characteristic way. If one is not aware of this possibility, the blackened mitochondria could be erroneously included as part of the Golgi apparatus. This finding is not new: Bowen (1928) quotes numerous examples of cells in which the mitochondria or 'chondriomes' blackened if post-osmication or silver impregnation were prolonged. Some investigators have had difficulty in demonstrating the Golgi apparatus of the parietal cell (Beams & King, 1932; Menzies, 1949; Moussa & Khattab, 1957), and this may have been due to the tendency of the numerous mitochondria to blacken and thus

obscure the 'true' Golgi apparatus - the agranular endoplasmic reticulum. Indeed, Moussa & Khattab (1957) - in a light microscopic study - describe a 'degenerate' Golgi apparatus in many parietal cells, and show micrographs where the cytoplasm is packed with blackened granules, many of which are probably mitochondria.

There has been much controversy about the detailed morphology of the Golgi apparatus in the parietal cell. Kolster (1913) describes a true reticulum dispersed throughout the cytoplasm: Beams & King (1932) found chiefly isolated filaments scattered fairly generally throughout the cytoplasm: and more recently, Menzies (1949) found in the majority of parietal cells 'slightly osmophilic granules spread throughout the cytoplasm and osmophilic strands between the granules'. This result of Menzies' is identical with the present findings: the 'slightly osmophilic granules' being the mitochondria, and the 'strands' the agranular endoplasmic reticulum. The appearance of the Golgi apparatus - consisting as it does of the cytoplasmic vacuoles of the agranular endoplasmic reticulum - depends on the thickness of the section. The individual vacuoles are too close-packed to be resolved by the light microscope, and therefore in aggregates appear as strands or large granules between the mitochondria. Moreover, superimposition in 5μ sections leads

in some instances to an apparently complete reticulum throughout the cell.

The main finding of the present study, however, is that the Golgi apparatus of the gastric parietal cell is the agranular reticulum and not - as in other cells - the Dalton complex.

Although Baker and his colleagues (Baker, 1957a) have shown conclusively that histochemically the term 'Golgi material' is meaningless, in recent years the classical methods have been somewhat restored to favour by the discovery that they consistently blacken one or more components of the Dalton complex. The parietal cell is an exception, however, and thus one cannot assume that a cell contains a Dalton complex because classical Golgi methods have demonstrated that it possesses a Golgi apparatus.

Further, the fact that the Golgi methods blacken the Dalton complex in most cells and the agranular reticulum in the parietal cell, indicates that both may be related.

The gastric parietal cell is not unique in possessing a diffuse vacuolar type of agranular endoplasmic reticulum, as cells in the adrenal cortex have a similar reticulum (Lever, 1955; Palade, 1956; Zelander, 1959; Carr, 1959). According to Carr (1959), the

'compact' cells of the human adrenal cortex contain numerous vacuoles of the agranular reticulum lying between abundant mitochondria. He further observed, with the light microscope, that the classical Golgi apparatus consists of black granules which appear to have a similar distribution to the vacuoles. Zelander found within the cells of the adrenal cortex of mouse numerous discrete cytoplasmic vacuoles in addition to groups of Dalton complex. He was unable to differentiate between the cytoplasmic vacuoles of the agranular reticulum and those belonging to the Dalton complex, as they were morphologically identical. Lever (1955) had tentatively suggested that these cytoplasmic vacuoles within the cells of the adrenal cortex were the Golgi apparatus, but this hypothesis has never been tested.

Cytogenetic studies are also relevant, as Clermont (1956) concluded that within the developing rat spermatid, the Dalton complex gave rise directly to the cytoplasmic vacuoles of the agranular endoplasmic reticulum. Palade (1956), in a separate study of the rat spermatid, also produced evidence of the essential unity of the agranular reticulum and the Dalton complex.

Thus the above results and those of the present study provide evidence that the Dalton complex is a particular form of the agranular endoplasmic reticulum.

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# ELECTRON MICROSCOPIC OBSERVATIONS ON CERTAIN SECRETORY EPITHELIAL CELLS OF THE GASTRO-INTESTINAL TRACT IN THE MOUSE.

## VOL. II : ILLUSTRATIONS.

# TABLE OF CONTENTS.



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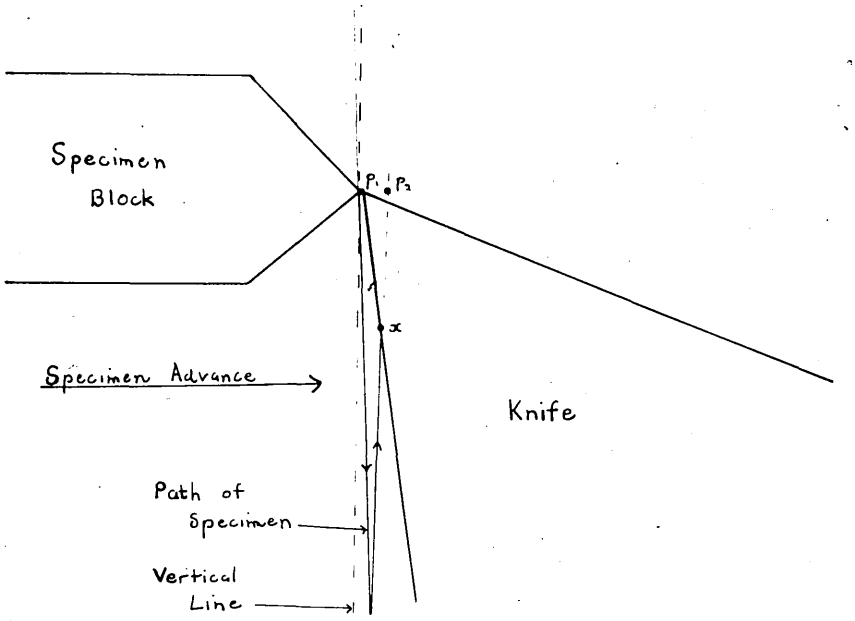



Fig. 1a.

Distortion of block on microtome without by-pass. The specimen block meets the cutting edge at $p_1$ on the downward cutting stroke. On the subsequent upward stroke it does not return exactly to $p_1$ but due to the continual advance would reach a point $p_2$ were it not deflected by the face of the knife at x, which tends therefore to distort the block.

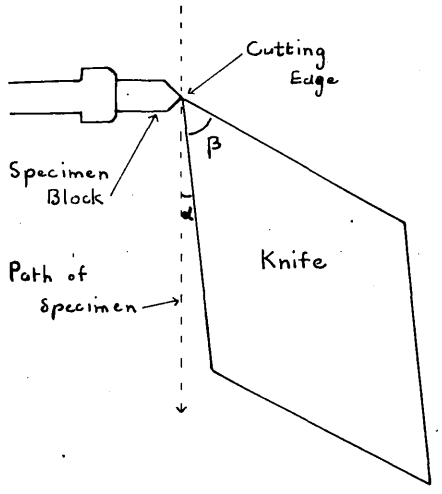



Fig. 1b.

Glass knife. A glass knife consists of a piece of $\frac{1}{4}$" thick glass in the form of a parallelogram.

$\alpha$ = clearance angle. $\beta$ = included angle.

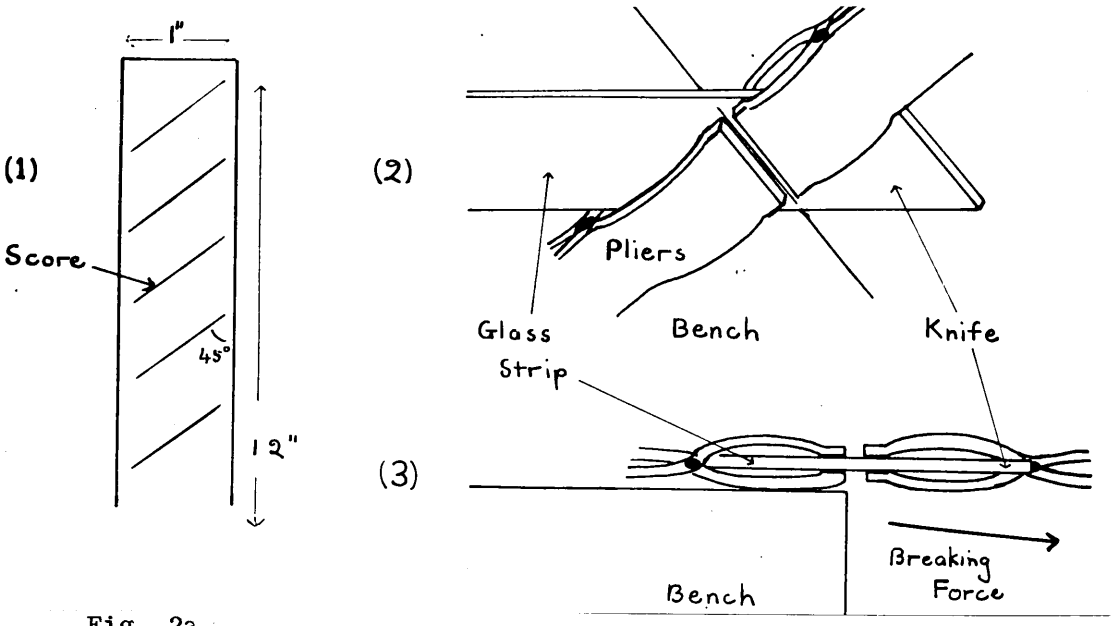



Fig. 2a.

Preparation of Glass Knives. (1) A glass strip ¼" thick is obliquely scored with a glass cutter at 1" intervals along its length. Two pairs of glass pliers are placed on either side of a score as shown in (2) and a steady pull applied by one pair, mainly in the plane of the glass (3), to distract the knife away from the remaining glass.

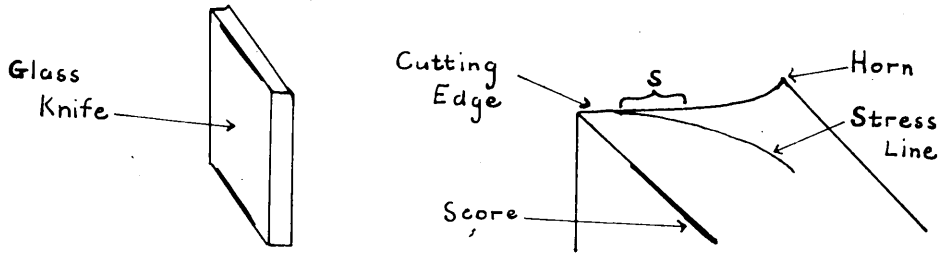



Fig. 2b.

Cutting edge of glass knife. A good edge is smooth, slightly concave and rises to a low 'horn' at the side of the knife remote from the original score. The sharpest portion (S) lies beyond where the stress line diverges from the edge.

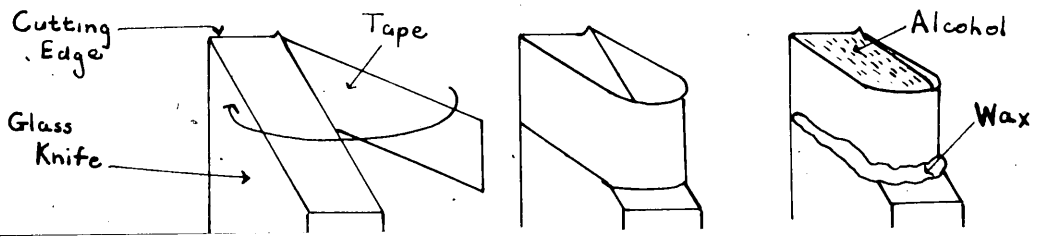



Fig. 2c.

Liquid Bath. A strip of adhesive electrical tape is attached to the glass knife, and sealed with molten dental wax.

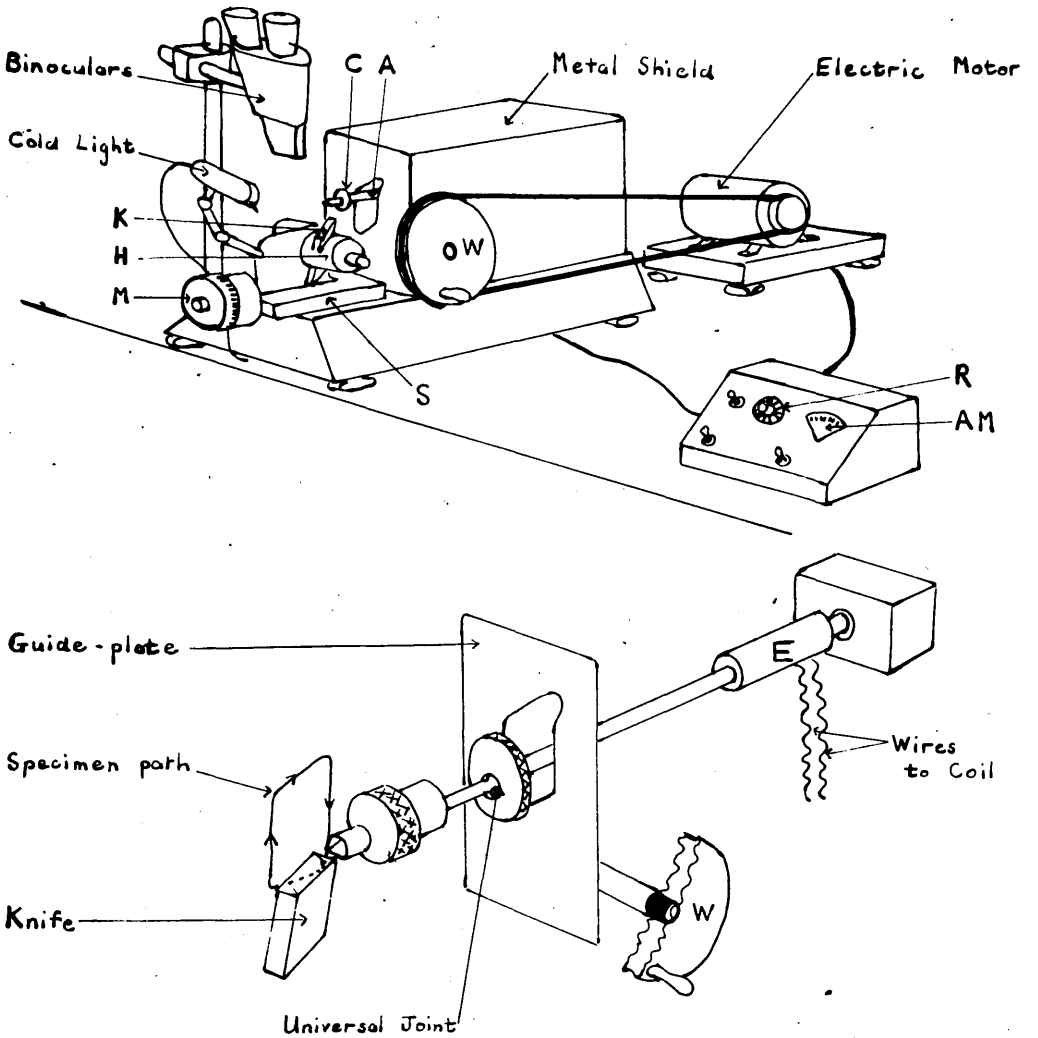



Fig. 3.

The Cooke and Perkins Ultramicrotome.
(a) General Features.
(b) By-pass mechanism and thermal expansion.
For explanation see text.

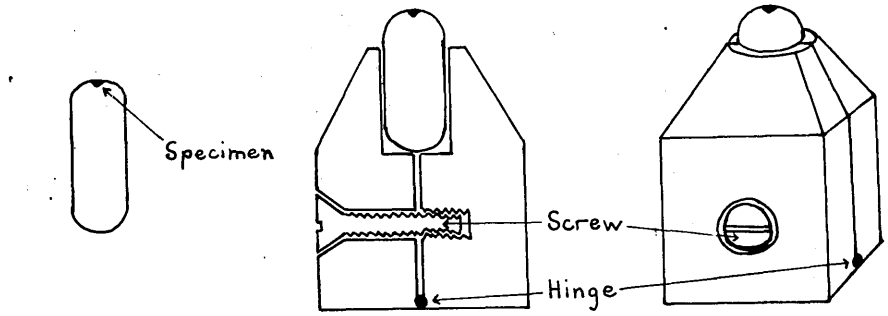



Fig. 4a.

Brass chuck for trimming specimen block. The block fits into a cylindrical recess in the apex of the chuck and is held firmly when the screw is tightened. The chuck is laid on its four sides in turn and trimmed to a pyramid with a sharp razor blade, under a binocular microscope.

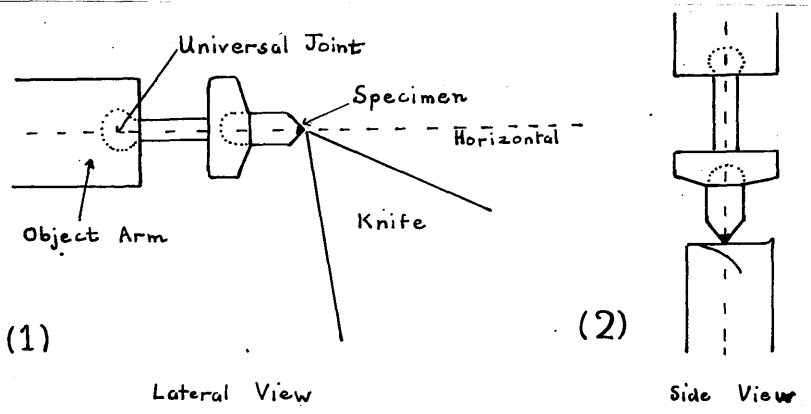



Fig. 4b.

Adjustment of block in relation to knife edge. (1) Vertical view. (2) Horizontal view.

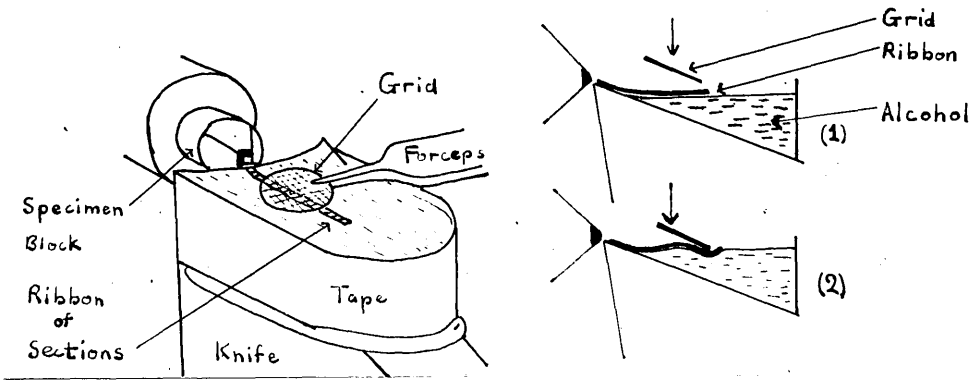



Fig. 4c.

Picking up a ribbon of sections on a grid.

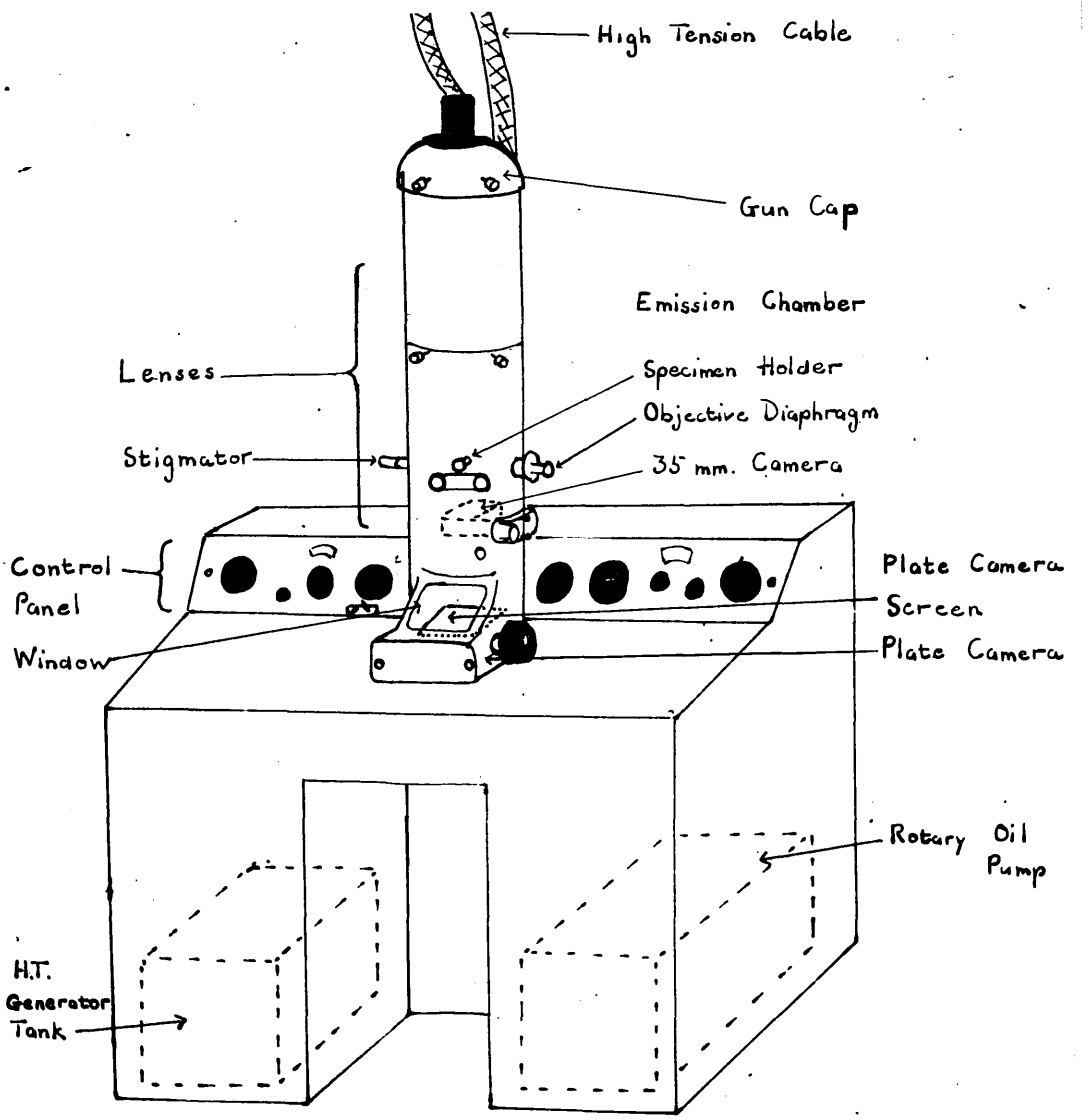



Fig. 5.

General features of Philips EM.75B electron microscope.

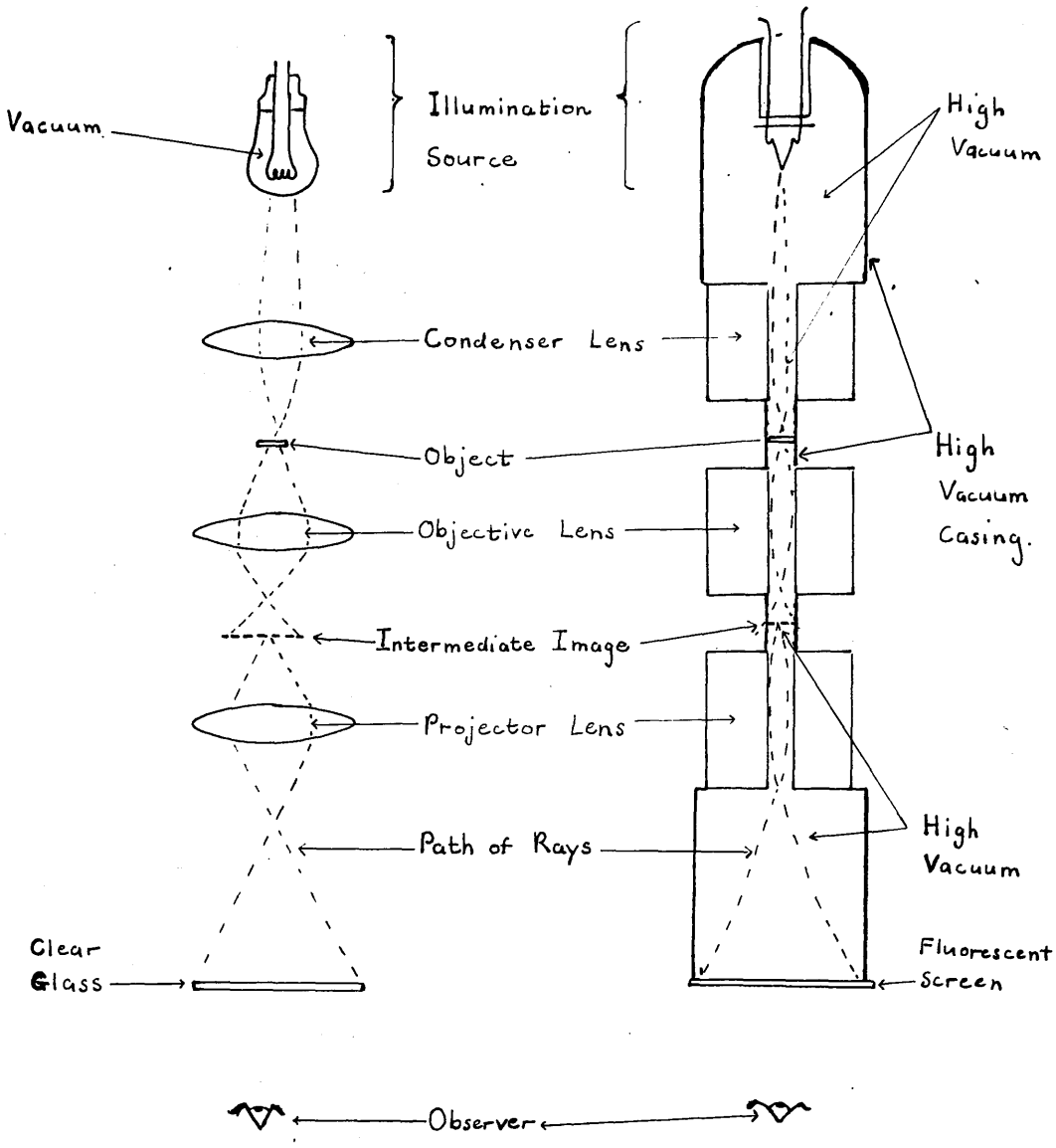



Fig. 6.

The optics of the light and electron microscopes.

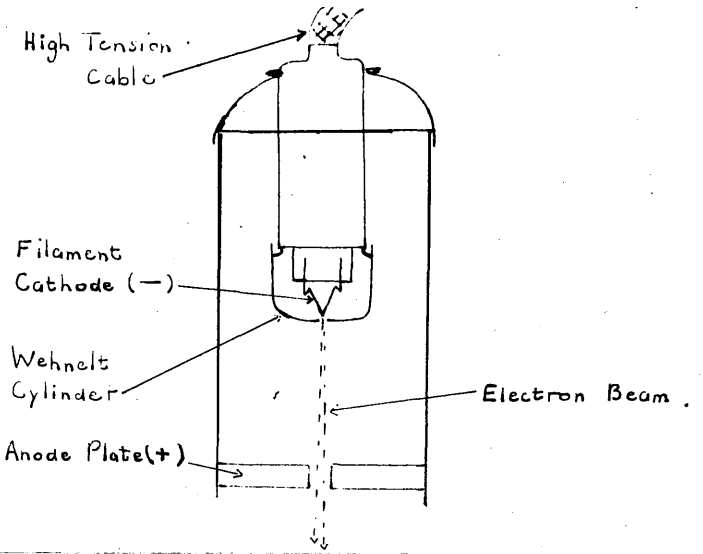
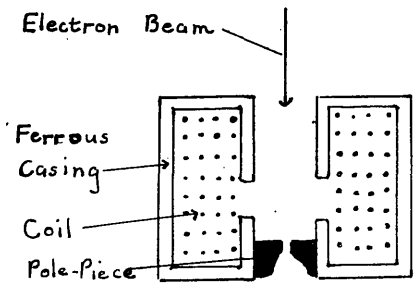
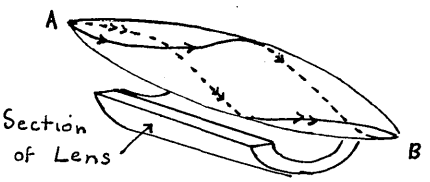



Fig. 7a.
Electron gun.




Magnetic Lens.




Helical Path of Electrons through Lens

Fig. 7b.
Magnetic lens.
(1) The form of the lens. (2) Helical path of electrons through a lens.

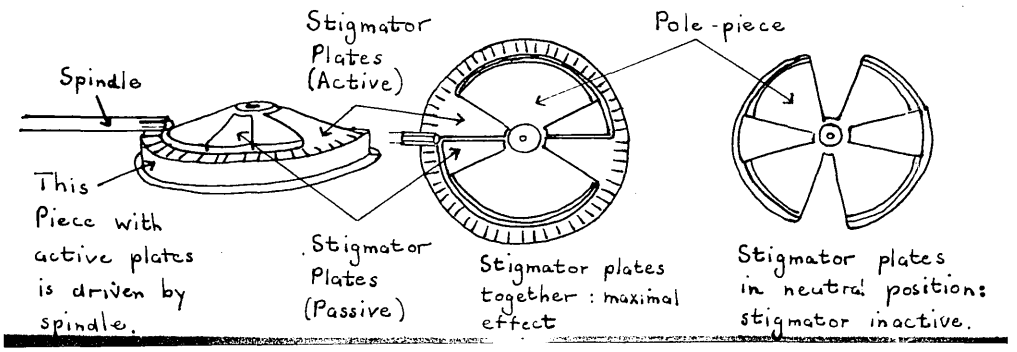



Fig. 7c.
Stigmator. The stigmator plates can be rotated by an externally adjustable spindle.

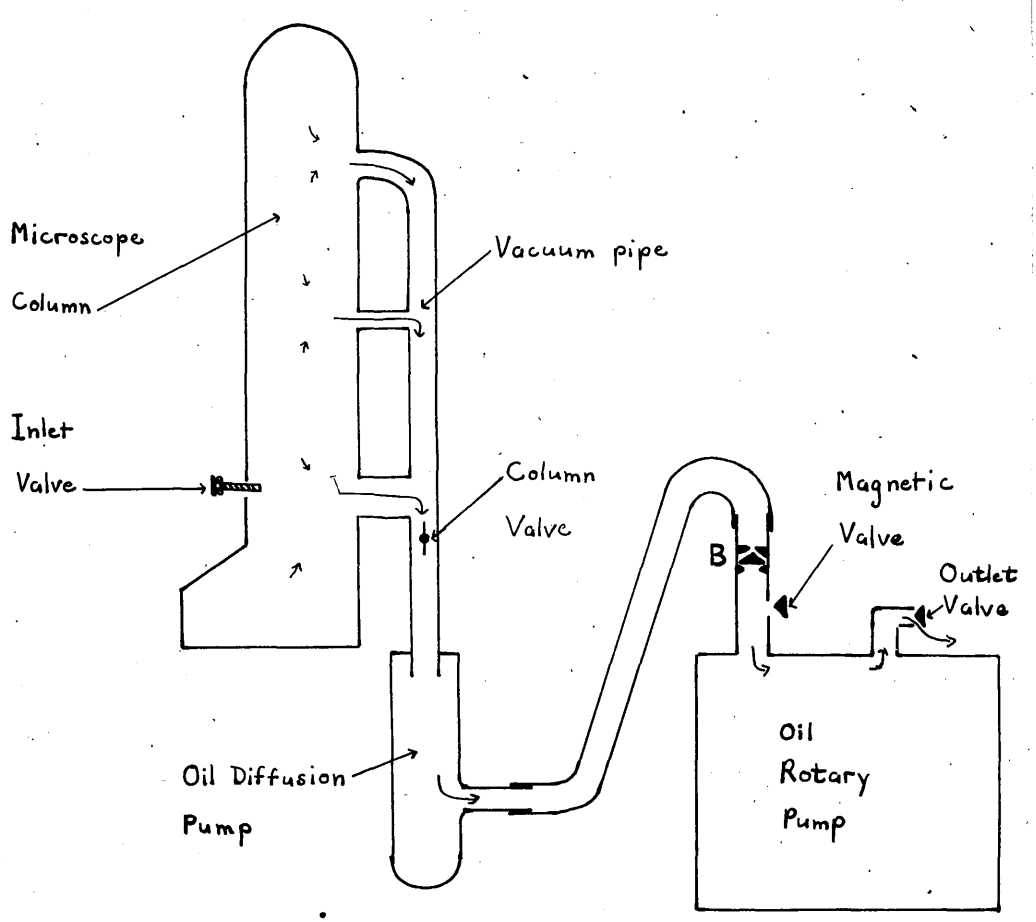



Fig. 8.

Vacuum system of EM.75B.

In the event of power failure the magnetic valve between the two pumps opens and the inrush of air closes a second valve B, thus isolating the oil diffusion pump and bringing the oil rotary pump to atmospheric pressure.

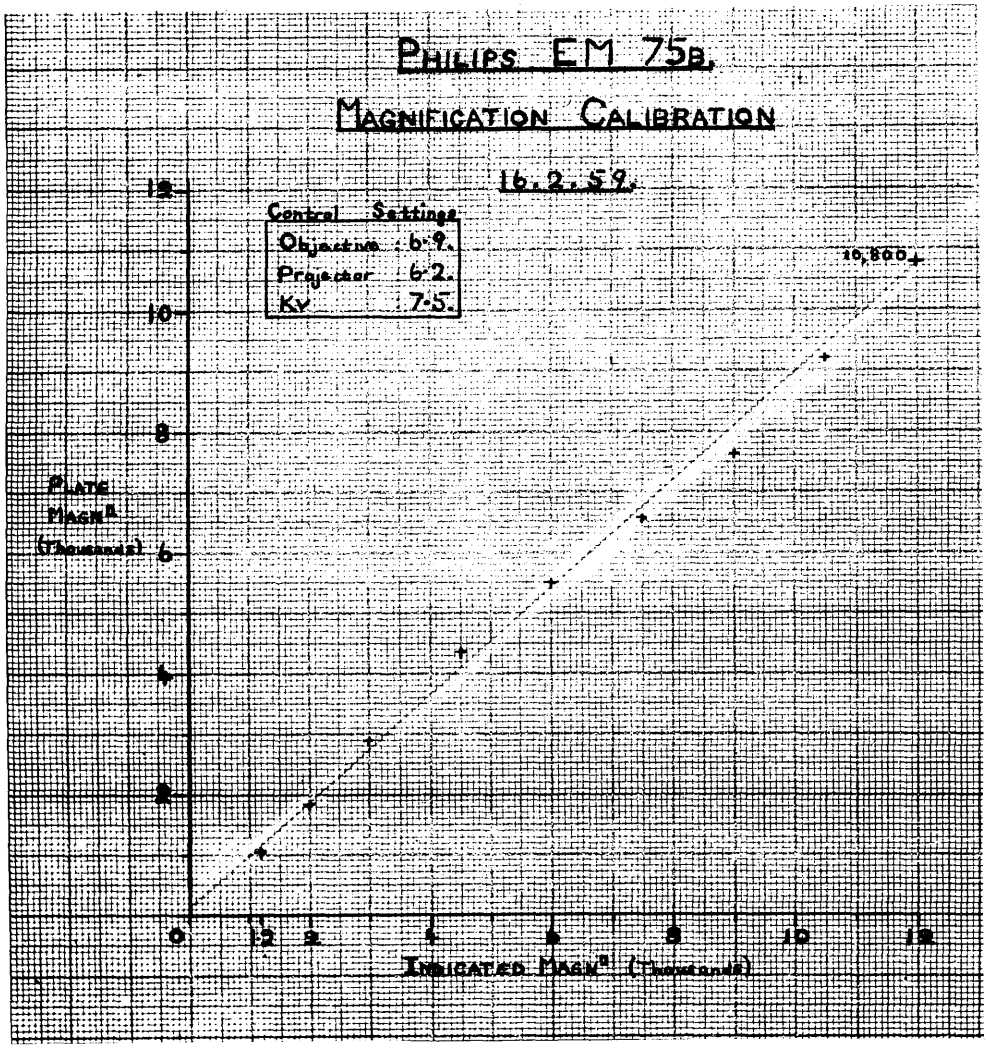



Fig. 9.
Calibration curve for EM.75B microscope.

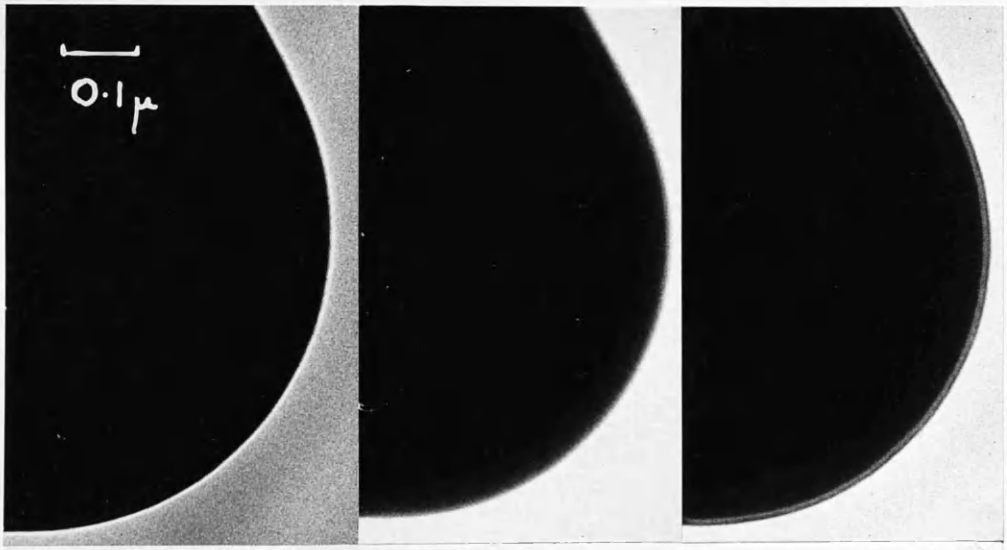



Fig. 10a.

Through-focus series of carbon particle (1μ steps), (1) under focus, (2) near focus, (3) over focus.

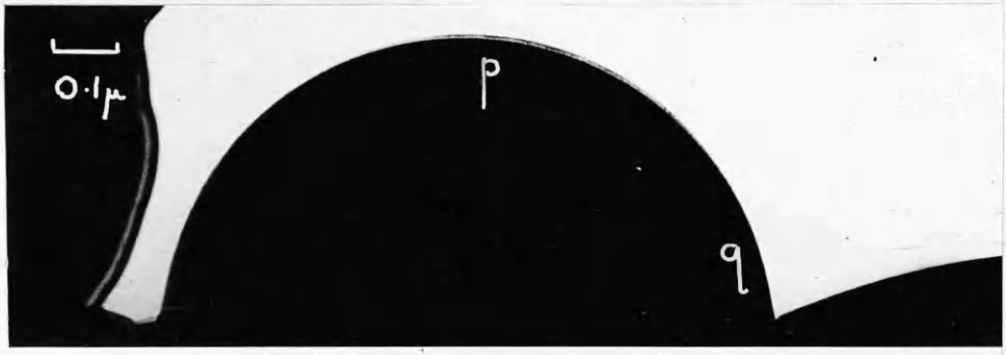



Fig. 10b.

Astigmatism. Grossly asymmetric over focus fringe, which is present at 'p' but diminishes and then vanishes towards 'q'.

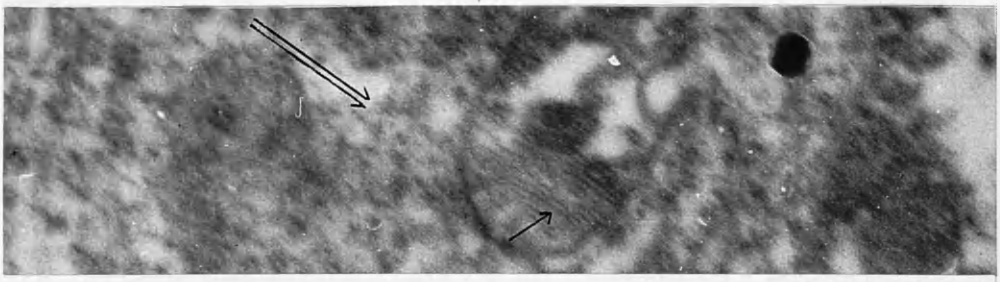


Fig. 10c.

Drift. Micrograph is blurred due to drift and only membranes (arrow) lying parallel to direction of drift (double arrow) are clearly defined.

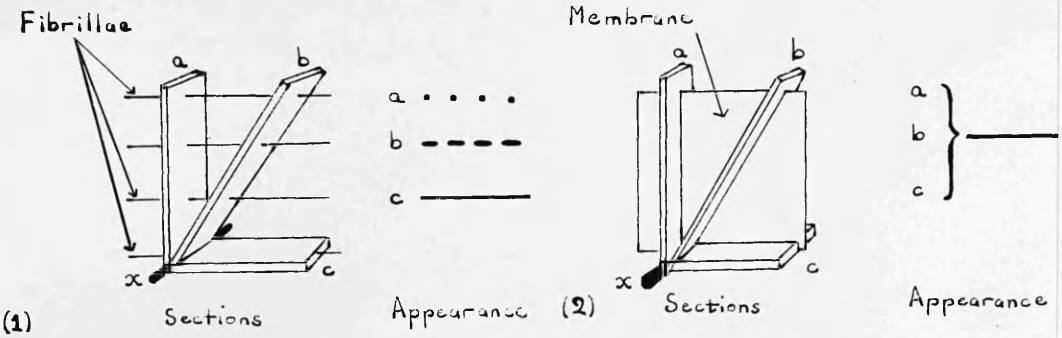



Fig. 11a.

Variation in plane of section through fibrillae and membranes. Consider only an alteration in the sectional plane around a fixed horizontal axis x : as the fibrillae are cut more obliquely their appearance alters (i), whereas that of membranes is unchanged (ii).

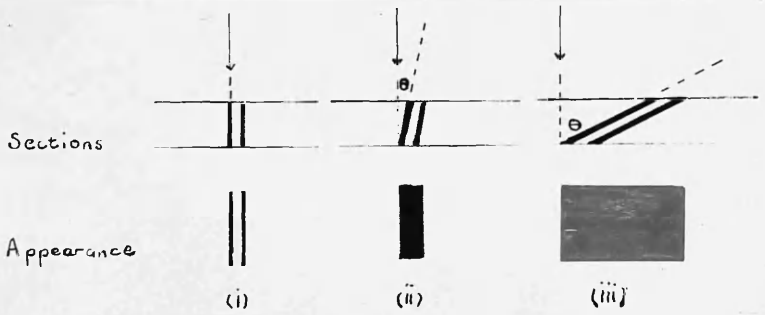



Fig. 11b.

Variation in plane of section through double membranes. As a pair of membranes is traversed more obliquely by the electron beam, i.e. as the angle Θ increases from $0^{o}$ (i), each membrane broadens and the interspace diminishes until the two are no longer resolved (ii). With a further increase in Θ the density of the membrane diminishes towards that of the surrounding ground substance (iii).

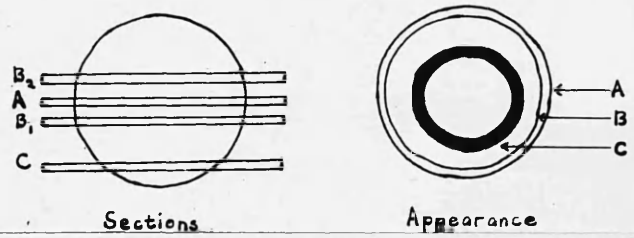



Fig. 11c.

Variation in plane of section through spherical organelle. The bounding membrane will be sharp in sections ranging from A - the equatorial diameter - to $B_1$ or $B_2$, but in a more eccentric section C it will be broadened and indistinct.

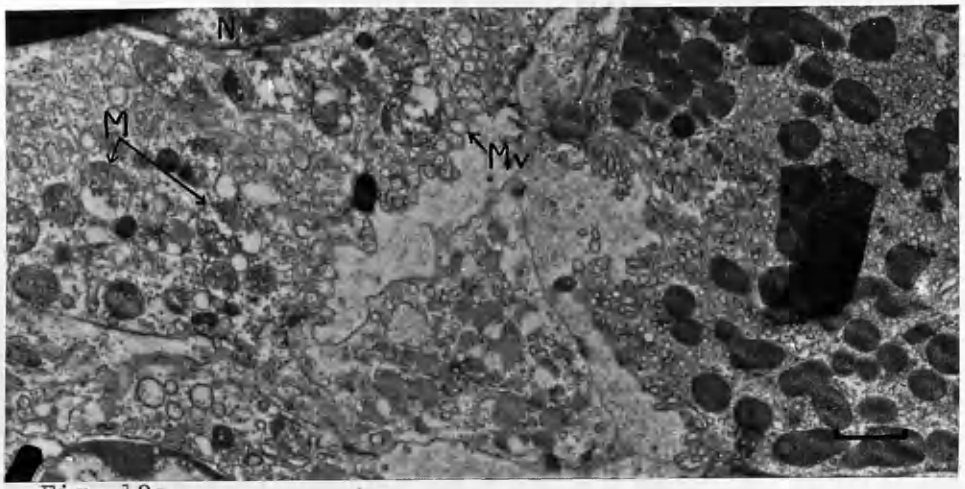



Fig. 12a.

Post mortem change. The damaged parietal cell to the left contains vacuolated mitochondria (M) and swollen microvilli (Mv).

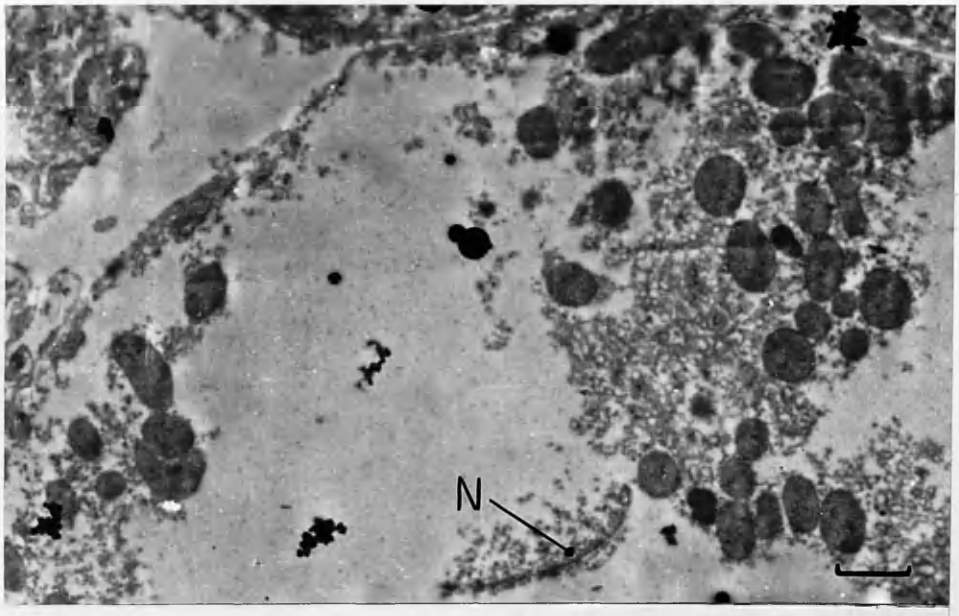



Fig. 12b.

Polymerization damage. Disrupted parietal cell with areas devoid of cytoplasmic ground substance, and a fragmented nucleus (N).

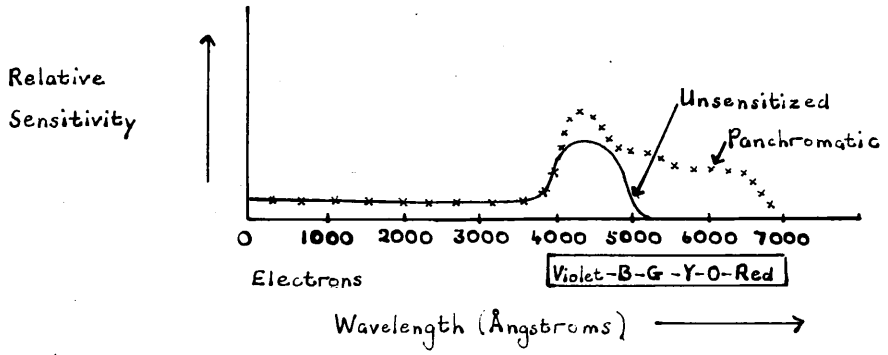



Fig. 13a.

Comparative sensitivity of blue-sensitive and panchromatic photographic emulsions.

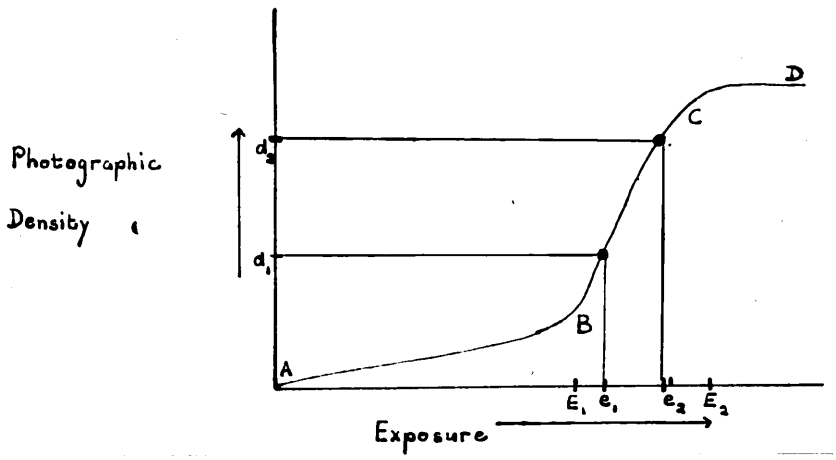



Fig. 13b.

Characteristic curve of a photographic emulsion.

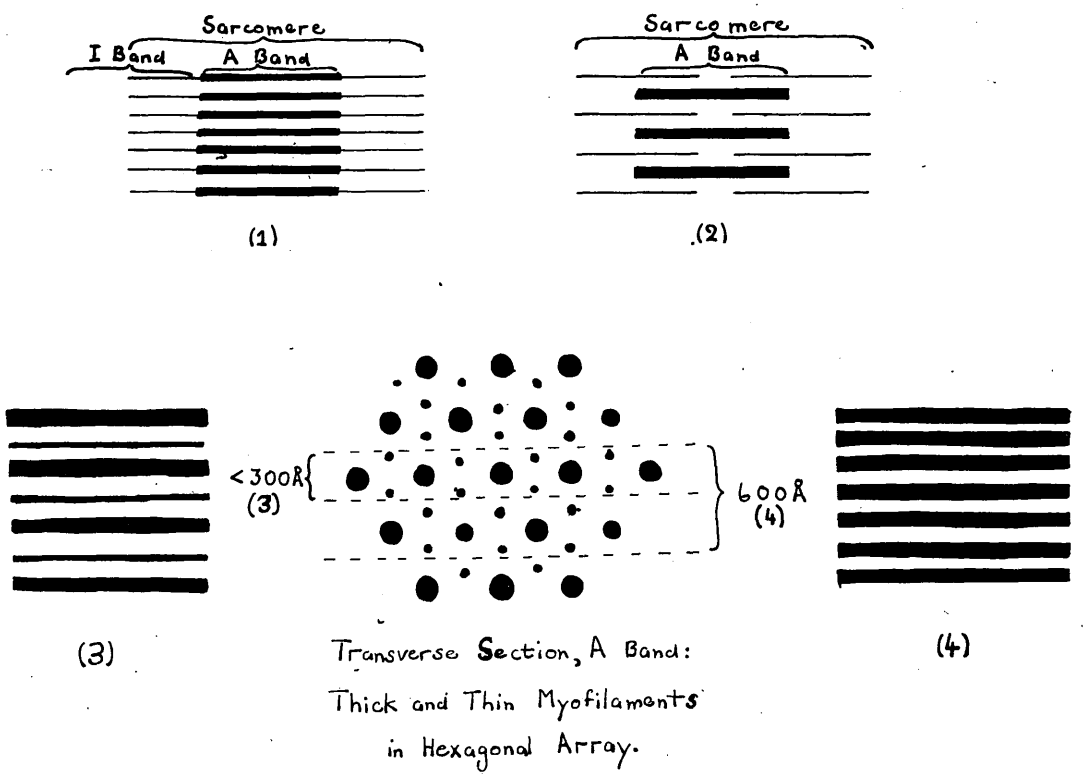



Fig. 14.

Arrangement of myofilaments in skeletal muscle. (1) Single filament model. (2) Huxley model. (3) 300Å-thick section through myofilaments of A band and resultant appearance. (4) 600Å section through myofilaments demonstrating that overlapping of filaments in this thicker section results in apparent absence of thin filaments from the A band.

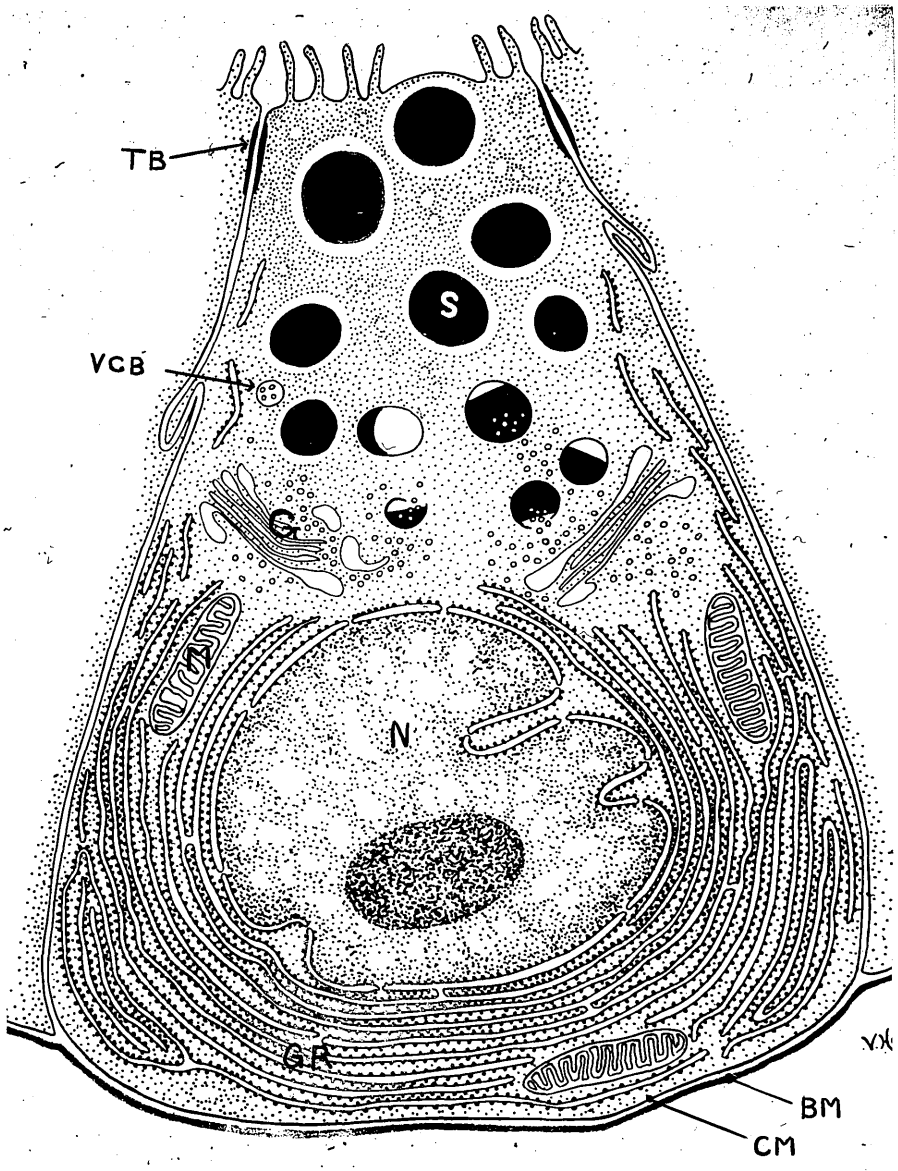



Fig. 15.

Diagram of a Paneth cell showing its main features. The irregular nucleus (N) is surrounded by the parallel membranes of the granular endoplasmic reticulum (GR). The secretory granules (S) in the upper half of the cell arise in close relation to the Golgi complex (G).

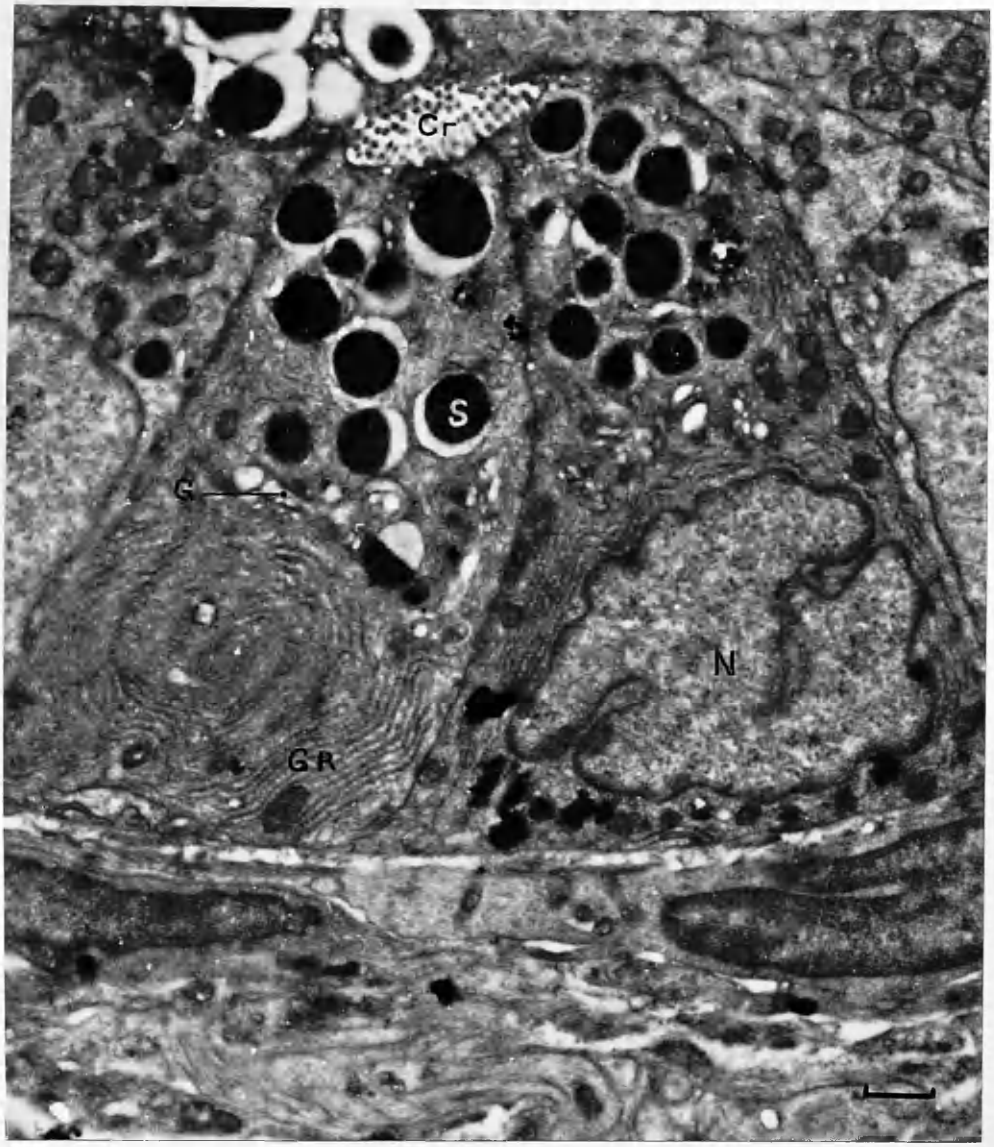



Fig. 16.

Survey micrograph through base of crypt (Cr), with two Paneth and several principal cells. The Paneth cells contain secretory granules (S), and extensive endoplasmic reticulum (GR), irregular nucleus (N), and a supranuclear Golgi complex (G).

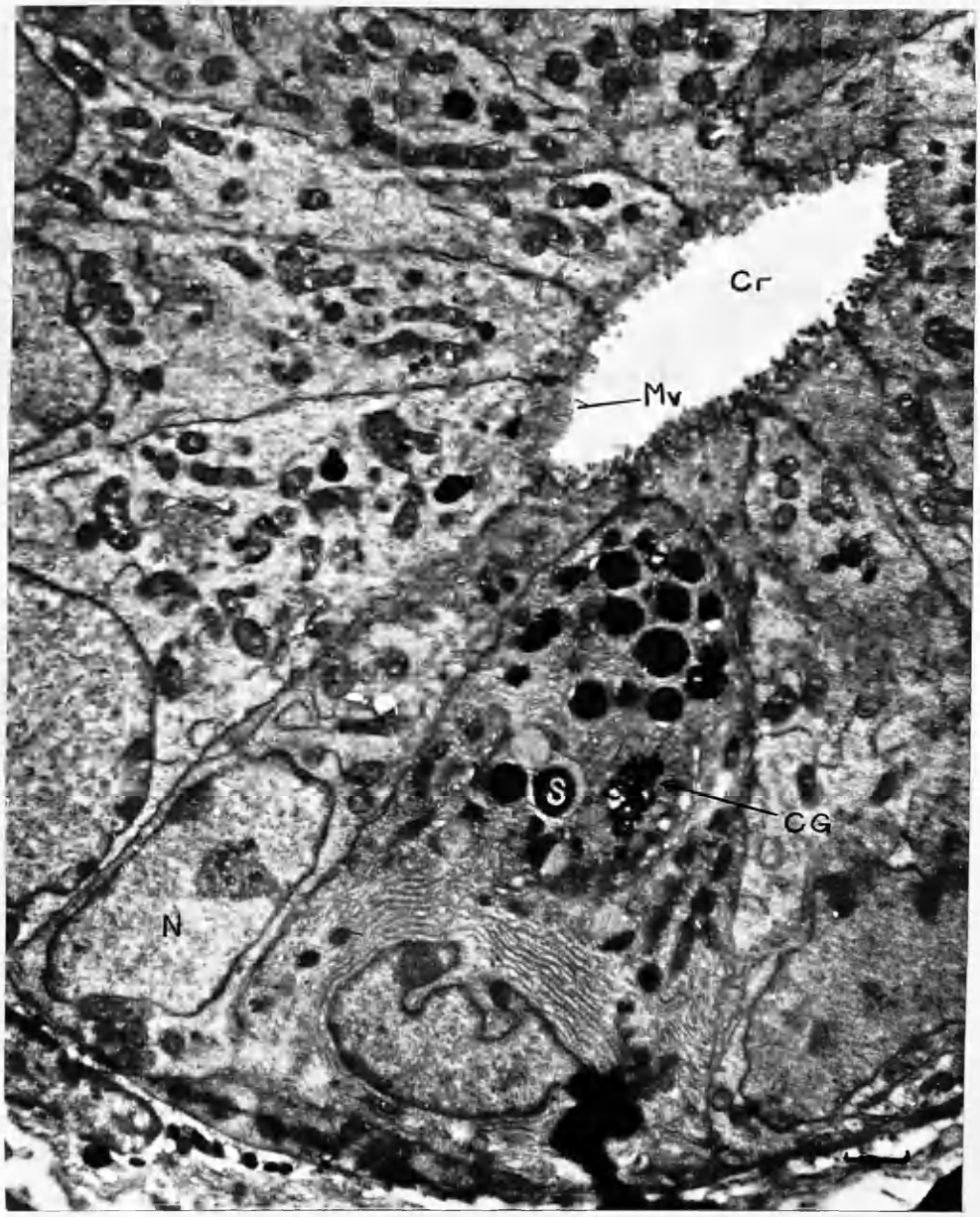



Fig. 17.

An unusual feature of this Paneth cell, lying amongst several principal cells, is the lack of a vacuole surrounding each secretory granule (S). There are dense clusters of small black granules (CG) in the upper half of the cell.

Microvilli (Mv) project into the crypt (Cr) from the apical surfaces of the epithelial cells.

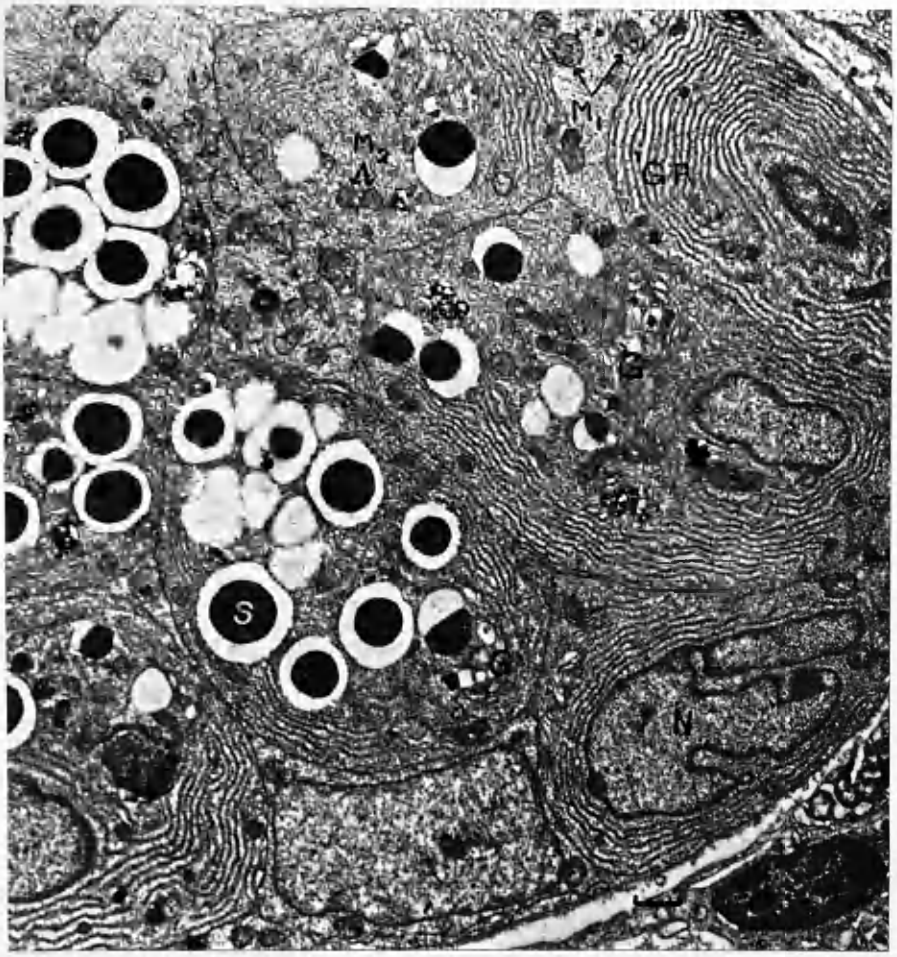



Fig. 18.

Micrograph of obliquely-sectioned Paneth cells. Small developing granules are present within the Golgi complex (G). The membranes of the granular endoplasmic reticulum (GR), are arranged concentrically on the nucleus. The mitochondria of the principal cells ($M_1$) contain dense granules, in contrast to those of the Paneth cells ($M_2$).

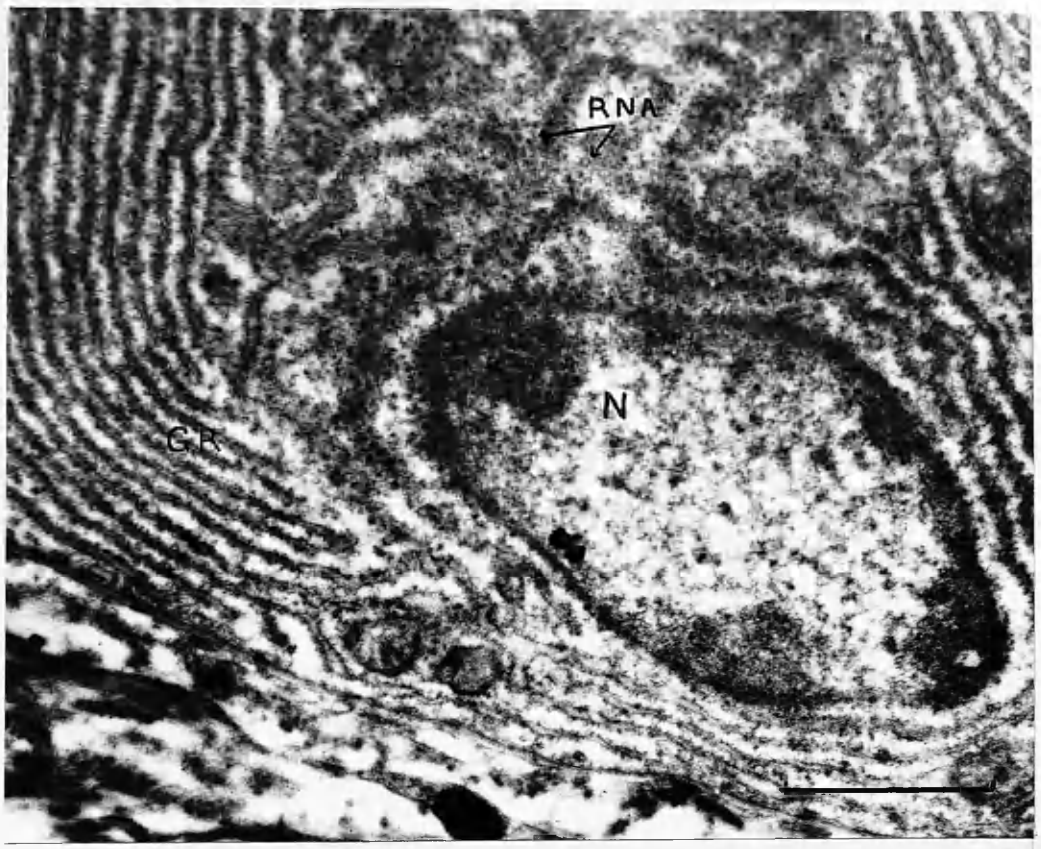



Fig. 19.

Basal half of Paneth cell with numerous membrane pairs of the granular endoplasmic reticulum surrounding the nucleus (N). The single membranes are distinct to the left of the nucleus, but above the nucleus - where they are cut obliquely - they are broadened and the attached RNA particles are apparently free in the cytoplasm.

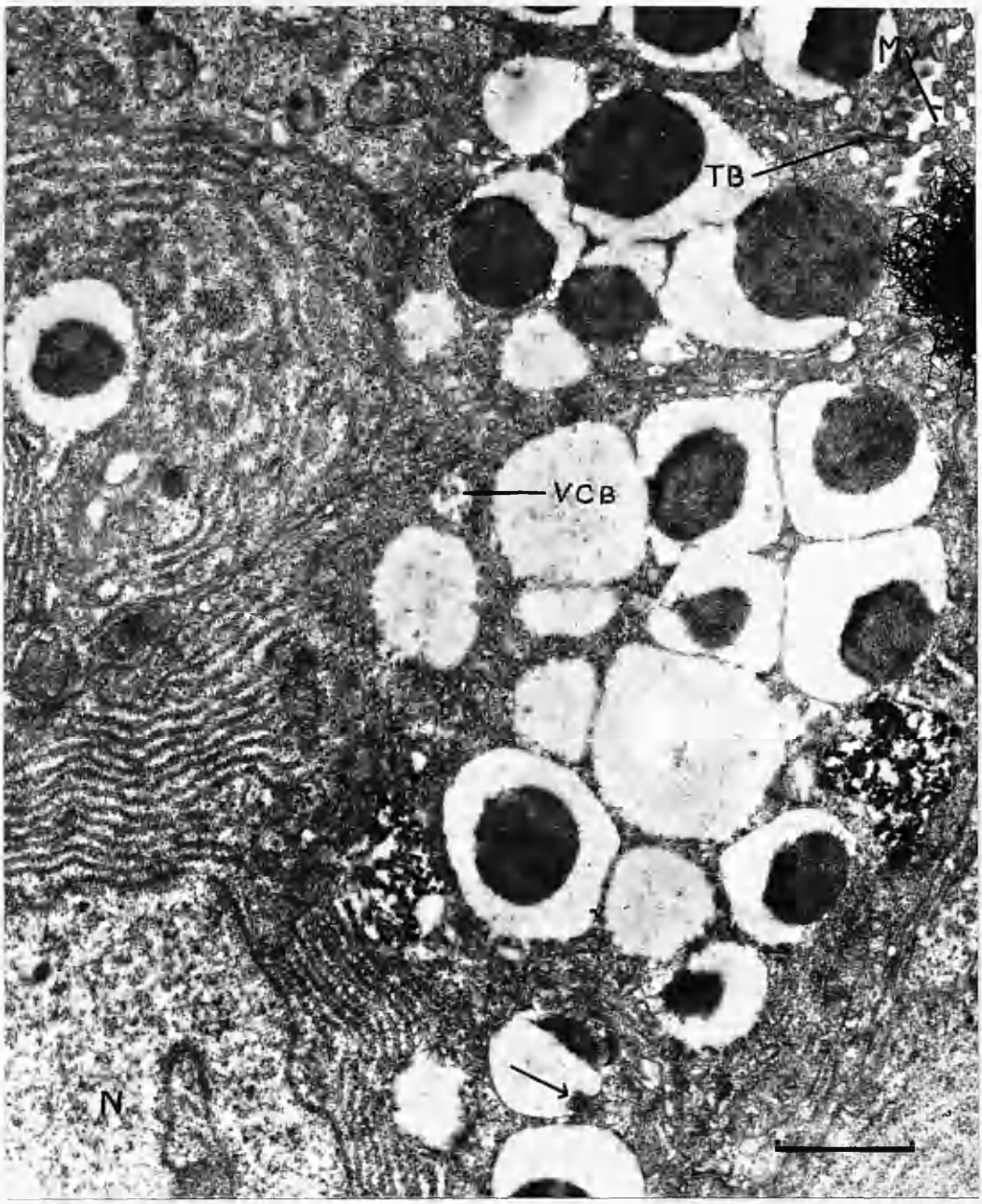



Fig. 20.

Upper portion of several Paneth cells converging on a crypt containing cross-sectioned microvilli (Mv). The cell membranes are evident, with a terminal bar (TB) near the free surface of the cell. A small vacuole-containing body is present (VCB). A developing secretory granule contains both secretory material and a cluster of small vesicles (arrow).

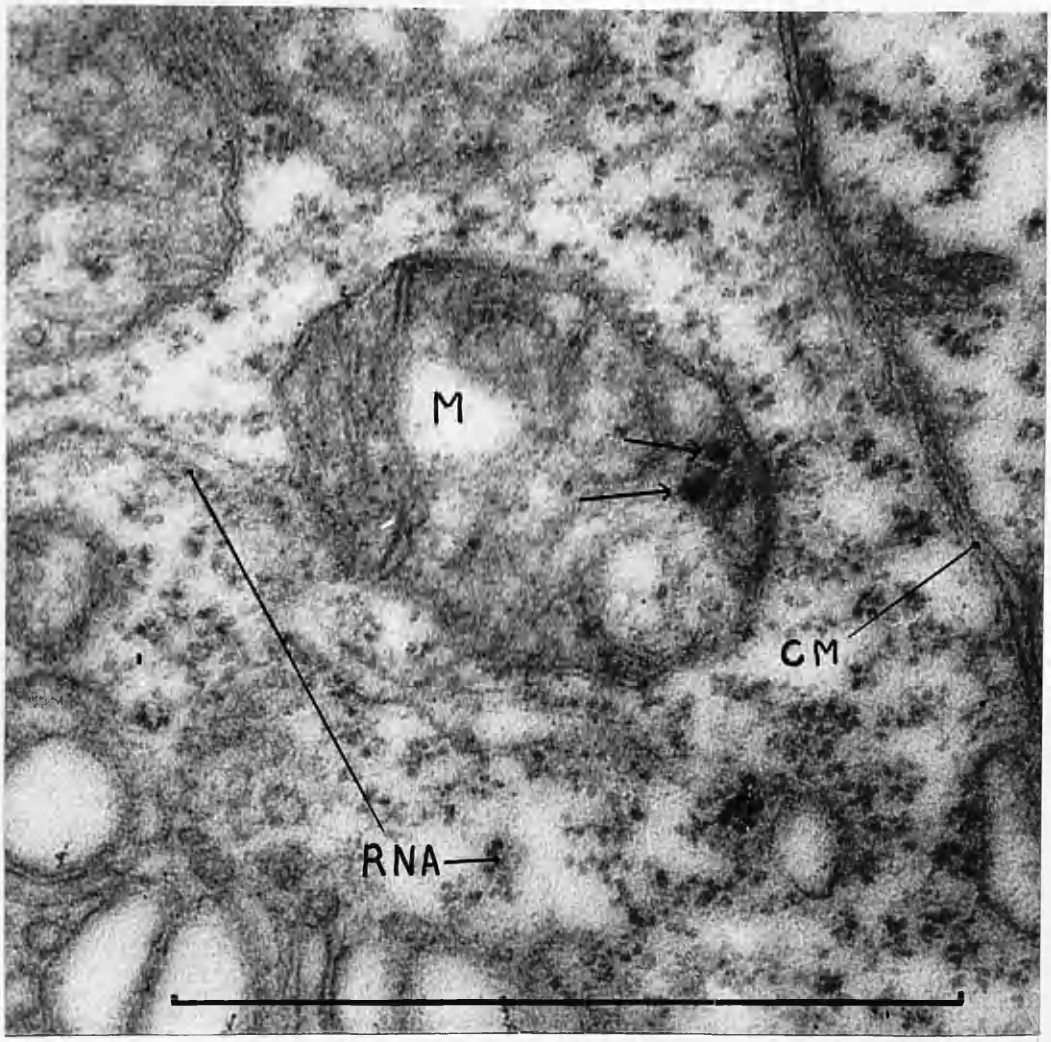



Fig. 21.

A mitochondrion (M) in a principal epithelial cell, with double limiting membrane and internal cristae, and containing two dense granules (arrows).

Small groups of RNA particles lie both free in the cytoplasm and attached to the membranes of the granular endoplasmic reticulum.

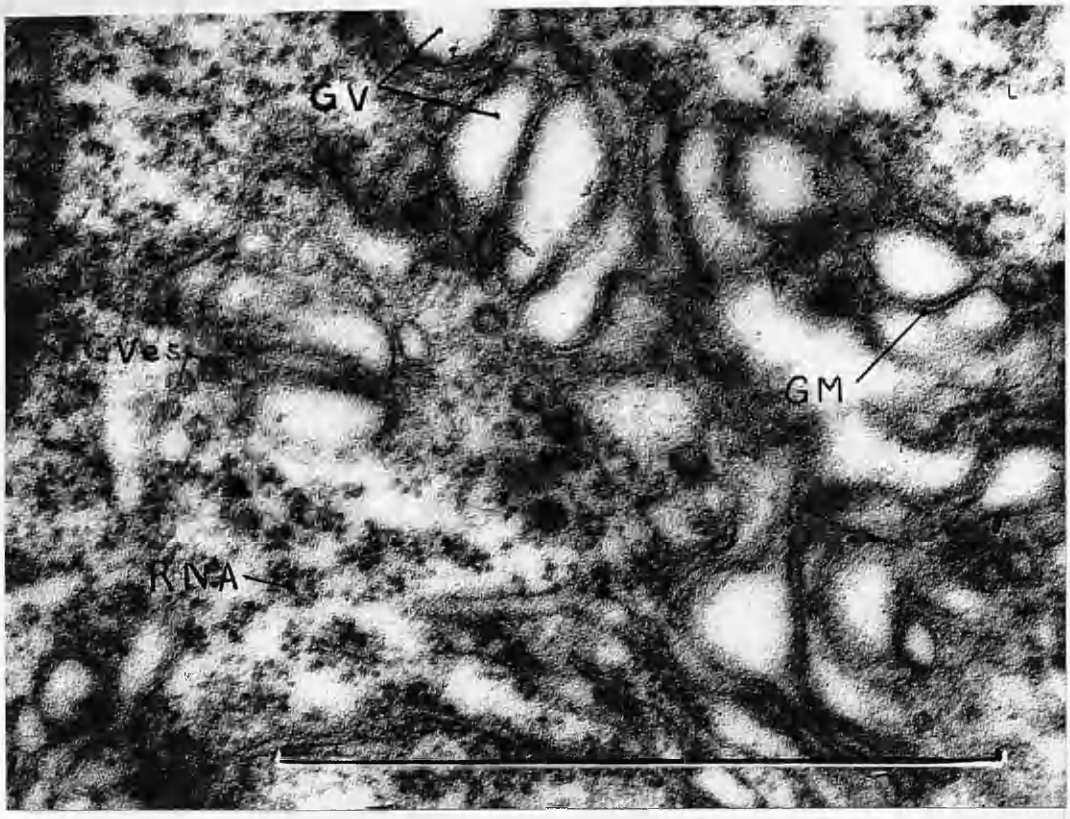



Fig. 22.

Golgi complex in principal epithelial cell, composed of smooth Golgi membrane pairs (GM), Golgi vacuoles (GV), and small vesicles (GVes) lying in the Golgi ground substance. This ground substance is free of RNA particles, which are found only in the adjacent cytoplasm (RNA).

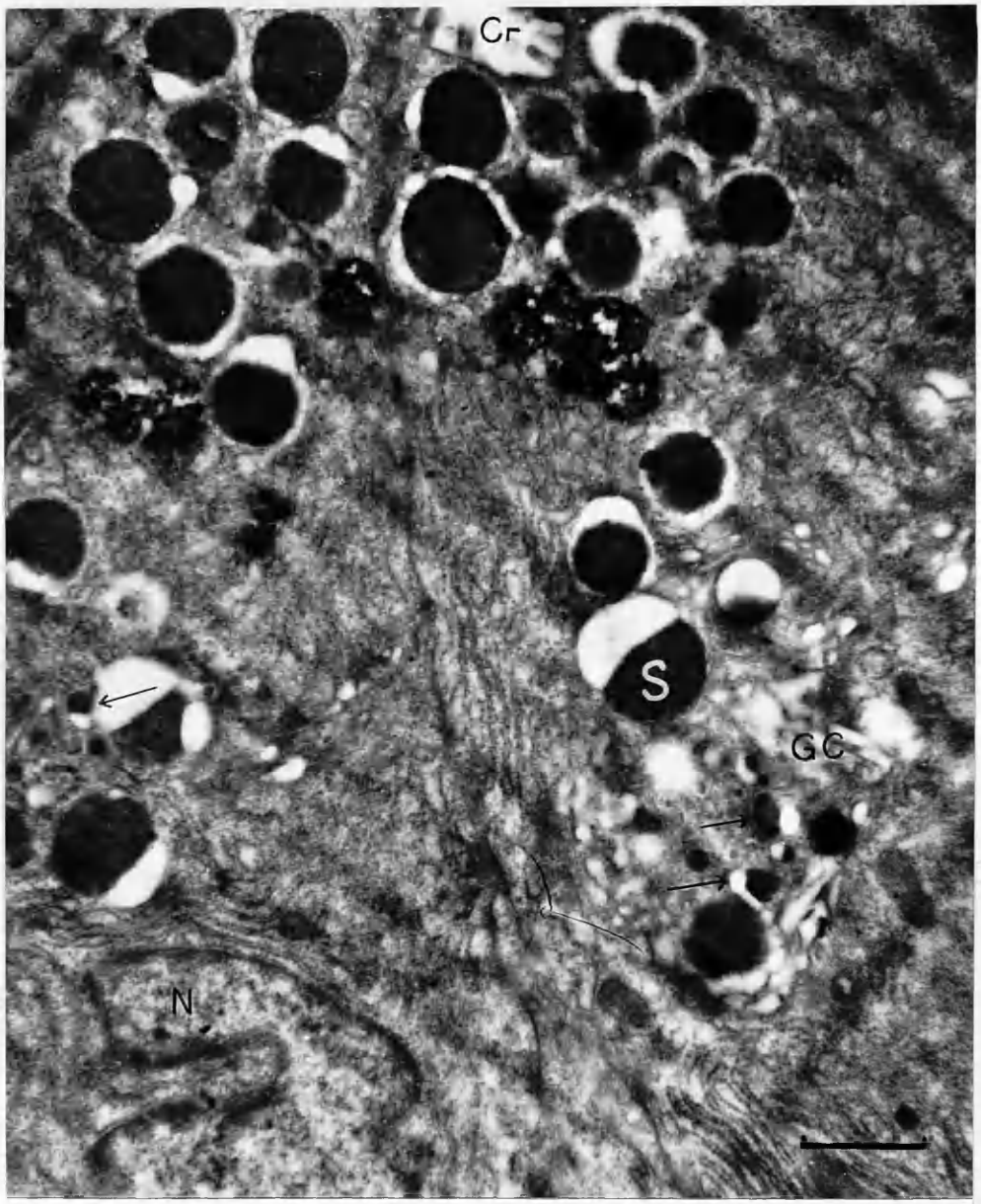



Fig. 23.

Two adjacent Paneth cells, with the crypt above (Cr). The developing secretory granules (arrows) within the Golgi complex (GC), are small and irregular in outline. One of the secretory granules (S), has a clearly defined enclosing membrane.

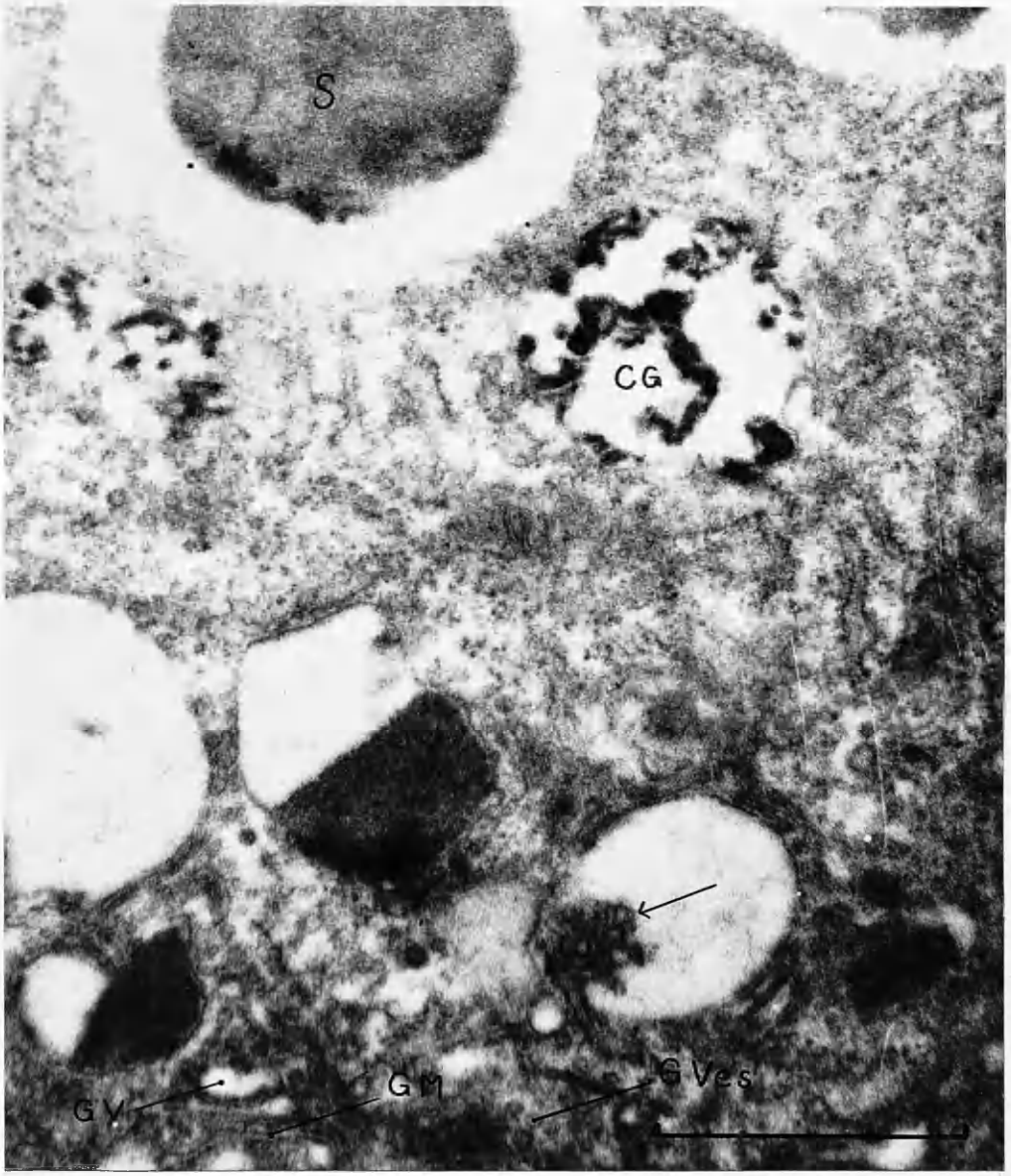



Fig. 24.

Supranuclear region of Paneth cell. The Golgi complex is basically similar to that in Fig. 22, and consists of membranes (GM), vacuoles (GV), and small vesicles (GVes). There is a secretory vacuole - with a cluster of vesicles (arrow) which resemble the Golgi vesicles - in close relation to the Golgi membranes.

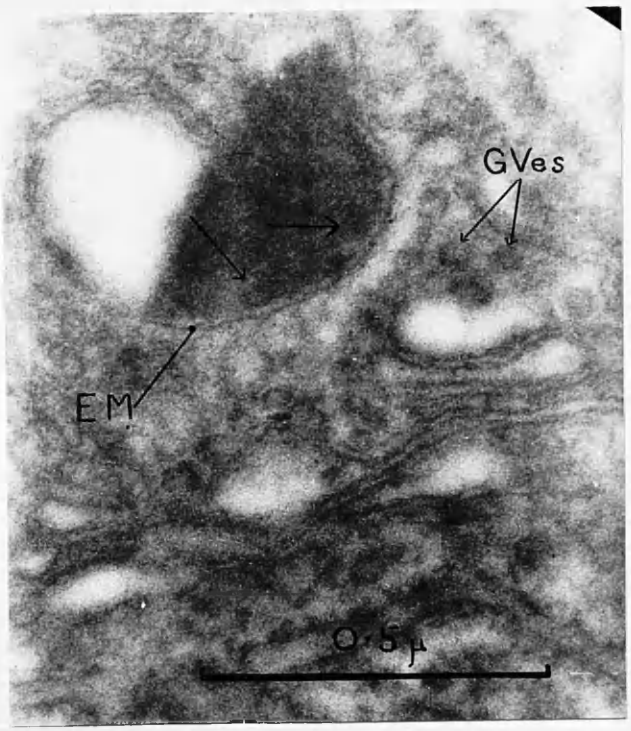



Fig. 25.

Enlarged portion of preceding figure, showing granule in close relation to Golgi complex. The granule is irregular in outline, and the enclosing membrane (EM) is deficient above, where the osmophilic granular material communicates freely with the Golgi ground substance and vesicles. Embedded in the granule are vesicles (arrow), similar to the Golgi vesicles (GVes).

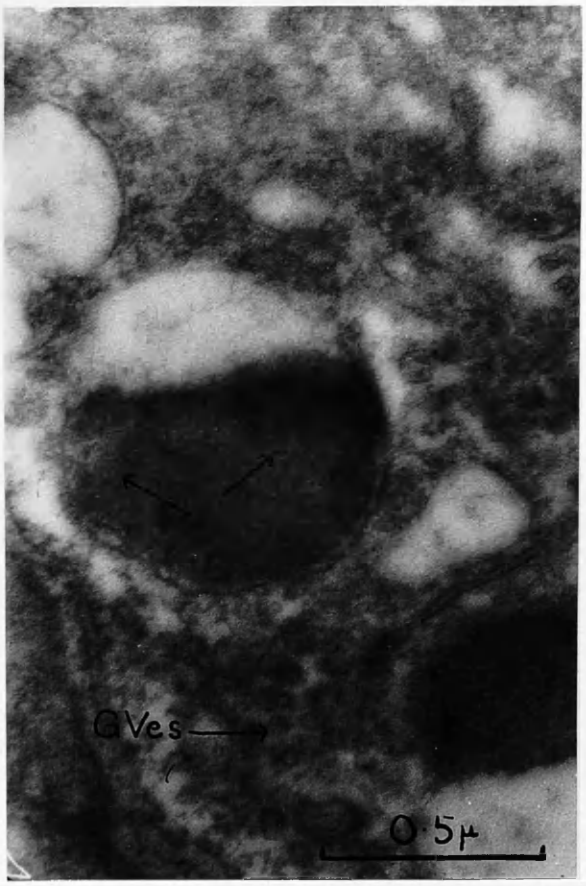



Fig. 26.

Another developing secretory granule within the Golgi complex, containing vesicles (arrows) similar to the Golgi vesicles.

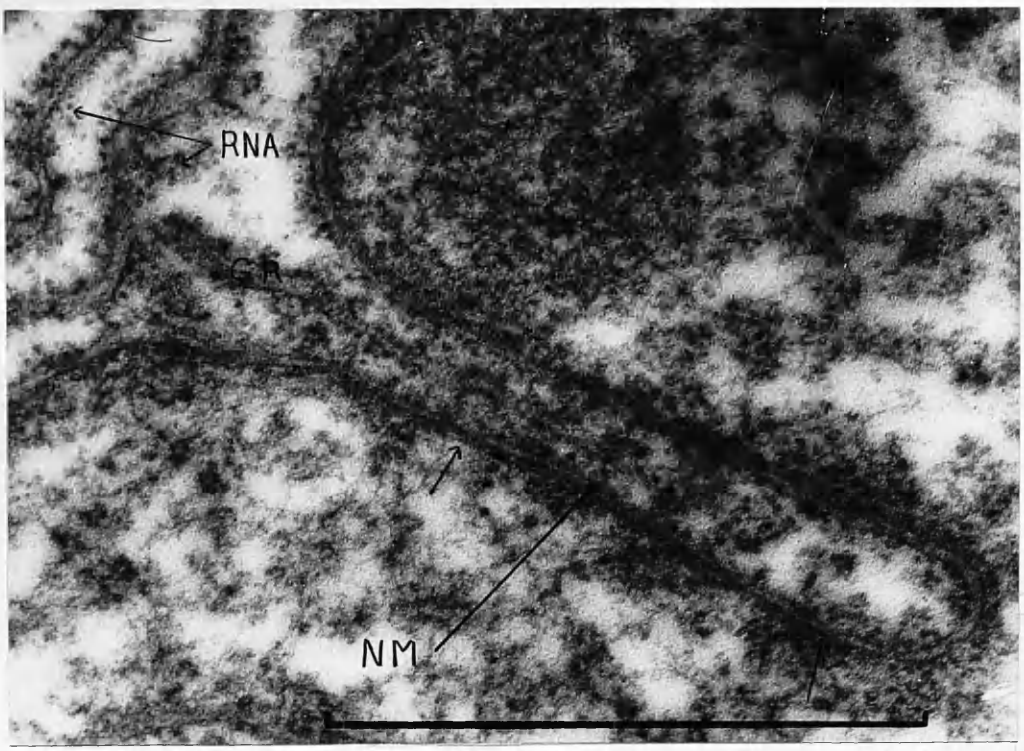



Fig. 27.

Part of a nucleus in a Paneth cell. The nuclear membrane (NM) is double, and interrupted by pores (arrows). It is invaginated into the nucleus by a long process of cytoplasm containing granular endoplasmic reticulum (GR). The membrane pairs of the reticulum have smooth inner surfaces, and outer surfaces studded with RNA particles (RNA).

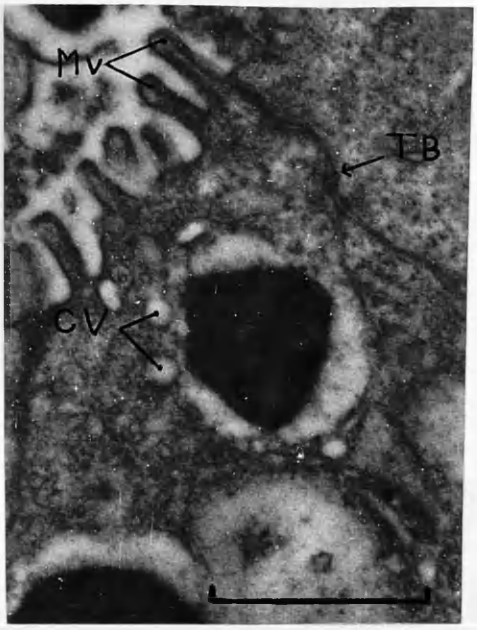



Fig. 28a.

Microvilli (Mv) project from the apical surface of this Paneth cell. Cytoplasmic vacuoles (CV), and a terminal bar (TB), are also visible.

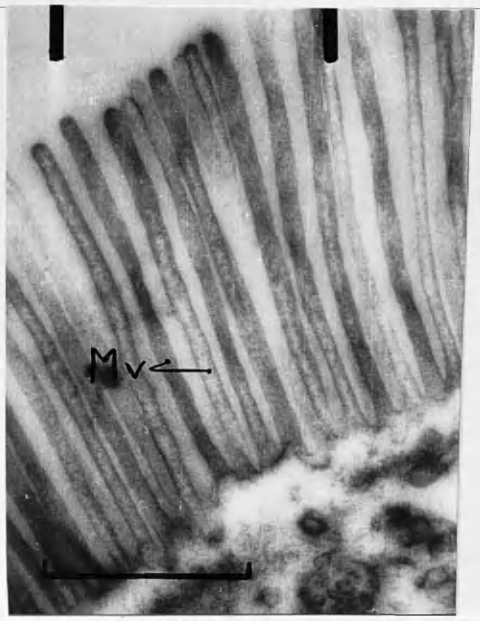



Fig. 28b.

Microvilli (Mv) of the striated border of the intestinal villus, which are more numerous, more regular, and longer than those of the Paneth cell in the above micrograph.

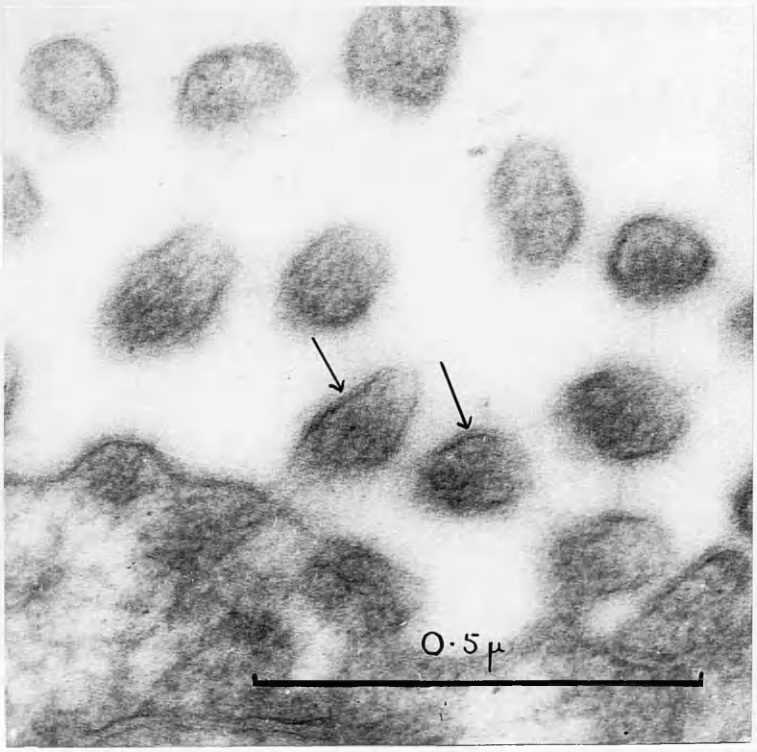



Fig. 29.

Obliquely-sectioned microvilli of Paneth cell. The cell membrane bounding the microvilli is triple-layered (arrows).

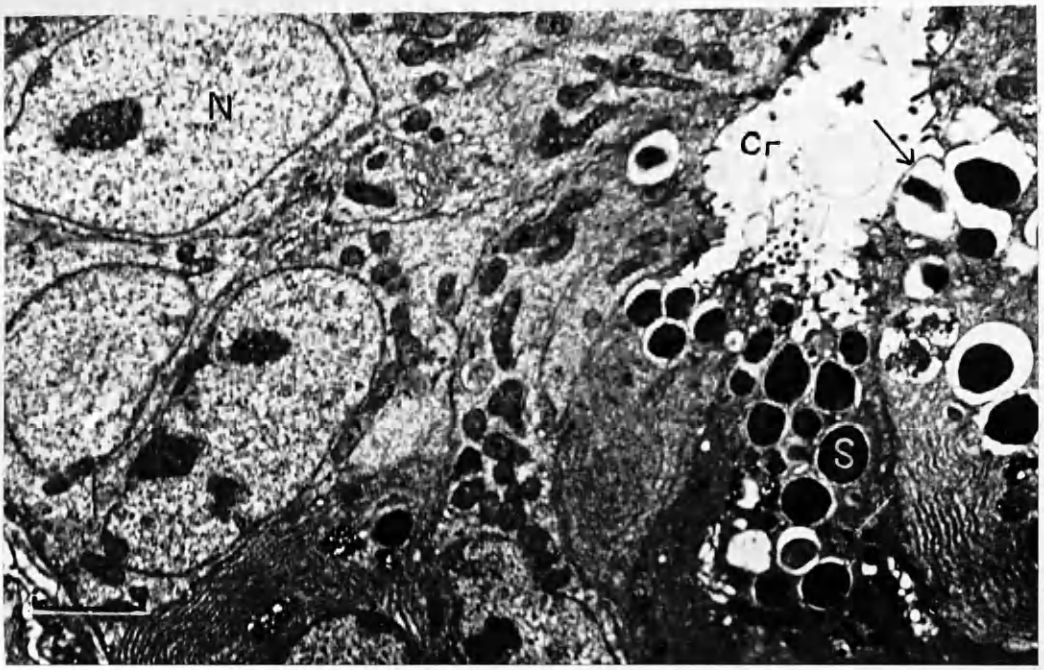



Fig. 30.

A crypt bounded by several Paneth cells. Microvilli are absent from the cell membrane (arrow), over a subjacent secretory granule.

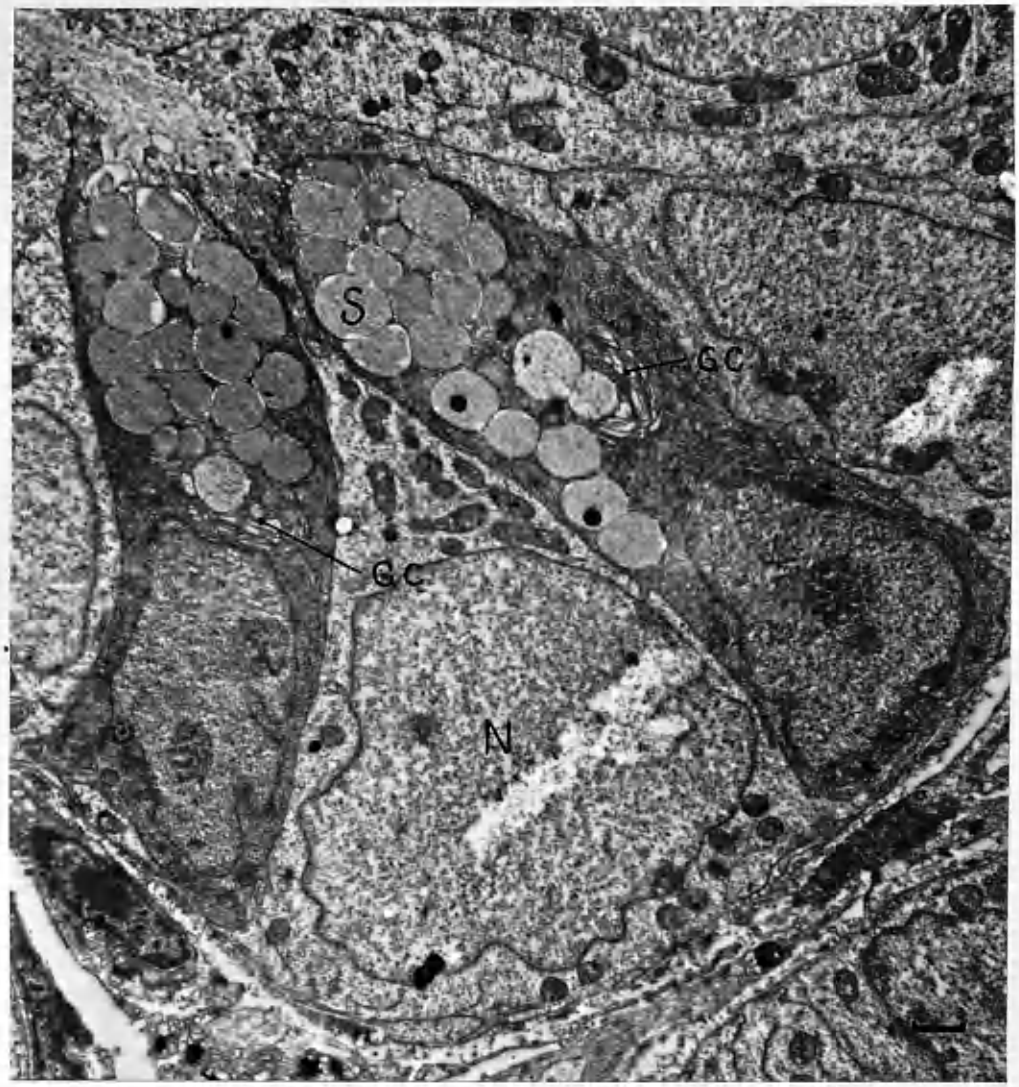



Fig. 31.

Two mucous cells in a crypt of the small intestine. Notice that the mucous granules within the Golgi complex (GC) are of lighter texture than those outwith it. The granular endoplasmic reticulum is sparse.

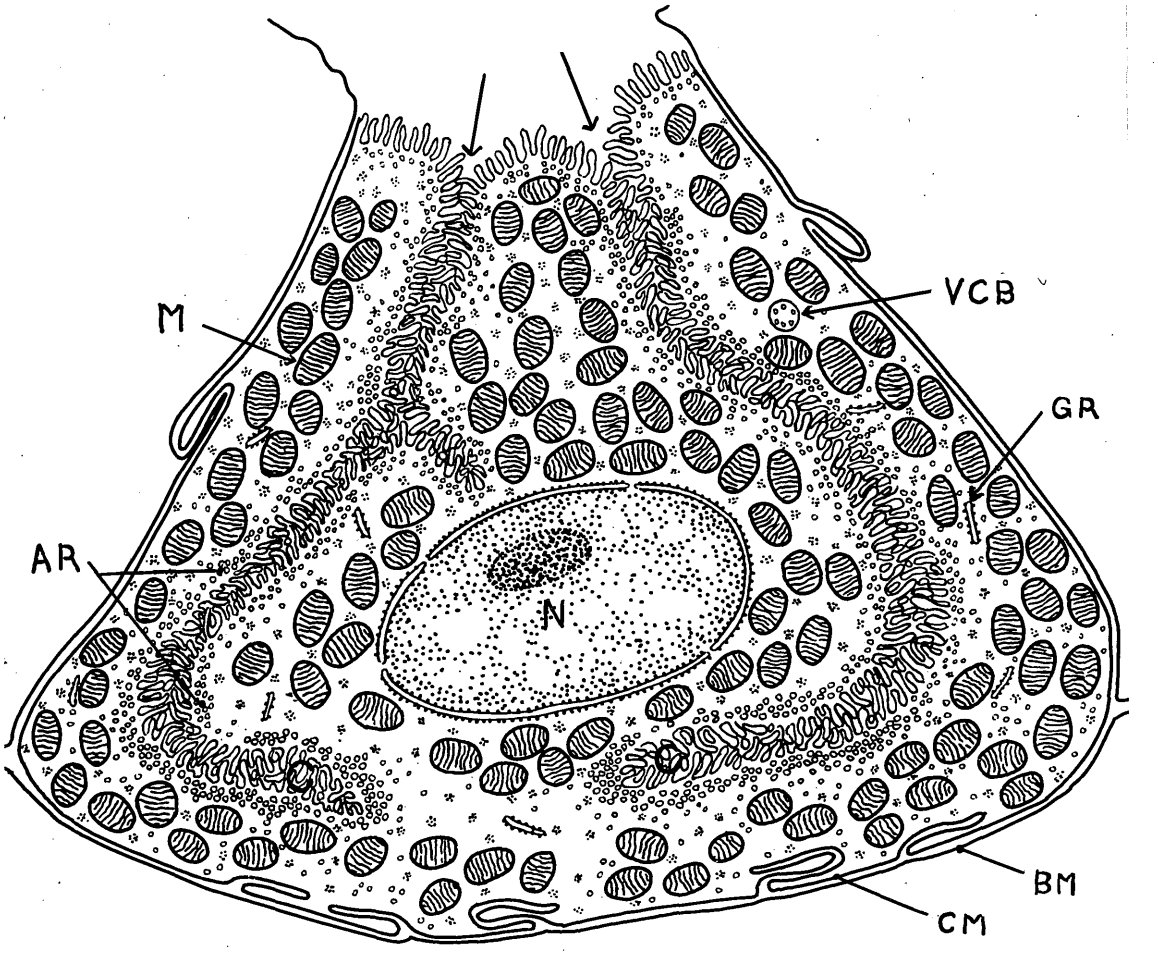



Fig. 32.

Diagram of a gastric parietal cell. The intracellular canaliculi (C), begin basal to the nucleus (N), and extending upwards, open at the apical surface of the cell into the lumen of the gland (arrow). The lumen of each canaliculus is largely filled with microvilli. The mitochondria (M) are abundant, and there are numerous cytoplasmic vacuoles of the agranular endoplasmic reticulum (AR). A small vacuole-containing body (VCB), and a few profiles of granular endoplasmic reticulum (GR), are present.

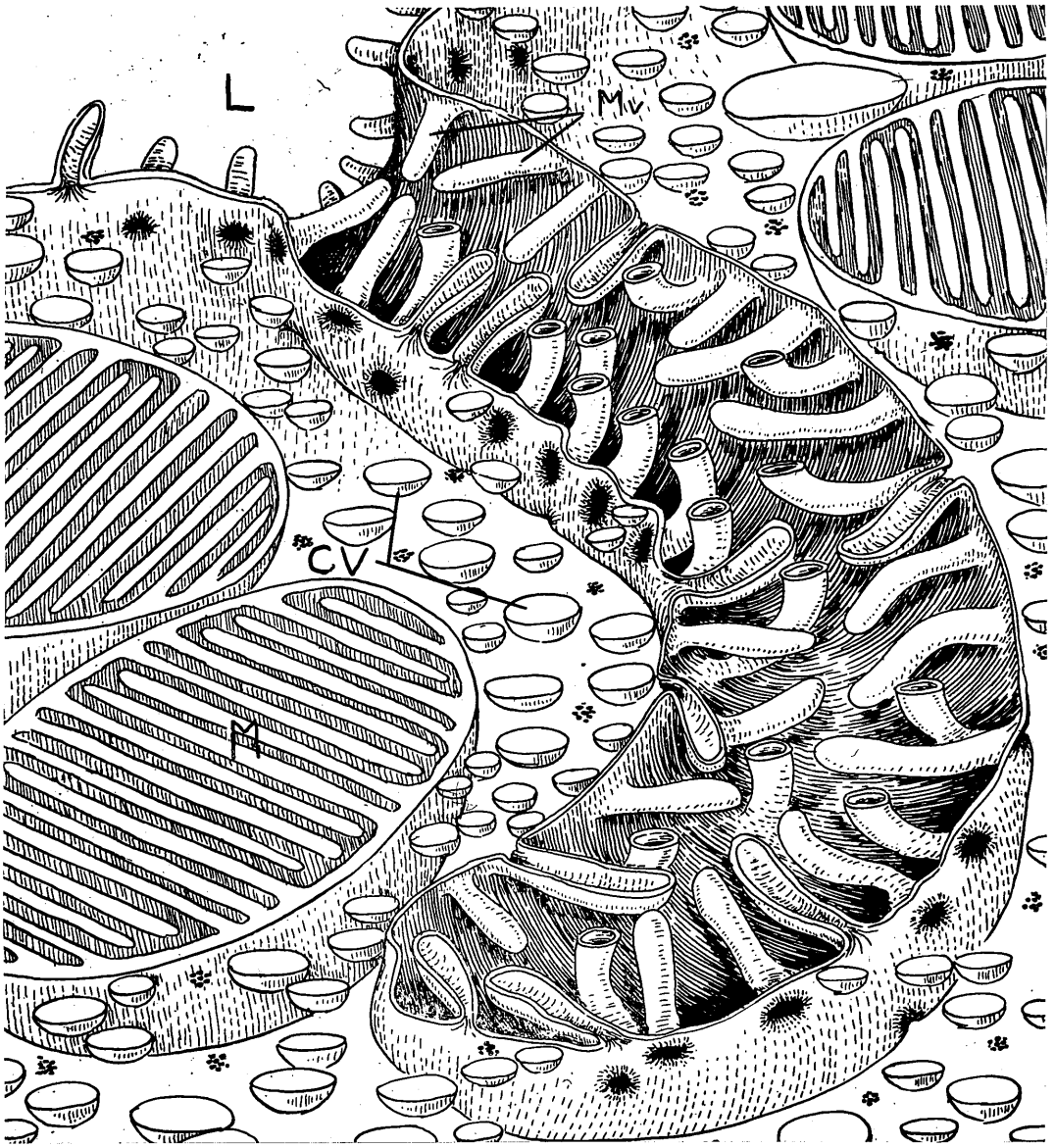



Fig. 33.

Diagram showing the apical portion of a gastric parietal cell. An intracellular canaliculus, lined with microvilii (Mv), opens at the free surface of the cell into the lumen of the gland (L). Numerous cytoplasmic vacuoles (CV) lie adjacent to the canaliculus. The mitochondria (M) contain close-packed cristae.

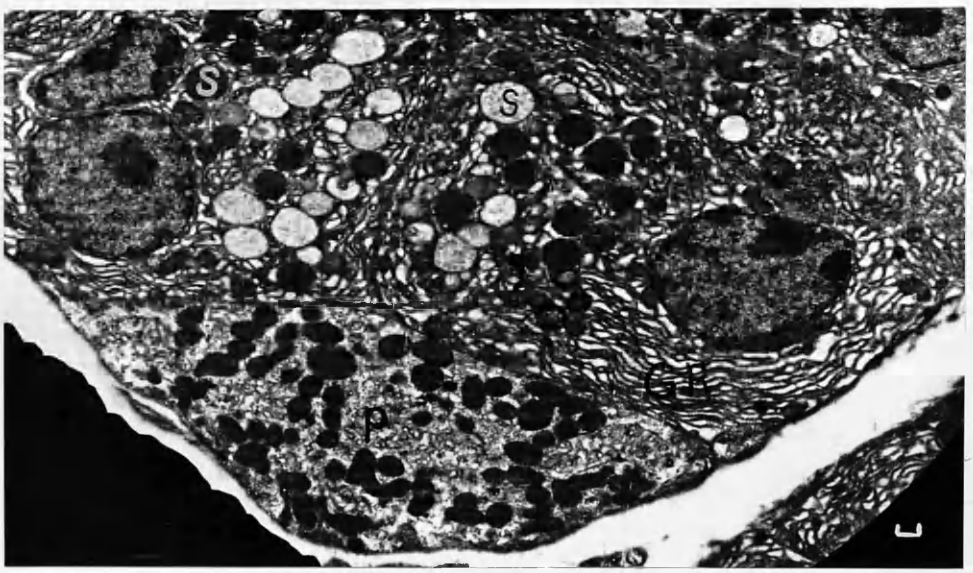



Fig. 34.

Section through the body of a gastric gland. A peripherally placed parietal cell (P) is adjacent to two zymogenic cells, which are characterised by a highly organised granular endoplasmic reticulum (GR) and secretory granules (S).

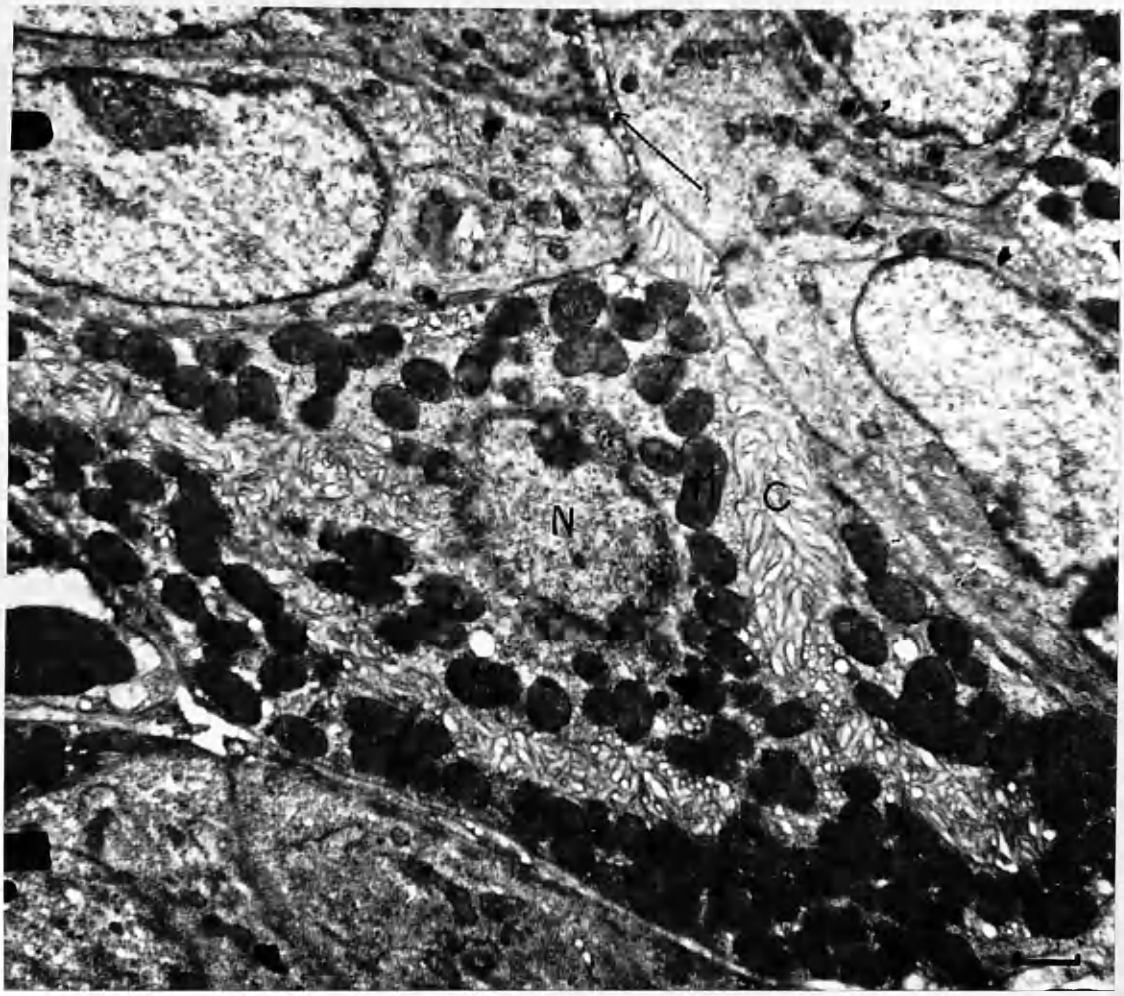



Fig. 35.

A parietal cell with large dense mitochondria (M), a centrally placed nucleus (N), and an intracellular canaliculus (C) which opens on the free surface of the cell to become continuous with a narrow cleft between adjacent mucous neck cells - an intercellular canaliculus (arrow).

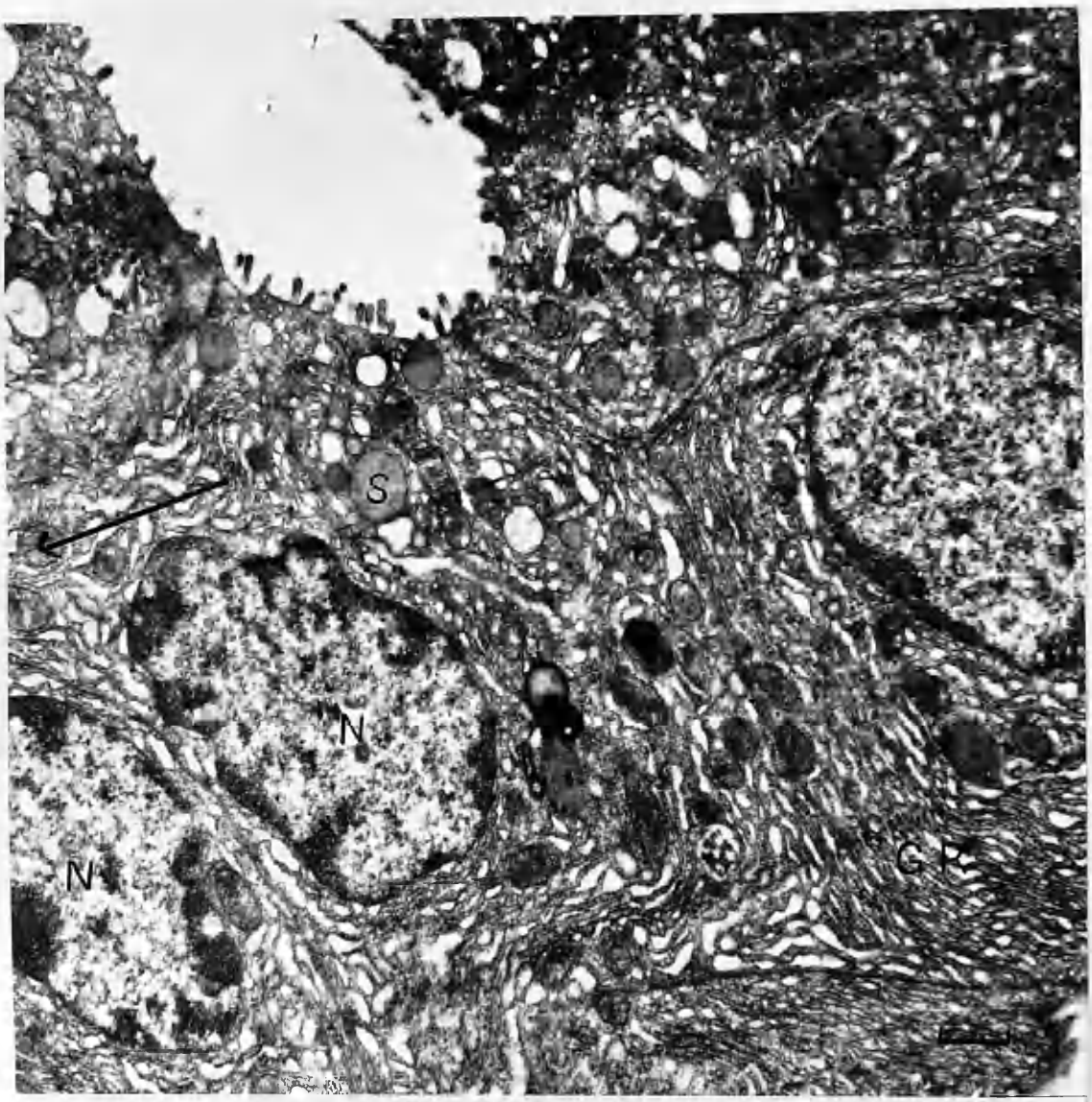



Fig. 36.

Section through the body of a gastric gland with several obliquely cut zymogenic cells, which have an extensive granular endoplasmic reticulum (GR) - similar to the Paneth cell - and secretory granules (S). An arrow indicates the direction of the knife. Note the compression of the nuclei, and the lines parallel to the arrow which arise from irregularities in the knife edge.

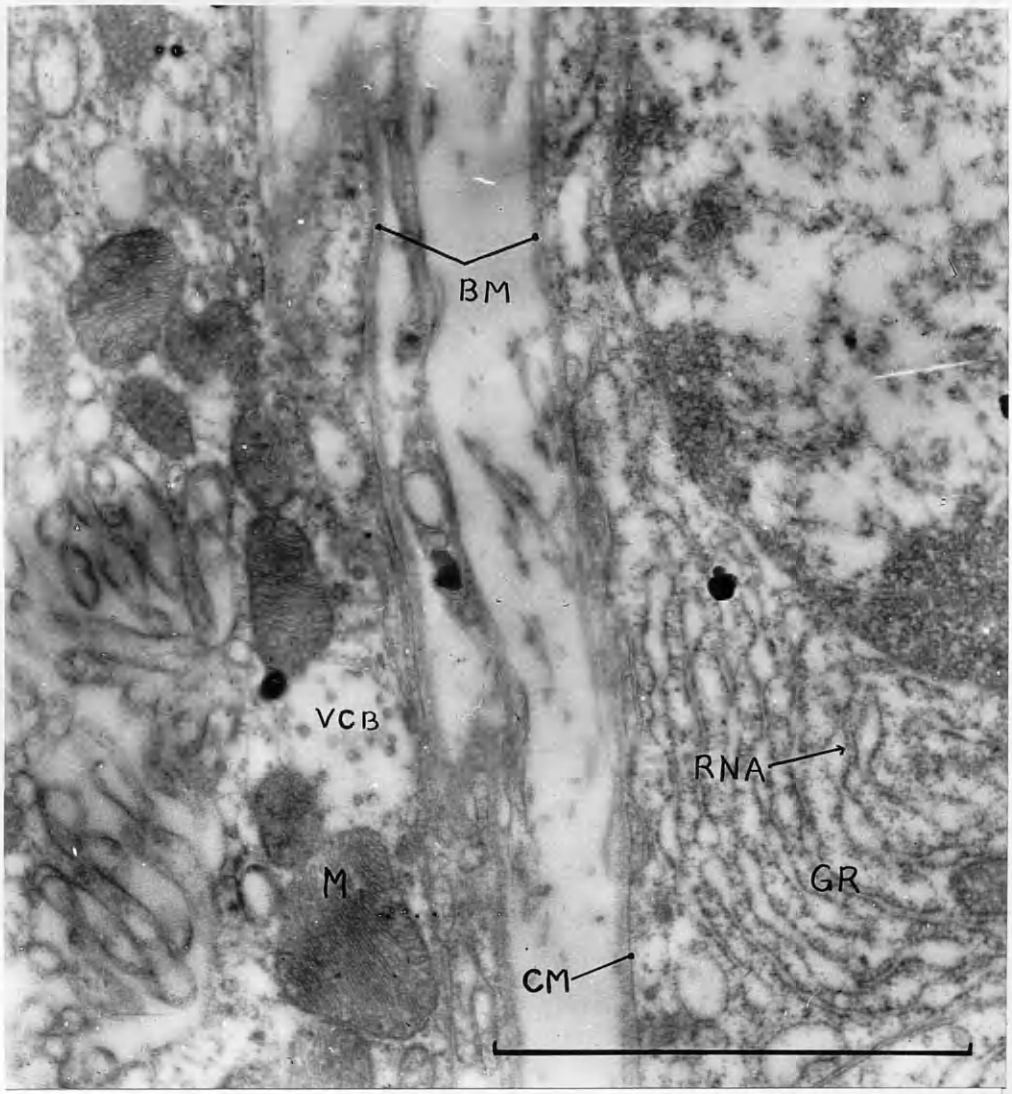



Fig. 37.

The basal portion of a parietal cell lies to the left, in this micrograph, and a zymogenic cell to the right. The membranes of the granular endoplasmic reticulum (GR) in the zymogenic cell are smooth on one surface, but studded with RNA particles on their cytoplasmic surfaces.

The basement membranes (BM) of the two cells are visible. Numerous cristae lie within the mitochondria (M) of the parietal cell. The print lacks contrast, as the negative was underexposed.

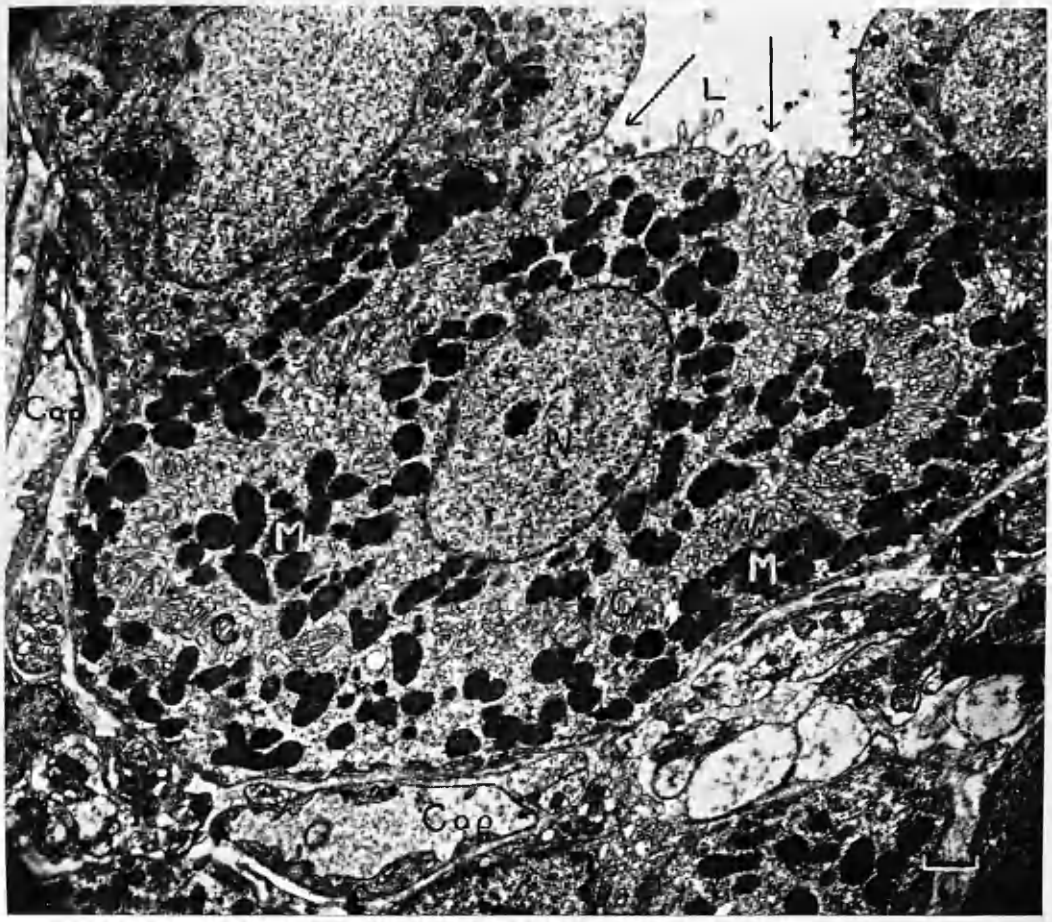



Fig. 38.

A parietal cell interposed between mucous neck cells, so that its free surface bounds part of the main lumen (L) of the gland. Basal to the nucleus (N) of the parietal cell are two intracellular canaliculi (C), which extend past the nucleus to reach the free surface of the cell and open directly into the lumen of the gland (arrows). Two capillaries (Cap) are subjacent to the parietal cell.

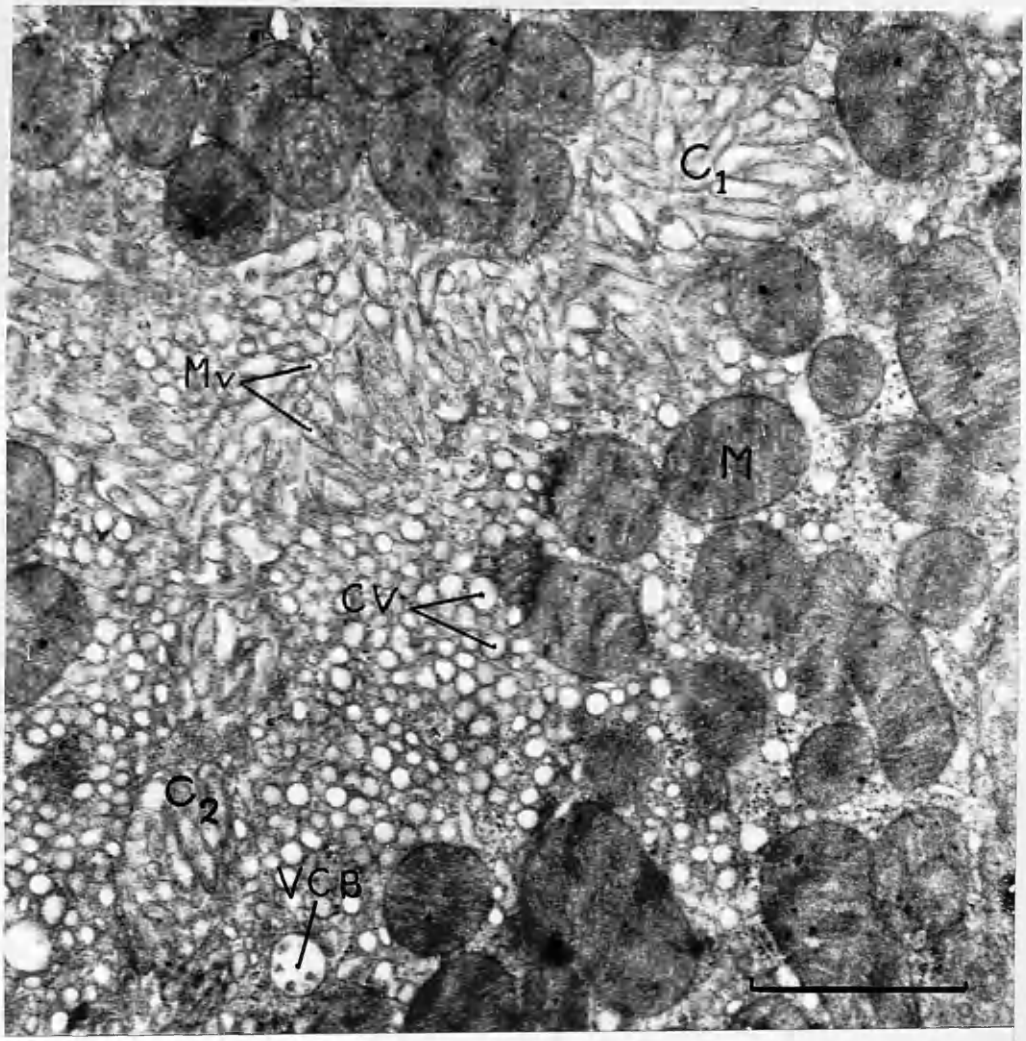



Fig. 39.

Part of a parietal cell, containing large ovoid mitochondria (M) with close-packed cristae and dense ground substance. An intracellular canaliculus extends from the upper right corner ($C_1$), downwards to the lower left corner ($C_2$). The lumen of the canaliculus is filled with microvilli (Mv). Many cytoplasmic vacuoles (CV) lie between the canaliculi and the mitochondria. A single vacuole-containing body (VCB) is also present.

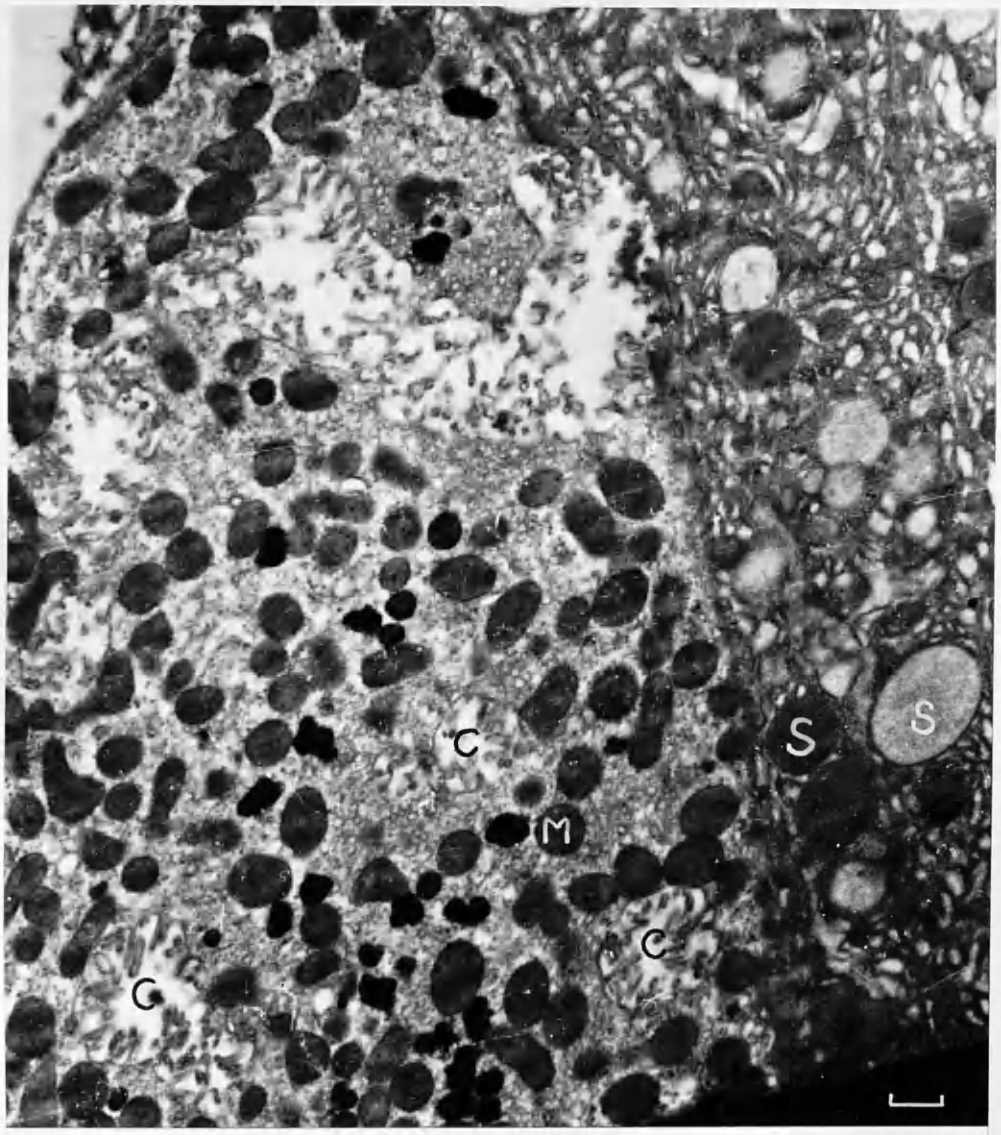



Fig. 40.

Within the parietal cell occupying the left half of this micrograph are several cross-sectioned intracellular canaliculi (C). Microvilli project into the lumen of each canaliculus, rendering its outline irregular.

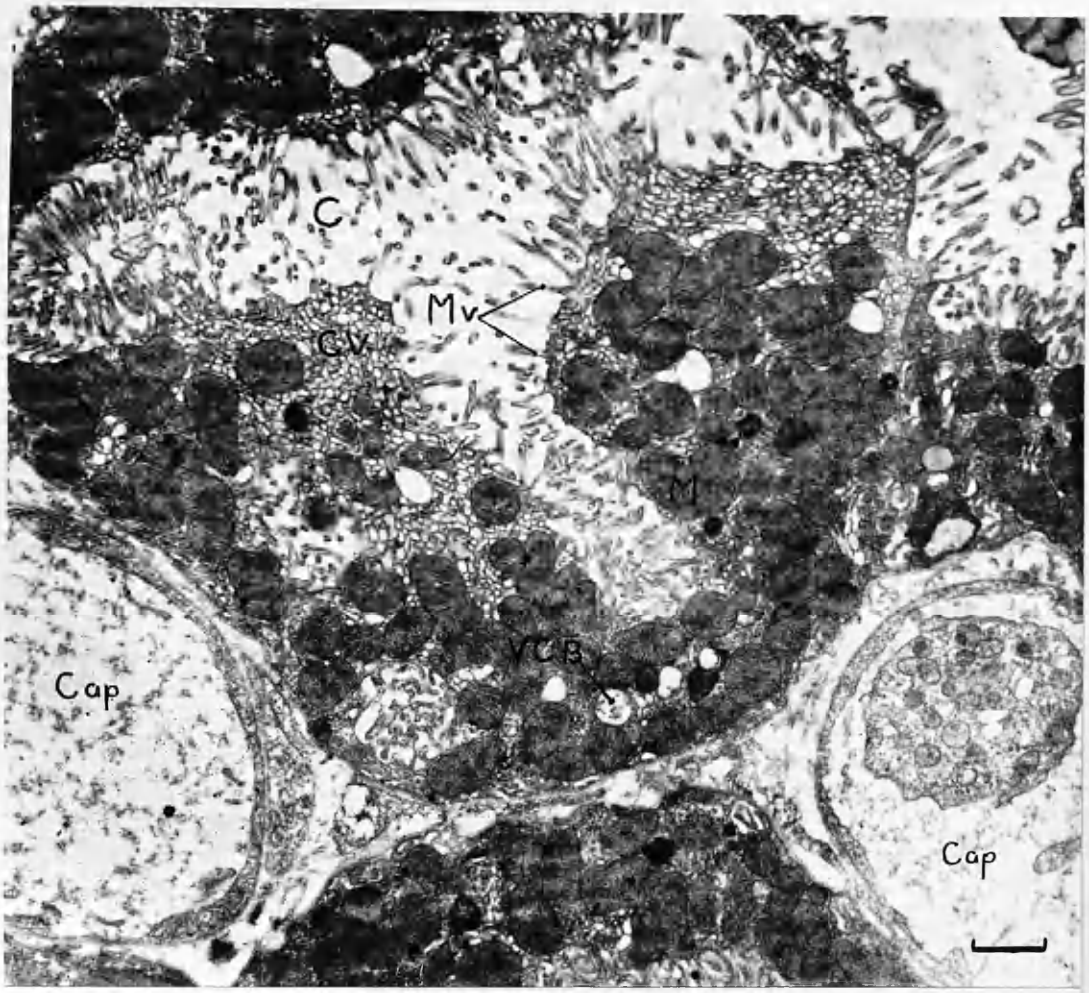



Fig. 41.

A gastric parietal cell from an animal which had been injected with histamine. The intracellular canaliculi are distended so that the microvilli (Mv) are prominent. The cytoplasmic vacuoles (CV) are particularly abundant.


Fig. 42.

A micrograph of the basal portions of two adjacent parietal cells. The cell membrane (CM), except where it is infolded (arrows) into the cell, is in close relation to the underlying basement membrane (BM). Small clusters of RNA particles (RNA) lie free in the cytoplasm and there are a few profiles of granular endoplasmic reticulum (GR). There is a paucity of cytoplasmic vacuoles in the peripheral cytoplasm near the cell membrane, compared to the large numbers (CV) found in the uppermost part of the micrograph, nearer the intracellular canaliculi.

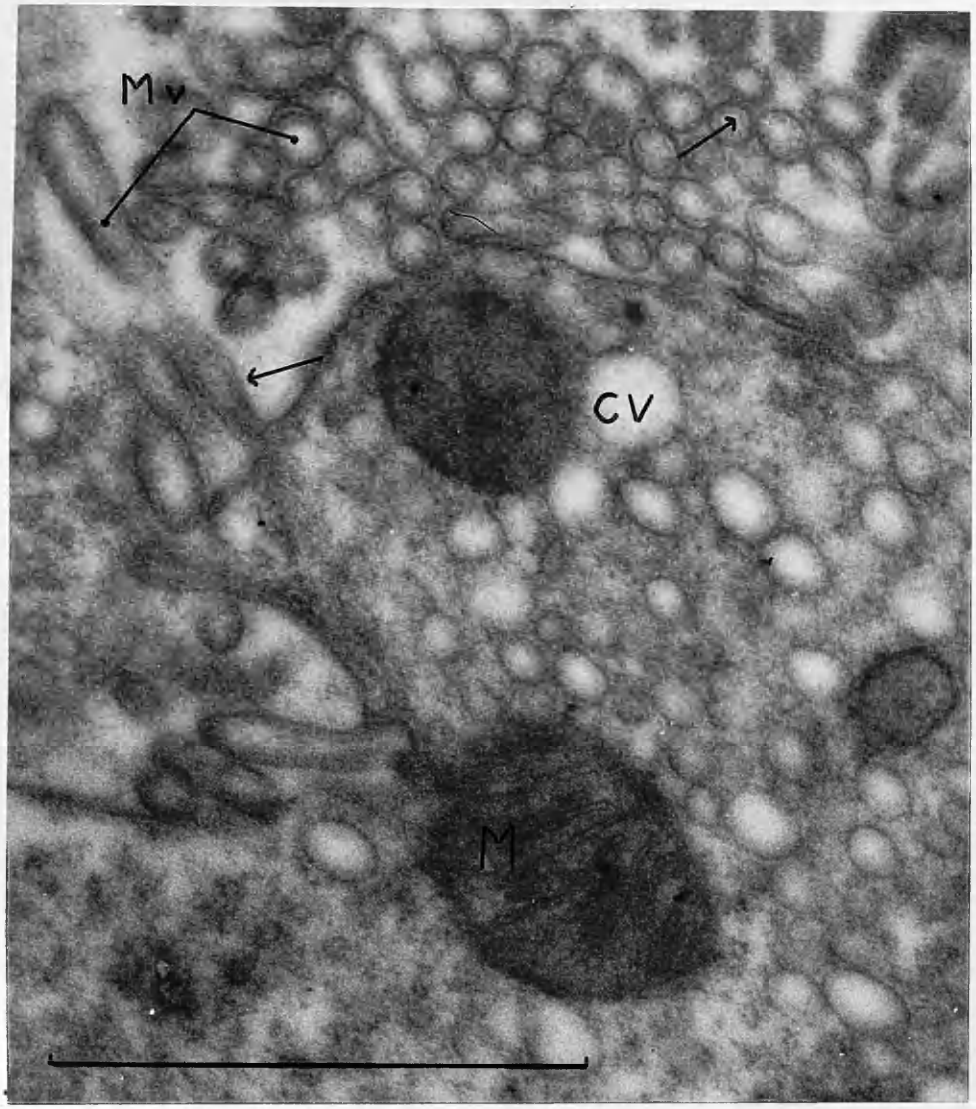



Fig. 43.

A micrograph of the apical portion of a parietal cell. The intracellular canaliculus in the upper part of this micrograph contains numerous circular profiles of cross-sectioned microvilli and other microvilli cut longitudinally, an example of each being indicated (Mv). In addition to the cell membrane bounding the microvillus, there is an underlying membrane, visible in both cross-sectioned and longitudinally sectioned microvilli (arrows).

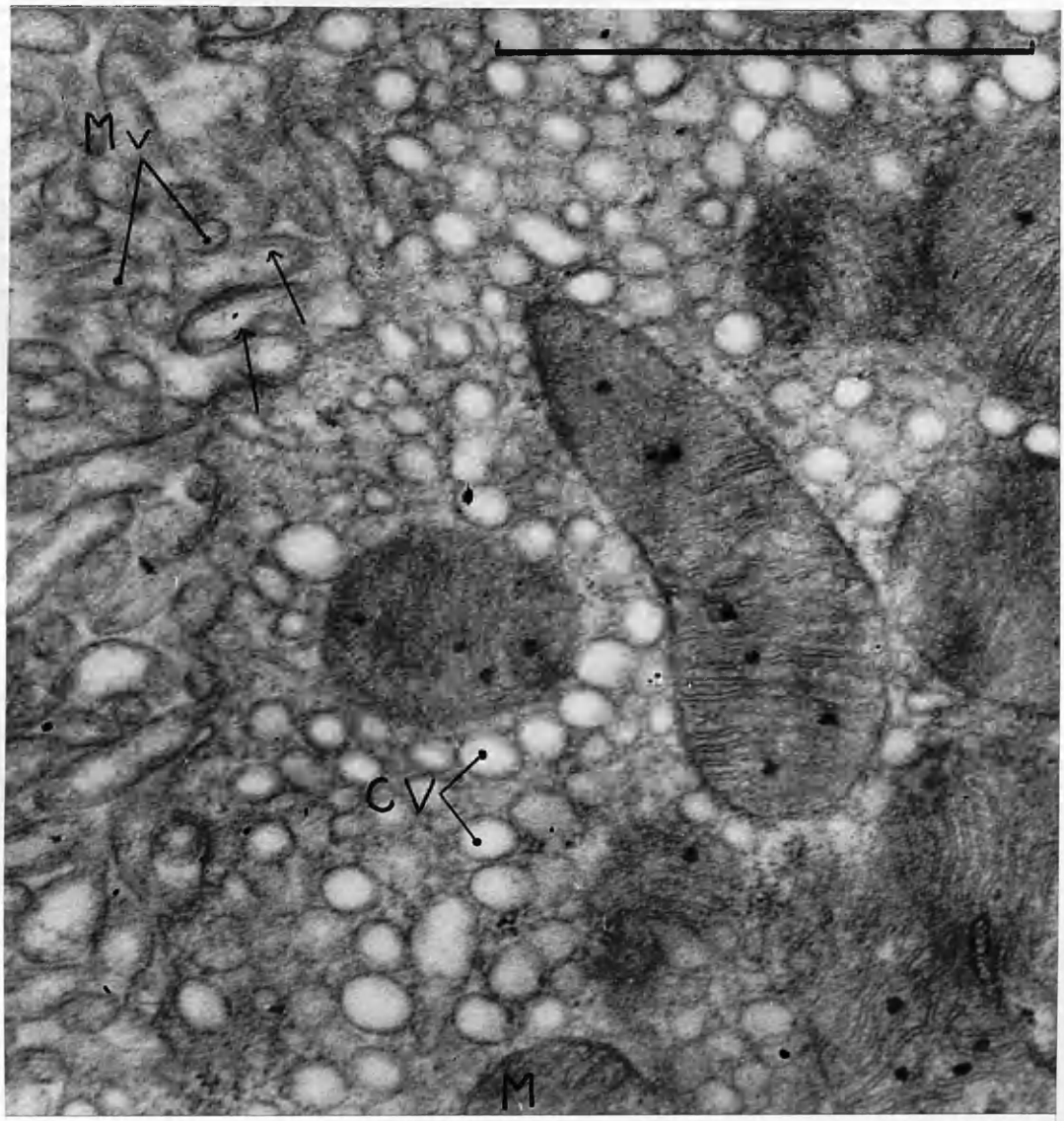



Fig.44.

An intracellular canaliculus - lined with inwardly projecting microvilli (Mv)- lies close to the left margin of this micrograph of a parietal cell. Most of the microvilli are cut longitudinally or obliquely, and some show the characteristic additional membrane (arrows) underlying the cell membrane. Cytoplasmic vacuoles (CV) pack the cytoplasm between the intracellular canaliculus and the mitochondria. Each spherical vacuole is bounded by a smooth single membrane, and is unconnected with neighbouring vacuoles.

The infoldings of the inner limiting membrane to form the cristae are clearly seen in a mitochondrion (M).

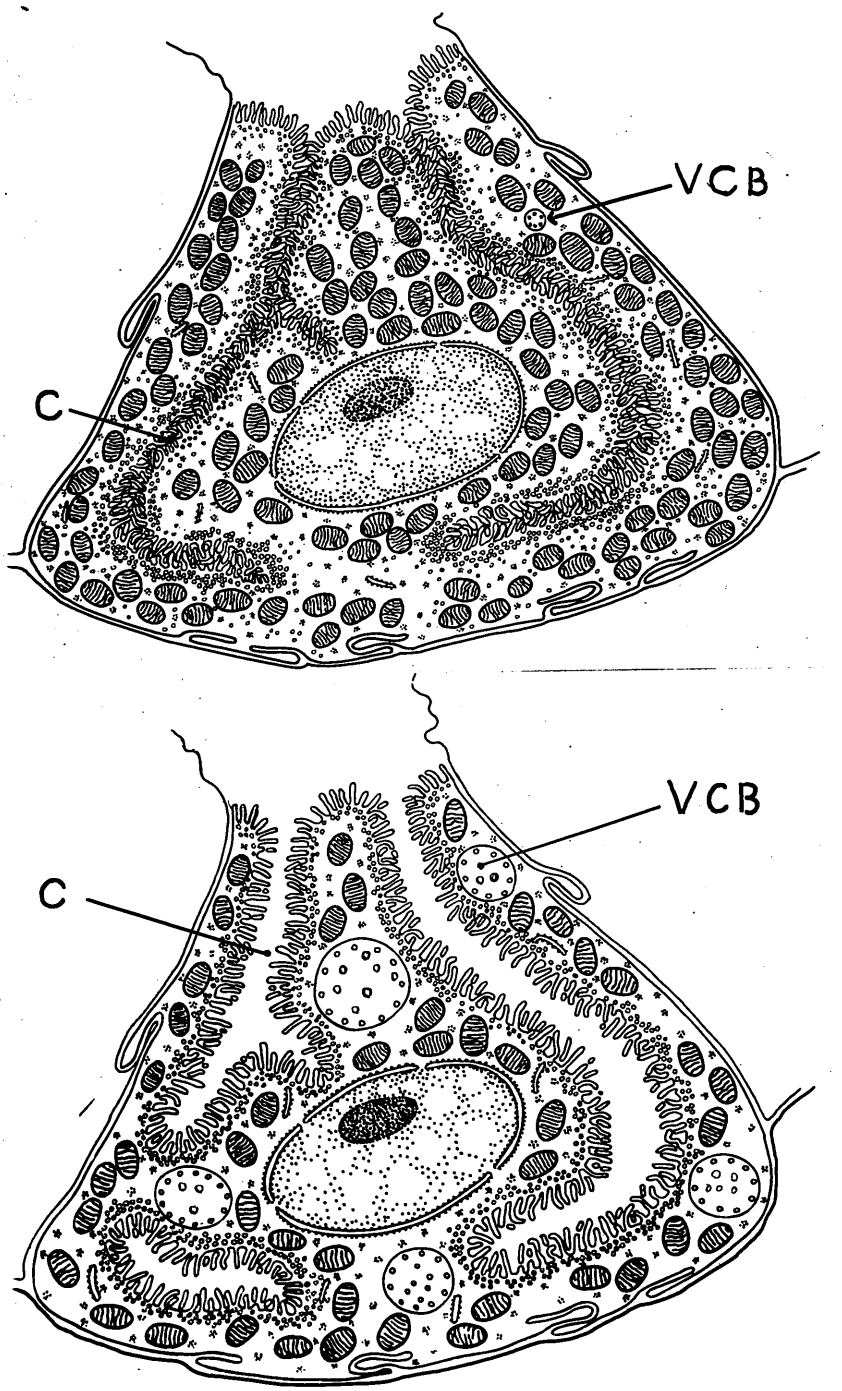



Fig. 45.

Diagrams of a parietal cell from a control animal (above) and a treated animal (below). The treated cell differs from the control in having distended intracellular canaliculi (C), and larger and more numerous vacuole-containing bodies (VCB).

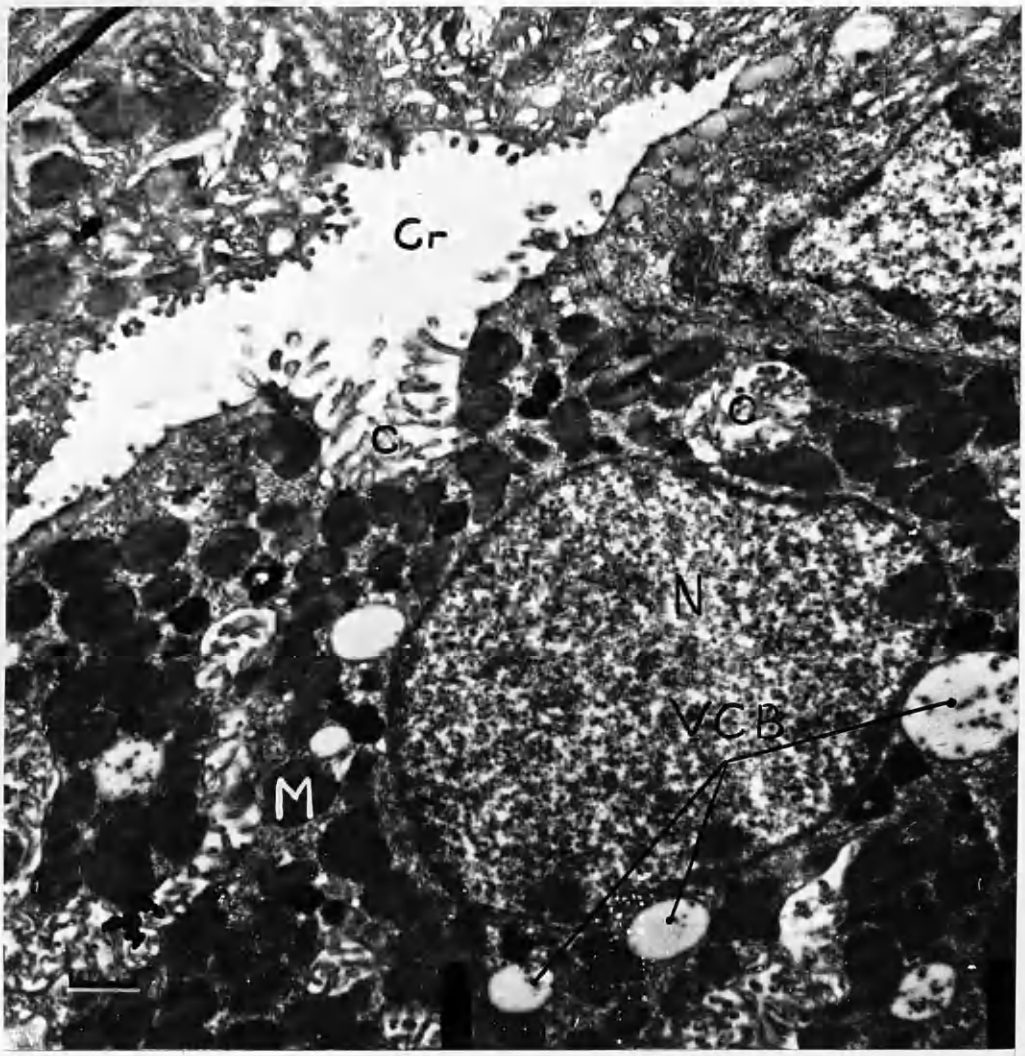



Fig. 46.

A parietal cell from a treated animal, with unaltered mitochondria (M) and nucleus (N).

The intracellular canaliculi (C), however, are moderately distended, and the vacuole-containing bodies (VCB) larger and more numerous. The adjacent cells are zymogenic.

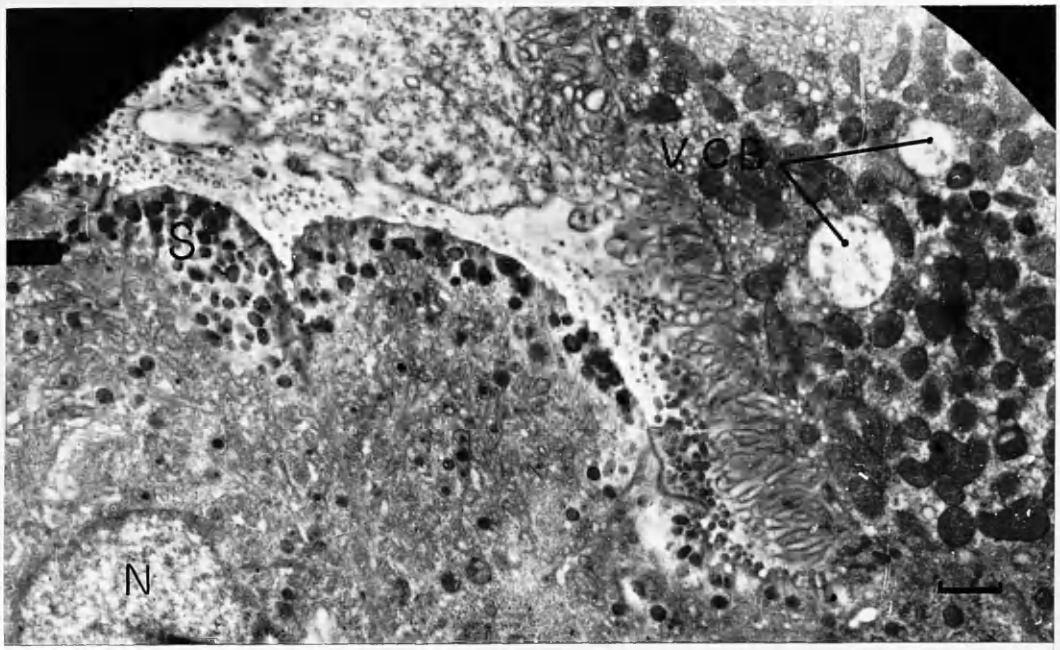



Fig. 47.

This micrograph through the neck of a gastric gland shows a single parietal cell containing two enlarged vacuole-containing bodies (VCB), and several mucous neck cells with small secretory granules (S).

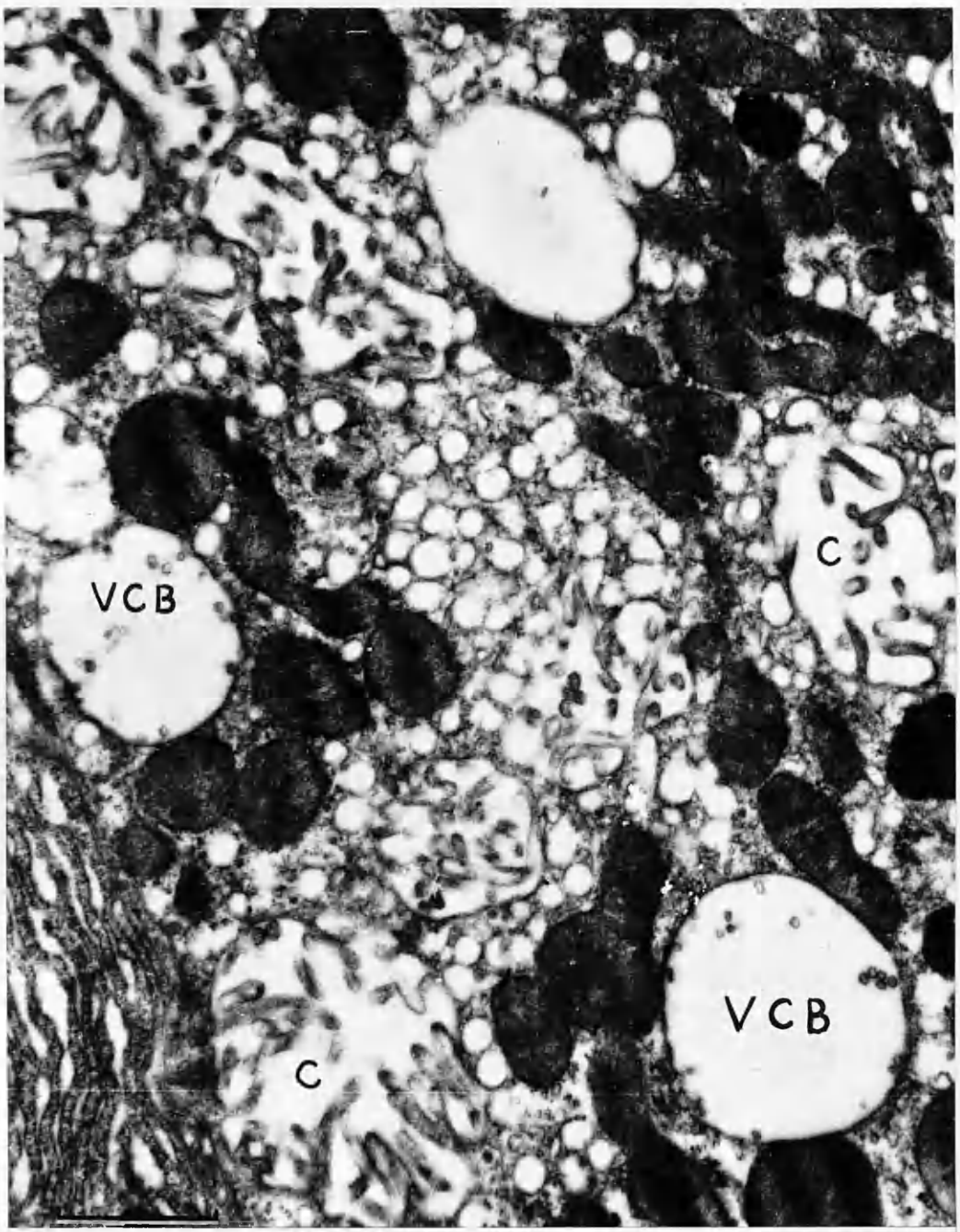



Fig. 48

Another treated parietal cell, with enlarged canaliculi (C) and vacuole-containing bodies (VCB).

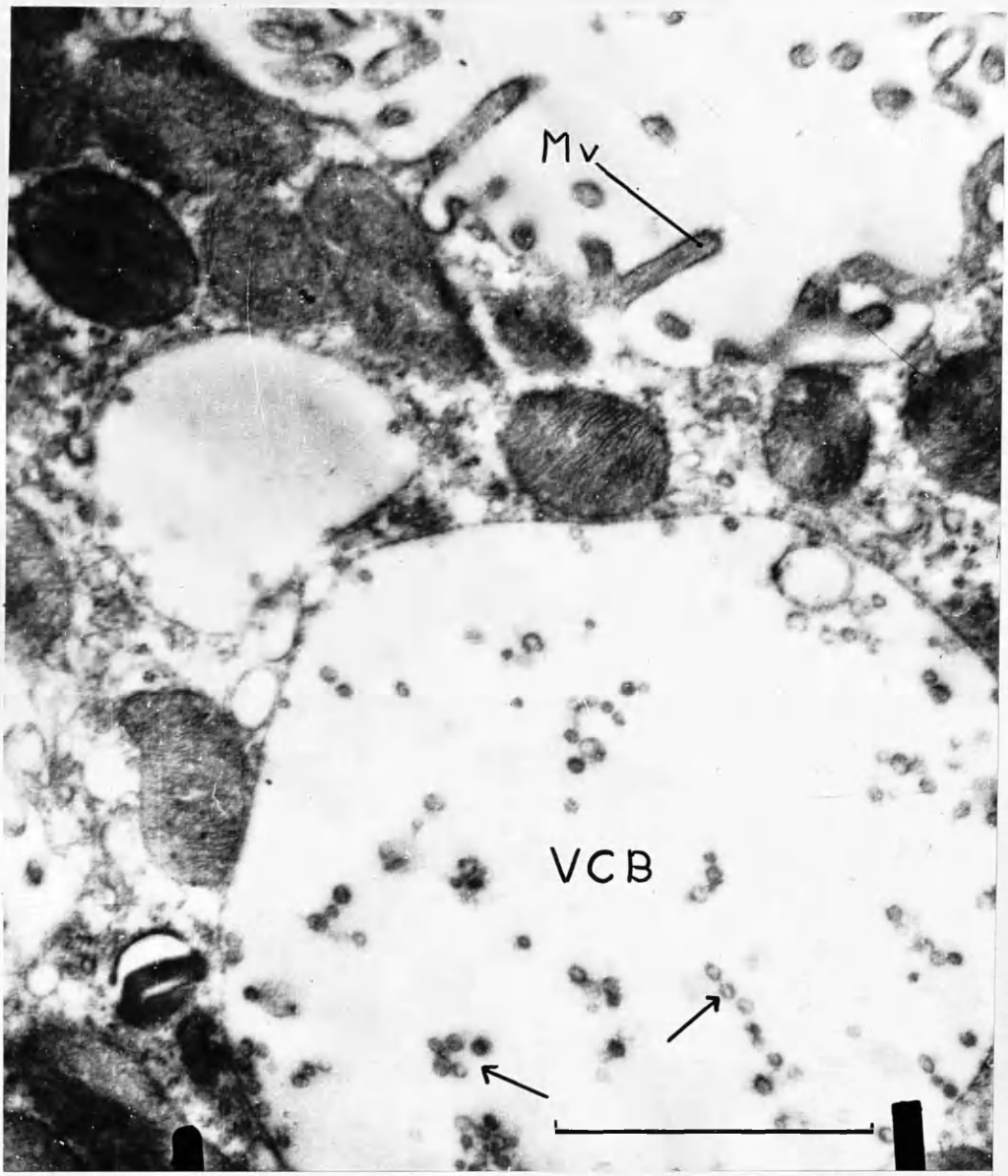



Fig. 49.

Treated parietal cell with a grossly enlarged vacuole-containing body almost 3μ in diameter, containing large number of small vacuoles (arrows).

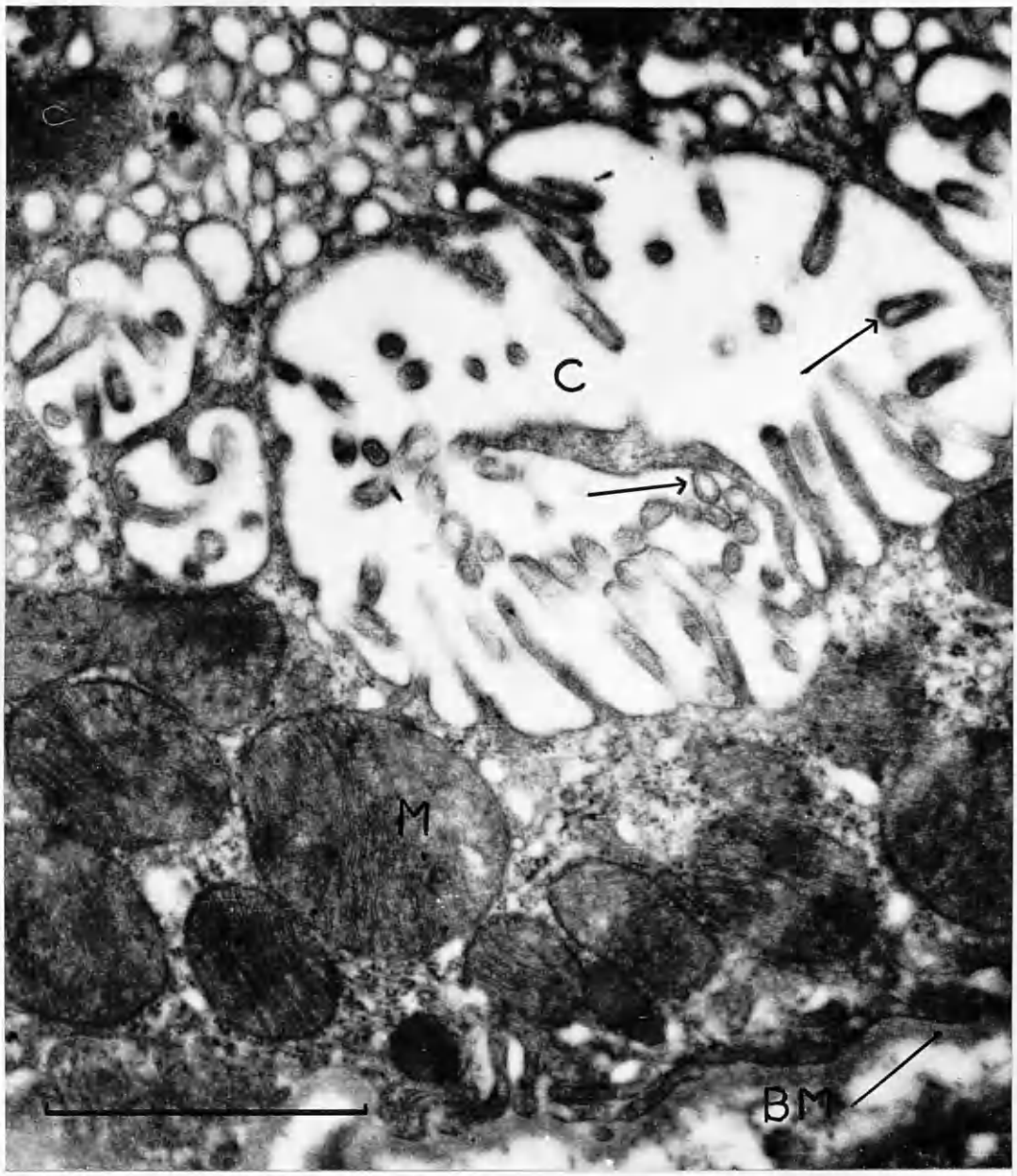



Fig. 50.

Basal portion of treated parietal cell with distended canaliculus (C). The underlying membrane in the microvilli is visible (arrows). The mitochondria are also unchanged, and contain numerous cristae.

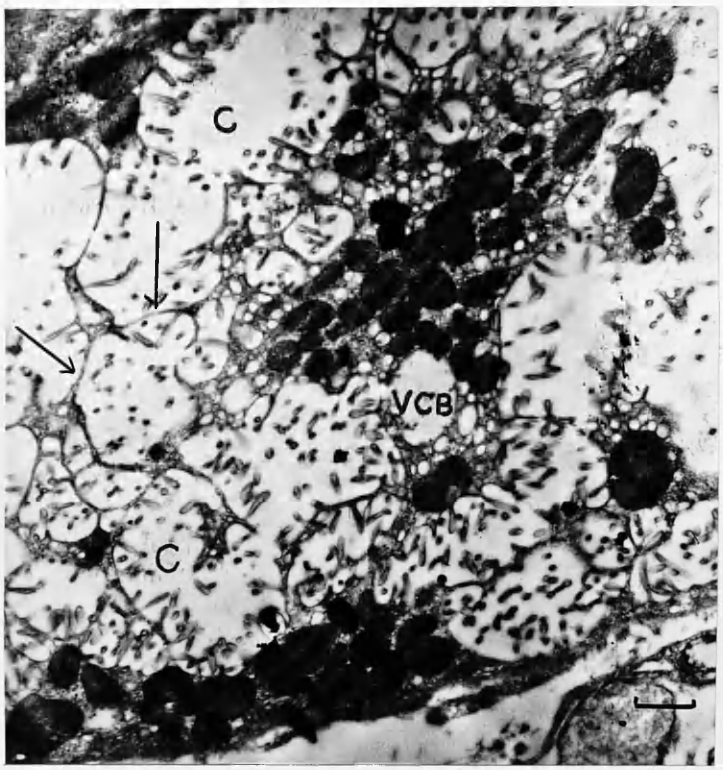



Fig. 51.

Part of a treated parietal cell, with a very complex system of dilated intracellular canaliculi. The evaginations from the canaliculi are separated from each other by thin sheets of cytoplasm (arrows). Randomly sectioned microvilli lie within the canaliculi and evaginations. There is a single vacuole-containing body (VCB), amongst the mitochondria (M).

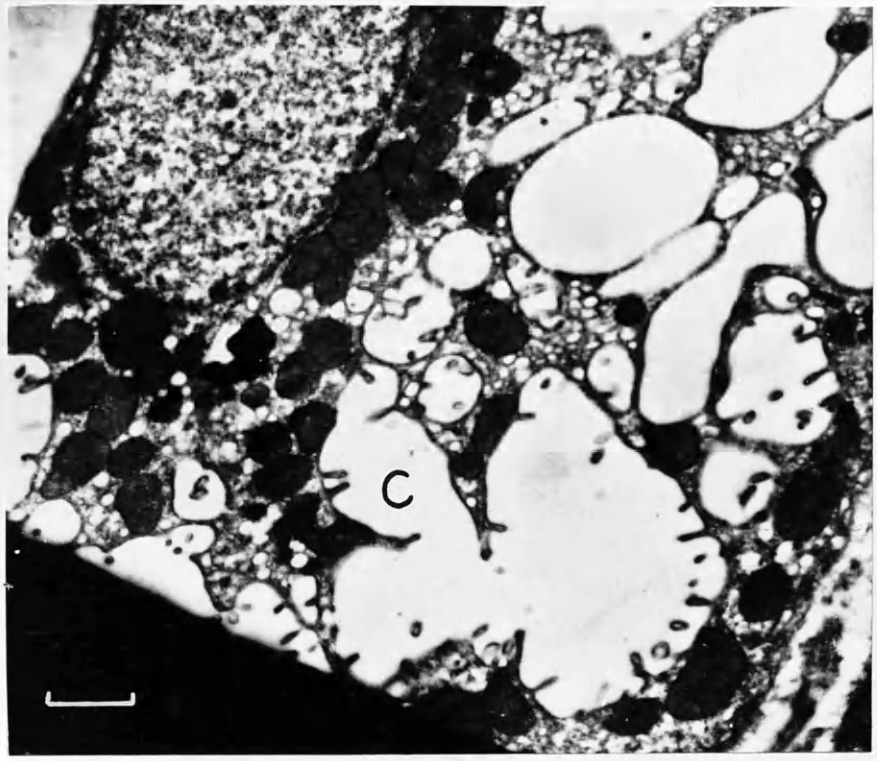



Fig. 52.

Treated parietal cell with grossly distended intracellular canaliculi (C), in which the microvilli are much reduced in number, or absent.

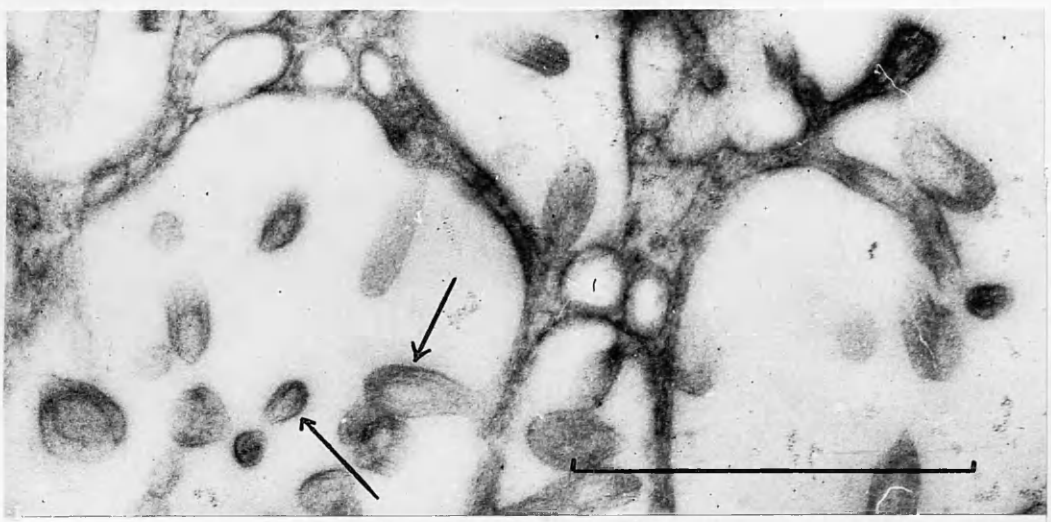


Fig. 53.

A micrograph of the complex intracellular canaliculi with their numerous small evaginations. Within such an evagination lie microvilli, which have a second membane underlying the bounding cell membrane (arrows).

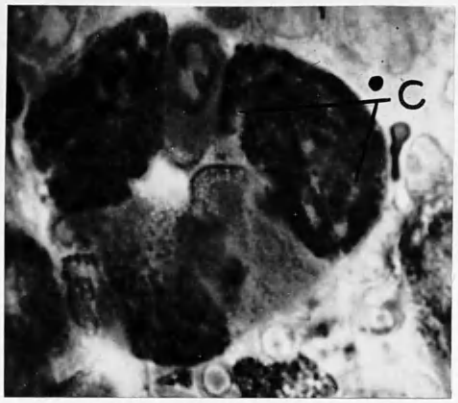



Fig. 54a.

Light micrograph of gastric tubule with three heavily impregnated parietal cells. Their cytoplasm is filled with black granules, which contrast sharply with the clear intracellular canaliculi (C).

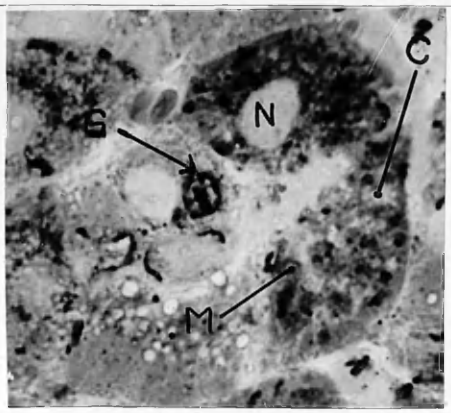



Fig. 54b.

Light micrograph of gastric gland with two moderately impregnated parietal cells. Black granules and strands - constituting the Golgi apparatus - are scattered throughout the cytoplasm among the grey mitochondria (M), and clear intracellular canaliculi (C). A cell adjacent to the upper parietal cell contains a typical localised Golgi apparatus (G).

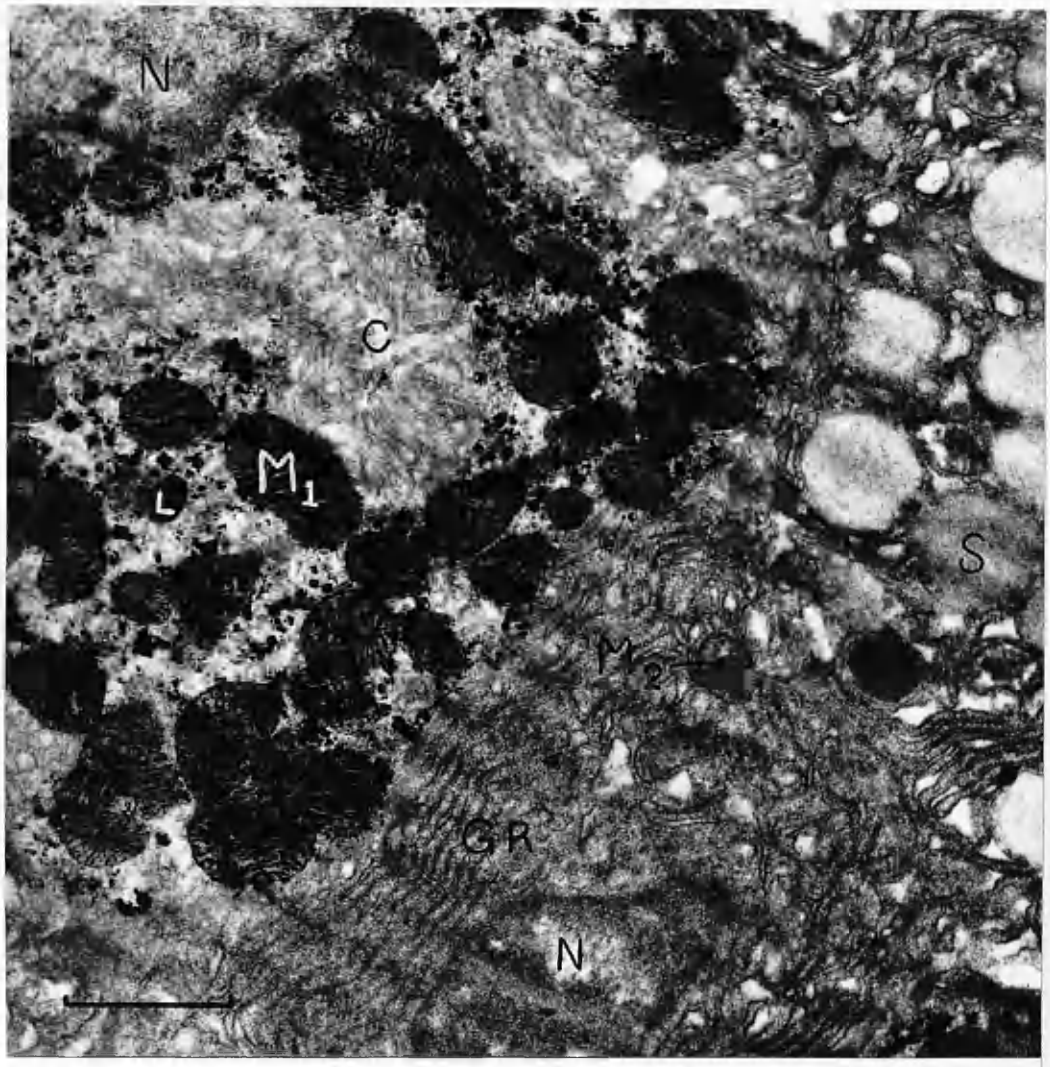



Fig. 55.

Electron micrograph of heavily impregnated parietal cell comparable to Fig. 54a. Black granules lie between the blackened mitochondria ($M_1$). The intracellular canaliculi (C) and nucleus (N) are unblackened. A small lipid globule (L) is present. Neither the granular endoplasmic reticulum (GR), nor the mitochondria ($M_2$), within the adjacent zymogenic cell are blackened.

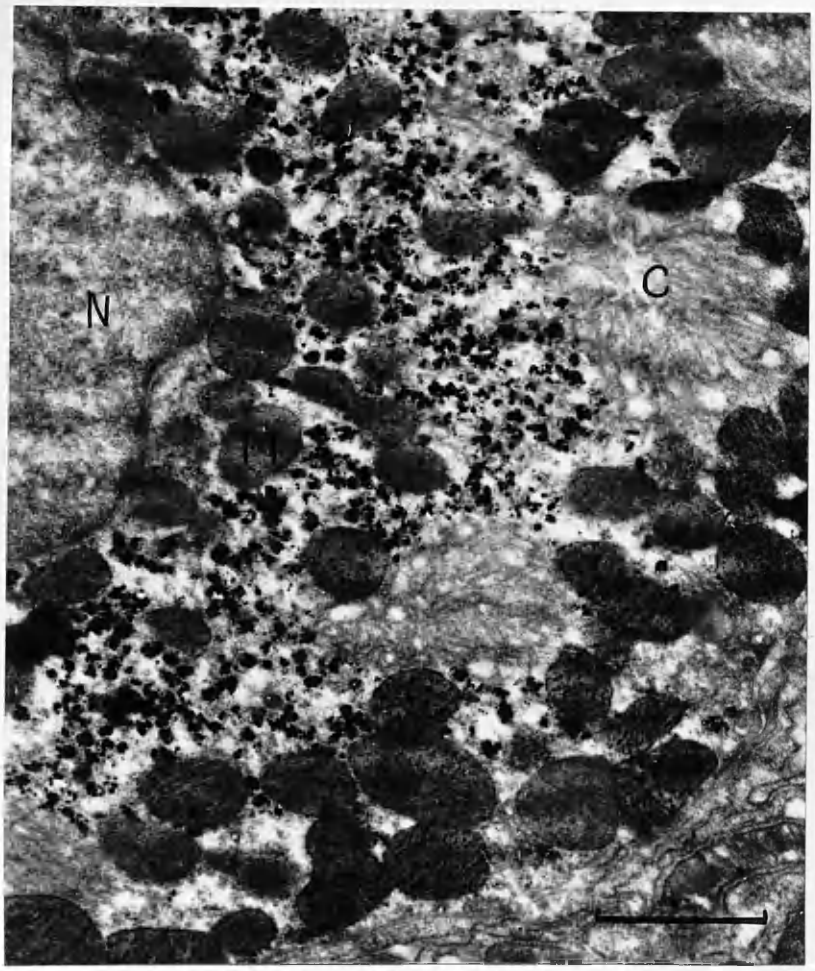



Fig. 56.

Electron micrograph of moderately impregnated cell comparable to Fig. 54b. Numerous black granules are found in the cytoplasm, but the mitochondria (M) are scarcely blackened.

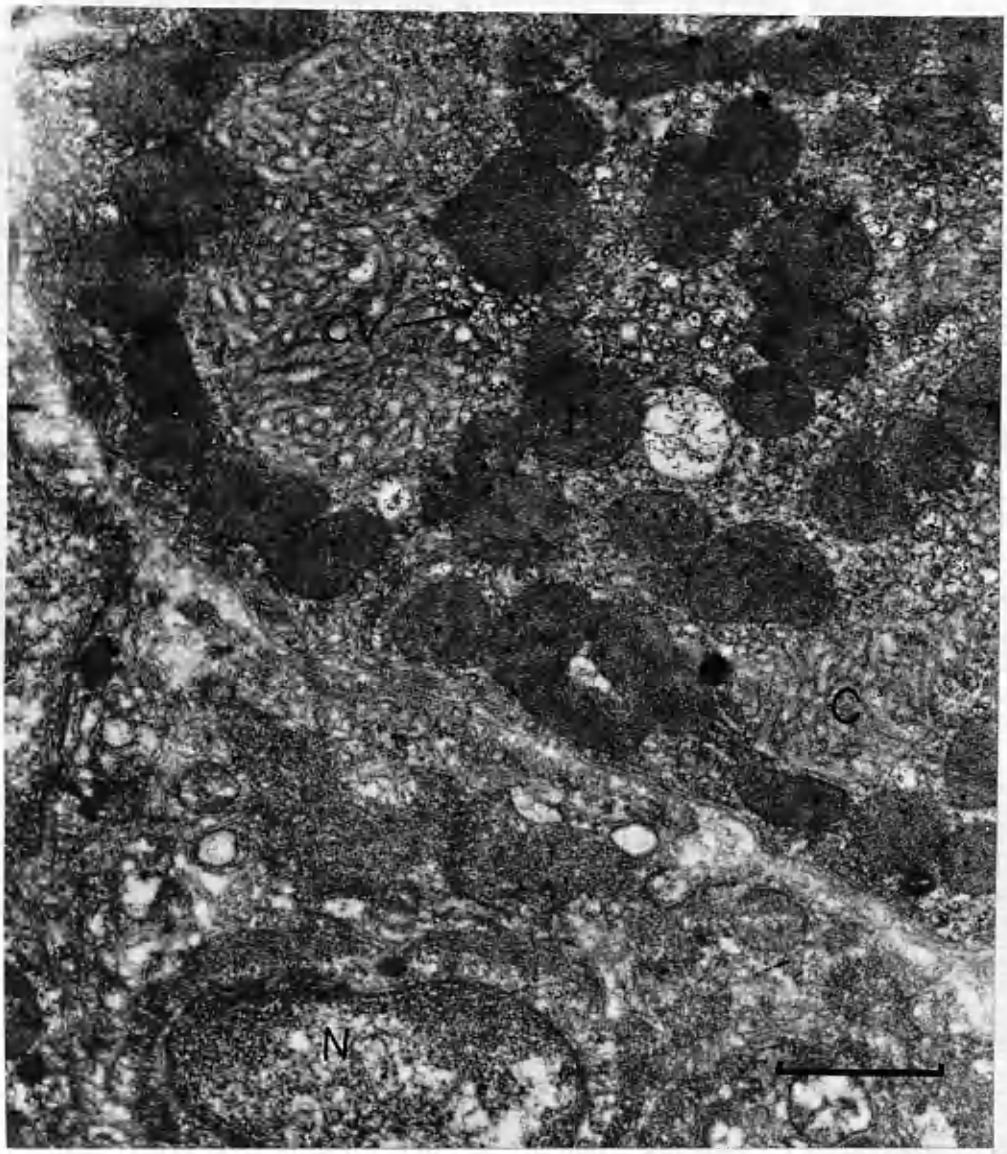



Fig. 57.

Parietal cell after short post-osmication. Although numerous tiny particles lie within the cytoplasmic vacuoles (CV), they are rare in the mitochondria (M) and intracellular canaliculi (C).

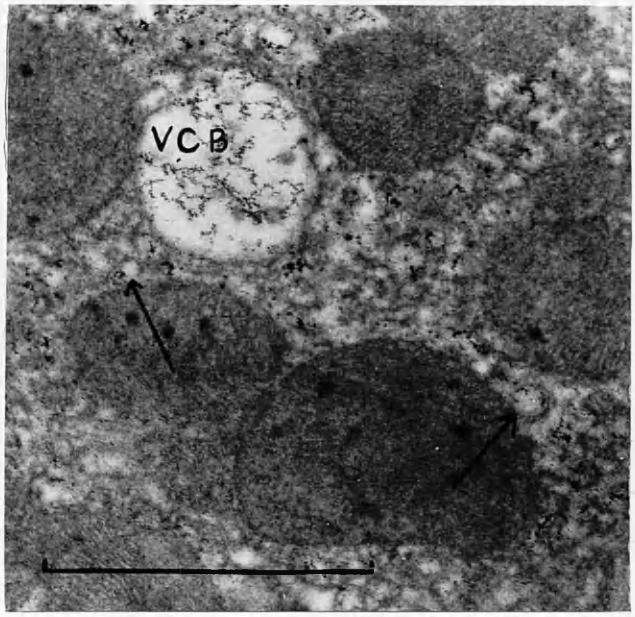



Fig. 58.

Enlarged portion of Fig. 57. Small black particles lie inside the cytoplasmic vacuoles of the agranular reticulum (arrows). A vacuole-containing body (VCB) also contains black particles, which are often arranged in delicate strands.

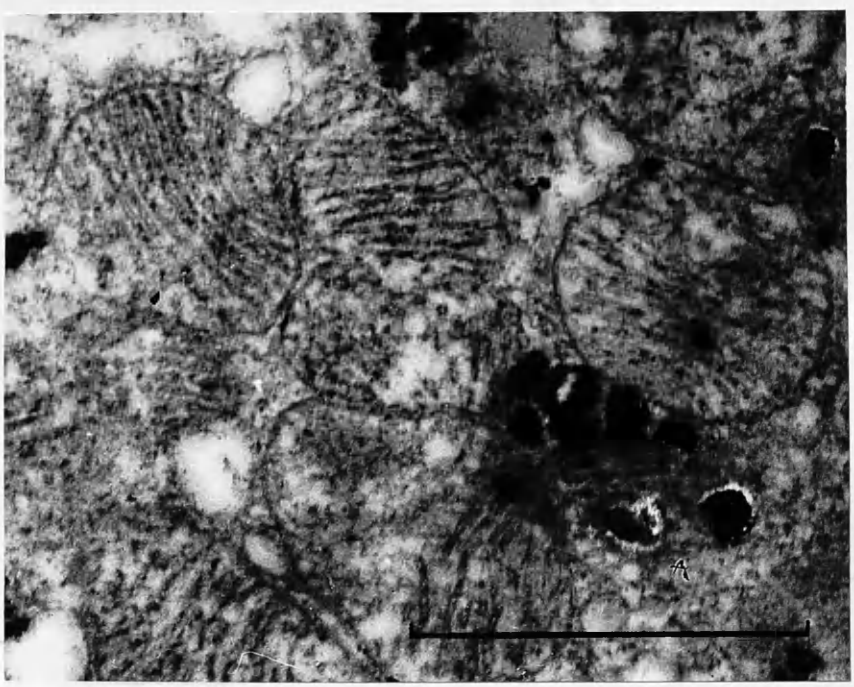


Fig. 59.

Mitochondria in a post-osmicated parietal cell which contain black particles between the outer and inner mitochondrial membranes, and within each crista.

## GENERAL KEY TO ELECTRON MICROGRAPHS.

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| | |
|---|---|
| AR | Agranular endoplasmic reticulum |
| BM | Basement membrane |
| C | Intracellular canaliculus |
| Cap | Capillary |
| Cr | Crypt |
| CG | Clusters of black granules |
| CM | Cell membrane |
| CV | Cytoplasmic vacuoles |
| EM | Enclosing membrane |
| G | Golgi complex |
| GM | Golgi membrane |
| GV | Golgi vacuole |
| G Ves | Golgi vesicle |
| GR | Granular endoplasmic reticulum |
| L | Lumen |
| Li | Lipid globule |
| M | Mitochondrion |
| Mv | Microvilli |
| N | Nucleus |
| NM | Nuclear membrane |
| P | Parietal cell. |
| RNA | RNA particles |
| S | Secretory granule |
| TB | Terminal bar |
| VCB | Vacuole-containing body. |

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The magnification mark on each micrograph represents one micron unless stated otherwise.