

THE ULTRASTRUCTURE OF A MARINE LUMINOUS  
BACTERIUM

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## SUMMARY

An electron microscope study of normal and L-forms of a marine luminous bacterium was carried out. In addition to the features found normally in Gram-negative bacilli, observed in cells from solid medium, cells from actively luminescent liquid culture were found to possess certain peculiar features. The most notable of these were tetrads, cells in which ingrowth of the cytoplasmic membrane causes the contents to be divided up into four protoplasts within the cell wall. Small cytoplasmic spherules which arise from the cytoplasmic membrane were also seen to occur. The cell walls which were three-layered and typical of Gram-negative bacteria often had blebs on the surface. The cytoplasmic membrane, typical of a 7.5 nm unit biological membrane in appearance in normal cells seemed to be composed of a number of laminations in some L-form cells. These laminations consisting of alternate dark and light bands may consist of numbers of unit membranes with adjacent protein layers fused. The occurrence of L-form cells was shown to be associated with luminescence and aeration in liquid sea-water medium.

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## CHAPTER 1.

### INTRODUCTION

While considerable attention has been paid to the biochemistry of luminescence\*, the ultra-structure of luminous bacteria has not been given much consideration. In early investigations Johnson et al. (1943) used the electron microscope to study cells and cytolysates of a number of luminous species, but this was before metal-shadowing, negative-staining and ultra-microtomy had been developed. Johnson and Gray (1949) studied the effects of salt concentration, temperature and urethane on the cells of Photobacterium fischeri and P. splendidum, using the light microscope. These authors described the development of large bodies of P. fischeri and they noted that small granules were to be seen inside and on the surface of such cells; they thought the granules also sometimes appeared to be partly on the outside. Recently, Hendrie et al. (1965) and Hodgkiss and Shewan (1968) have conducted electron microscope studies of the flagellation, which has taxonomic significance, of luminous bacteria.

No account of ultra-structural features peculiar to luminous

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\*Harvey et al. (1948), McElroy et al. (1953), Terpstra (1963), Hastings et al. (1964), and others.

bacteria has been published and this may be due to the fact that distinctive features are only developed under certain conditions of growth. The organism used in this study was found to be luminous when a number of cultures, which were part of a routine collection of marine bacteria, were examined for this property.

The original aim of the study reported here was to investigate the possible existence of ultra-structural features peculiar to luminous bacteria which might be correlated with luminescence. No such features were found in bacteria from solid medium. On this medium, however, the colonies were only faintly luminescent, and therefore cultures were grown in aerated liquid medium; under such conditions luminescence was most striking.

When cells from these cultures were examined under the light microscope, and later, the electron microscope, the results were most unexpected. These luminescent cultures contained cells which in no way resembled those from the agar plates and the differences were so great that at first contamination was suspected. The cells appeared to be L-forms and were a constant feature of aerated liquid cultures. Generally, the cells were spherical, but often amoeboid shapes with bud-like protrusions of the surface were present. Some cells when viewed under the phase-contrast microscope appeared to have tiny spherical globules attached to them by means of thread-like connections and many cells appeared to contain smaller spherical parts and granules. Many cells were seen in which the protoplasm appeared sub-divided, and cells in which four protoplasts were arranged to form a tetrad within the cell wall

were common. A most interesting observation was that although no normal cells were seen in any of these cultures, when transfers were made to solid medium the resulting growth contained normal cells only. These observations were confirmed by further experiment, and the organism was selected as being suitable for investigation with a slightly modified goal, the intention now being to elucidate the nature of the peculiarities observed in the cells from liquid culture.

This investigation was intended to be primarily ultra-structural though certain physiological observations were made. Such observations, while they may be significant, are purely incidental and much more experimental work would be required before they could be substantiated.

## CHAPTER 2

### MATERIALS AND METHODS

#### A. THE STUDY ORGANISM

##### Collection and isolation

The culture which is the subject of this study was isolated from a collection made at a depth of 50 metres in March 1968 at Station 52\* off the Cape Coast. The temperature of the water was 9.96 C, the salinity 34.80‰, and the oxygen concentration was 4.48 ml/L. The sampler used was a submerged membrane filter apparatus (Williams, 1969). The sample was collected under aseptic conditions. After culture of the membrane on a nutrient agar sea-water (NAS) plate, colonies were picked and streaked out on this medium a number of times until pure cultures were obtained. The subject of this investigation is Culture Number 734 of a series of collections.

Preparation methods and formulae for all culture media and reagents used in this section will be found in Appendix A.

##### Identification

Using the identification scheme of Shewan, et al. (1960), the organism was identified as a Vibrio sp. However, when plate cultures

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\*'Slangkop' fixed station (No. 52), Department of Oceanography, University of Cape Town.

were examined in the dark, as part of a routine procedure, the colonies were found to be faintly luminous. Subsequent culture in aerated liquid medium (nutrient broth sea-water, NBS) showed the bacterium to be highly luminescent. The cultural and biochemical characteristics of the organism coincide most nearly with those of Photobacterium fischeri. However Spencer (1955) is of the opinion that P. fischeri ought to be placed in the genus Vibrio. Since the rods of the study organism have been shown to be slightly curved on solid medium the author is in agreement with this opinion. However, at present the organism must be classified as Photobacterium sp.

#### Description of the organism

The organism is a thick rod 0.5 - 0.7 by 1.0 - 2.5  $\mu\text{m}$  in size, with rounded ends and occurring singly or in pairs. The rods may be slightly curved. A capsule is visible under phase contrast. The rods are motile by means of a single polar flagellum and they are Gram negative.

Colonies on sea-water agar (NAS) are 1.0 - 1.5 mm in diameter after 24 hours; they are circular, raised, smooth, greyish-white and shiny. Colonies on 'Kings' medium are luminescent but produce no pigment. Growth on MacConkeys medium occurs at 20 C but is poor. The optimum growth temperature on NAS or NBS is 25 C, the minimum is 9 C and no growth occurs at 37 C. Sodium chloride is required and the organism will not grow unless the sea-water concentration is greater than 25%  $\text{v/v}$ . The best growth occurs at sea-water concentrations of 75%  $\text{v/v}$  or more. The organism is fermentative in Mitchell's glucose medium, producing acid only, and it is Kovacs' oxidase positive. Indole is not produced. Plate tests show the organism to be sensitive to pteridine

compound 0/129, streptomycin, tetracycline and chloramphenicol; it is not sensitive to penicillin. The GC ratio is 45.1%, ( $\pm 3\%$ ). Under conditions of active growth in young luminescent cultures, the cells become highly pleomorphic.

## B. GROWTH CONDITIONS

### (1) Cells for electron microscopy

The optimum growth temperature was investigated by growing the organism in tubes of 75% sea-water nutrient broth (NBS). The tubes were incubated in a temperature gradient device (see Appendix C). The gradient was linear between 38 and 6.4 C with intervals of 2.6 C. Temperature variation was less than 0.5 C for the duration of the growth period of 18 hours. The optimum growth temperature was found to be between 23 and 26 C.

The optimum sea-water concentration for growth on solid medium was determined in the following way. The organism was grown on nutrient agar plates containing five different concentrations of sea-water (0%, 25%, 50%, 75% and 100%). The plates were incubated at 25 C. No growth occurred on the 0% plates and the best growth took place on the 75% sea-water medium. Growth on nutrient agar made with five different concentrations of sodium chloride (0‰, 8.7‰, 17.5‰, 26.2‰ and 35‰) in tap-water gave the same result. A 75% sea-water medium (NAS) was, therefore, used to grow the cells for electron microscopy.

The optimum sea-water concentration of liquid medium was determined in the following way. The organism was grown in nutrient broth made with five different concentrations of sea-water (0%, 25%, 50%, 75% and 100%). The flasks were incubated at 25 C on a rotary shaking machine. No growth took place in the medium without sea-water and the best growth and light output occurred in the medium containing 75% sea-water. This medium, NBS, was therefore used to grow luminescent cells for electron microscopy.

A number of 1 litre flasks containing 500 ml of NBS were inoculated with a few drops of an overnight culture in the same medium. The flasks were incubated at 25 C on a rotary shaking machine. At intervals the light output was measured. For this purpose the photo-cell and amplifier from a Zeiss photomicrographic exposure meter was used. The photo-cell was placed directly against the bottom of the culture flask. Maximum deflection (light output) was attained after 20-22 hours incubation.

## (2). Cells for physiological investigation

Six one litre culture flasks fitted with bubbler tubes and containing 500 ml amounts of NBS were inoculated with a few drops of an unshaken overnight NBS culture of the organism. Duplicate flasks were incubated at 23 C for 3 days under different conditions: (i) with continuous aeration, (ii) with nitrogen bubbled through the medium, and (iii) with no aeration. The gas used was of medical quality and free of carbon dioxide. Cells were harvested by centrifugation, washed twice with 0.2 M phosphate buffer pH 7.2 and resuspended in the same buffer. The optical density of the suspensions was adjusted to 0.360 in a

Beckman DB spectrophotometer.

## C. METHODS FOR ELECTRON MICROSCOPY

### Fixation and embedding

Preparation methods and formulae for all materials mentioned in this section will be found in Appendix B.

Cells were removed from the surface of agar plates by washing with cold (4 C) 0.2 M cacodylate buffer at pH 7.0, after which the cells were washed twice more in fresh buffer. Cells from liquid culture were removed by centrifugation and washed three times in the same buffer. The washed cells were fixed in the cold in 4% glutaraldehyde in cacodylate buffer at pH 7.0 for two hours. After two 15 minute changes in fresh cold fixative the cells were washed in three changes of buffer. Post-fixation was then carried out in 1% osmium tetroxide. The cells, suspended in about 4 ml of cold cacodylate buffer were squirted into an equal volume of cold 2% osmium tetroxide with a Pasteur pipette. The tip of the pipette was always kept below the surface of the osmium tetroxide solution during this process. After two hours in the cold, the suspension was washed three times in fresh cold buffer, centrifuged at low speed, and the pellet dehydrated in 30%, 50%, 70% 96% and three changes of absolute ethanol at room temperature; the cells were kept in each ethanol concentration for 30 minutes. They were then treated in two changes of propylene oxide for 15 minutes each, after which a 1:1 mixture of propylene oxide and the embedding medium was pipetted onto the deposit of cells. The tube was

allowed to stand overnight at room temperature. When the propylene oxide had evaporated the deposit was embedded in Epon in 'Beems' capsules, gelatin capsules or flat 2 cm polythene lids. Polymerisation was standard, 24 hours each at 37 C and 45 C, and 3 days or more at 60 C. The flat embedded material was cut from the block with a coping saw and mounted on 5 mm 'Perspex' pegs with either 'Araldite' adhesive or embedding Epon.

### Sectioning

The blocks were prepared on an LKB pyramitome and sections were cut on a Reichert UM2 ultramicrotome, modified to accept the LKB orientation head. Sections between 60.0 and 80.0 nm were cut onto water, with a glass knife, and were picked up, from the edge, directly onto 400 mesh copper grids. The sections were not flattened with chloroform or other solvent. It was not necessary to support the sections on formvar or carbon films, and the sections were stable under the beam.

### Staining

The sections were stained by floating the grids, section down, on small drops of stain on a sheet of dental wax. The staining procedure used was as follows:

1% Uranyl acetate	20 mins.
Running water wash	10 secs.
Reynold's stain	7 mins.
Distilled water wash	2 mins.

Before use, the staining solutions were filtered to remove any deposit. After washing, the grids were dried at 37 C and stored in LKB grid boxes.

#### Examination of sections

Observations on the ultrastructure of the cells were made on a Philips EM 300 electron microscope. The objective aperture used was 50  $\mu\text{m}$  in diameter and the microscope was used in the double condenser mode. The microscope cold-finger device was used during all observations.

Photographs of the sections were made on Kodak 35 mm Release Positive Film at 60 or 80 KV. The films were developed in Agfa Rodinal 1:25 dilution at 20 C for 15 minutes. Prints were made on Agfa glossy single weight paper of a suitable grade at an optical magnification of 7 or 10. In some cases (Plates 45 and 47) the contrast range of the negative was too great to allow a print showing detail in all parts to be made. These negatives were therefore printed through density masks made on Kodak Photo-mechanical film (20.3 x 25.4 cm) which was developed to low gamma and laid over the enlarging paper when the exposure was made. The enlarger used was a 'Leitz Focomat' 35 mm with lamp housing modified and fitted with a low voltage point source lamp. The prints were developed in 'Agfa' neutol.

#### D. PHYSIOLOGICAL METHODS

A series of dilutions of each cell suspension from aerated, nitrogenated, and unaerated cultures were made in 0.2 M phosphate buffer

pH 7.2. This was done by adding cell suspension volumes of 2.0, 1.0, 0.75, 0.50 and 0.25 ml to the manometer flasks of a Warburg apparatus and making up the volume to 2.5 ml with buffer. To the side arm of each flask, 0.5 ml of 30 volume % stabilized  $\text{H}_2\text{O}_2$  solution was added, the flasks were fixed to the manometers and after equilibration at 25 C they were tipped and the rate of  $\text{O}_2$  evolution was measured.

## CHAPTER 3

### OBSERVATIONS

#### A. CELLS FROM SOLID MEDIUM

##### (i) General features of the cell

When slides of cells from NAS plates were examined in the light microscope, the cells appeared as normal rods. The size range was normal (0.5 - 0.7 by 1.0 - 2.5  $\mu\text{m}$ ). Both Gram-stained slides and living cells from in the culture medium were examined. Under phase-contrast the cells appeared to be encapsulated and motile. Polar flagella were observed under dark-field illumination.

Typical sections of Epon-embedded cells are shown in Plates 1 and 2. The outer envelope, or cell wall, does not appear to differ significantly from the inner cytoplasmic membrane. Both can be resolved into three-layered structures which are typical of unit biological membranes (Robertson, 1959). The overall thickness of each is between 7.0 and 8.0 nm. There is an electron transparent zone of about 10.0 nm between the wall and the cytoplasmic membrane of these cells.

The cytoplasm of sectioned cells appears densely granulated under moderate magnification due to the presence of ribosomes except in

the area of the DNA-plasm. Individual ribosomes are difficult to distinguish due to the densely packed nature of the cytoplasm. Higher magnifications show the ribosomes to be roughly spherical bodies of about 15·0 nm in diameter.

The DNA-plasm appears normal for the method of fixation employed in that densely stained coagulates of DNA on a clear electron transparent area free of ribosomes can be seen. The DNA-plasm is normally centrally situated and clearly delineated from the rest of the cytoplasm.

No mesosomes or other intracytoplasmic membrane systems could be seen in these cells at any time. Cell division takes place by peripheral constriction of the rod, as is common in many Gram-negative bacteria (Plate 6).

#### (ii) Flagellation

The single polar flagellum, 18·0 - 24·0 nm in diameter, does not seem to be hollow, but appears to be composed of an outer sheath of protein sub-units surrounding a central core. The core gives the appearance of being composed of a number of fibrils arranged cylindrically within the sheath. Such flagella are much thinner than the ensheathed flagella of Photobacterium harveyi described by Hodgkiss and Shewan (1968).

P. harveyi, however, bears numerous fine lateral flagella as well. In Plate 5 the flagellum can be seen to originate within the cytoplasm and pass through the cytoplasmic membrane and the cell wall. Protein sub-units are visible in micrographs of flagella lying horizontally within the

sections. An area free of ribosomes at the place where the flagellum enters the cytoplasm can be seen in Plates 3, 5 and 6; this has been a routine observation.

## B. CELLS FROM LIQUID MEDIUM

### 1. General features of the cells

Cells from actively growing luminescent NBS cultures of various ages were suspended in a few drops of culture medium and examined by phase-contrast microscopy. The cells from all such cultures were abnormal. Most of them were spherical, ranging in size from 1.5 - 6  $\mu\text{m}$  in diameter. A few oval and one or two elongated cells, but very few normal cells were seen. Finger-like processes were observed on many of the cells. This was the case in all the young actively growing luminescent cultures grown with continuous shaking.

Cells from these liquid cultures were streaked onto NAS plates and incubated for 18 hours at 25 C; the resulting growth was normal in all cases. This procedure was repeated many times with a number of liquid cultures. In all cases, when cells from these cultures were streaked onto solid medium, only normal, rod-shaped cells occurred.

A number of shaken liquid cultures were left to stand on the

bench (21 C) for 6 days. When examined in the light microscope it was found that a large number of normal cells were present. The procedure was repeated and 4 shaken liquid cultures (18 hours old) containing only abnormal cells were incubated at 24 C without shaking for 10 days. When the cultures were examined most of the cells observed were normal bacilli.

Brown, Drummond and North (1961) used glycine to produce spheroplasts of a marine pseudomonad. Using this method it was found that normal cells could be converted to spheroplasts by incubation in glycine plus sea-water. Large inoculations of cells grown on NAS were made into 50 ml amounts of 0.5% glycine in sea-water. The flasks were incubated at 25 C for three days without shaking. The cultures were not luminescent. These glycine spheroplasts were very similar to the spherical cells of luminescent cultures in NBS although they differed in certain respects which will be discussed later.

Typical sections of Epon-embedded, liquid-grown, luminescent cells are shown in Plates 7-10. The cells are all more or less spherical and most of them are surrounded by an outer wall and an inner cytoplasmic membrane. The DNA-plasm of these cells is more diffuse than in the normal cell. Many cells appear nearly empty and are clearly undergoing autolysis. The range in size is much greater than can be observed in the light microscope. Many small bodies which, however, cannot be described as cells, some less than 0.2  $\mu\text{m}$  in diameter, are present. Vesicular, finger-like processes or blebs can be seen on many of the cells. Flagella are absent and living cells are not motile. In some cells, structures which appear to be parts, or short lengths of flagella, can be seen within the cytoplasm.

## 2. Extracellular structures

### (i) The cell wall

The walls of the cells from luminescent cultures do not appear different from those of normal cells in section; they retain the appearance and dimensions of unit membranes. The walls do, however, appear to lack the rigidity necessary to maintain the rod-shaped morphology and the volume enclosed by the wall is much greater than normal. Sometimes this increase in size is accompanied by an increase in size of the protoplast volume as well, but often the protoplast is smaller in volume than the space enclosed by the wall, which results in a considerable amount of space between the wall and the protoplast. Sometimes only a small area of the protoplast membrane appears to be in contact with the inner surface of the cell wall. The large difference in surface area between the wall and the protoplast is connected with another kind of abnormality, the blebs.

### (ii) Blebs

Sometimes contact between the wall and protoplast membrane is retained over large areas, as shown in Plate 11. Instead of the wall being a large, loose, bag-like structure, many such cells have pseudopodium-like outgrowths, or vesicular blebs, on them. These blebs appear to start as small bulges in the wall which become increasingly large with the wall constricting behind a vesicular bulge to form a long tubular neck with a balloon-like sac at the end. Such structures are shown in Plates 11 - 17. One such cell, viewed under phase-contrast, appeared to have at least ten blebs attached to it. Blebs most often appear to contain

the same granular electron-opaque material which is present between the cell wall and the protoplast (Plate 13). Sometimes blebs contain small, membrane-bound inclusions as seen in Plate 18; this phenomenon will be discussed in the section on the cytoplasmic membrane.

Large flattened folds were observed on the walls of some cells and were often of considerable size. Plate 13 shows a section of a cell which has this feature. It is possible that some of the blebs seen on cells were in fact similar folds of the cell wall which had been cut transversely. However serial sections did not confirm this and living cells were observed to have small spherical structures attached to them by means of thread-like connections when viewed in the phase contrast microscope.

In some cells, large blebs which give the cell an amoeboid appearance are produced (Plate 9). Large blebs like this usually involve the cytoplasmic membrane as well as the wall and thus differ from the small blebs. This process could be responsible for the small cells seen in many of the preparations (Plates 8 and 9). Such small cells can be distinguished from free protoplasts since they are surrounded by a wall and a cytoplasmic membrane.

### (iii) Wall fragments

Another peculiar feature observed in electron-micrographs of sections of spherical cells from NBS culture is shown in Plates 19-23. A progressive disintegration of the wall is accompanied by a rolling-up of the edges to form the spiral figures shown in these electron-micrographs.

This rolling-up of the edge may take place outwards (Plates 19 and 20) or inwards towards the protoplast (Plates 21 and 22). In Plate 24 one of these rolls of cell wall is shown cut partially in a longitudinal direction and it would appear that considerable lengths of wall are involved. It is suggested that this is a tension phenomenon and the direction in which rolling occurs is determined by the relative tensions in the outer and inner layers of the wall.

Sometimes, when the rolling takes place in an inwards direction, some of the small cytoplasmic elements or spherules which lie between the wall and the cytoplasmic membrane may become rolled up within the wall fragment. Such inclusions are not uncommon and several may be seen in Plate 24. The same phenomena are observed in sections of glycine spheroplasts (Plate 25). Sometimes the wall fragments roll up against and within one another to form complex figures, as seen in Plate 26. The walls of lysing cells and of those releasing what appear to be viable protoplasts do the same thing.

### 3. Tetrad-like bodies

A large number of sections were cut of Epon-embedded cells from luminescent cultures in NBS. One of the most striking observations made when these sections were examined in the electron microscope was the constant presence of apparent dividing cells. Cells in which the protoplast had divided into a number of parts, usually four, were present in all sections examined. A number of such cells are shown in Plates 7-10. It can be seen from the plates that the geometrical arrangement of the components is tetrahedral and is consistent with such structures found in

other organisms. Each protoplast of the tetrad shown in Plate 27 is completely surrounded by a single membrane. Naturally, only three components are visible in most cases, since a section in which all four components of a tetrahedron are visible is improbable. Plate 28 and the accompanying diagram shows a tetrad in which all four components are partly visible, although the membrane separating two of the protoplasts has been cut at an angle which makes it difficult to see. Plates 29 and 30 show sections of tetrads cut on different planes. Upon disintegration or rupture of the wall, it appears that the protoplasts are released.

#### 4. Intracellular structures

##### (i) The cytoplasmic membrane

Plate 31 shows an electron micrograph at high magnification (264,000 X) of the membrane of a protoplast. The overall width (approximately 7.0 nm) and appearance of the membrane is normal. The cytoplasmic membrane is responsible for the division of the cytoplasm of certain cells into tetrad units. These divisions, however, are not always symmetrical and sometimes result in protoplasts of unequal size. See Plates 32 and 33.

The cytoplasmic membrane is also responsible for the production of two other interesting structures, membrane-bound vesicles within the protoplasm itself, and small cytoplasmic elements or spherules which are found in the space between the cytoplasmic membrane and the cell wall.

Plates 34 and 35 show cells in which both of these features are present.

(ii) Invaginations of the cytoplasmic membrane

The intercytoplasmic vesicles and the small cytoplasmic spherules seem to arise from invagination and folding of the membrane at the periphery of the protoplast. Various stages of these processes are shown in Plates 36-40. In many of these large cells the DNA-plasm is very diffuse and coagulates of the material are to be seen all over the cells. Many of the cytoplasmic spherules appear to contain material indistinguishable from DNA-plasm. Ribosomes too are often observed in them. Dense accumulations of ribosomes are sometimes seen in the areas where these bodies arise (Plates 35 and 36). Small cytoplasmic spherules can be included in blebs of the cell wall as shown in Plate 18. They may also become rolled up in cell wall fragments (Plates 24 and 25) but more often are merely released from the cell when the wall fragments. (Plates 41-43). Plates 42 and 43 are electron micrographs of sections of glycine spheroplasts.

(iii) Multiple membranes

Spherical cells from luminescent cultures often accumulate multiple intracytoplasmic membranes in association with the cytoplasmic membrane (Williams, 1969). These structures consist of vesicular or whorled laminated structures which arise in close proximity to the cytoplasmic membrane. In fact they often appear to be part of the cytoplasmic membrane (Plate 44). In some cells, concentric (vesicular) or parallel (whorled) layers of dark and light bands occur. The dark bands

appear wider (4.0 - 4.5 nm) than the dark bands of the cytoplasmic membrane (3.0 nm). Plate 44 shows a section of a cell with a considerable amount of multiple cytoplasmic membrane and Plate 45 shows a section of a cell with a whorled multiple membrane. A vesicular type of multiple membrane can be seen in Plate 46. Plate 37 shows a feature which is believed to be an early stage in the assembly of multiple membrane of the vesicular type. Weigand et al. (1970) found that a temperature sensitive strain of Escherichia coli (strain 0111a<sub>1</sub>) accumulated "extra membranes" when grown at 40 C. They found that such membranes appeared as vesicles or whorls depending upon the time of growth at 40 C. The whorl-type of membrane is indistinguishable from the "multiple membranes" found in cells of the study organism. Using a microdensitometer on electron-micrograph plates, Weigand et al. found apparent centre-to-centre distances between dark bands of 7.1 - 8.8 nm. Measurement made on prints of the study organism show the dark bands to be 7.0 - 7.5 nm apart (centre-to-centre). The dimensions of multiple membranes suggest that the laminations could consist of stacks of unit membranes with the adjacent electron-dense bands fused.

The vesicular type of "extra membrane" described by Weigand et al. which is observed early during growth of the culture at 40 C is also found in cells of the study organism. These structures are composed of a vesicle bounded by a single layer of unit membrane. However these vesicles can be converted to multiple structures and Plate 47 shows a number of stages of the process.

The multiple membranes are therefore of two types: (i) those in close association with the original cytoplasmic membrane (Plate 44), in

which the membrane itself appears to have become multi-layered and (ii) those which arise from vesicular structures (Plates 44-47) and which may be found in situations removed from the original membrane.

(iv) Ribosomes

Since the appearance of ribosomes is neither uniform nor constant, it is not possible to distinguish different sorts and sizes of particles which might correspond to ribosomal particles of different sedimentation rates in thin sections of normal cells. The spheroplasts of the study organism possess protoplasm which is much less dense, and ribosome-like particles of a number of different sizes can be observed in thin sections. These particles can be seen in many of the plates but are very clear in Plates 35 and 36. In Plate 36, some with an apparent diameter of 16.0 nm, 25.0 nm and 30.0 nm are seen. A number of particles of 18.0 nm, a few about 25.0 nm and one or two 30.0 nm in diameter can be seen in the sections on Plate 35. It is not suggested that these figures represent the true diameter of these particles, since the fixation method could increase or decrease the apparent diameter of ribosomes. Only densely stained particles were measured as these were more likely to be completely within the section and not cut by the microtome knife in sectioning.

C. GLYCINE SPHEROPLASTS - GENERAL OBSERVATIONS

Examination of Epon-embedded sections of glycine spheroplasts showed that these cells were in many ways similar in appearance to cells

from luminescent NBS cultures. Under the light microscope the living cells, suspended in glycine/sea-water, appear completely spherical. Glycine spheroplasts produce large bud-like blebs and also small vesicular ones, as can be seen in Plates 48 and 49. Plate 43 shows small cytoplasmic spherules produced by these cells, but no invaginations of the cytoplasmic membrane were seen in the preparations.

While the cytoplasm of such spheroplasts was often highly vacuolated (Plates 48-50) the vacuoles did not appear to be bounded by a membrane as in the case of luminescent cells from NBS. As a rule the cytoplasm of the glycine spheroplasts contained many more vacuoles than were seen in preparations of luminescent cells from NBS. The cytoplasm of glycine spheroplasts was observed to be much denser than that of cells from luminescent liquid culture.

Wall fragments of glycine spheroplasts appeared curled up in a most pronounced way and inclusion of small particles was evident. Plate 50 shows a cell which is probably at an early stage of spheroplast formation, the cell appears swollen and there are a number of vacuoles in the cytoplasm. No luminescence was observed in these glycine cultures.

#### D. PHYSIOLOGICAL OBSERVATIONS

When aerated cultures, cultures grown under nitrogen, and unaerated cultures were examined it was found that the aerated cultures were luminescent, the nitrogen-grown ones were not, and the unaerated

cultures only luminesced when shaken. Examination by phase-contrast microscopy revealed that the nitrogen-grown cultures contained very few pleomorphic cells, most of them being normal rods, cells from the aerated luminescent cultures were all pleomorphic, while the unaerated cultures contained cells of both morphological types.

Since pleomorphism appeared to be connected in some way with aeration and luminescence, the involvement of a peroxide was suspected. The organism is catalase positive, but it was considered possible that under conditions existing in the cultures the enzyme could have been inactivated or inhibited in some way. Another possibility was that the amount of enzyme present in the cells was low for some reason. An experiment was therefore carried out to measure relative catalase activity in cells grown under different conditions of aeration. Catalase activity as measured by oxygen evolved from hydrogen peroxide in a Warburg apparatus at 25 C was found to be highest in nitrogen grown cells. The table overleaf shows the rate of oxygen evolution for dilutions of cells from each type of culture.

An attempt was also made to detect peroxide using peroxidase, which has a greater affinity for peroxides than catalase. When peroxidase (with benzidine as H acceptor) was added to suspensions of cells in the Warburg apparatus, small amounts of oxygen were released in the case of luminescent cells from aerated culture which had been harvested a short time before. When the experiment was repeated, no oxygen was evolved and the results must be regarded as inconclusive.

Catalase activity of cell suspensions

Ml cell suspension*	Cells from N <sub>2</sub> culture	Cells from aerated culture	Cells from unaerated culture
	μL/O <sub>2</sub> /min**	μL/O <sub>2</sub> /min	μL/O <sub>2</sub> /min
2.0	-	18.0	51.0
1.0	39.7	8.1	27.0
0.75	28.5	5.8	21.5
0.50	18.7	2.6	18.1
0.25	9.7	2.0	7.2

\* Cells suspended in 0.2 M Phosphate buffer pH 7.2, made up to total volume of 2.5 ml.

\*\* O<sub>2</sub> released from 0.5 ml 30 volume % H<sub>2</sub>O<sub>2</sub>.

Dropwise additions of 30 volume hydrogen peroxide were made to suspensions of normal cells in NBS at intervals of a few hours. Within twelve hours, numbers of cells, morphologically similar to those from aerated liquid medium were observed in the suspensions by light microscopy. The cells used were grown on NAS plates and suspended in NBS in screw-cap bottles which were filled up to the top. Control

cultures showed no change. It was not possible to measure the amount of hydrogen peroxide added accurately since each addition resulted in the evolution of oxygen. However, a total volume of about 3.5 ml was added to the culture (30 ml) in which the most significant changes took place.

structure formed from three helically wound strands of identical sub-units. Alternatively the same hollow structure can be formed from five parallel strands of sub-units. No provision is made in these models for any internal structure nor has one been proposed.

Abram et al., (1965) have shown that the flagella of Proteus vulgaris arise from a basal granule attached to the cytoplasmic membrane. This does not seem to be the case in the organism studied. The flagella appear to arise well within the cytoplasm and not from the membrane.

The cells from luminescent liquid cultures are clearly L-forms and have a remarkable similarity to the L-forms of many other bacteria. Dienes (1968) is of the opinion that bacteria with defective cell walls may grow either as pleomorphic bacterial cultures or as L-forms. The size and shape of the cells is altered in pleomorphic cultures and large bodies may be produced, but the mode of multiplication is unchanged. L-form cultures on the other hand contain no regular bacterial forms and the structure of the cells and the mode of multiplication is different. Dienes considers that the observation of elementary corpuscles in cultures of L-forms is particularly significant. In this respect there is close similarity between L-forms and the mycoplasmas. He makes a distinction between elementary corpuscles, small spherical particles ( $0.5 \mu\text{m}$  and up) and granules, which are spherical, elongated or irregular structures which range in size from that of the corpuscles to  $1 \mu\text{m}$ . The corpuscles and the granules are enclosed within unit membranes and contain ribosomes.

Cells from luminescent liquid cultures of the bacillus studied are quite different from those from solid medium. Liquid cultures contain

no normal rod-shaped cells and the mode of reproduction is by sub-division of the cytoplasm within a cell wall which is defective. Elementary particles (spherules) are also produced, and many contain DNA-plasm and ribosomes. The larger spherules may be reproductive structures. Weibull (1963) found that the minimal size of reproductive units for L-forms of Proteus sp., Staphylococcus sp. and Corynebacterium sp. was 0.6 - 1.0  $\mu\text{m}$ .

The cytoplasmic spherules observed in cells of the organism studied seem to arise from evagination or folding of the cytoplasmic membrane (Plates 34-36) and larger bodies (protoplasts) arise from unequal division of the cytoplasm brought about by ingrowth of the cytoplasmic membrane. One feature, which seems to be unique, is the occurrence of tetrads which are interpreted as being attempts by the cytoplasmic membrane to mediate normal divisions of the cytoplasm. It would seem that the internal divisions of the cytoplasm which form the tetrad units and larger spherical bodies are the means of reproduction of the L-form cultures. However, large bud-like blebs which are seen sometimes, and probably represent an early stage in L-form production, are quite likely to be reproductive as well. These large blebs probably result from turgor pressure on small areas of cell wall which have become defective.

The L-form cultures in liquid medium invariably revert to cultures of bacilli when transferred to solid medium and are therefore to be regarded as unstabilized forms (Dienes 1968). This author recognizes two types of L-form cells in Proteus, A and B. Type B cells retain both the cytoplasmic membrane and cell wall whereas the A cells are bounded by the cytoplasmic membrane only. Type A cells are therefore really protoplasts.

Furthermore type B cultures retain the ability to produce normal bacilli but type A cultures produce them only occasionally. Since cells of the organism studied are L-forms only under certain conditions of culture, the use of the descriptive term metabolic L-form is suggested.

The peculiarities observed in the walls of pleomorphic cells from liquid culture could result from deficiencies or malfunction of wall assembly enzymes and a consequent failure of the cell to terminate wall synthesis after the protoplast has stopped growing. The blebs and folds observed on such cells could be early stages of a progressive deterioration, reversible up to a certain stage only, which eventually results in a complete disruption of the wall. Very early in this process of degeneration the wall loses its ability to mediate normal division of the cell.

The cytoplasmic membrane also appears to be subject to a similar process of deterioration, but this seems to be more gradual than in the case of the cell wall. Thus the cytoplasmic membrane is capable of sub-dividing the cell contents without the participation of the wall and tetrads could result from such division processes. The smaller protoplasts and cytoplasmic spherules could be accounted for in the same way but probably result from divisions by membranes which have become considerably disorganised.

Glycine spheroplasts have certain features in common with the L-forms from liquid culture, notably blebs and the rolled-up wall fragments seen in many sections. More wall fragments are seen in preparations of glycine spheroplasts than in L-forms. This is probably indicative of an earlier degradation of the cell wall. While the presence

of glycine in the medium affects wall synthesis it does not seem to affect the cytoplasmic membrane much, since multiple membranes have not been observed in the cells. However the cytoplasm of glycine spheroplasts is denser than that of L-forms and if multiple membranes were present they might be difficult to see. The effect of glycine seems to be the production of spheroplasts only, and eventually results in removal of nearly all the wall material from the protoplast. Brown et al. (1962) found that the cell envelopes of glycine-modified Pseudomonas NCMB 845 contained no detectable diamino-pimelic acid (normal 0.4%) and were low in protein, 40% compared with 80% in normal bacilli.

An indirect effect of glycine is the production of vacuoles which have no boundary membranes, within the cytoplasm of the spheroplasts. This effect is noticeable in early stages of spheroplasting (Plate 50) as soon as the cell wall begins to lose its rigidity. This demixing process may be related to osmotic phenomena which are at least partly responsible for the observed increase in size. Cells of the organism stop dividing shortly after being placed in glycine sea-water and apart from an initial increase in size do not grow and no divisions of the cytoplasm have been observed. Cytoplasmic spherules are produced however, and it would seem that this is an inherent property of the organism which is common to all bacteria capable of becoming L-forms.

The process involved in the deterioration of cells in aerated liquid culture seem to have a much more extensive effect than glycine and many cells eventually reach a state of complete disorganisation which appears to be irreversible. However, there are always some cells with dense cytoplasm present and these are presumably responsible for the continued viability of such cultures.

The abnormal growth of the membranes of L-form cells seen in many sections seems to be the result of deranged metabolic processes. The most extensive multiplication of the membranes is seen in cells which appear to be senescent and undergoing lysis. It may be that such abnormalities exist in the membrane systems of other cells, but the dense nature of the cytoplasm makes the feature difficult to detect. Some cells with dense cytoplasm do appear to have a certain amount of multiple membrane (Plate 37) but this is thought to be an early stage of development.

Weigand et al. (1970) have shown that a strain of E. coli accumulated "extra membranes" when grown at 40 C. The membranes developed were very similar to those found in the organism studied. Both vesicles and whorls were developed but Weigand et al. were unable to show that vesicles developed into multiple whorled structures although they made the suggestion. In sections of the study organism (Plate 47) it can be clearly seen that vesicles derived from the cytoplasmic membrane, develop into multiple membranes and various stages of the process are visible. Much of the multiple membrane observed in L-form cells is associated with the cytoplasmic membrane itself and often shows continuity with it (Plate 52). Weigand et al. have proposed that the accumulation of "extra membrane" by E. coli is a result of failure of the cell to cease membrane synthesis after growth has stopped. A similar explanation is offered in the present case.

The walls of cells from aerated liquid cultures appear to be subject to a similar phenomenon and the blebs and folds seem to be the result of wall growth which outpaces the growth of the protoplast.

It is widely postulated that luminescence in bacteria is merely a mechanism for the disposal of oxygen (McElroy et al. 1953). When cells are grown in highly aerated liquid medium luminescence is intense and the incidence of L-forms is practically 100%. When grown in the same medium without aeration the incidence of L-forms is much lower. Cultures in medium through which nitrogen was bubbled contained very few L-forms, most of the cells were normal rods and the cultures were non-luminescent.

Hastings and Gibson (1963) have shown that the first step in the bio-luminescent system of P. fischeri involves the reduction of enzyme by flavin mononucleotide which is followed by the combination of reduced enzyme with molecular oxygen. The reaction scheme of Hastings et al. (1964), proposes that aldehyde then reacts with an hypothetical peroxide intermediate to form a complex which results, after a number of steps, in oxidized enzyme.

Under conditions of high oxygen concentration, such as existed in shaken and aerated cultures of the organism studied, it is possible that an accumulation of peroxide could occur. When cells from aerated and nitrogenated cultures were assayed for catalase activity it was found that nitrogen grown cells had six times (or more) the activity of cells from aerated culture. It is possible that the discrepancy in catalase activity between aerated and nitrogenated cells is due to enzyme inhibition in the case of the aerated cells. This could come about by product feedback ( $O_2$ ) onto the enzyme which results in inhibition. A possible result of this could be an accumulation of peroxide under the highly oxygenated conditions of the cultures which would result in metabolic disturbance of the cells.

The addition of hydrogen peroxide to suspensions of normal cells in unaerated medium had the effect of converting these cells to spheroplasts but also caused the lysis of considerable numbers of cells.

While it seems that a connection between aeration and pleomorphism in this organism has been established, further physiological investigation is indicated but this was considered to be beyond the scope of the present study.

APPENDIX A

Note: All media were sterilized by autoclaving at 1 atm. for 15 minutes.

Antibiotic discs

Antibiotic sensitivity was examined by the use of oxoid 'Multodiscs' (special order) having eight arms with antibiotics in the following concentrations.

Penicillin	P,	(1.5 units)	P+,	(5.0 units)
Streptomycin	S,	(10 µg)	S+,	(50 µg)
Tetracycline	TE,	(10 µg)	TE+,	(50 µg)
Chloramphenicol	C,	(10 µg)	C+,	(50 µg)

'Kings' medium (King et al., 1954).

'Oxoid' peptone (L 37)	20 g
Glycerol	10 g
K <sub>2</sub> SO <sub>4</sub>	10 g
MgCl <sub>2</sub>	1.4 g
'Oxoid' agar No. 3	20 g
Sea-water	750 ml
Distilled water to	1,000 ml

'Kovacs' oxidase reagent (Kovacs, 1956).

This is a 1% solution of Dimethylparaphenylenediamin base. The reagent is used on 5 cm filter papers upon which a loopful of growth from a plate culture is rubbed. A red to purple colour which eventually goes black is indicative of a positive reaction.

'Mitchells' sugar medium\*

'Oxoid tryptone	0.10%
" yeast extract	0.01%
Bromocresol purple	0.002%
'Ionagar' No. 2	0.15%
Sea-water	75.0%
Distilled water to	100%

The sugar (glucose), sterilized by filtration is added after the medium has been dispensed into tubes (to a depth of 7 cm) and autoclaved. The final sugar concentration should be 1%. The medium is inoculated while still liquid (45 C) with a young culture grown in NAS. A heavy inoculation is made and the medium is mixed well. Oxidative organisms produce acid first at the surface of the medium. Fermentative organisms produce acid at the bottom of the tube first. In the case of heterofermentative action gas bubbles are usually trapped in the medium. No wax seal is necessary and results are obtained in a few hours.

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\* A modification of Hugh and Leifson's medium, (Hugh and Leifson, 1953) by T. Mitchell of Torry Research Station, Aberdeen. (Personal communication).

Nutrient agar sea-water (NAS)

'Oxoid' nutrient agar	28 g
Sea-water	750 ml
Distilled water to	1,000 ml

Nutrient broth sea-water (NBS)

'Oxoid' nutrient broth	13 g
Sea-water	750 ml
Distilled water to	1,000 ml

Sea-water

Sea-water for culture media is collected and stored in the dark for at least 6 months before use. It is then autoclaved, filtered and stored in 2.5 L quantities (in the dark) until required for use.

Sensitivity to compound 0/129\* (Shewan and Hodgkiss, 1954).

The compound was applied as a sterile 0.1% solution in acetone to 'Whatmans' 6 mm AA paper discs, which had been sterilized by dry heat. A volume of 0.2 ml was applied to each disc which was

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\* 2:4-diamino-6:7-di-isopropyl-pteridine.

then dried at 37 C.

For the tests these and the antibiotic discs were applied aseptically to the surface of freshly seeded NAS plates. Zones of inhibition were observed after 18 hours' incubation.

Sloppy agar sea-water (SAS)

'Lab-lemco' beef extract	2 g
'Oxoid' yeast extract	1 g
'Oxoid' proteose peptone	3 g
'lonagar' No. 2	3 g
Sea-water	750 ml
Distilled water to	1,000 ml

This medium contains no fermentable carbohydrates and dispensed in 7 ml amounts in 15 ml screwcap bottles is used as a storage medium for stock cultures.

## APPENDIX B

Cacodylate buffer (Ledbetter and Porter, 1963., Sabatini et al., 1964.)

0.2 M Sodium cacodylate is adjusted to pH 7.0 with 0.2 M HCl.

### Epon

The embedding medium is made up as follows:

'LADD'	DDSA	240 ml
"	EPON 812	150 ml
"	MNA	30 ml
"	BDMA	8.2 ml

The ingredients are mixed for 1 hour by a motor driven glass screw in a 500 ml measuring cylinder; dispensed in 10 ml snap-cap vials and stored at -22 C. The complete embedding medium keeps for at least 12 months under these conditions.

Glutaraldehyde fixative (Sabatini et al., 1963)

25% Glutaraldehyde, neutralised with barium or sodium carbonate is added to 0.22 M cacodylate buffer pH 7.0 to give a final concentration of 4%.

Osmium tetroxide fixative (Claude, 1961).

0.5 g Osmium tetroxide in a clean washed ampoule is broken below the surface of 25 ml glass distilled water. Twenty four hours is allowed for complete solution. The solution is stored in an opaque bottle at -4 C.

'Perspex' rod

Perspex plastic material in the form of 6.25 mm diameter rods is manufactured by ICI plastics division. Short lengths (1.2 cm) are cut off with a parting-tool in a lathe and one end is chamfered at 45° leaving a 2.0 mm diameter face at one end.

'Reynolds' stain (Reynolds, 1963)

Pb(NO <sub>3</sub> ) <sub>2</sub>	6.65 g
Na <sub>3</sub> (C <sub>6</sub> H <sub>5</sub> O) <sub>2</sub> H <sub>2</sub> O	8.80 g
1N - NaOH	40 ml
Distilled water to	250 ml

The lead nitrate and sodium citrate are added to 120 ml distilled water in a 250 ml volumetric flask with constant shaking for 2 minutes. The solution is allowed to stand for 30 minutes with intermittent shaking. Then 40 ml of normal sodium hydroxide is added and the whole is made up to 250 ml. The solution is filtered before use. The pH is 12.0 ± 0.1.

APPENDIX C

## **A submerged membrane filter apparatus for microbiological sampling**

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### Abstract

The sampler is operated by hydrostatic pressure and consists of a stainless steel cylinder to which is attached a membrane filter holder. A glass inlet tube is broken by a messenger and a predetermined volume of water is filtered. Valves protect the membrane from flow-back and release the pressure as the sampler returns to the surface. The sampler works satisfactorily at 15 m and is strong enough to be used down to 6000 m. The filling rate is controlled by a jet behind the filter holder.

### Introduction

The use of the membrane filter in marine bacteriology is well established and the method has been used by OPPENHEIMER (1952), KRISS (1963) and many others. Bacteria collected on membranes may be examined directly and counted or they may be cultured. The quantitative aseptic collection of bacteria from a predetermined volume of water directly upon a filter membrane has advantages. There is no reduction in numbers through adsorption of cells to the walls of rubber, glass or plastic containers or due to the bactericidal effects of metals. The handling of samples is kept to a minimum and this reduces the risks of contamination or inaccuracies in rough weather. The opening mechanism is simple and reliable and is not subject to mechanical failure. The apparatus described has been devised to reduce the number of sources of error and the possibility of malfunction encountered in the use of samplers of more usual design.

### Description

The sampler body (Fig. 1 a) is made of 316 stainless steel and has an internal diameter of 56 mm. The cylinder is 603 mm long inside and has a capacity of 1520 ml. The walls and ends are 22 mm thick and the sampler vessel and valves will withstand 600 atmospheres (6000 m) without risk of deformation (factor of safety = 2). One end is bored to 32.5 mm and a modified Millipore XX45-047-00 high pressure membrane filter holder (Fig. 1 b) is fitted by means of 4  $\frac{3}{8}$ " whitworth stainless steel cap screws, (Fig. 2 a). The joint is sealed by a Viton A (2-30) "O" ring in a machined groove in the holder. Four  $\frac{1}{4}$ " whitworth cap screws (Fig. 2 b) keep the holder together when it is not on the sampler body. The outlet side of the filter holder is fitted with a check valve using a  $\frac{1}{4}$ " (5.8 mm) stainless steel ball and a light spring. This valve (Fig. 2 c) serves to protect the membrane from back pressure as the sampler returns to the surface. The

cap of this valve has a small outlet which serves as a jet to control the rate of filling; the cap is inter-



Fig. 1. Apparatus assembled ready for use. a: sampler body; b: high pressure membrane filter holder; c: gland; d: 7 mm heavy walled glass tube; e: knurled nut; f: inlet tube; g: latex pressure tubing; h: sliding plate

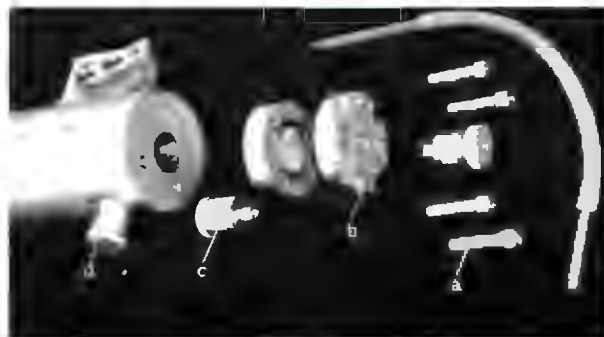


Fig. 2. Exploded view of filter vessel. a, b: cap screws; c, d: check valves

changeable. An identical valve on the outside of the sampler body (Fig. 2d) 20 mm from the top operates to release the pressure as the sampler comes up. A gland (Fig. 1c) is screwed into the inlet side of the filter holder. This gland has 2 small neoprene "O" rings which seal a short piece of 7 mm heavy walled pyrex glass tube (Fig. 1d) in place. There is a stainless steel spacer between the rings and the gland is tightened by means of a knurled nut (Fig. 1e). In the base of the gland there is a stainless steel mesh screen which holds back small pieces of glass which may enter when the inlet tube is broken. The inlet tube is 7 mm heavy walled pyrex glass (Fig. 1f), flame sealed at one end. It is joined to the tube in the gland by a short piece of latex pressure tubing (Fig. 1g). The sealed tube fits into a hole in a sliding plate (Fig. 1h) on the top clamp of the sampler body. The sampler has two clamps to fit 5 to 8 mm hydrographic wire.

### Assembly

The filter holder, inlet tubes and glands (assembled) and inlet valve are autoclaved separately wrapped in Kraft paper. Before use a sterile filter is placed in the holder and it is bolted together. Tape covering the outlet hole of the filter holder is removed and the inlet valve is screwed into place. The gland and valves are fitted with neoprene "O" rings and are screwed up tight against machined faces using a spanner. The gland is put on next, but the inlet tube is kept wrapped. This assembly is now bolted to the body. When the sampler is on the wire the top clamp and sliding plate are sprayed with ethanol and flamed; a handigas burner is used for this purpose. The inlet tube is put into the sliding plate using the paper to hold it. The latex tube is just long enough to be slightly stretched and holds the glass tube in place.

### Operation

The messenger strikes the sliding plate and the inlet tube is broken. The latex tube (which has been autoclave folded) has a permanent bend arranged to flip away from the wire and other parts of the sampler. Filtration will continue until the pressures are equalised. The volume filtered will depend upon the depth of water and a graph (Fig. 3) can be used to determine this. As the sampler is raised to the surface the inlet valve prevents any flow-back and contamination of the sample. The valve on the side operates and pressure is released as the sampler rises. The balls, valves and springs must not be allowed to remain wet after use. The valves are opened, washed in fresh water and dried, preferably in an oven. If the balls and seats of the valves become corroded the valves will leak and contamination of the sample may result. When the sampler has been brought to the surface the filter assembly is unbolted and another is secured in place. The filter is removed aseptically in the ship's laboratory after carefully drying the holder. If the membrane is

to be cultured it is usual to filter volumes smaller than 1500 ml, and this is achieved by adding measured amounts of water to the sampler before it is assembled.

This sampler is suitable for use as a series sampler by the attachment of a simple messenger release device to the sliding plate. Duplicate samples may be taken by the use of 2 samplers in tandem. In this case 1 glass inlet tube is passed through the sliding plate and the rubber tubes of the samplers are connected one to each end. This arrangement makes it possible to filter a small and a large volume at the same time.

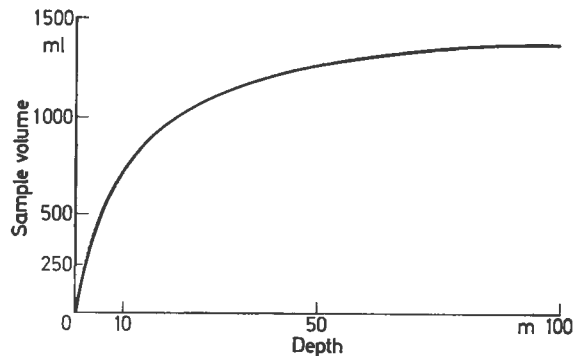


Fig. 3. Relation of sample volume to water depth

### Discussion

The entry speed of water into the vessel is controlled by a small hole in the cap of the inlet valve below the membrane filter. The size of this hole can be varied to suit the depth at which samples are to be collected. Since this jet is below the membrane, bacterial cells are not subjected to any great shearing force as the entry tubes can be quite large. Sudden decompression is also avoided with this arrangement because the pressure drop takes place as water enters the main vessel, after the cells have been removed. It is true that, if the inlet tube is empty when the sampler is opened, the first few millilitres entering are subject to a sudden pressure drop. This can be avoided by filling the filter apparatus and inlet tube with sterile sea-water. This also prevents a sudden rush of water which might damage the membrane.

If the sampler is used at depths greater than a few hundred metres a stainless steel inlet tube leading directly from the top of the filter holder is used in place of the rubber. This tube has a gland to hold a short sealed piece of hard glass tube which passes through the sliding plate.

For routine use, to collect samples down to 1000 m, much smaller vessels with thinner walls (12 mm) can be used. A useful volume is 500 ml.

### Summary

1. The ease of operation and reduction in the number of possible sources of error make this apparatus

particularly suitable for the collection of marine bacteria.

2. In South African waters, rough weather makes it very difficult, and often impossible, to filter or to handle water samples aboard.

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### Temperature gradient apparatus

The device consists of a 95 cm long bar of 'Duraluminium L 6' 12.70 cm high by 7.65 cm deep. Two rows of 12 mm diameter holes 2.5 cm apart were drilled into the top of the section down the length of the bar at a centre-to-centre distance of 7.65 cm. Between each pair of holes a smaller hole was drilled to accommodate the stem of a precision thermometer. The apparatus holds 11 duplicate pairs of 12 mm culture tubes. One end of the bar is heated by circulating light mineral oil pumped from a precision thermostat at 50 C through a stainless steel heat exchange tank bolted to the end of the bar. Cold ethylene glycol (-5 C) is circulated through a similar tank at the other end of the bar.

The apparatus was insulated on all sides and the ends by enclosing it in a 5 cm thick layer of foam insulation. The temperature gradient was linear between 38 and 6.4 C and was maintained to within 0.5 C for the duration of the experiments. The optical density of the cultures was measured in a Klett colorimeter.

## EXPERIMENTAL BIOLOGY GROUP : SUMMARIES OF SCIENTIFIC PAPERS

The following is an abstract of paper read at the 32nd Scientific Meeting of the Experimental Biology Group (EBG) which was held at the University of Cape Town Medical School, Observatory, Cape, on 29 August 1969:

### L FORMS OF A MARINE PHOTOBACTERIUM SPECIES

E. D. F. WILLIAMS, *Department of Microbiology, University of Cape Town*

The fine structure of a marine bacterium assigned to the genus *Photobacterium* has been studied. Cells were fixed in glutaraldehyde (2%), postfixed in osmium tetroxide (1%) and embedded in Epon 812. Sections were cut (600-750 Å) on a Reichert ultramicrotome and the mounted sections were stained in uranyl acetate and lead citrate. These were examined and photographed in a Philips EM 300 electron-microscope.

L forms predominate in old cultures (3-4 days) and in young actively luminescent cultures in 75% sea-water nutrient broth. In addition to the normal features found in Gram-negative bacteria the cells have many structural peculiarities. The diameter of the L bodies is 2-4 microns. Ingrowth of the cytoplasmic membrane on a single plane causes the cell contents to be divided into two. Further division results in the production of tetrads and these are a common feature of the cultures. Small intracytoplasmic elements occur in the cells which are believed to arise from invagination of the cytoplasmic membrane.

The cell wall is 3 layered and typical of Gram-negative bacteria but the walls of the cells have vesicular evaginations or blebs which often have small cytoplasmic elements inside them. These small bodies are surrounded by unit membranes and are similar to the intracytoplasmic bodies. Numbers of them are to be seen between the outer wall and the plasma

membrane from which they probably arise by extravasation of the cytoplasm. The extracytoplasmic elements frequently have dense contents and also less dense areas with fibrils similar in appearance to those of the nucleoplasm. Many of the small bodies are to be seen free in the sections, probably released by rupture of the cell wall. In this case they are enclosed in a single unit membrane. Some bodies are also seen which appear to be contained within a cell wall as well. This could be the result of a bleb containing one or more of these small bodies becoming detached from the cell.

The cytoplasmic membrane appears as two dense bands 25-30Å wide, separated by a light layer of about the same width. In some cells, however, concentric or parallel layers of dark and light bands occur. The dark bands appear wider (40-45Å) in this case and the laminations may consist of a number of stacked unit membranes with the adjacent protein layers fused. These laminated structures appear to arise from the plasma membrane and large numbers of ribosomes and polysomes occur in close proximity to the sites at which these structures appear. In these cells there is a complex 3-dimensional array of multiple membranes which is associated with the cytoplasmic membrane. The structures are often vesicular, sometimes appear as tubules or spheres and may consist of 10 or more alternate dense and light layers.

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PLATE 1

Typical field seen in section of Epon-embedded normal bacilli from solid medium (NAS). (Note the presence of some curved cells). Electron micrograph X 17,160.

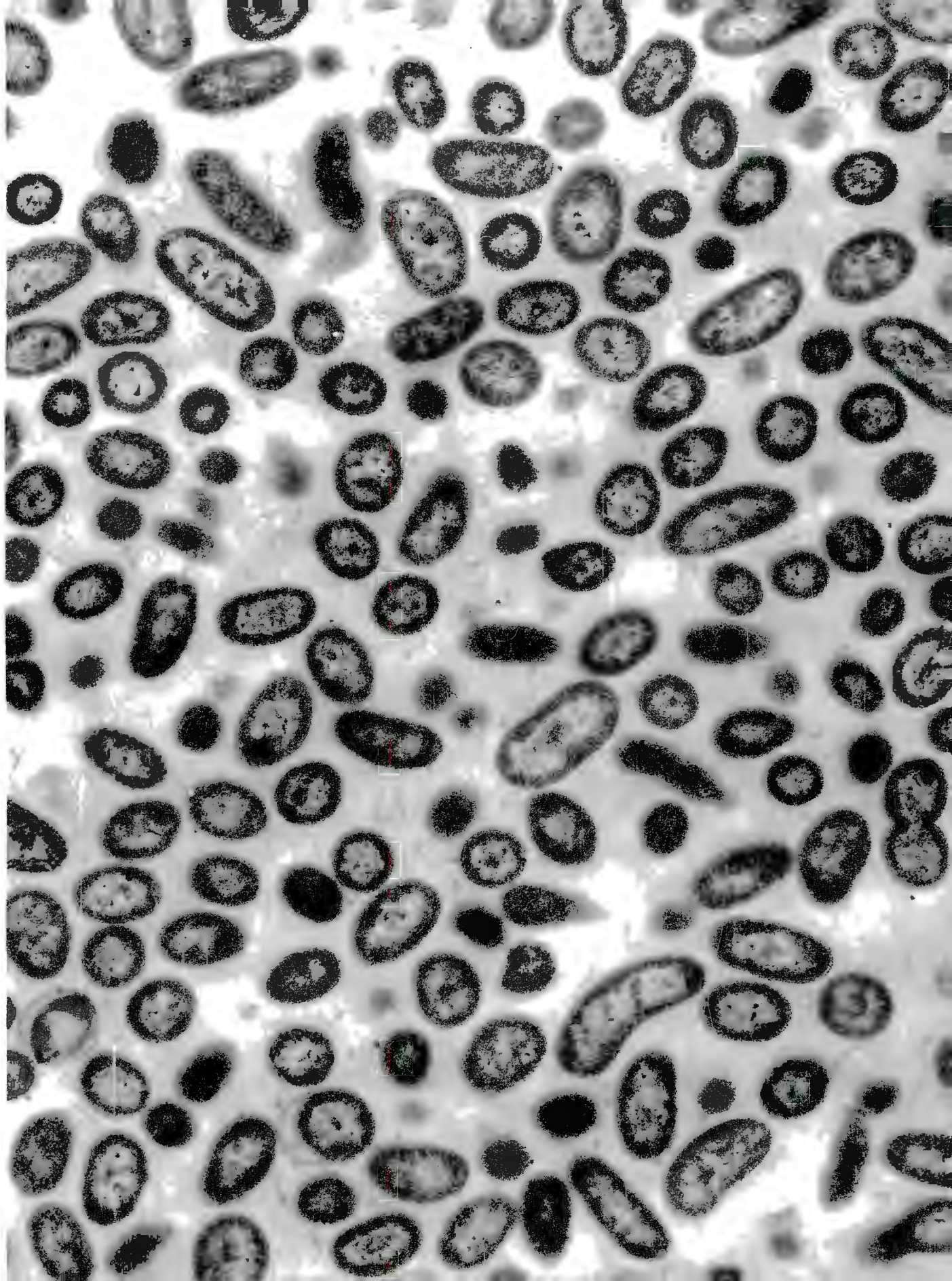


PLATE 2

Normal cells from solid medium. One early L-form cell (▷) is shown.  
Electron micrograph X 17,160.

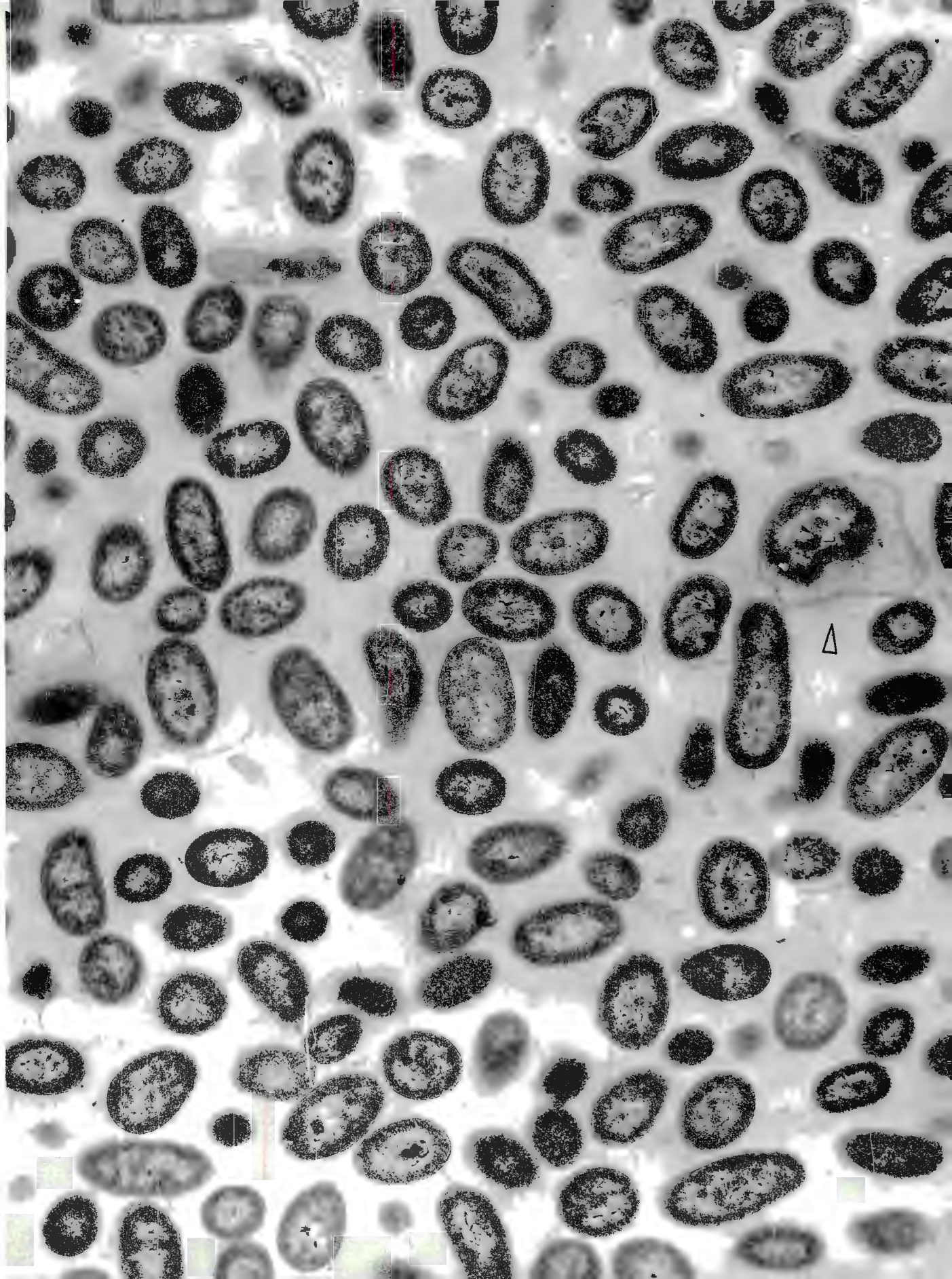
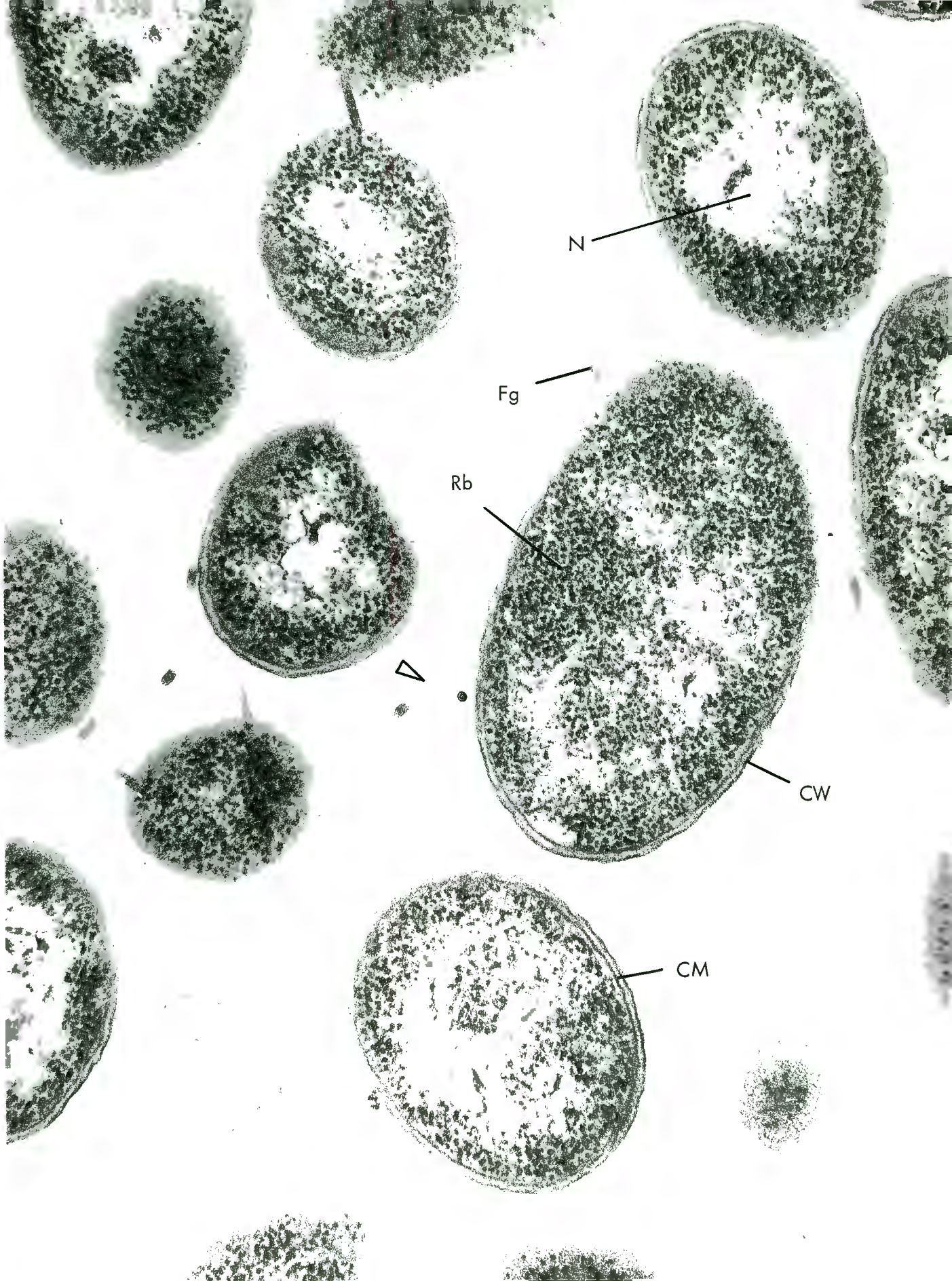


PLATE 3

Normal cells from solid medium. One flagellum ( $\blacktriangleright$ ) is cut in transverse section and shows presence of a central core surrounded by an outer sheath. Further optical magnification (10 X) can be used on the plate. Electron micrograph X 78,660.



N

Fg

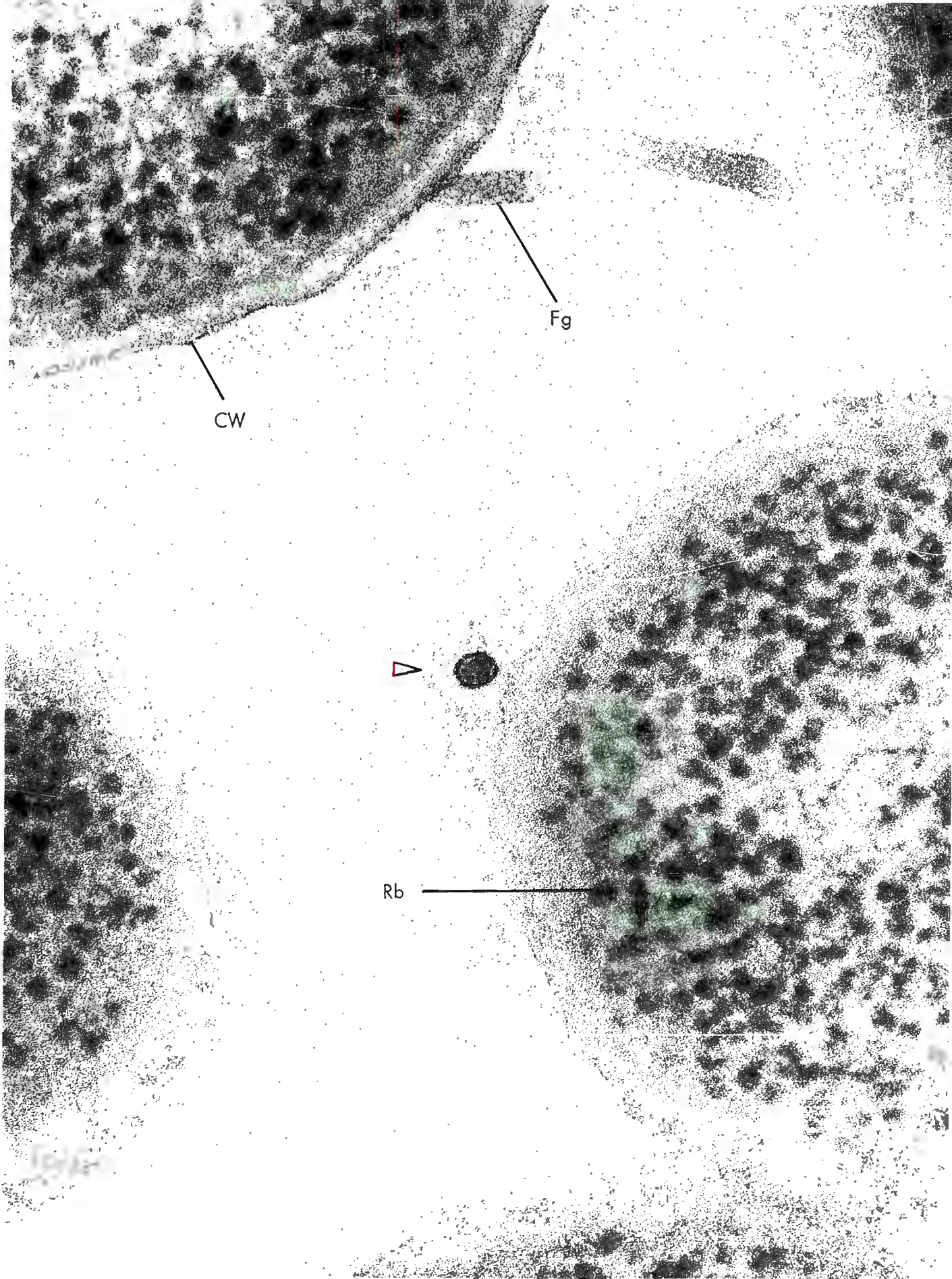
Rb

CW

CM

PLATE 4

Transverse section of flagellum (▷) at high magnification. The sheath is clearly shown. Small dense particles which may represent protein subunits possibly in a helical coil, appear to form a cylinder within the sheath. Electron micrograph X 323,330.



CW

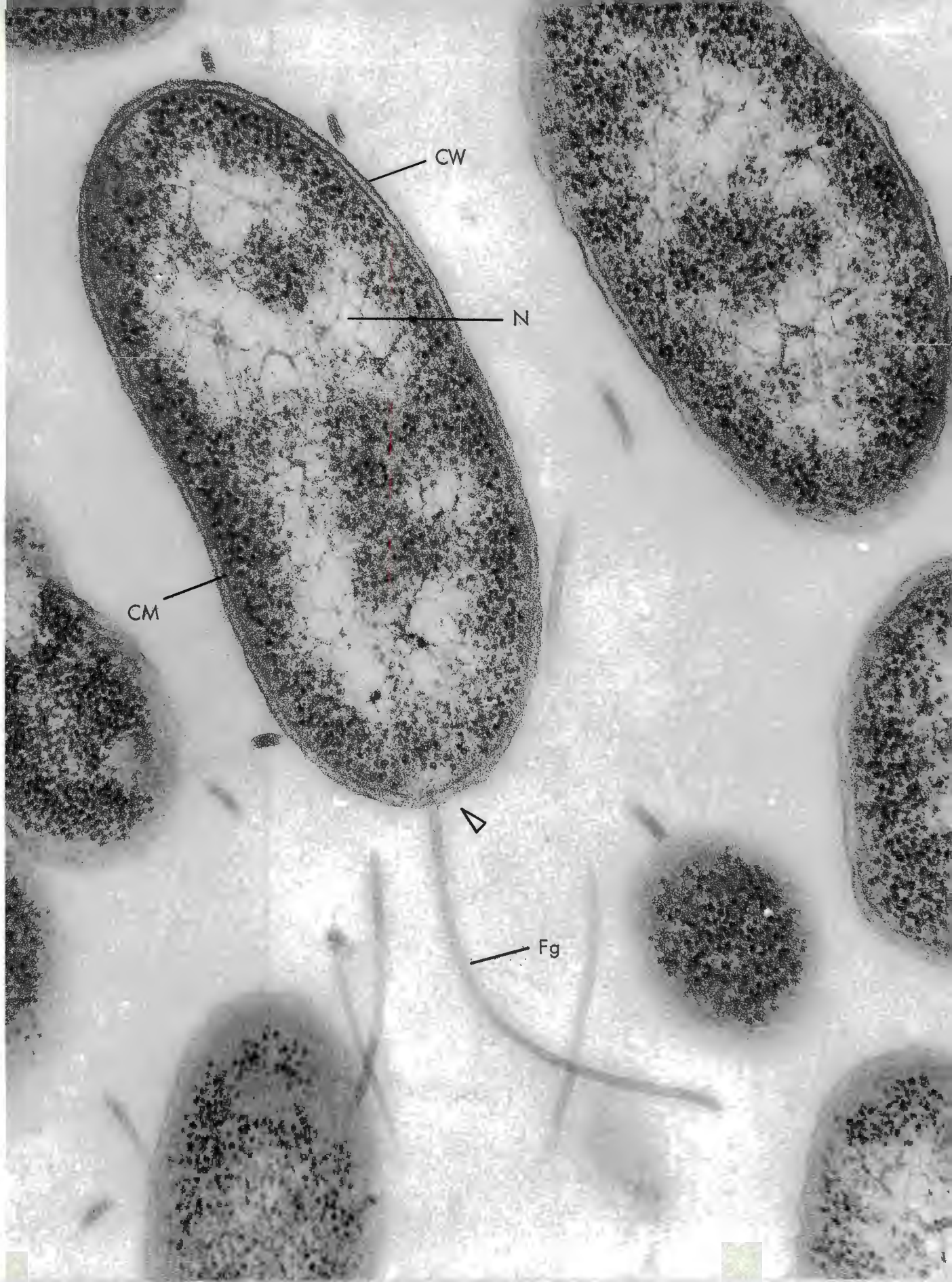
Fg



Rb

PLATE 5

Longitudinal section of normal cell showing attachment of the flagellum. The flagellum passes through the wall and ends in an area free of ribosomes (▷) at one pole of the cell. Sub-structure of both the sheath and core of the flagella is visible. Electron micrograph X 126,660.



CW

N

CM



Fg

PLATE 6

Dividing cell from solid medium. Division takes place by means of a peripheral constriction (▷) of the wall. Both daughter cells have flagella attached to them. Electron micrograph X 78,660.



PLATE 7

Typical section of Epon-embedded cells from luminescent liquid culture in NBS. Tetrads, spherical cells, and cells undergoing autolysis (▷) are indicated. Electron micrograph X 7,100.

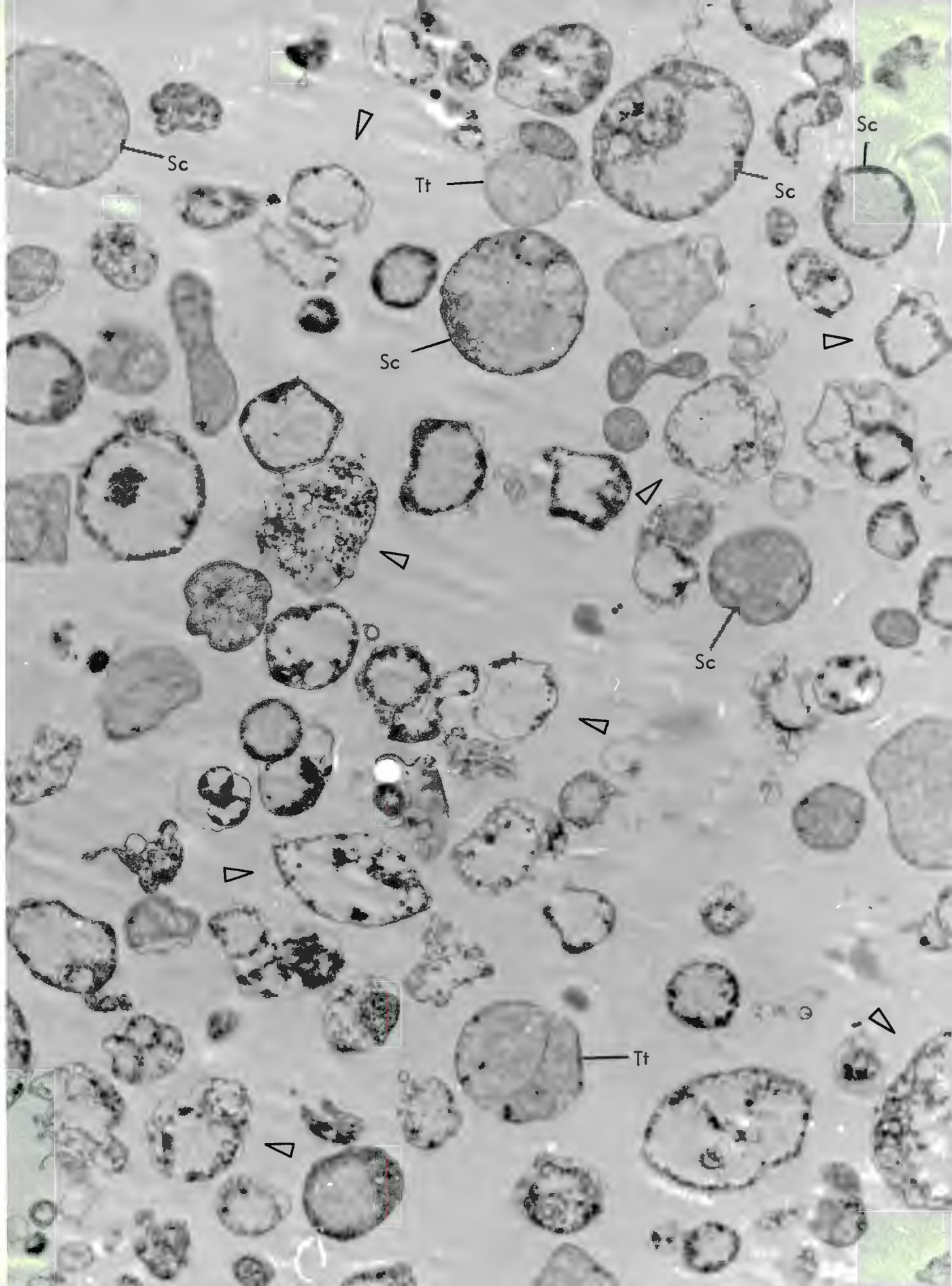


PLATE 8

Section of Epon-embedded cells from liquid culture in NBS. A number of cells have blebs (▷). Electron micrograph X 7,100.

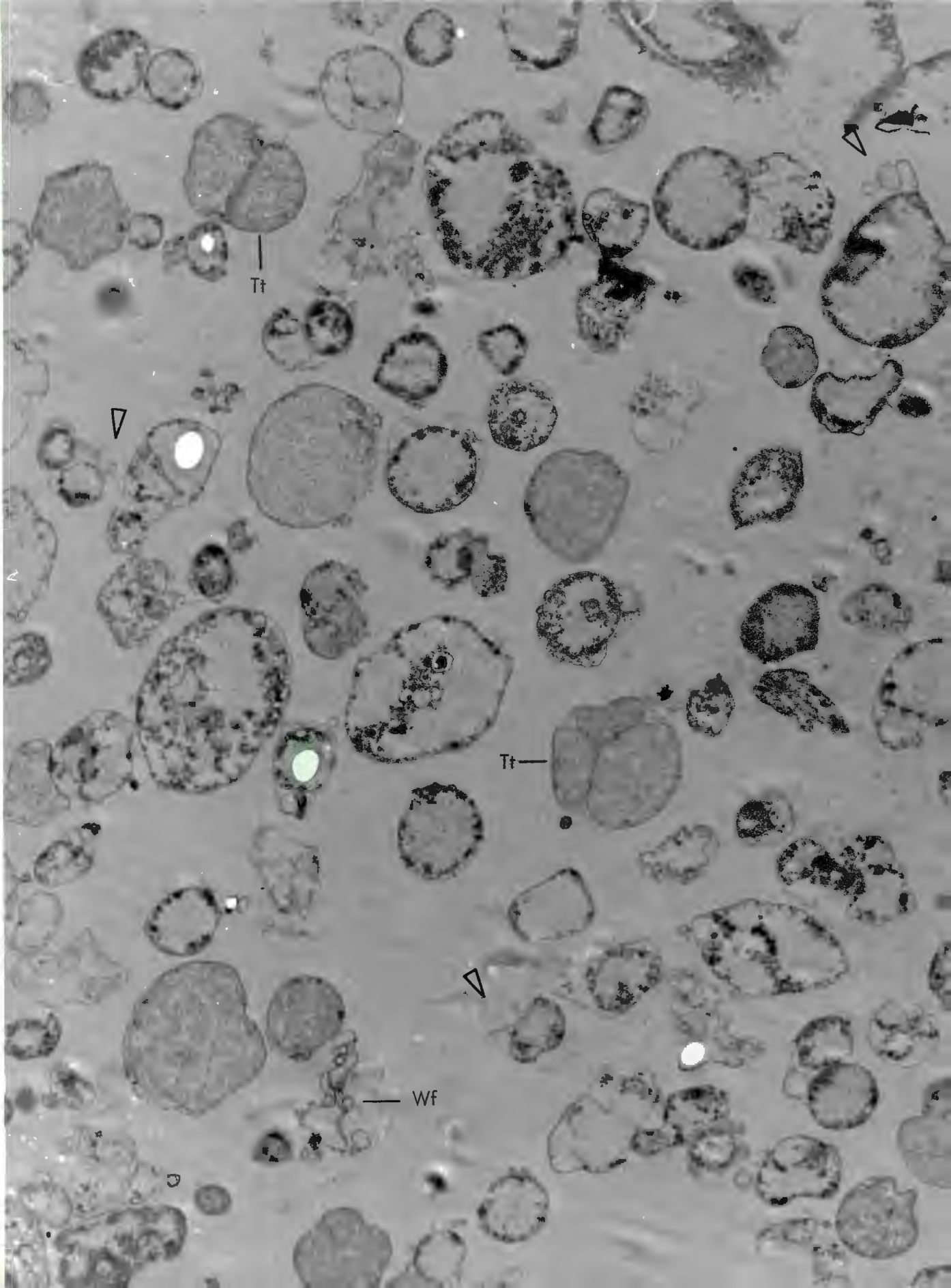


PLATE 9

Section of cells from luminescent liquid culture. One cell ( $\blacktriangleright$ ) shows a large pseudopodium-like bleb which involves the cytoplasm and the cell wall. Electron micrograph X 7,100.

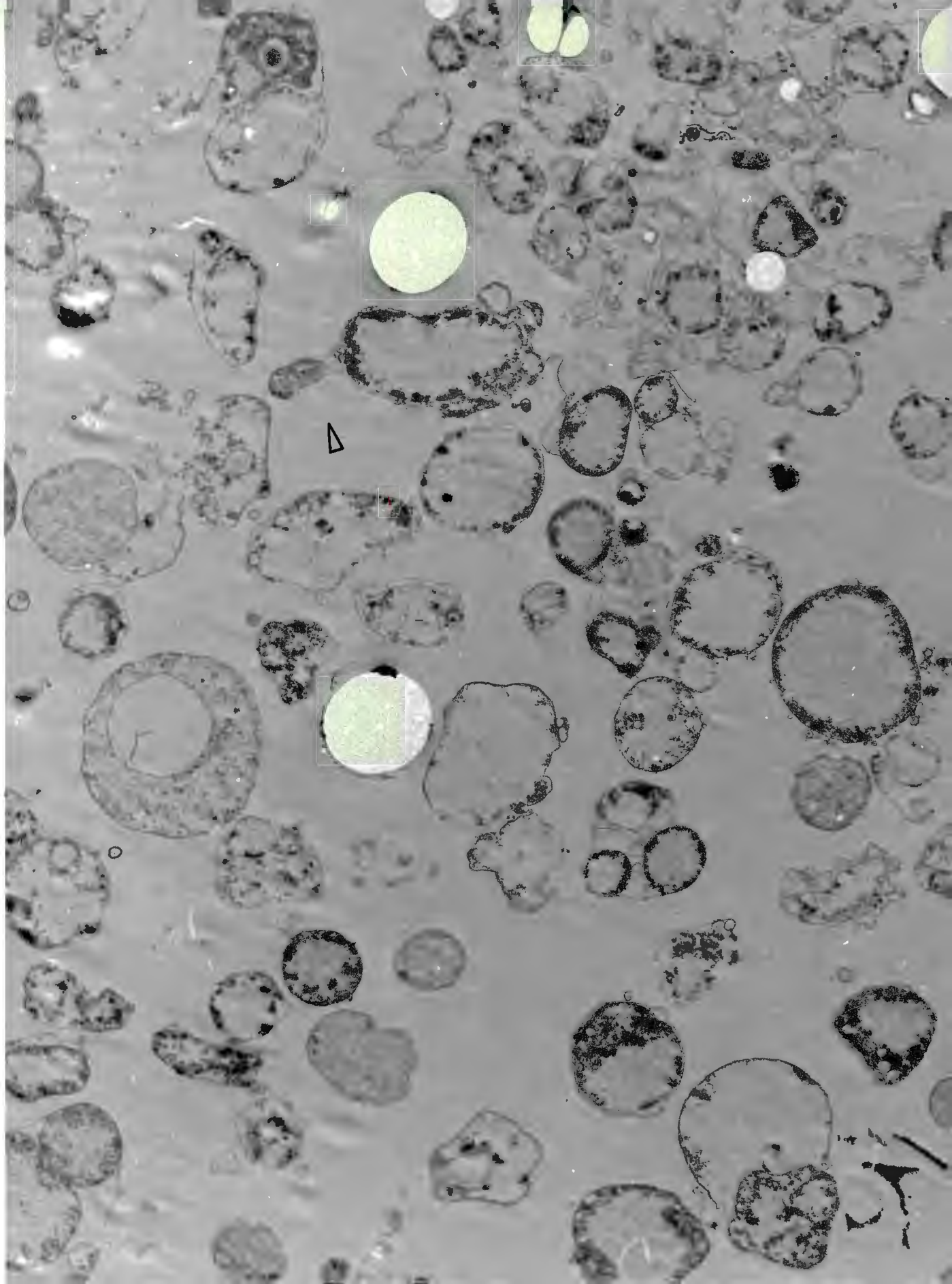


PLATE 10

Section of cells from 3 day old luminescent culture. A number of cells in the section, including one tetrad, appear to be undergoing autolysis (▷) and have lost a large part of their contents. Electron micrograph X 11,100.

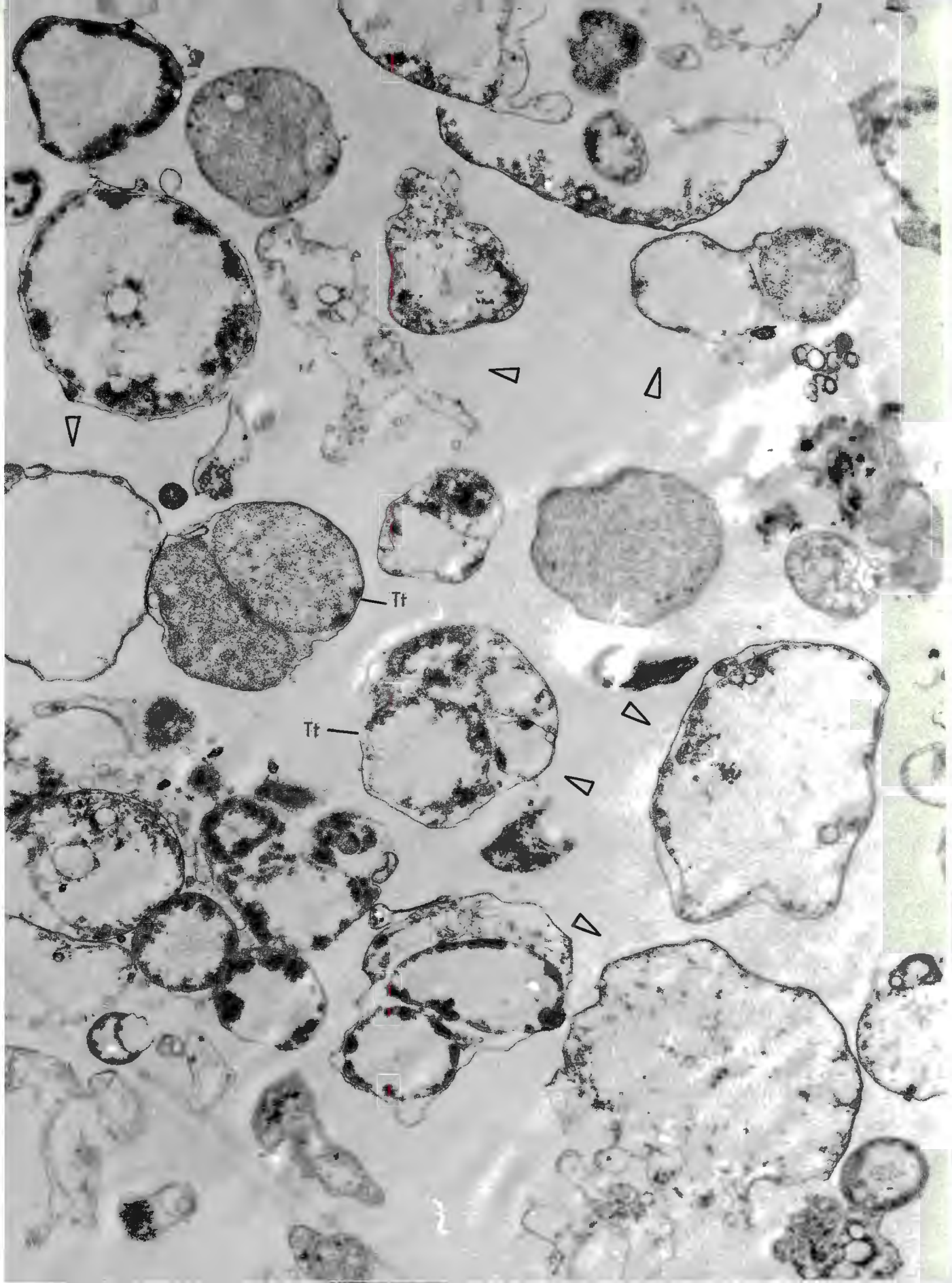


PLATE 11.

Section of L-form cell showing blebs (▷). Electron micrograph  
X 101,330.

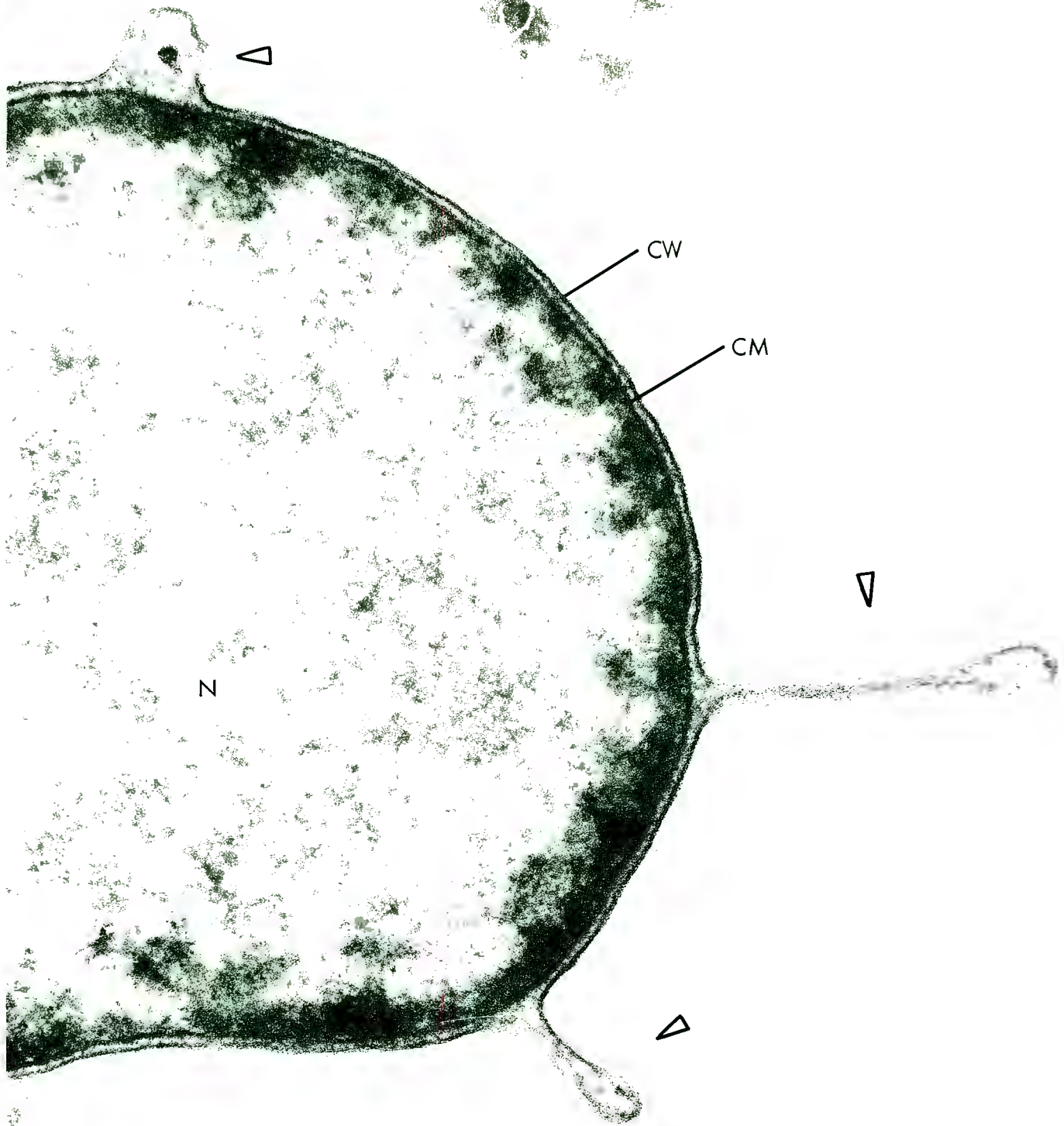
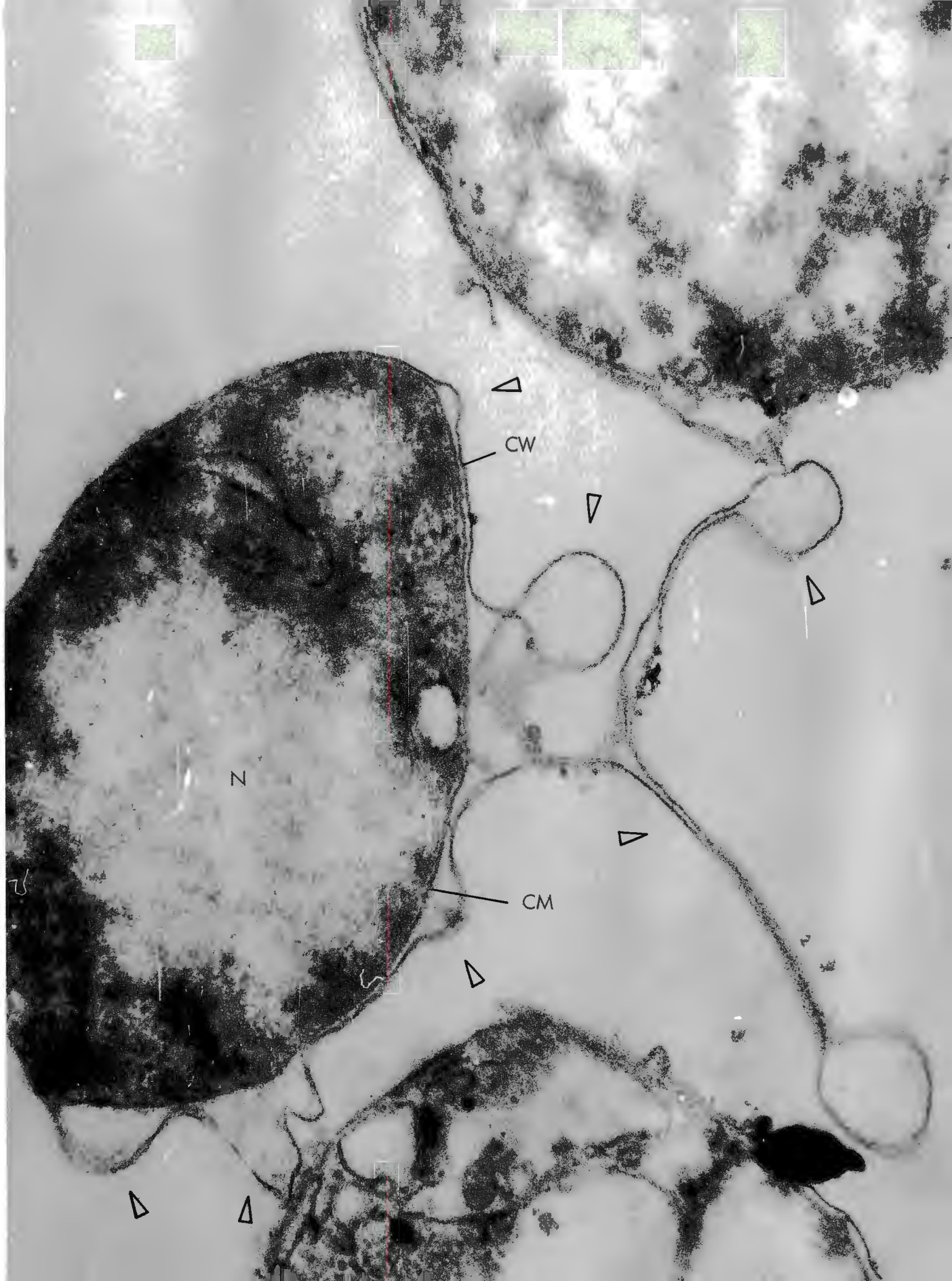


PLATE 12

Section of cell from NBS culture showing extensive blebbing and folding (▷) of the cell wall. Electron micrograph X 78,660.



Z

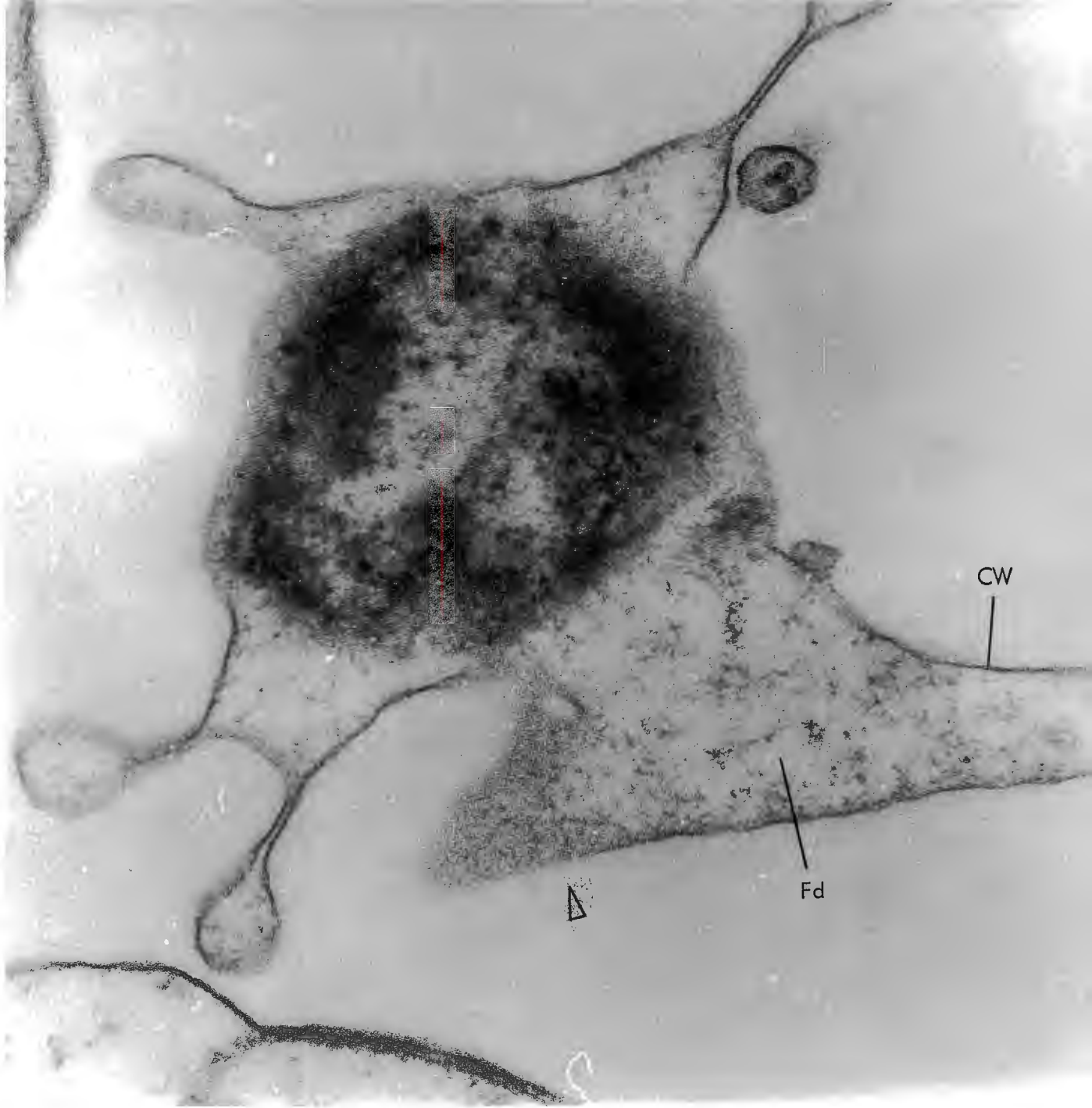
CW

CM



PLATE 13

Section of cell which shows blebbing and folding of the cell wall. The large fold at the bottom of the plate shows the cell wall partly in surface view (▷). Some surface detail is discernable. The bleb at the top right hand corner of the plate has been cut transversely and contains small dense bodies. Electron micrograph X 88,300.



CW

Fd



PLATE 14

Section showing a small blebbing cell from liquid culture. Note the dense aggregations of ribosome-like particles in the cell above, ( $\blacktriangleright$ ).

Electron micrograph X 45,660.

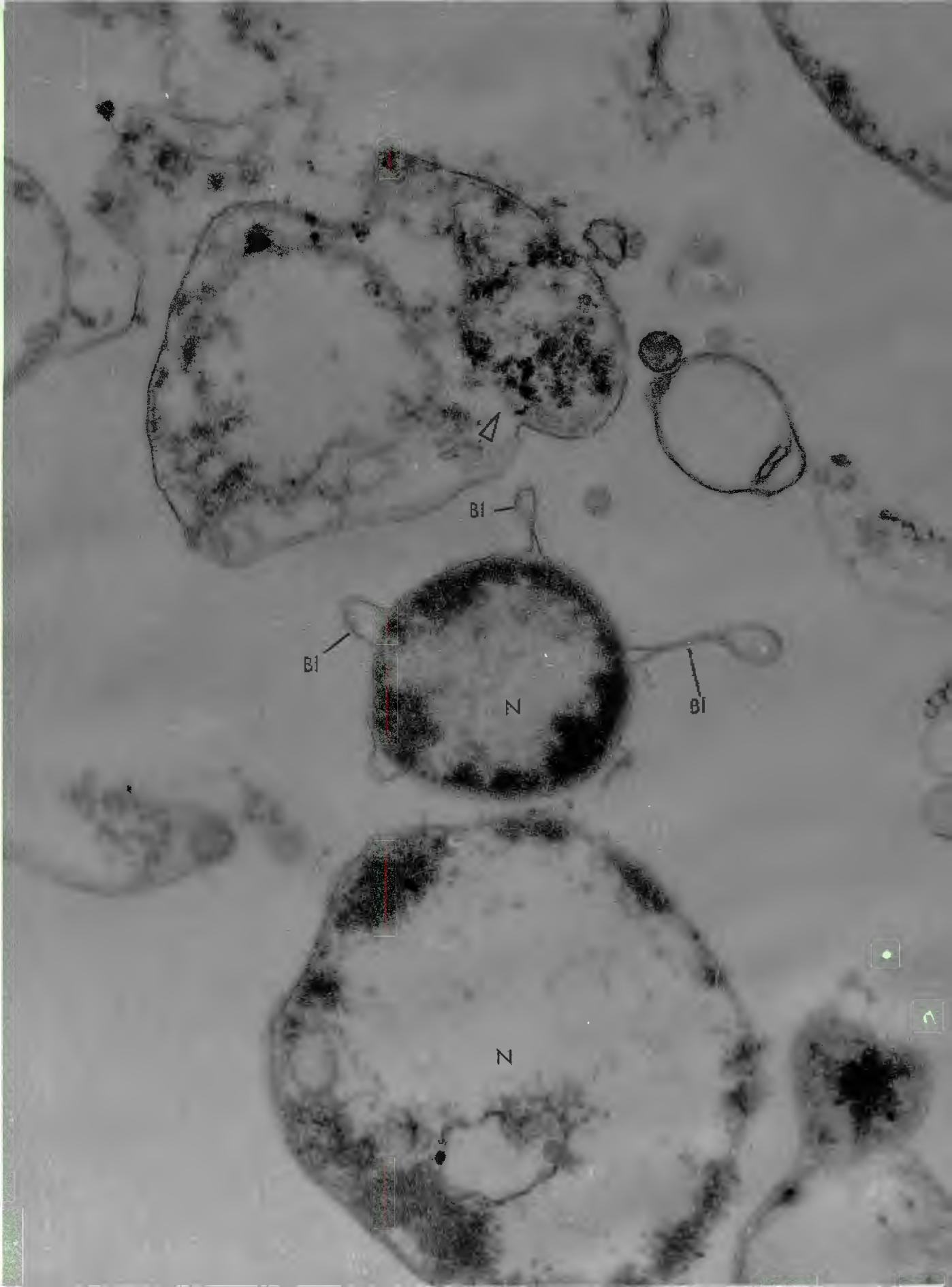
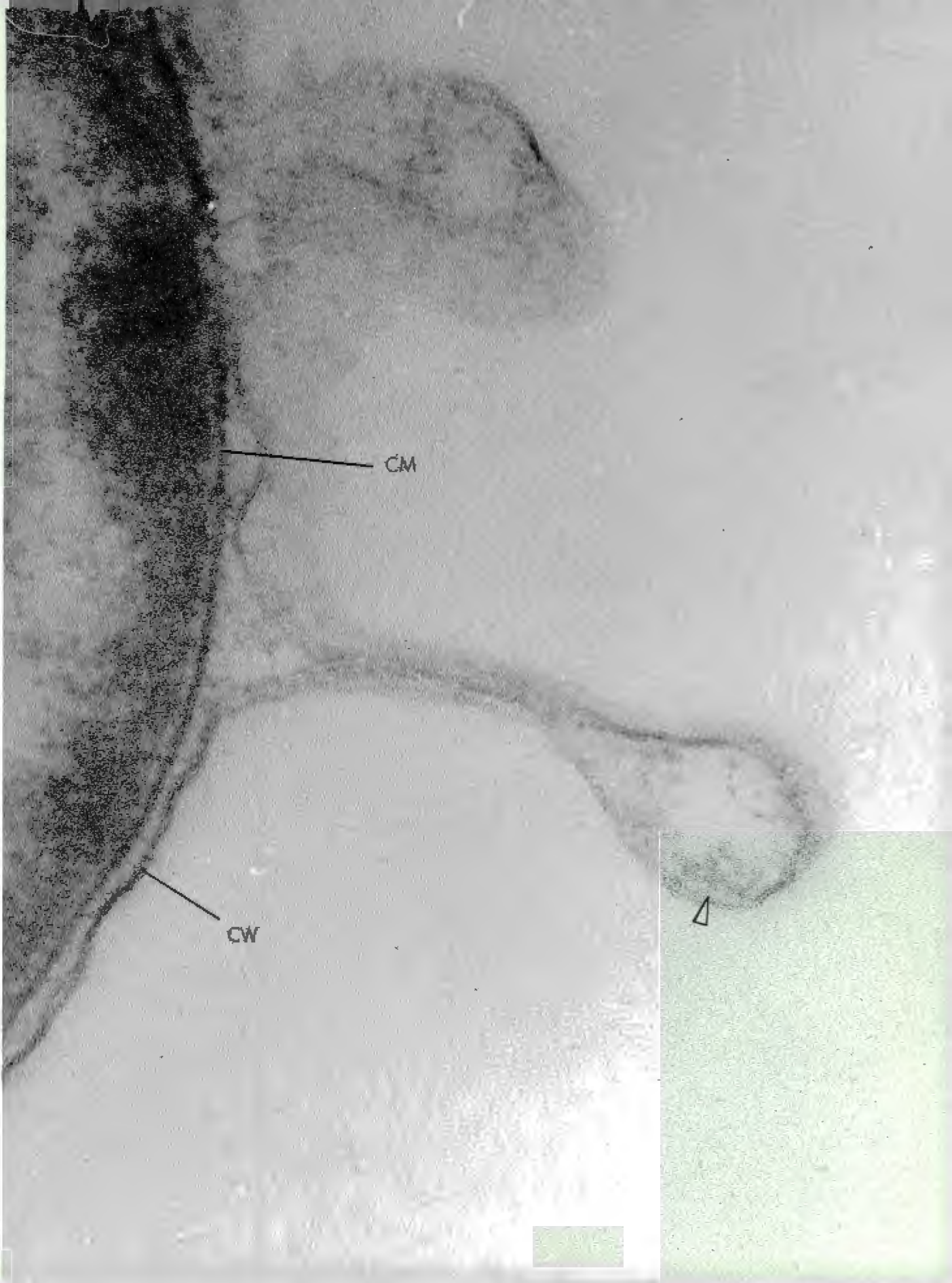


PLATE 15

Section of part of an L-form cell from liquid culture showing blebs. Blebs appear to be filled with a slightly granular electron transparent material (▷) which is found between the wall and the cytoplasmic membrane. Electron micrograph X 205,330.



CM

CW



PLATE 16

Detail of bleb on an L-form cell. The dimensions of the wall are normal, typical of a unit biological membrane, 7.0 to 8.0 nm wide ( $\blacktriangleright$ ).

Electron micrograph X 406,660.

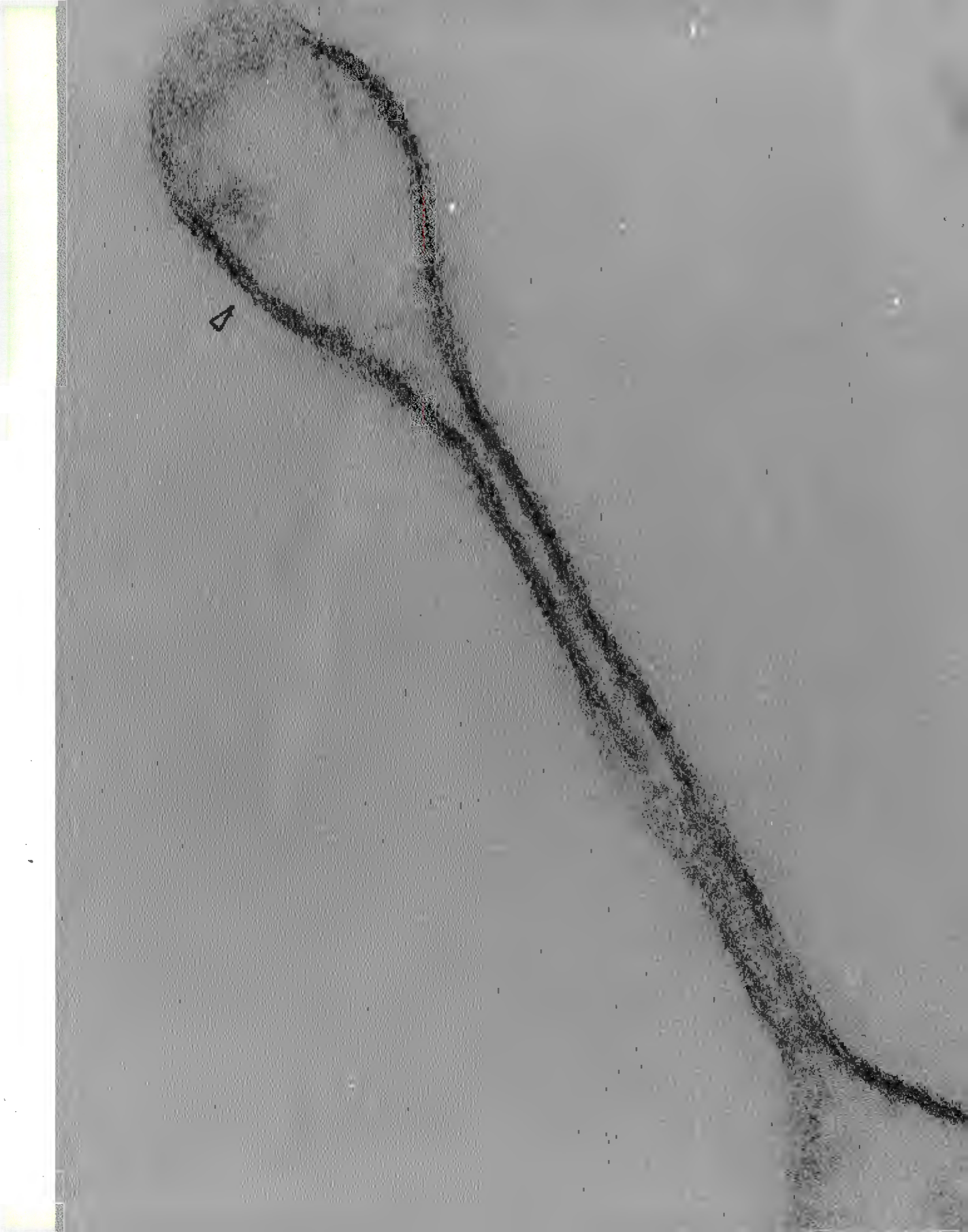
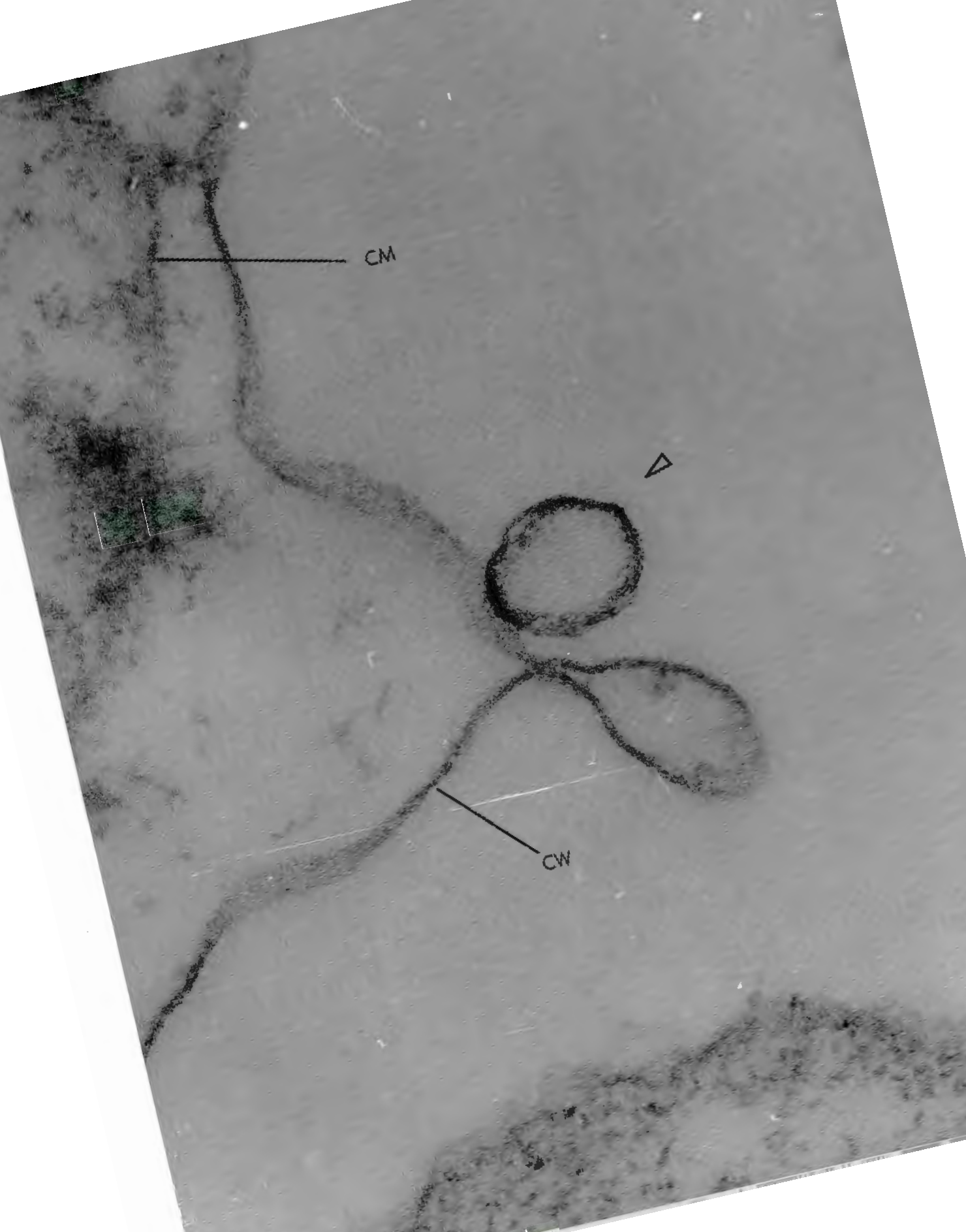


PLATE 17

Detail of blebs on wall of L-form cell from liquid culture. One bleb is cut in a transverse direction ( $\triangleright$ ). Electron micrograph X 205,330.



CM



CW

PLATE 18

Blebs on surface of L-form cell which contain small cytoplasmic spherules  
(▷) derived from the cytoplasm by evagination of the membrane.  
Electron micrograph X 205,330.

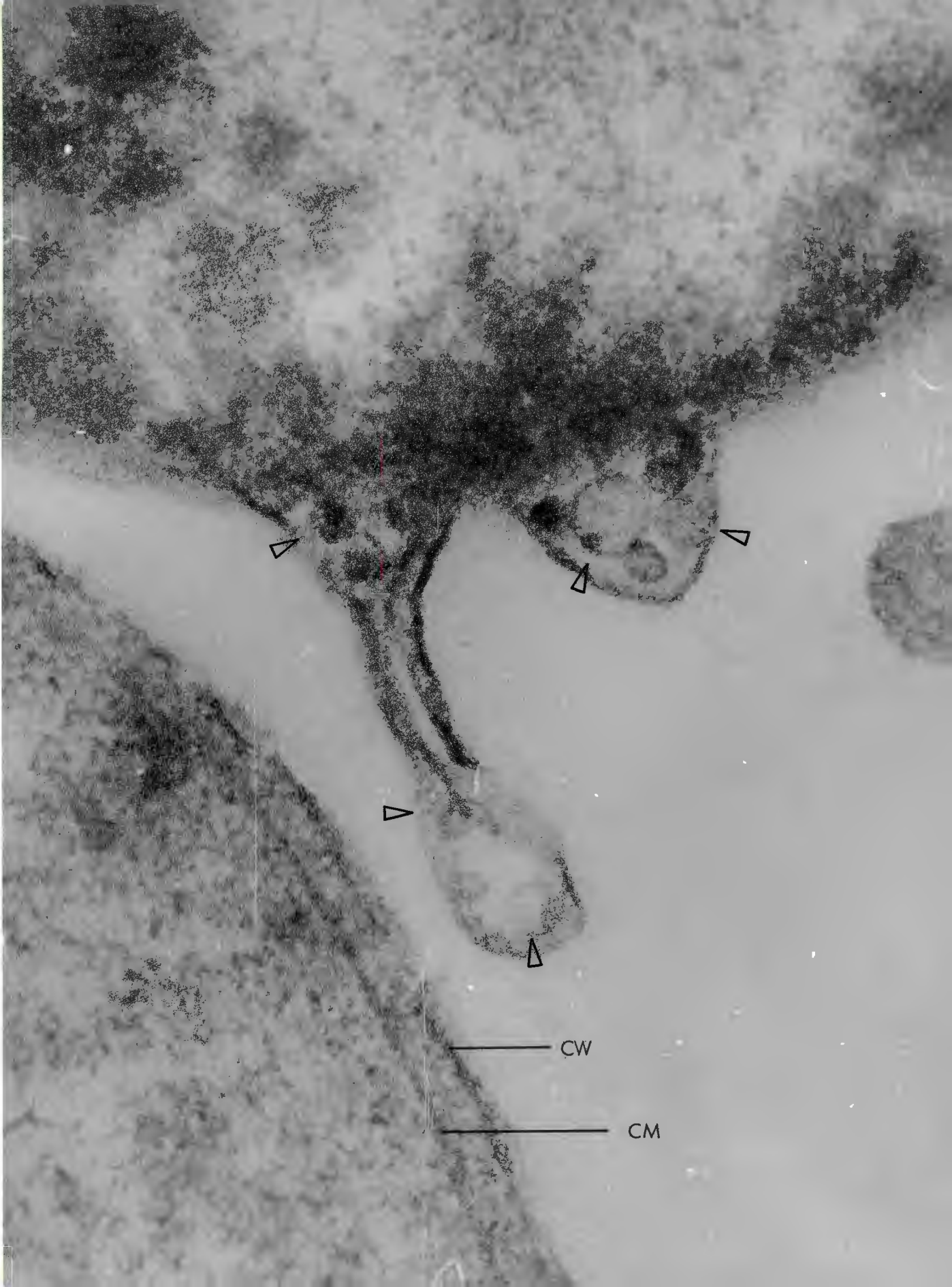
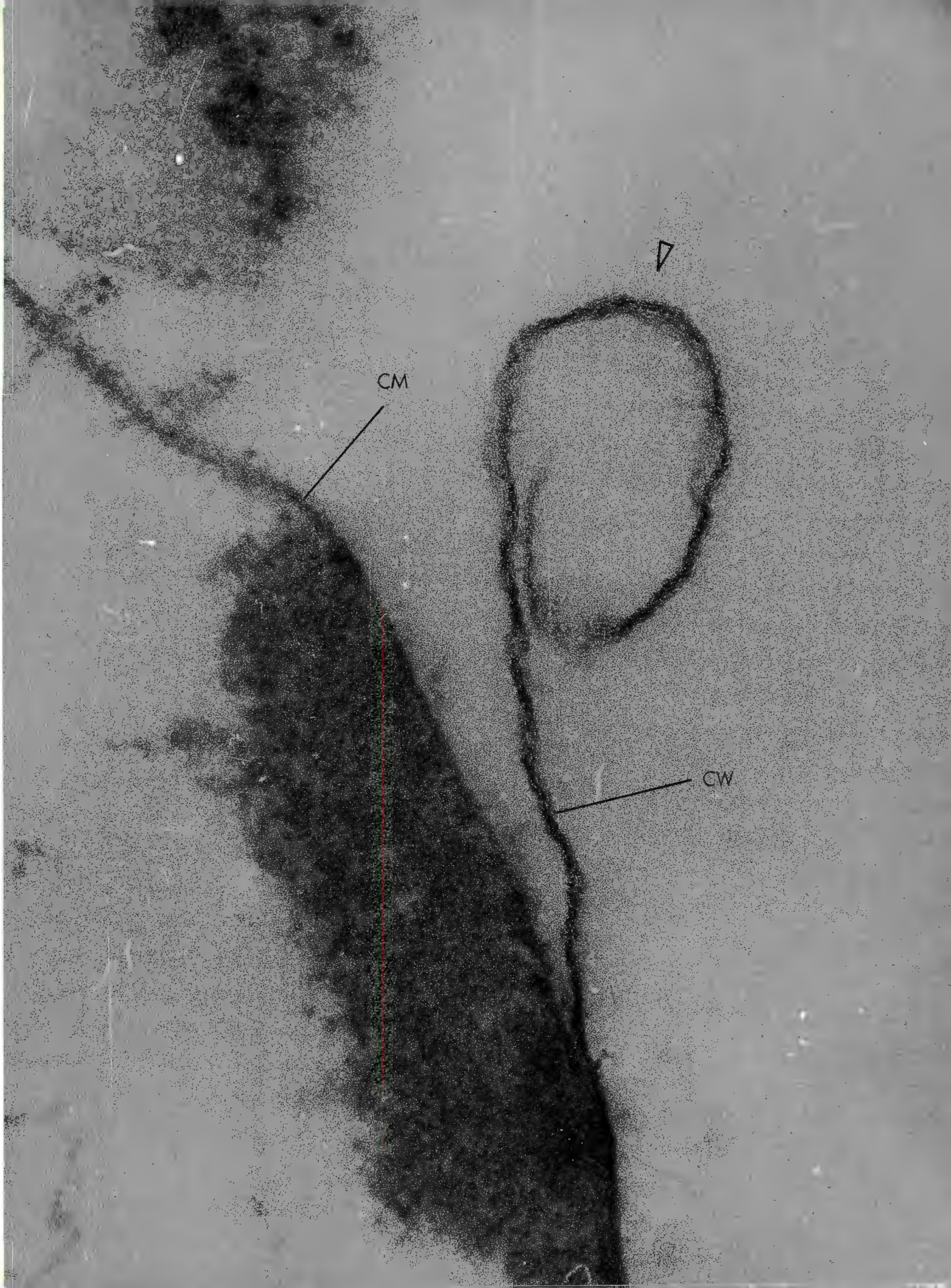


PLATE 19

Section of part of an L-form cell which shows an early stage of wall fragmentation (▷) which eventually results in the rolled up wall fragments seen in many sections. Electron micrograph X 205,330.



CM



CW

PLATE 20

Section showing an L-form cell in which a large part of the protoplast has been exposed by disintegration of the wall (▷). Electron micrograph X 78,660.

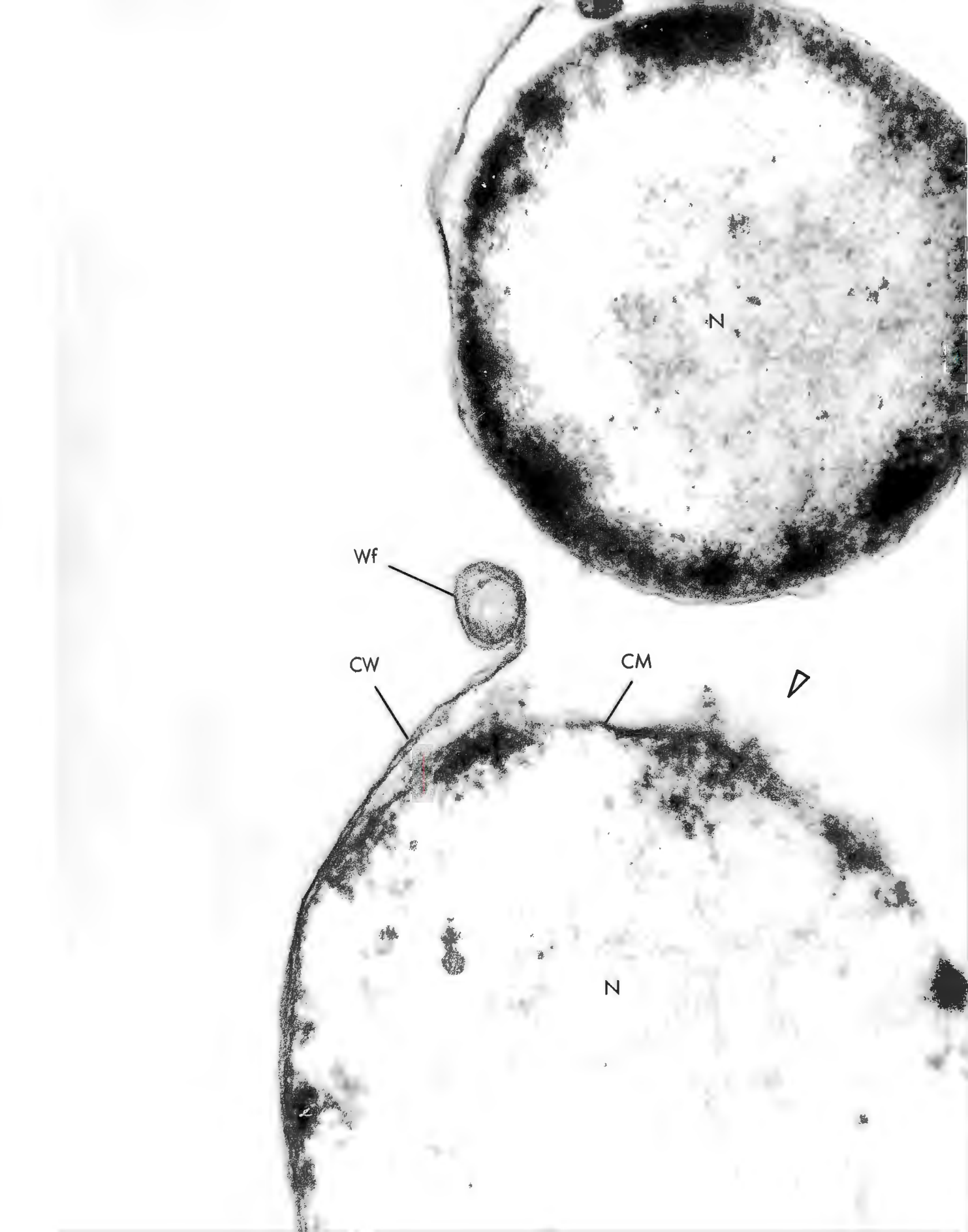


PLATE 21

Section of L-form cell with large attached wall fragments. The exposed cytoplasmic membrane appears to have developed into multiple membrane at one place (▷ ). Electron micrograph X 126,660.



WF

Δ

CW

WF



PLATE 22

Section of L-form cell with attached wall fragments. Note the aggregation of ribosomes in the cell at top (▷). Electron micrograph X 78,660.

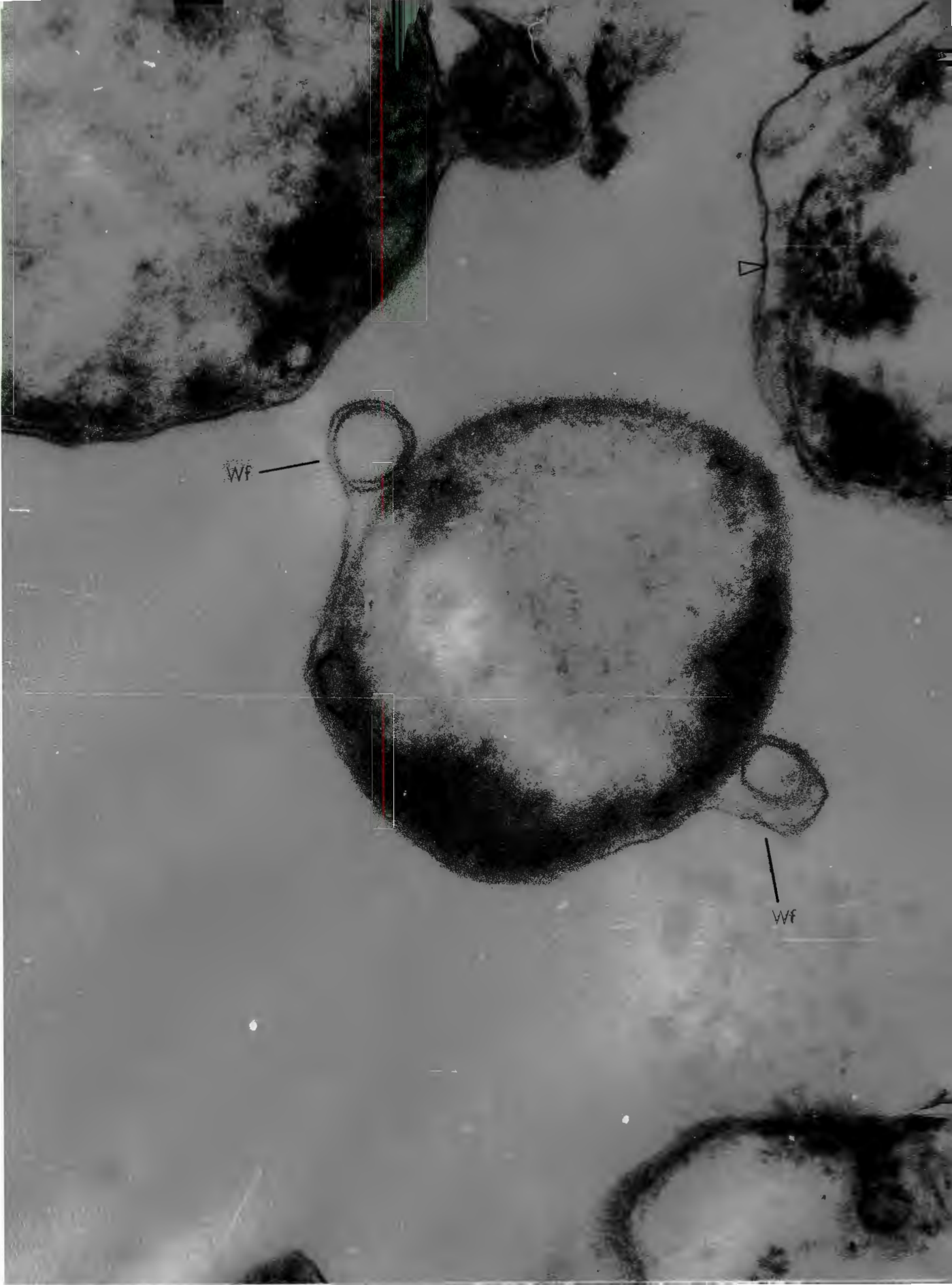
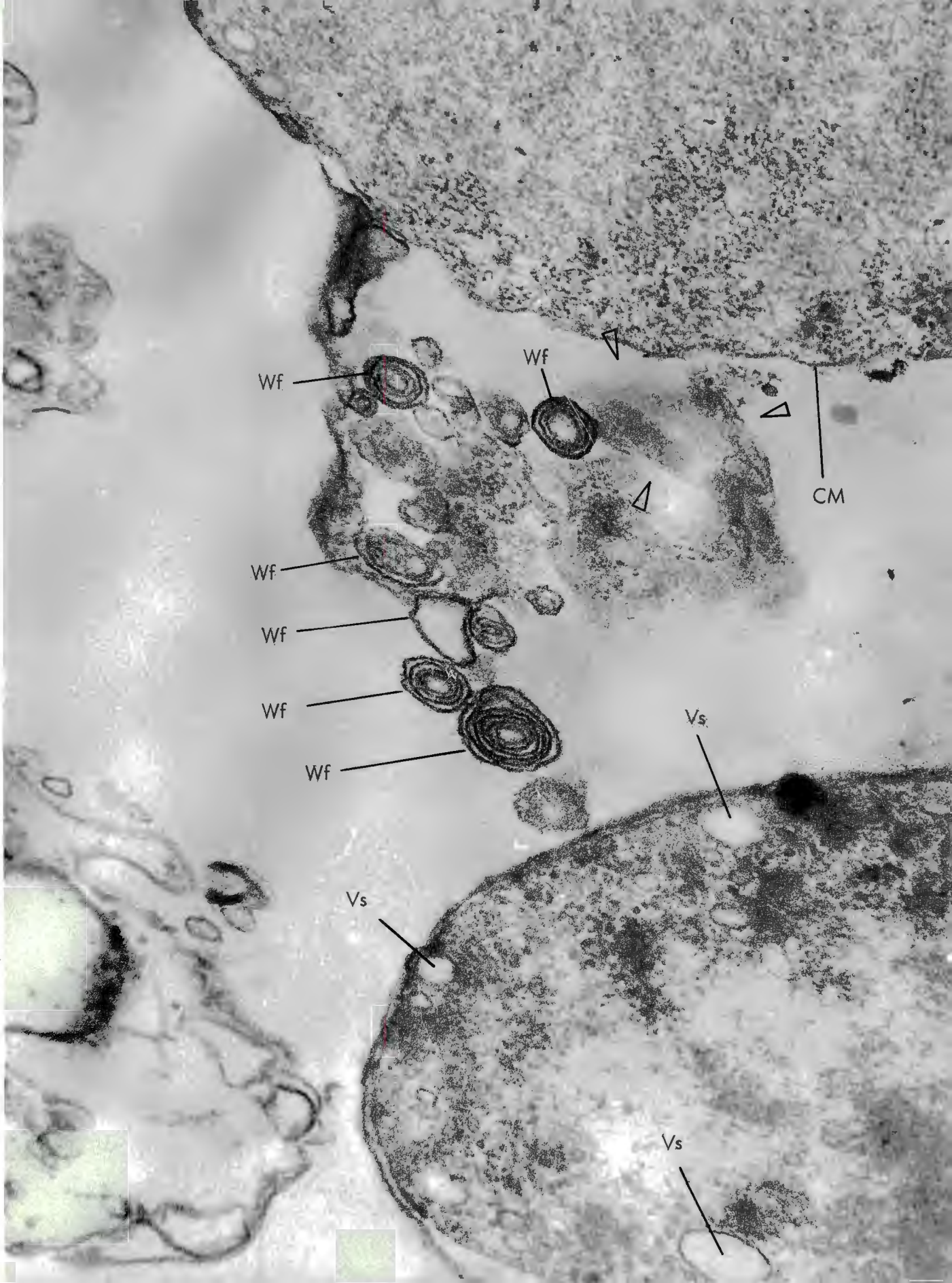


PLATE 23

Tightly rolled wall fragments which have become separated from the cell.  
Part of a wall fragment, less tightly rolled, is seen in surface view ( $\triangleright$ ).  
Electron micrograph X 36,660.



Wf

Wf

CM

Wf

Wf

Wf

Wf

Vs

Vs

Vs

PLATE 24

Wall fragments seen in section of L-form cells. Small cytoplasmic spherules have become enclosed in some of the fragments. One wall fragment lying horizontal to the plane of sectioning has been cut longitudinally over part of its length ( $\blacktriangleright$ ). Electron micrograph X 78,660.



Wf

Sp

Wf

Wf

Sp

Sp

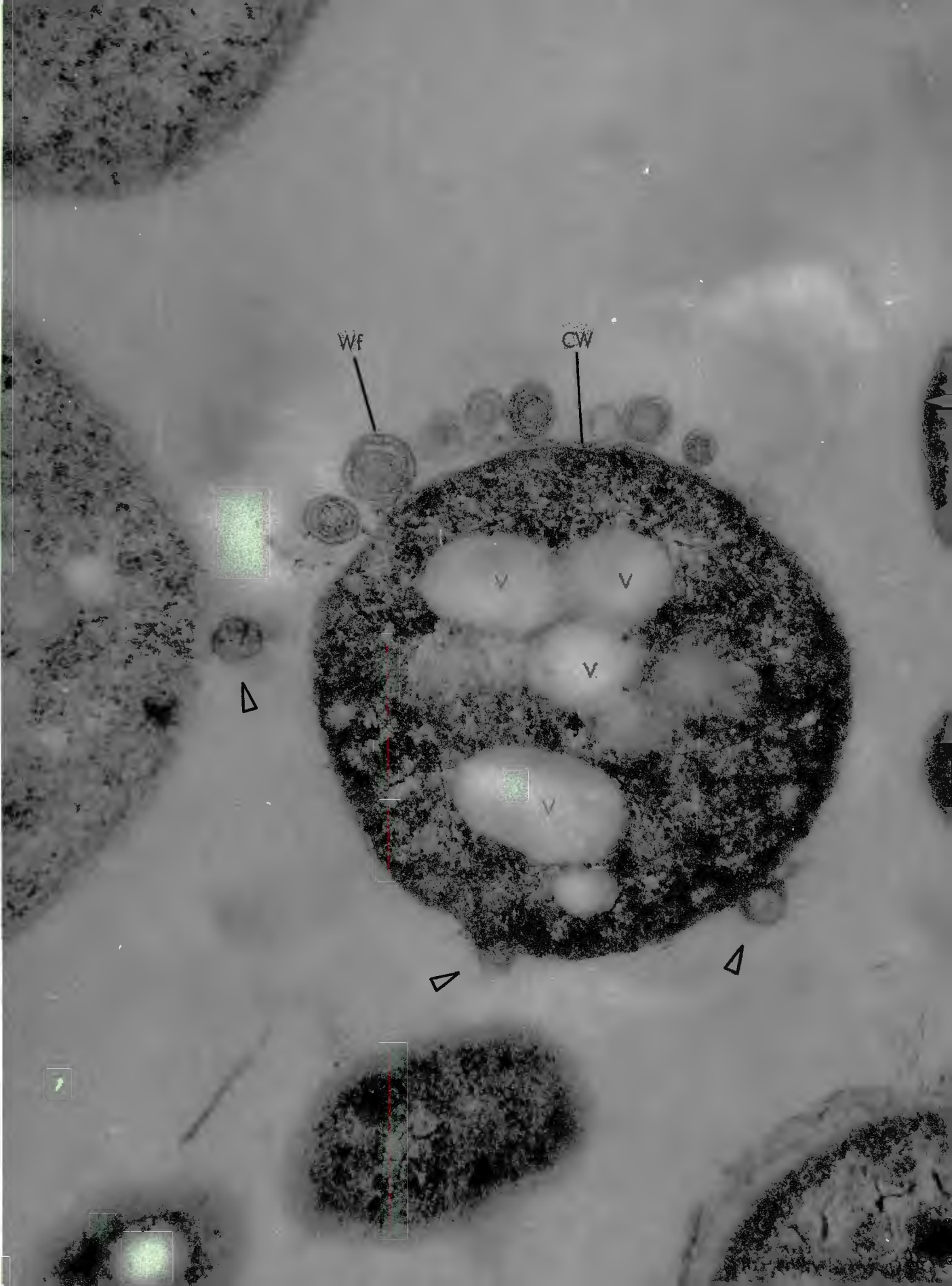
Sp

Sp

PLATE 25

Glycine spheroplast in section. The cell wall has been almost completely removed and the remaining fragments are adherent to short sections of wall still in place. Note the presence of small cytoplasmic spherules (▷).

Electron micrograph X 78,660.



WF

CW

V

V

V

V



PLATE 26

- **Complex figures made by rolled-up wall fragments in a section of L-form cells. The fragments often roll up around and within each other making interpretation difficult. Electron micrograph X 406,660.**

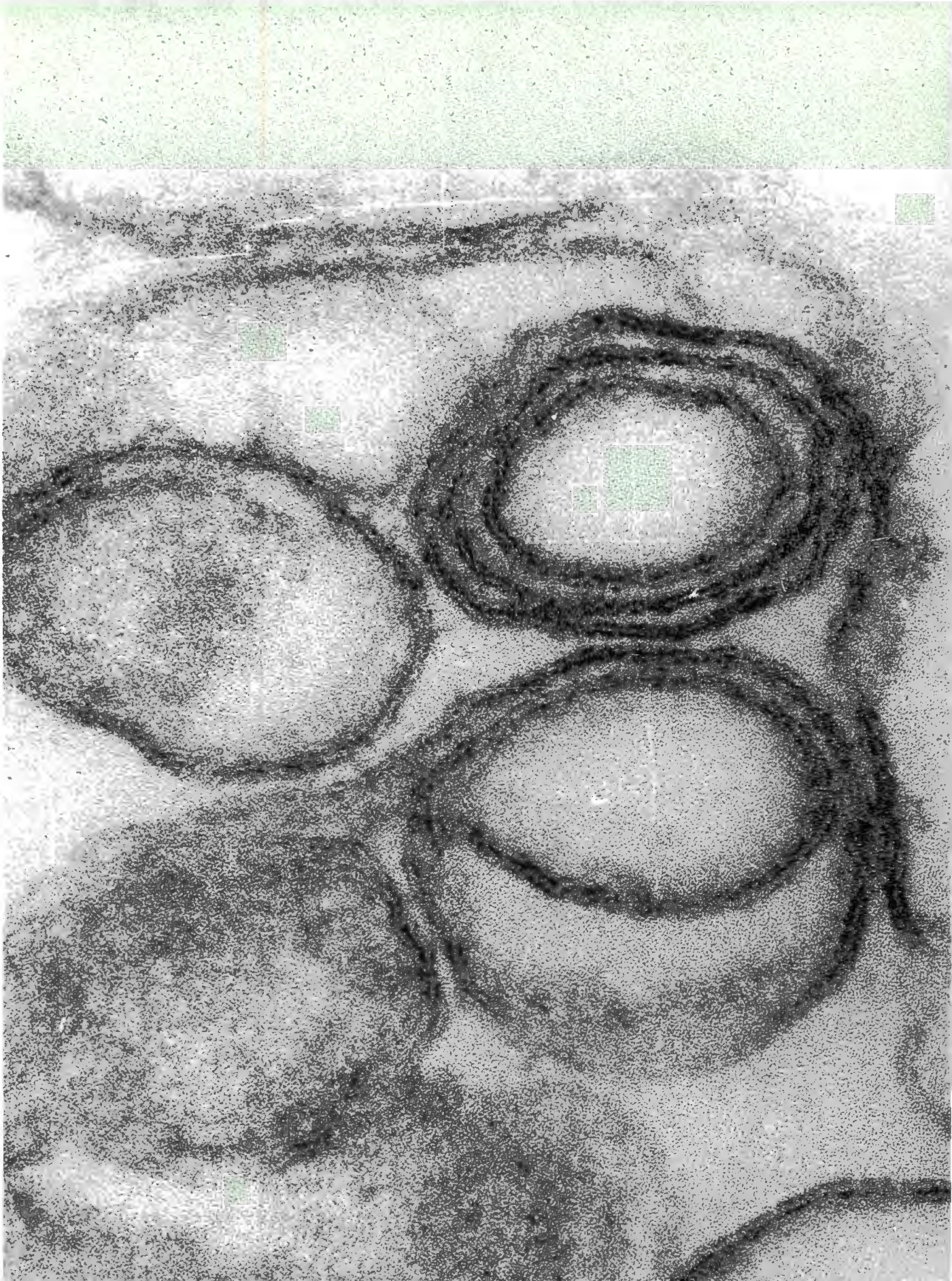
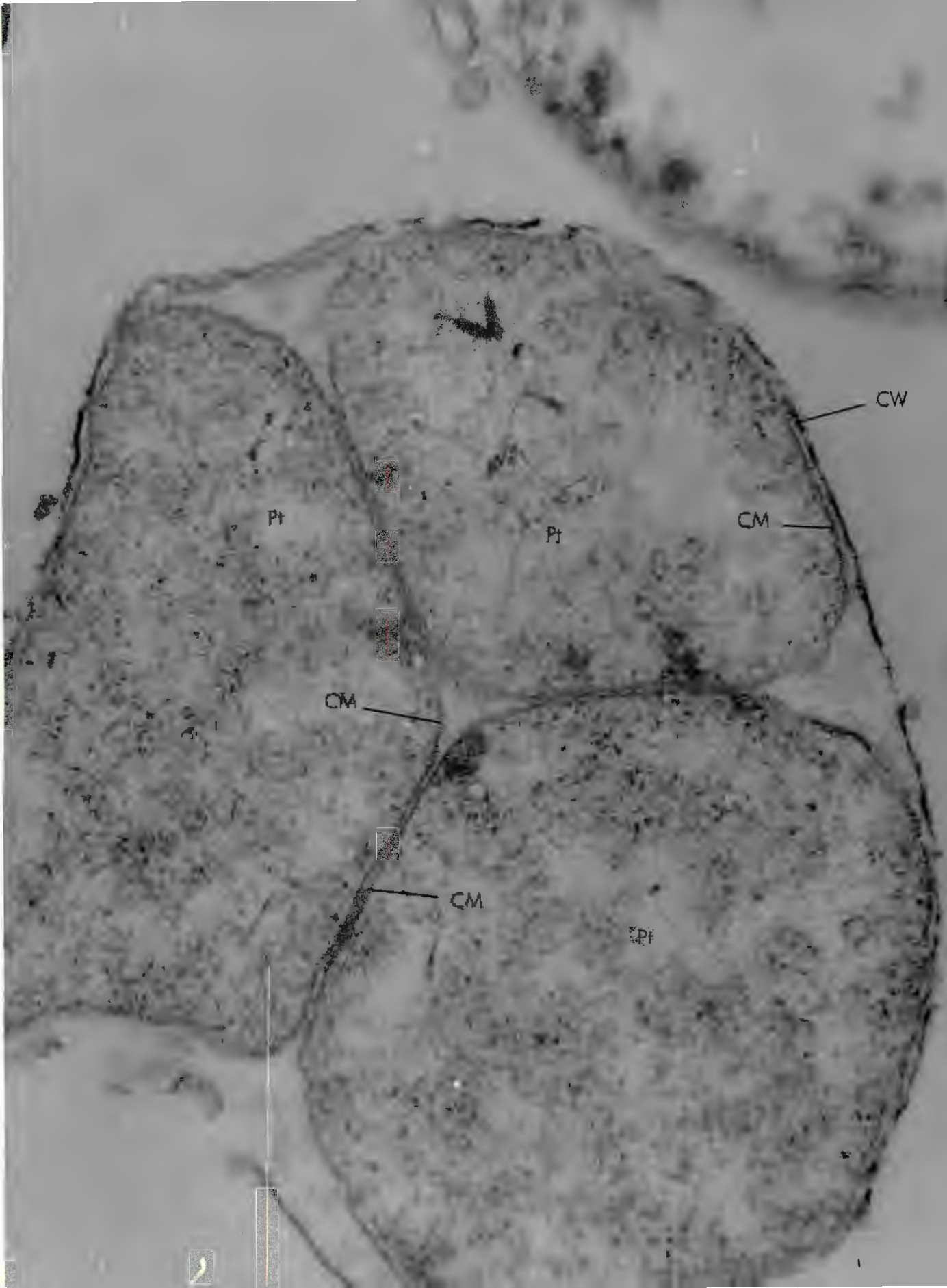


PLATE 27

Tetrad from culture of L-form cells. The cell is cut through a plane including three of the tetrad units. Electron micrograph X 101,330.



CW

CM

Pt

Pt

CM

CM

Pt

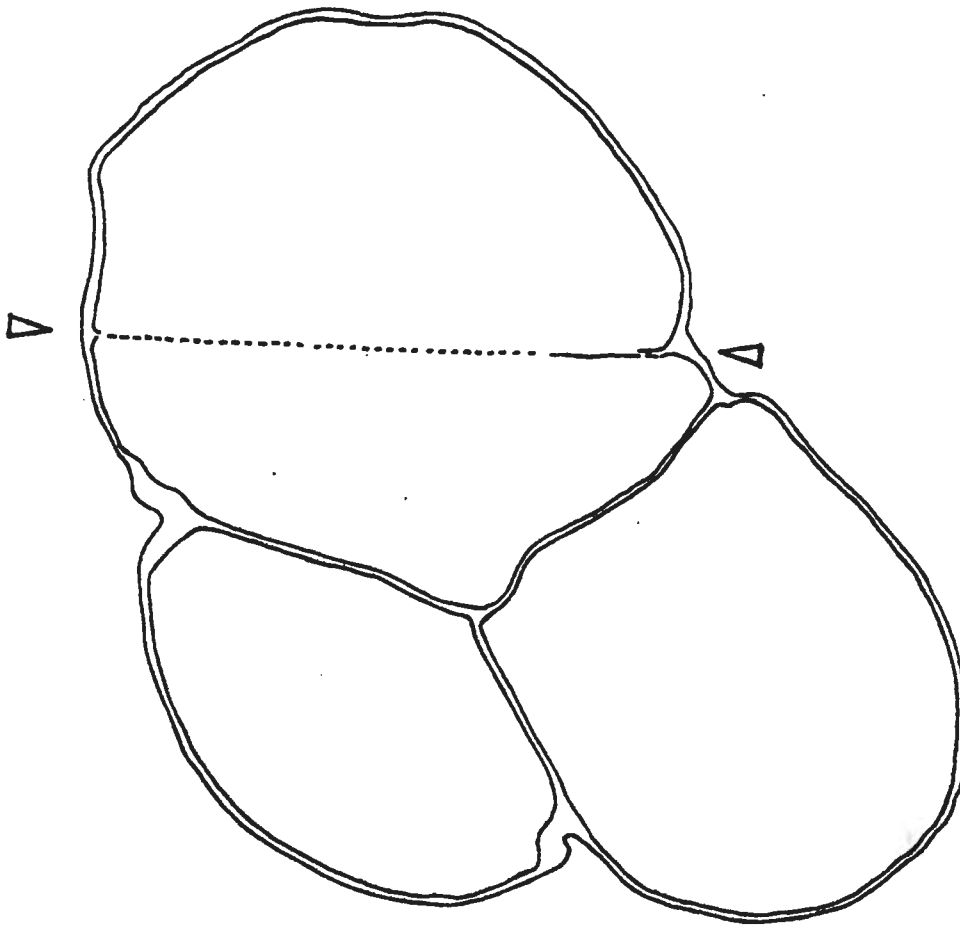


PLATE 28

The tetrad shown, from an L-form culture, has been cut on a plane which makes all four units of the cell partly visible. The diagram above indicates the position of the cytoplasmic membranes bounding the two units in the upper part of the plate. The division between the units is not visible along the entire length either because it had not been completed or was not normal to the electron beam all the way along. Electron micrograph X 36,600.

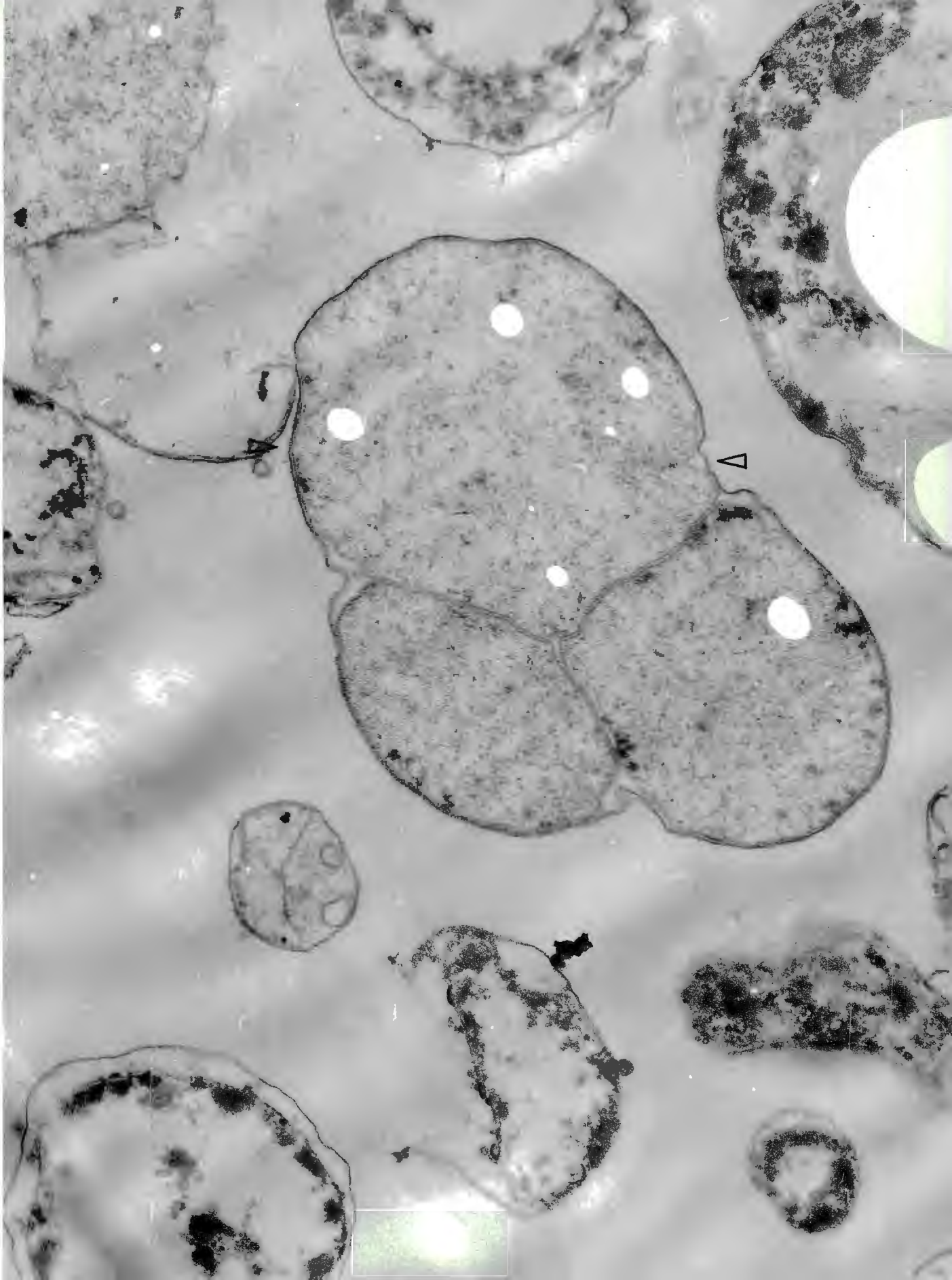


PLATE 29

Section of tetrad from culture of L-form cells showing three units. Also seen in this plate is a cell (▷) which has a number of small cytoplasmic spherules between the cytoplasmic membrane and cell wall. Electron micrograph X 36,660.

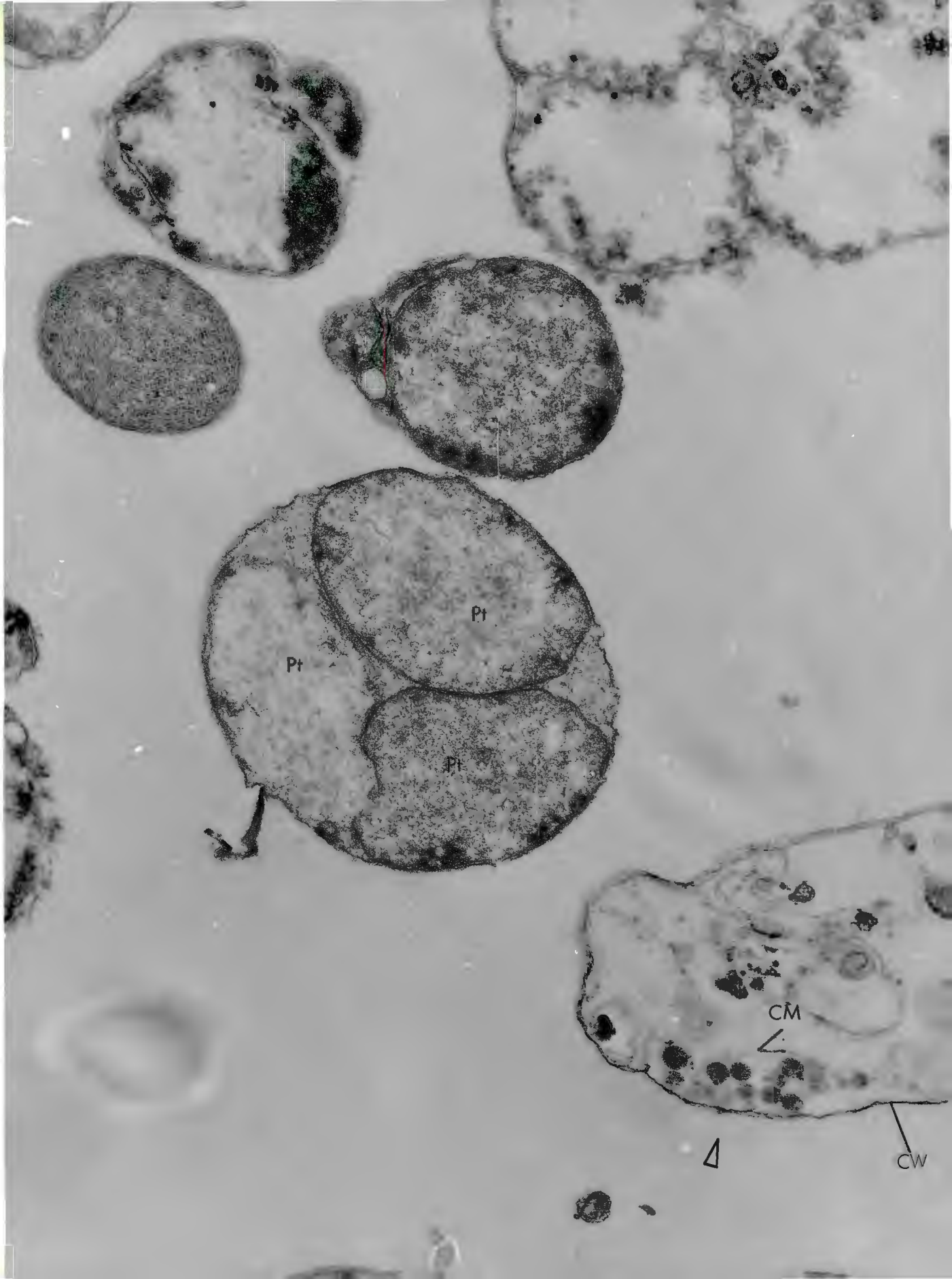


PLATE 30

Section of L-forms from liquid culture. One tetrad is visible, cut on a plane showing two units. The other cell which shows four units (▷) appears to have undergone unequal divisions resulting in protoplasts of different sizes. This cell appears to have undergone considerable autolysis. Electron micrograph X 13,100.

Note: The tetrad in the centre of the plate may be at the two cell stage.

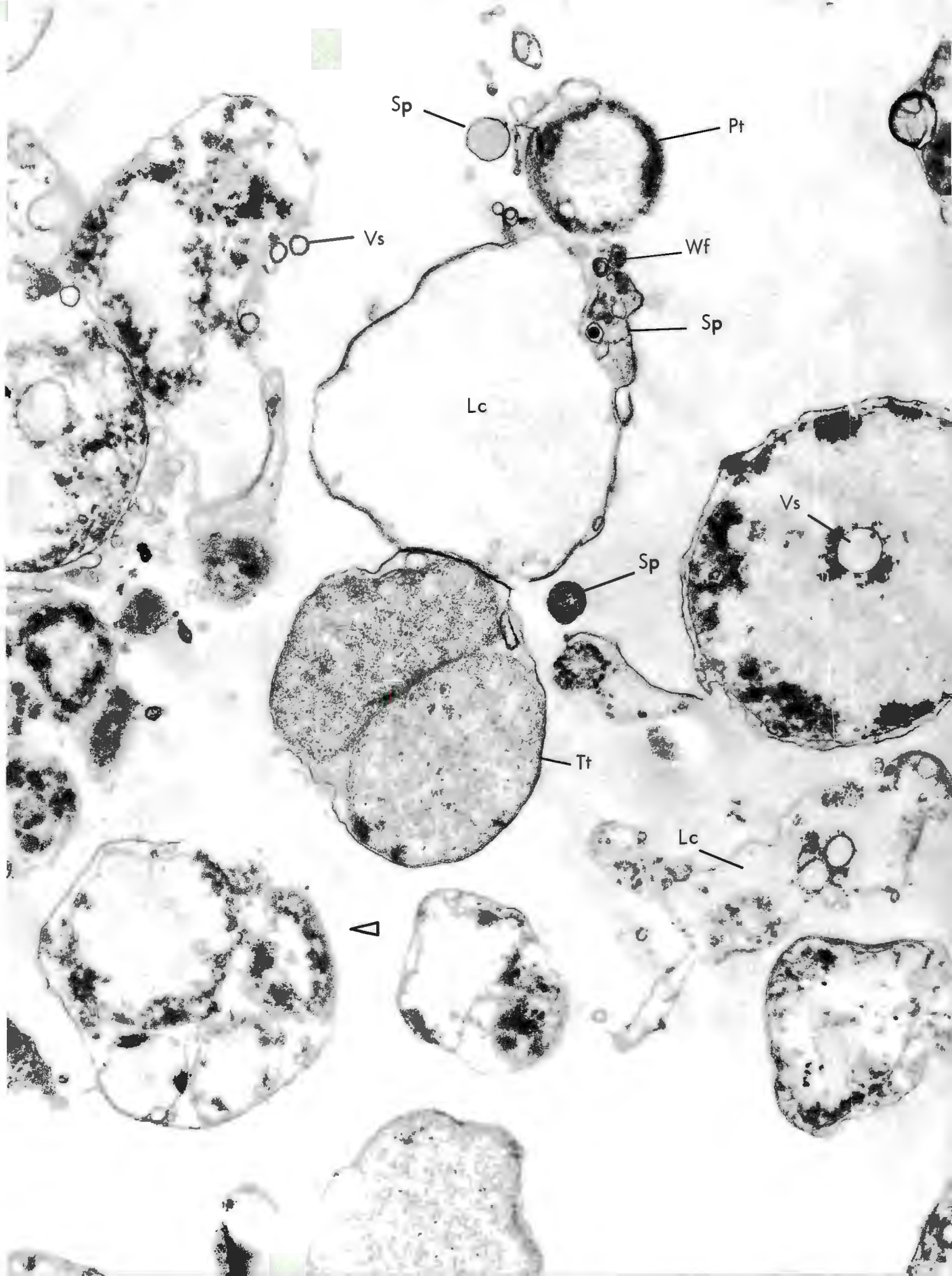
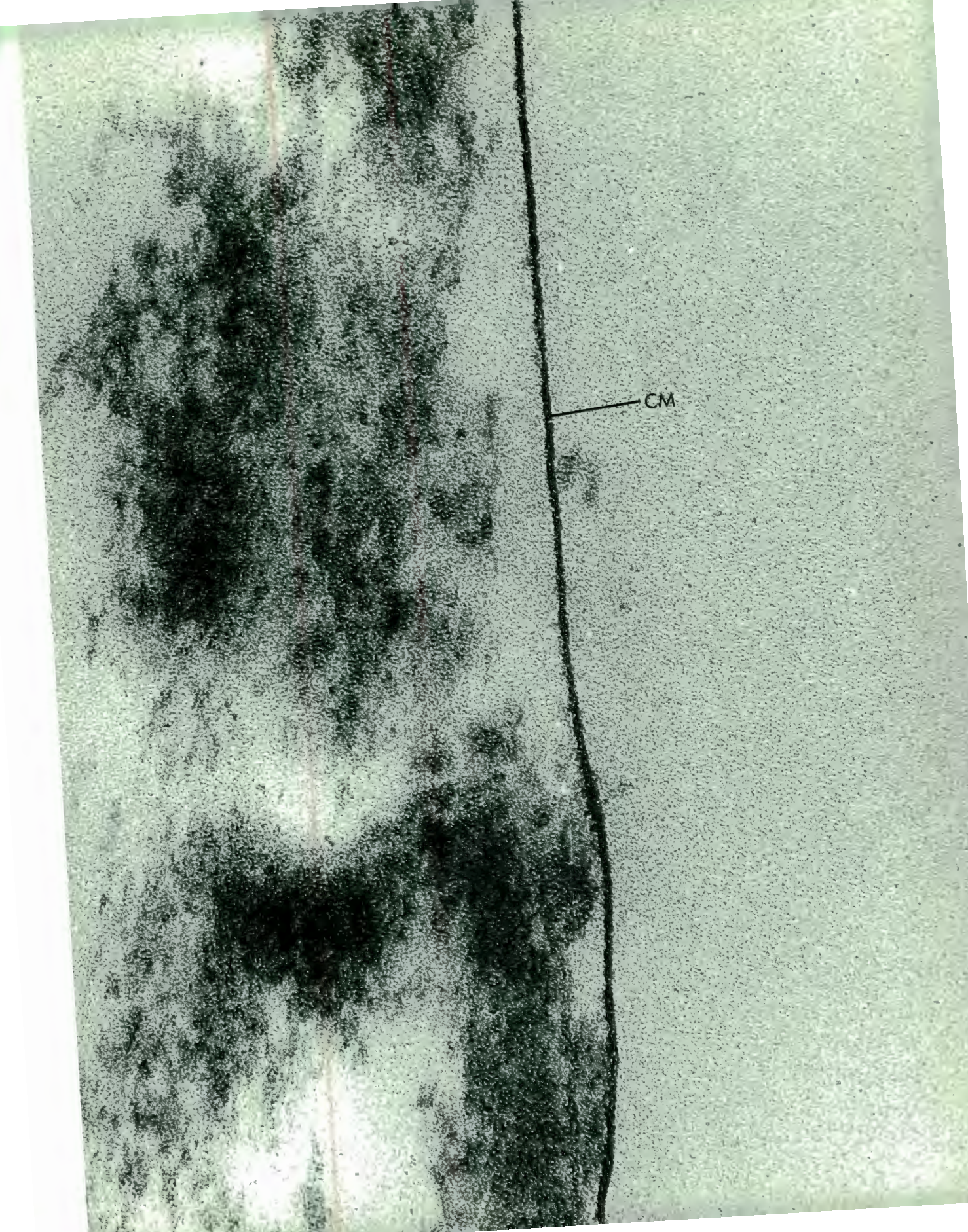


PLATE 31

High magnification electron micrograph of a section of a protoplast from an L-form culture, showing the cytoplasmic membrane. Electron micrograph X 264,000.



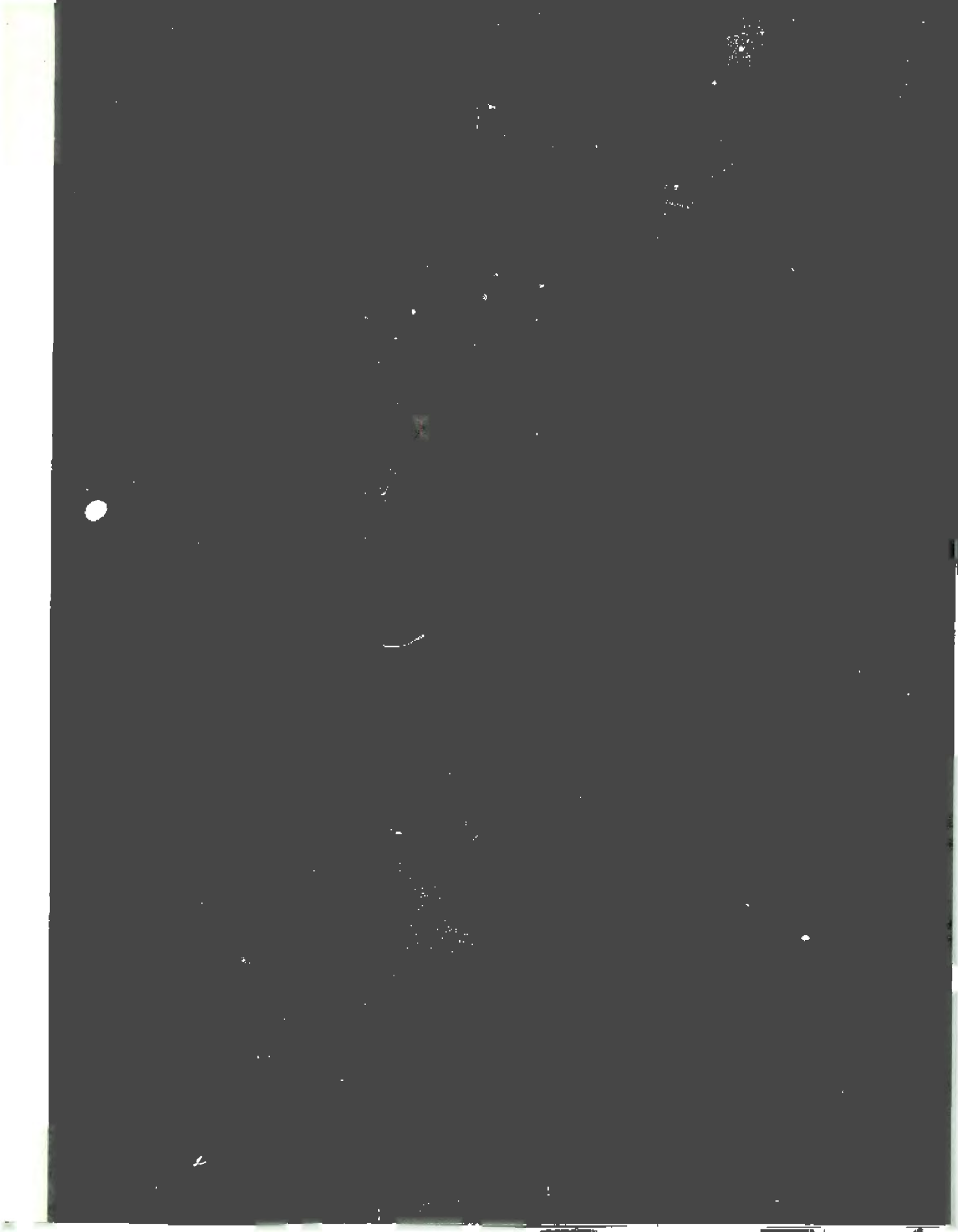
CM

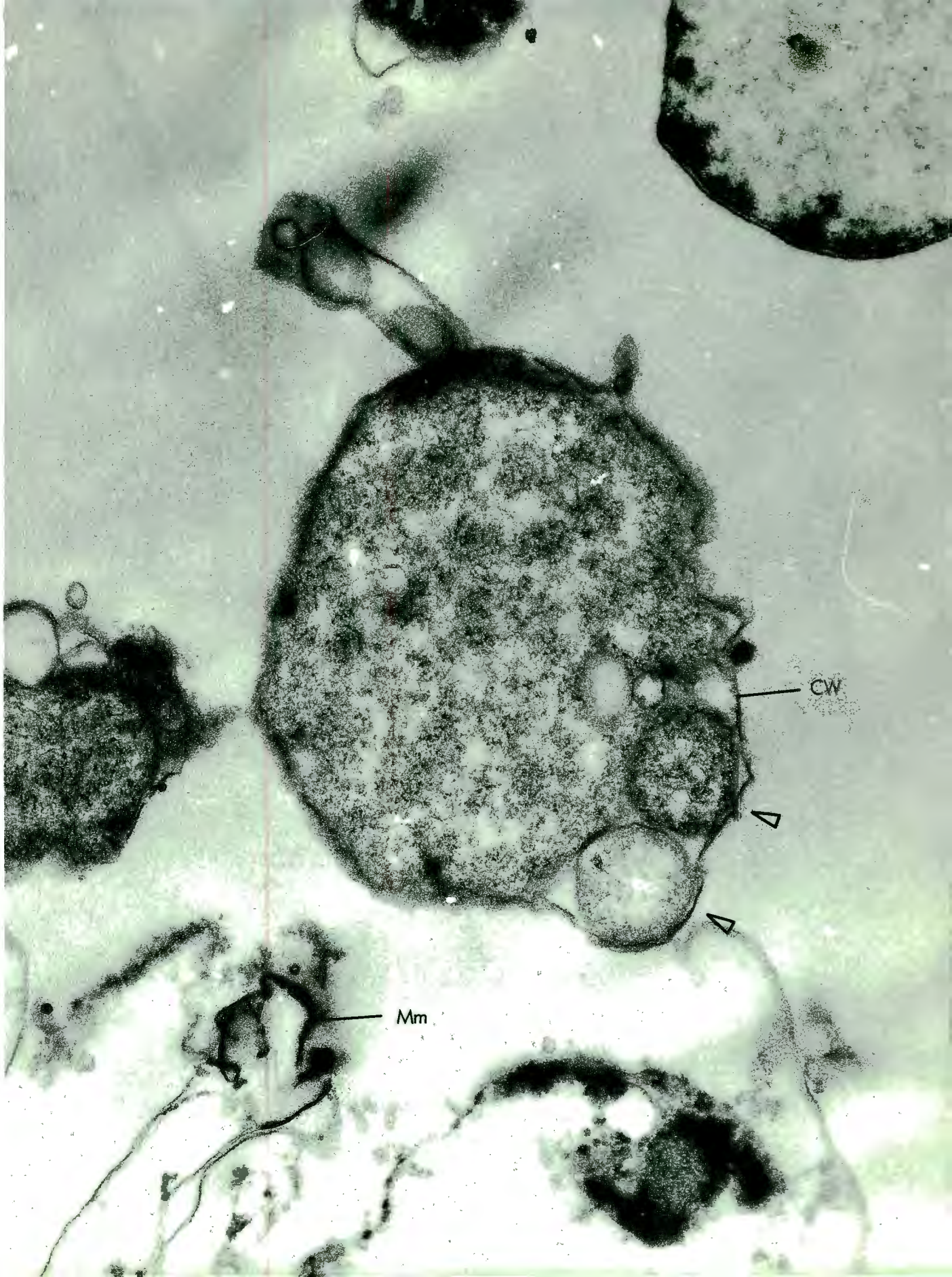
PLATE 32

Section of L-form cell showing unequal division of the cytoplasm.

Note also the presence of folds in the cytoplasmic membrane (▷).

Electron micrograph X 78,660.





CW

Mm

PLATE 34

Section of L-form cell showing the presence of small cytoplasmic spherules between the cell wall and the cytoplasmic membrane. One spherule (▷) is still attached to the protoplast. Numerous membrane-bound vesicles are also seen in the cell. Note the presence of ribosome-like particles. Electron micrograph X 78,660.

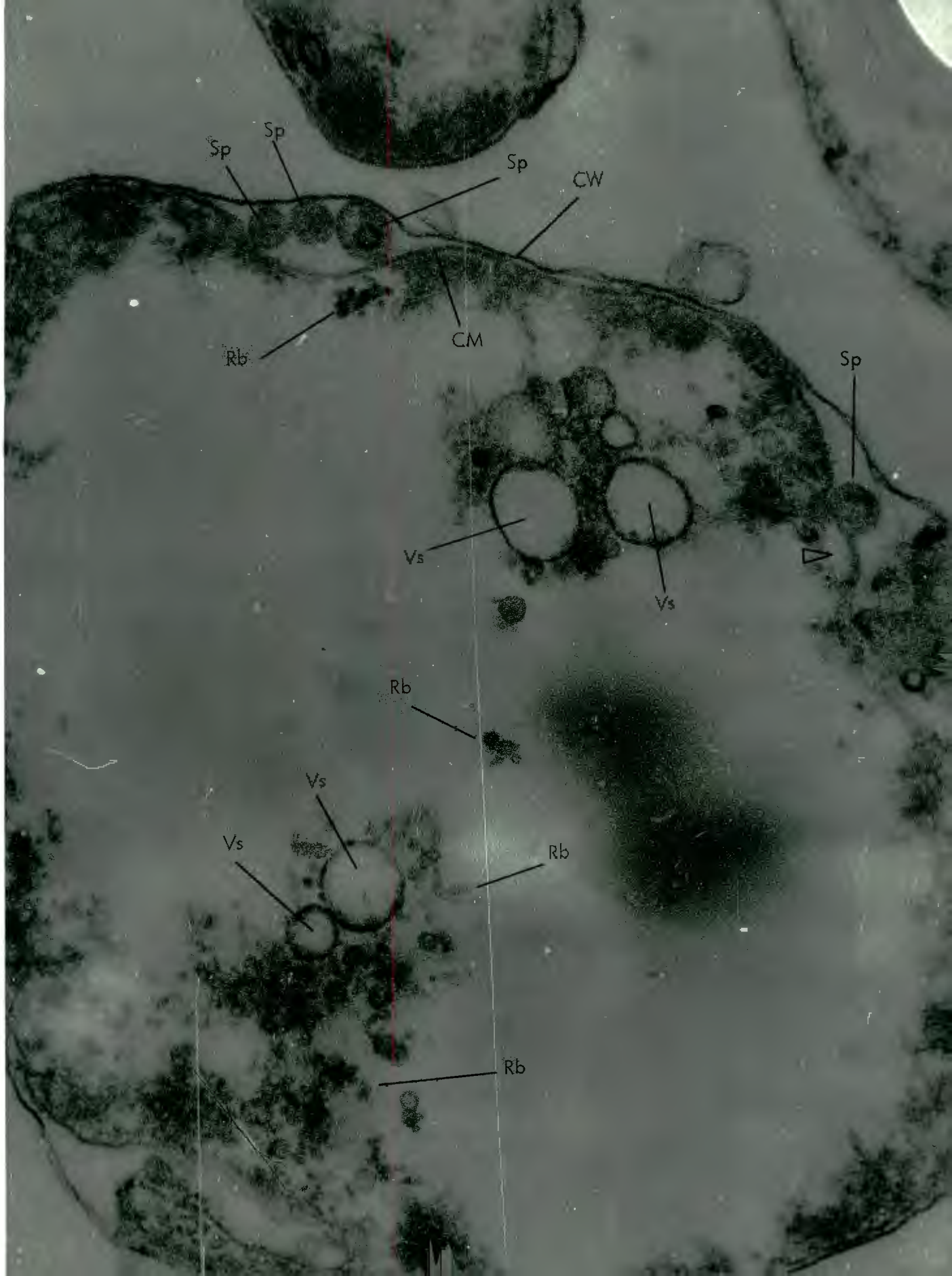


PLATE 35

Section of L-form cell in which numerous small cytoplasmic spherules are seen between the cell wall and the cytoplasmic membrane. Some of the spherules have ribosomes in them and one spherule (▷) has a vesicle inside it. There are a number of membrane-bound vesicles in the cell and aggregations of ribosomes are seen in association with them and also at the periphery of the cell. The cytoplasmic membrane is folded in at one place (▶). Electron micrograph X 101,330.

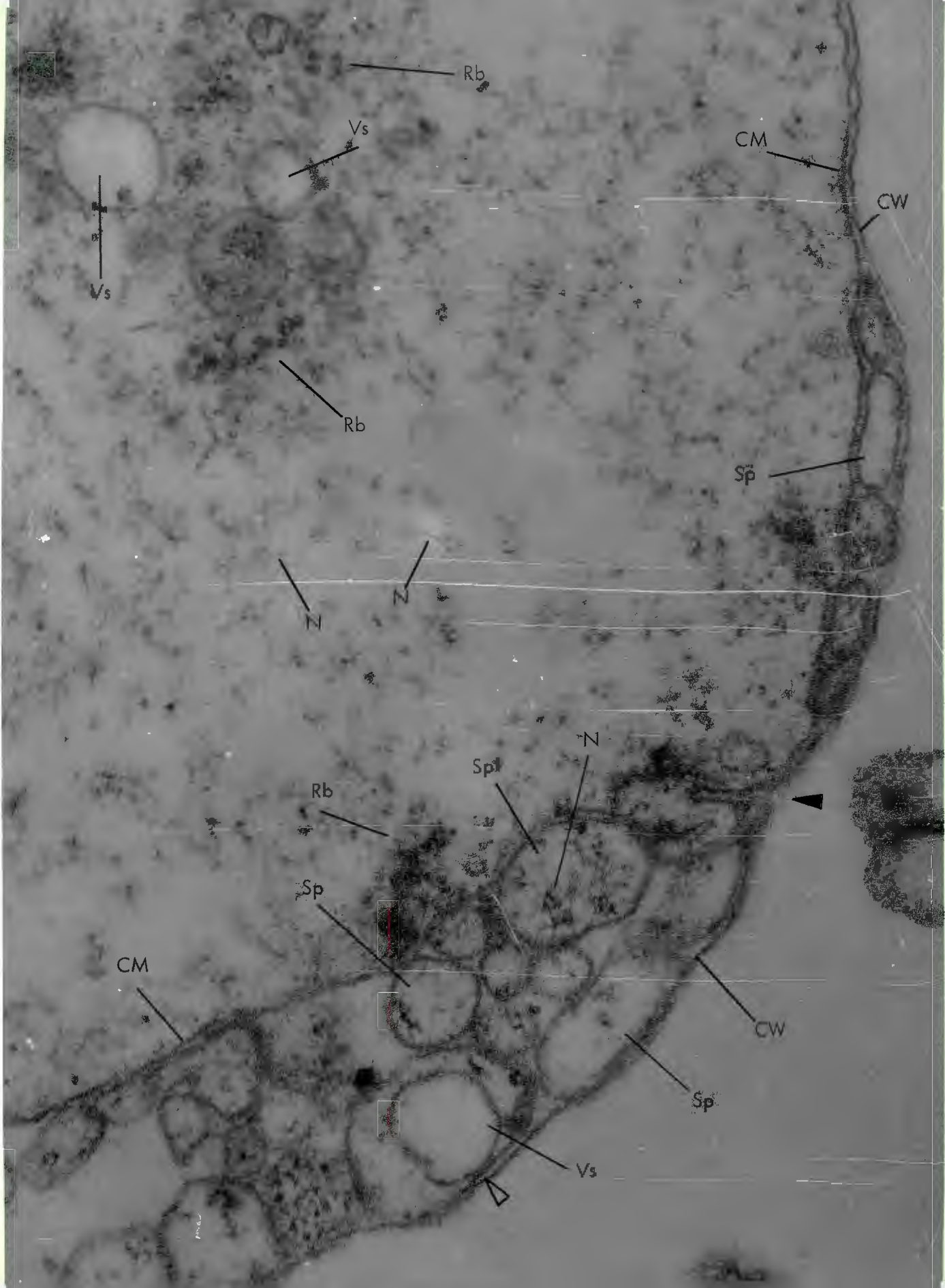
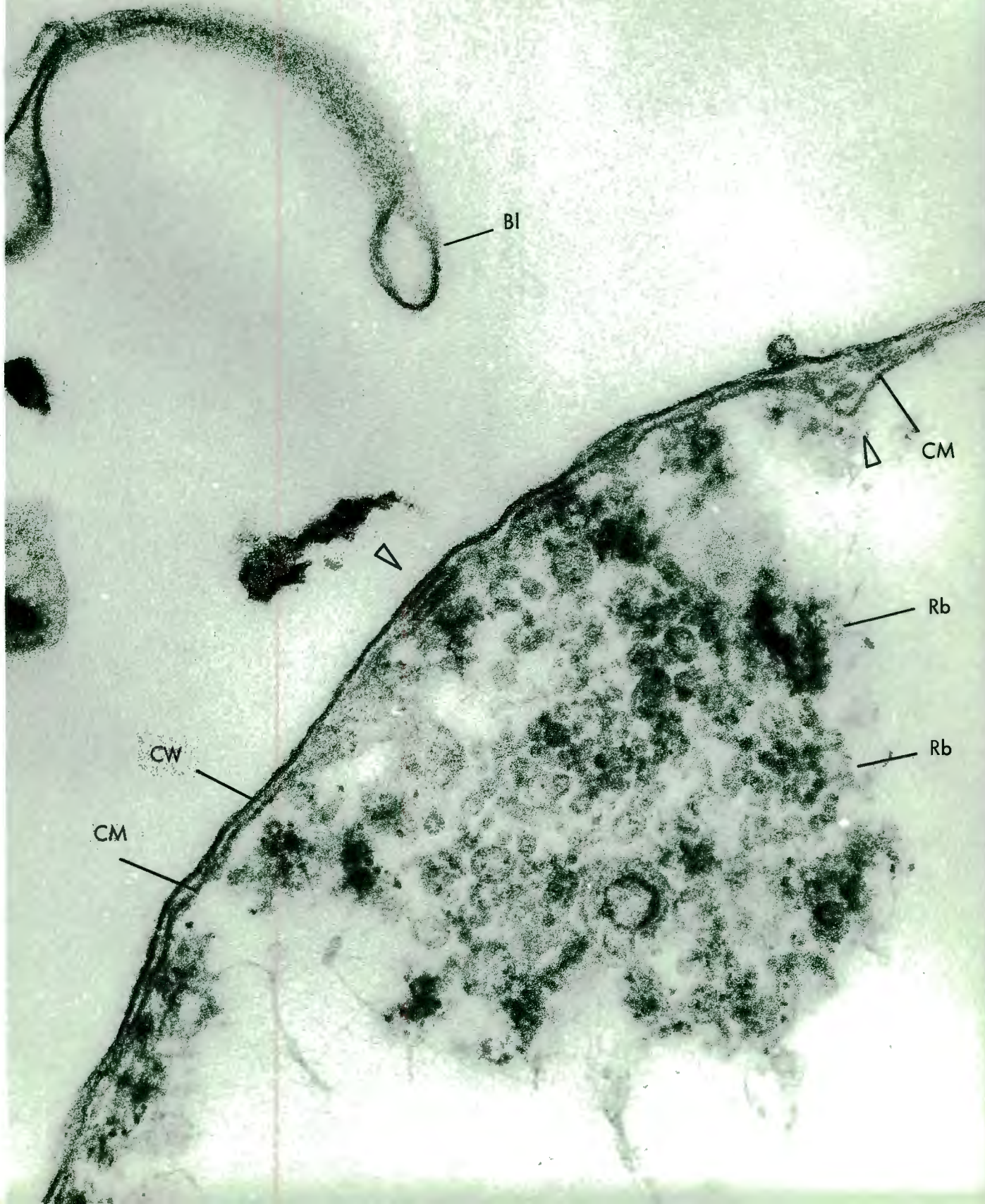


PLATE 36

Section of L-form cell showing early folding (▷) of the cytoplasmic membrane. Electron micrograph X 126,660.



BI

CM

Rb

Rb

CW

CM

PLATE 37

Section of L-form cells showing early stages of vesicle formation (▷). Also visible is an early stage of multiple membrane formation. Electron micrograph X 126,660.

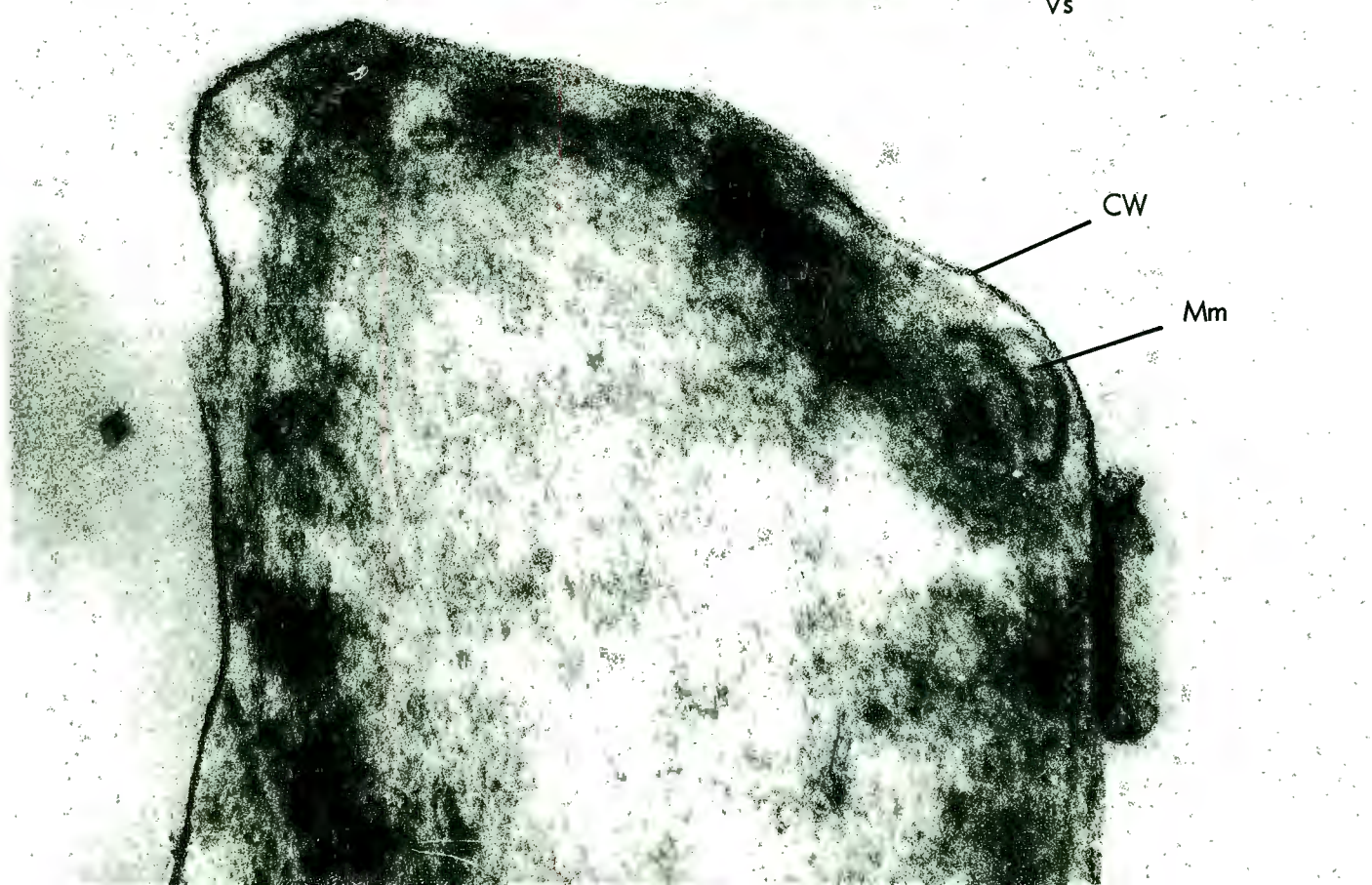
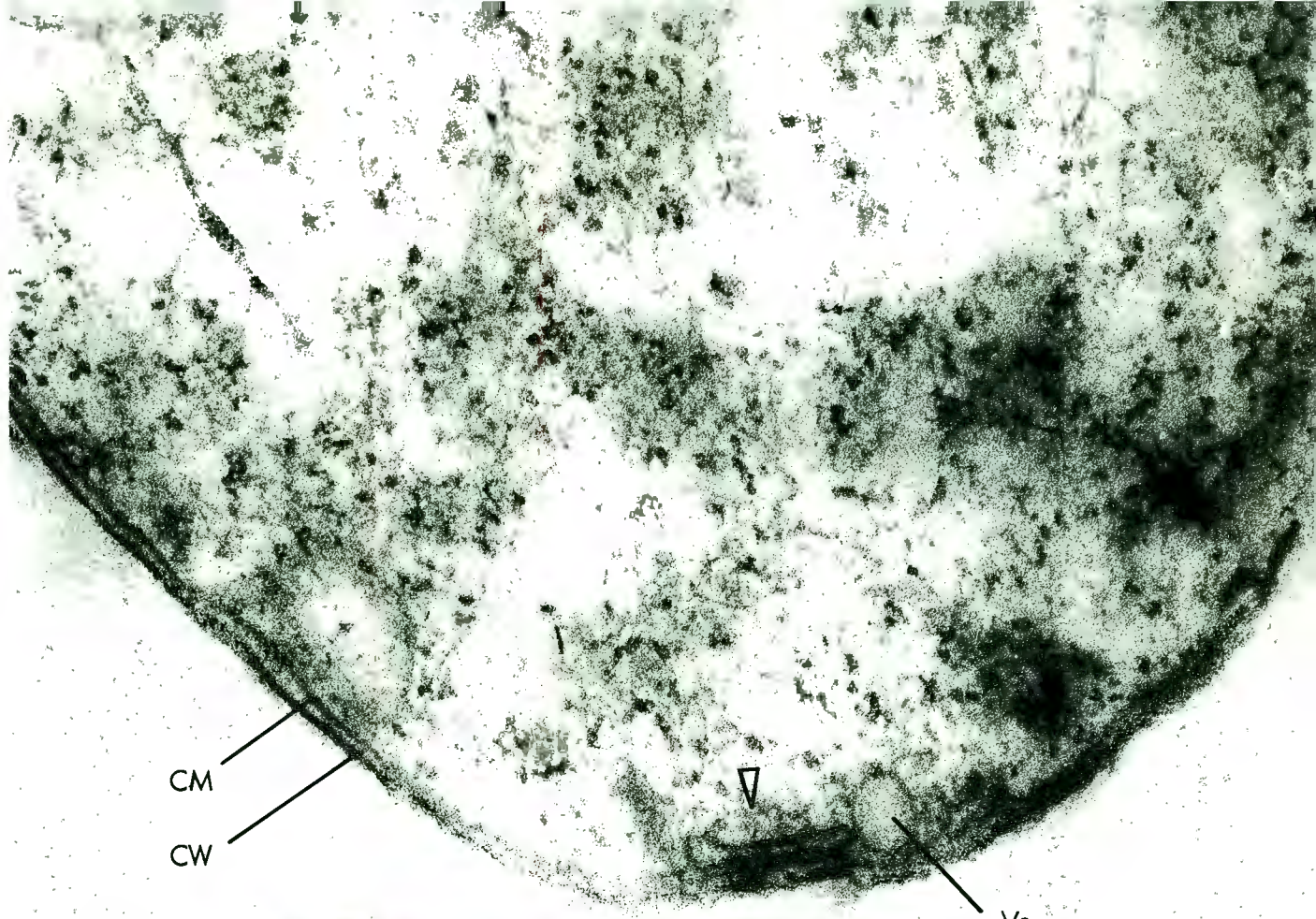


PLATE 38

Section of L-form cell showing early stage of vesicle formation (  $\blacktriangleright$  ).  
Electron micrograph X 101,330.

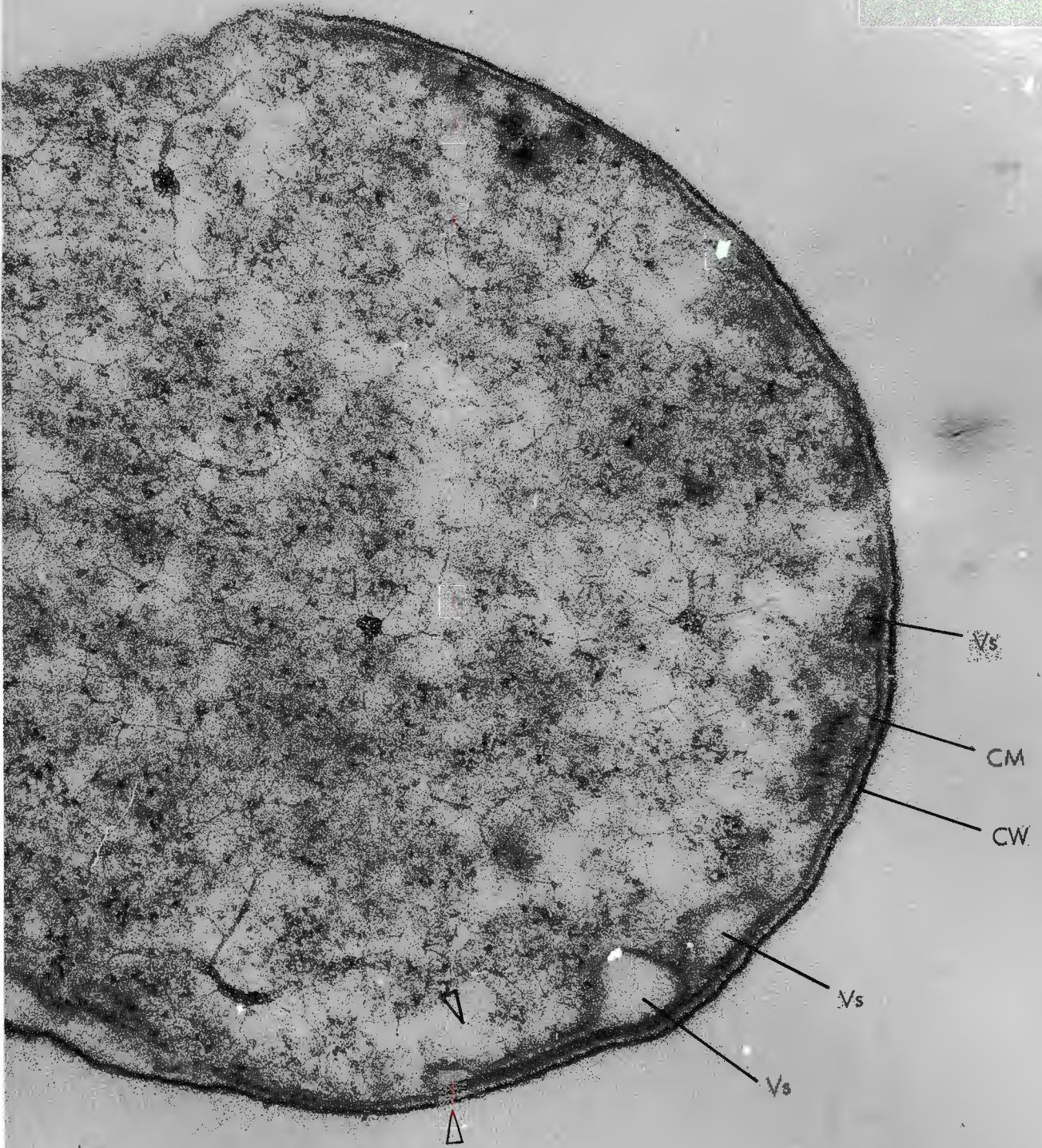


PLATE 39

Electron micrograph of L-form cell showing early stage of formation of cytoplasmic spherule (▷). Electron micrograph X 101,330.

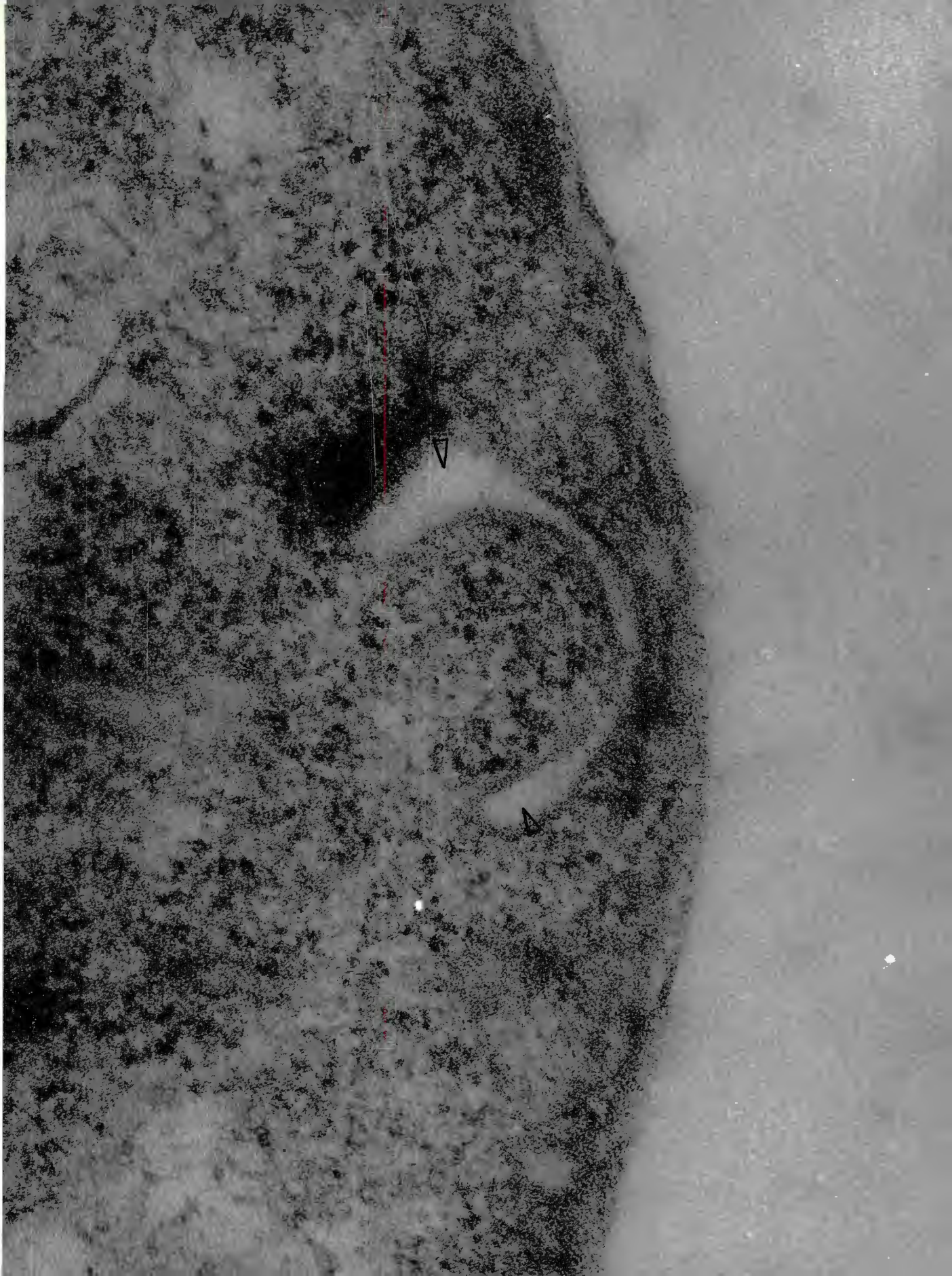
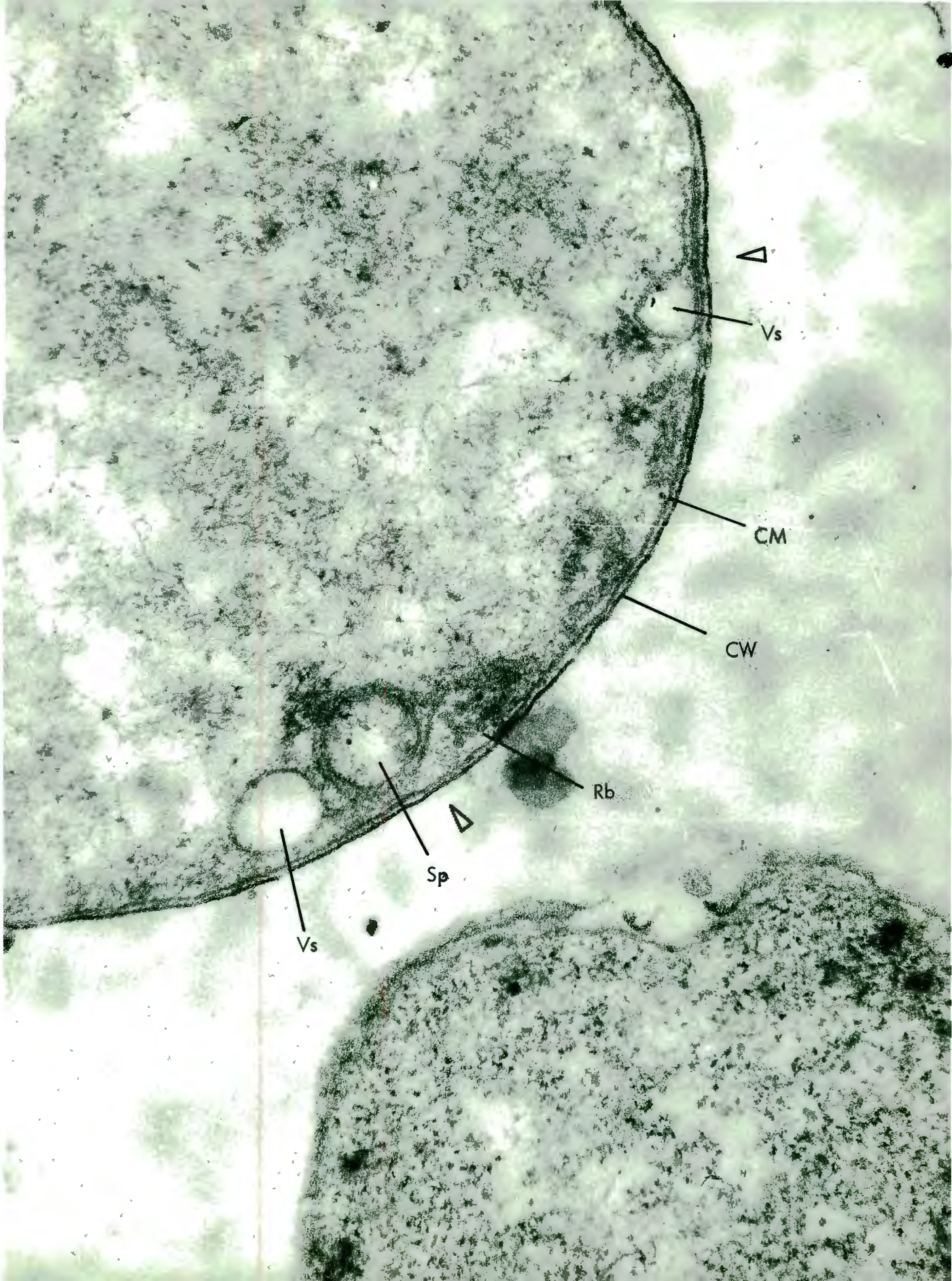


PLATE 40

Section of L-form cell showing stages of formation of cytoplasmic spherule (▷). Electron micrograph X 101,330.



Vs

CM

CW

Rb

Sp

Vs



PLATE 41

Section of L-form cell with partly disintegrated wall (▷). A number of small cytoplasmic spherules are to be seen. Electron micrograph X 78,660.

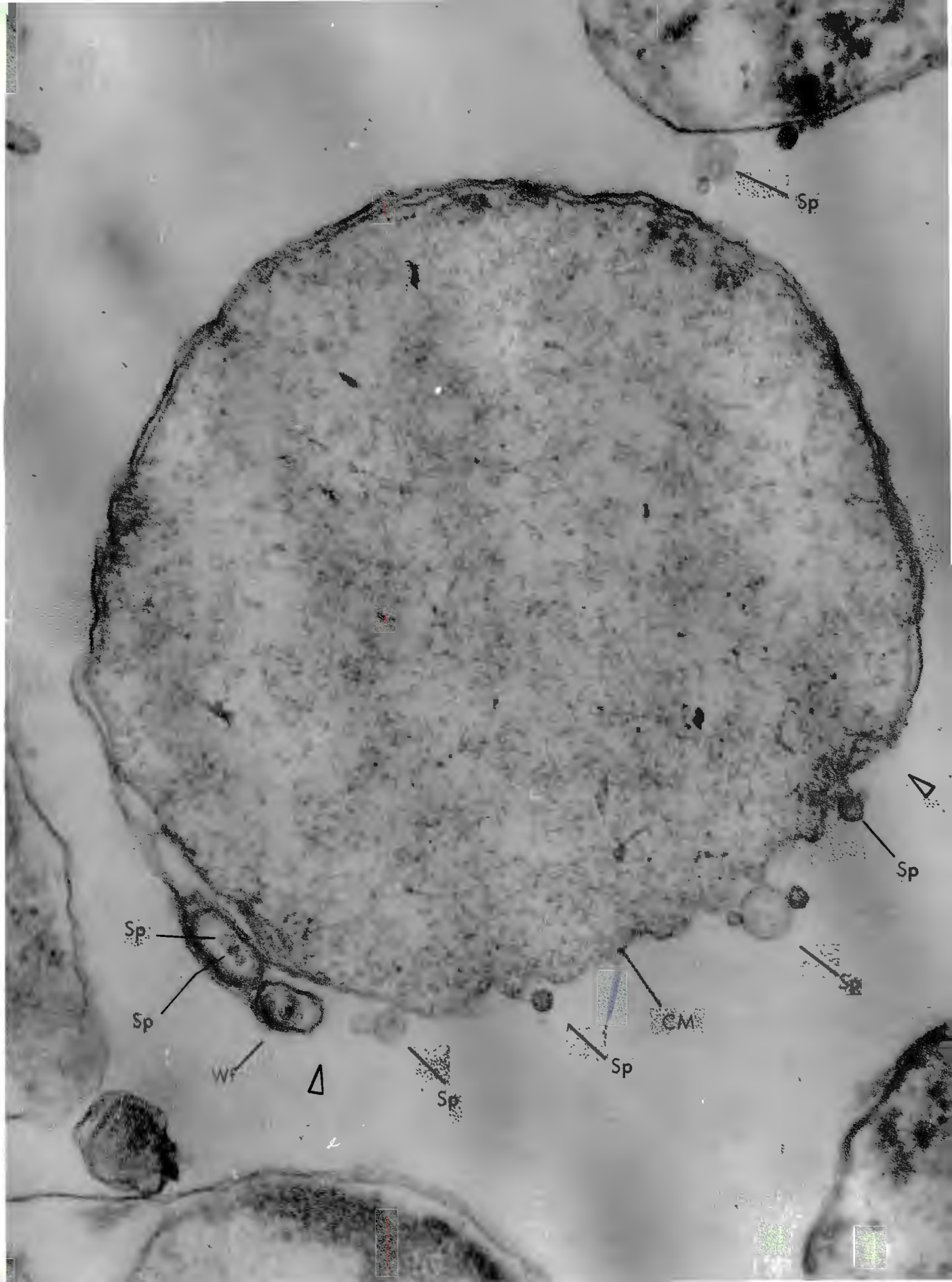
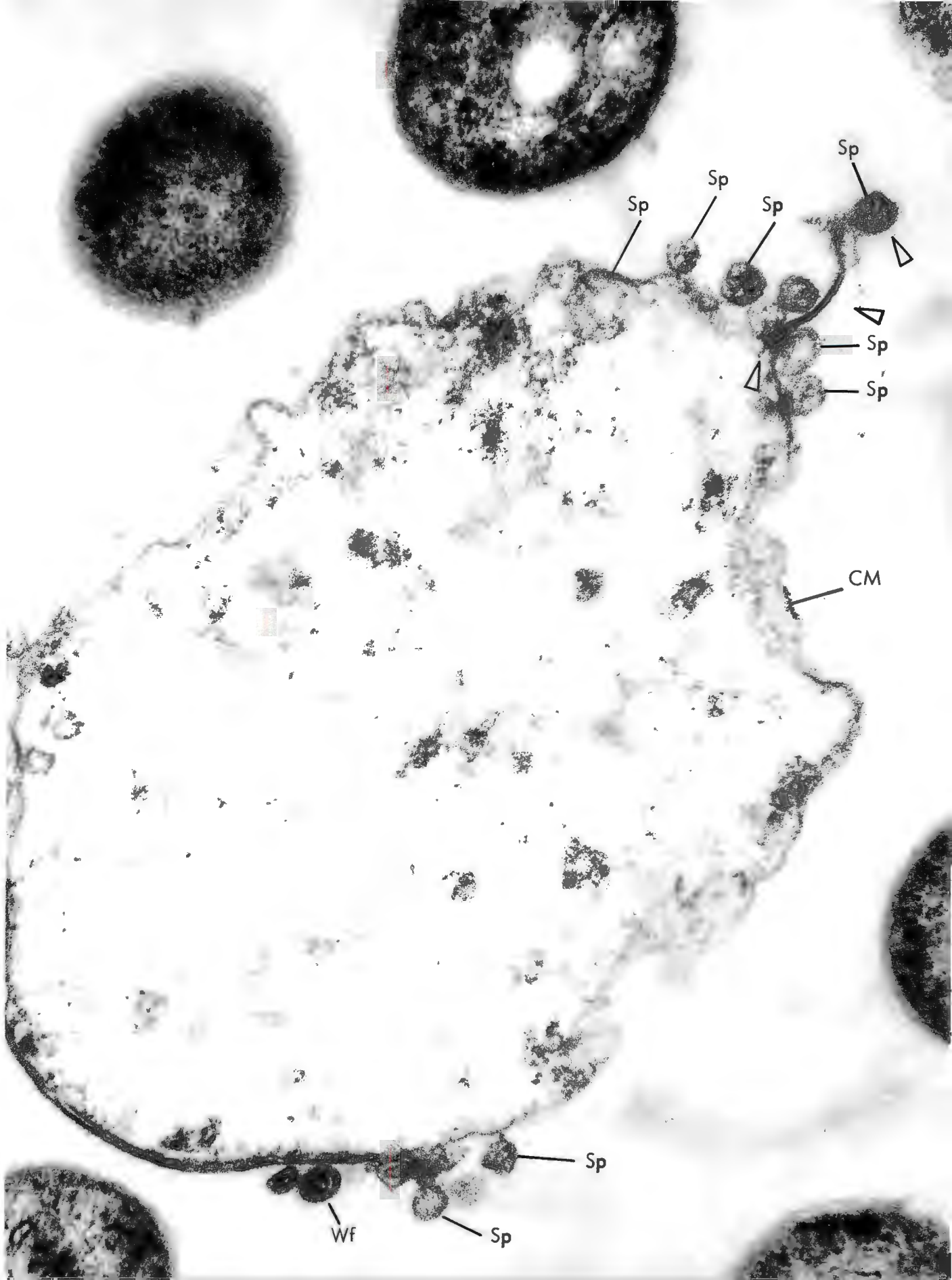


PLATE 42

Section of Epon-embedded glycine spheroplast. A number of small cytoplasmic spherules have been released from the cell as a result of disintegration of the wall. One spherule still appears to be attached to the protoplast (▷). cf. Plate 34. Electron micrograph X 78,660.



Sp

Sp

Sp

Sp

Sp

Sp

Sp

Sp

CM

Sp

WF

Sp

PLATE 43

Section of glycine spheroplasts in which a number of cytoplasmic spherules which have been released from the cells are seen. Electron micrograph X 78,660.

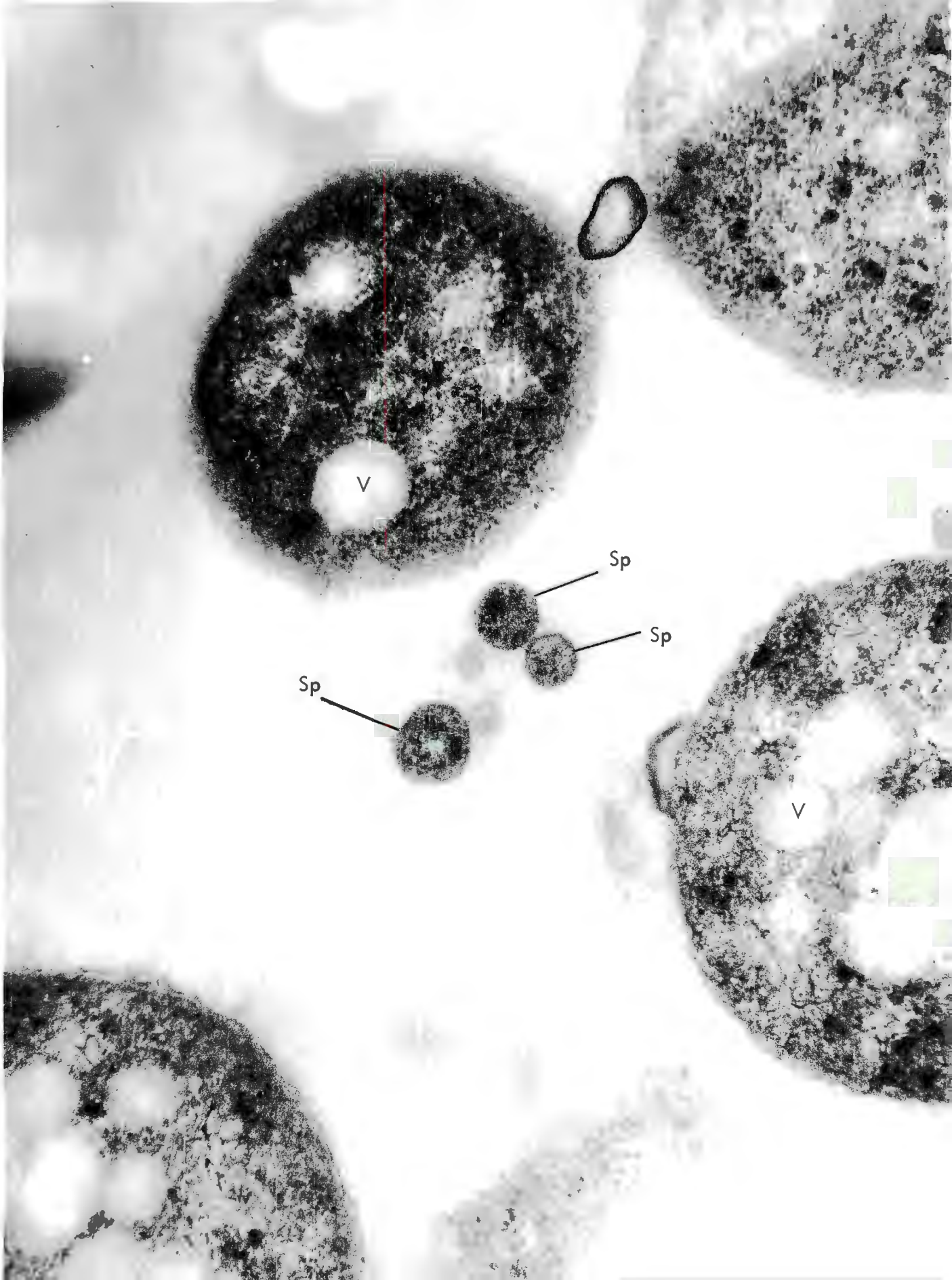


PLATE 44

Section of an L-form cell in which multiple membrane, both associated with the cytoplasmic membrane (▷) and of the vesicular type (▶) is seen.  
Electron micrograph X 45,660.

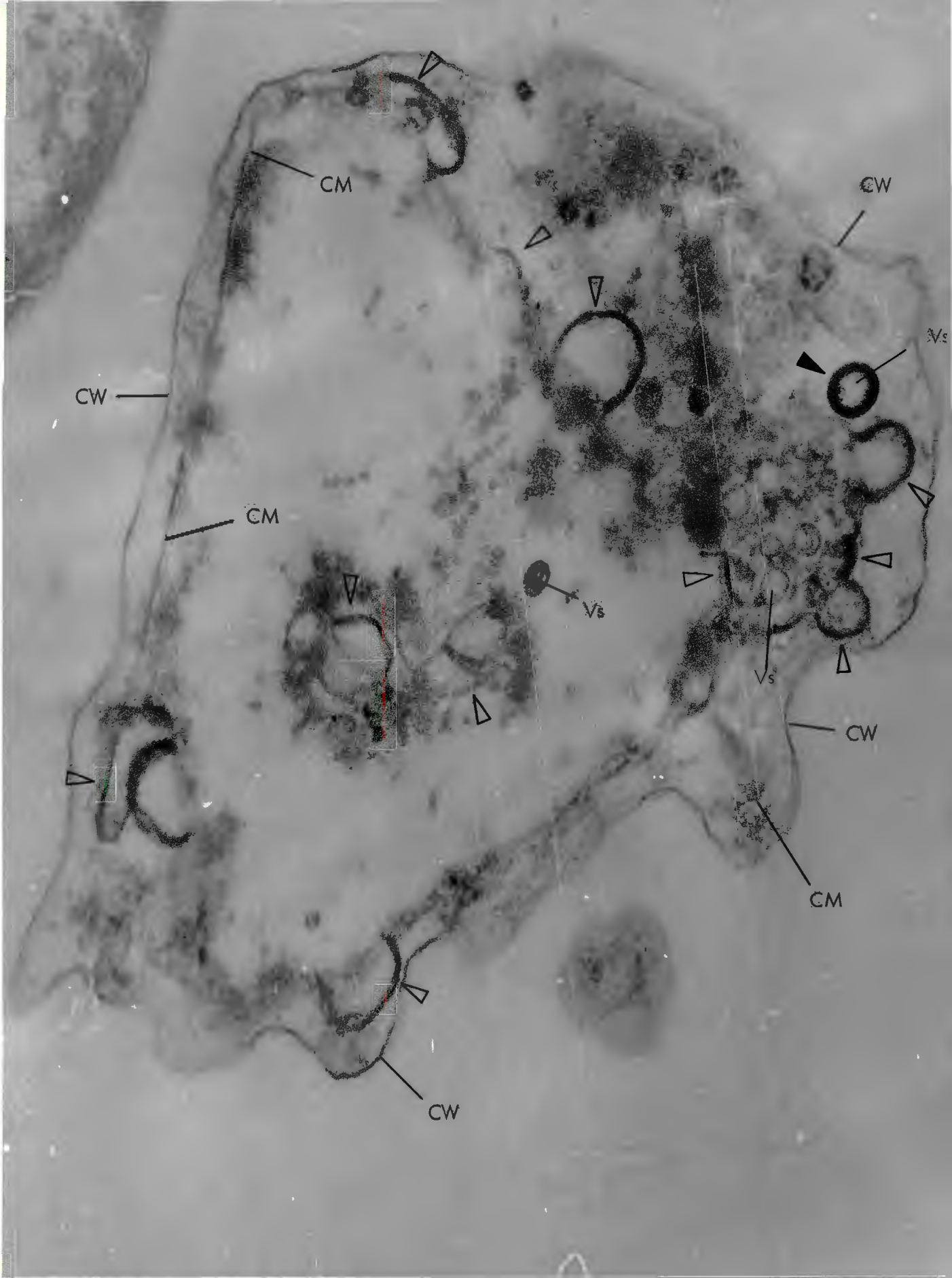


PLATE 45

Multiple membrane of the whorled type found in association with the cytoplasmic membrane of an L-form cell from luminescent liquid culture in NBS. Electron micrograph X 503,330, printed through a density mask.



PLATE 46

Vesicular multiple membrane seen in L-form cell. There are eight separate layers visible (▷). Electron micrograph X 264,000, unmasked print.

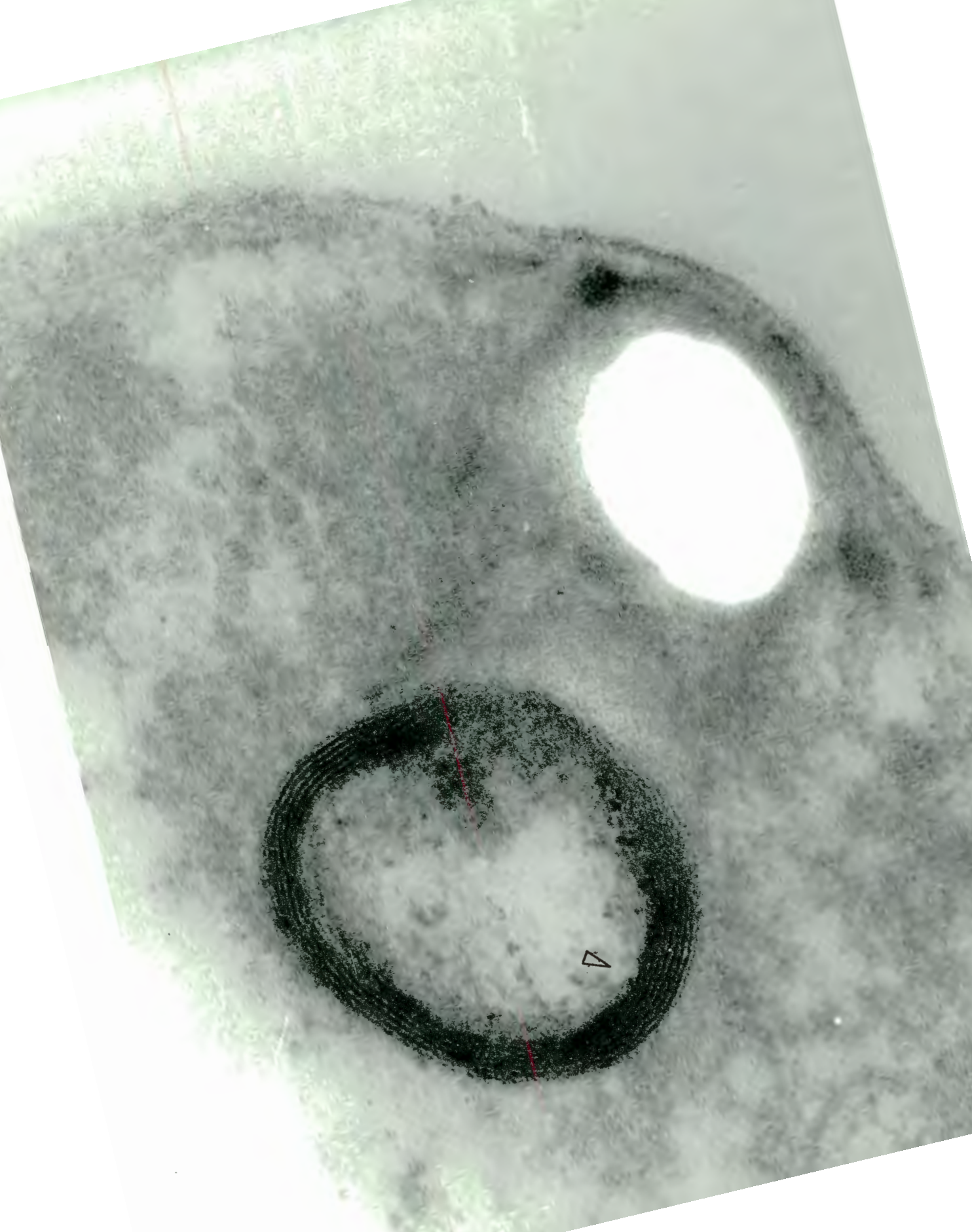
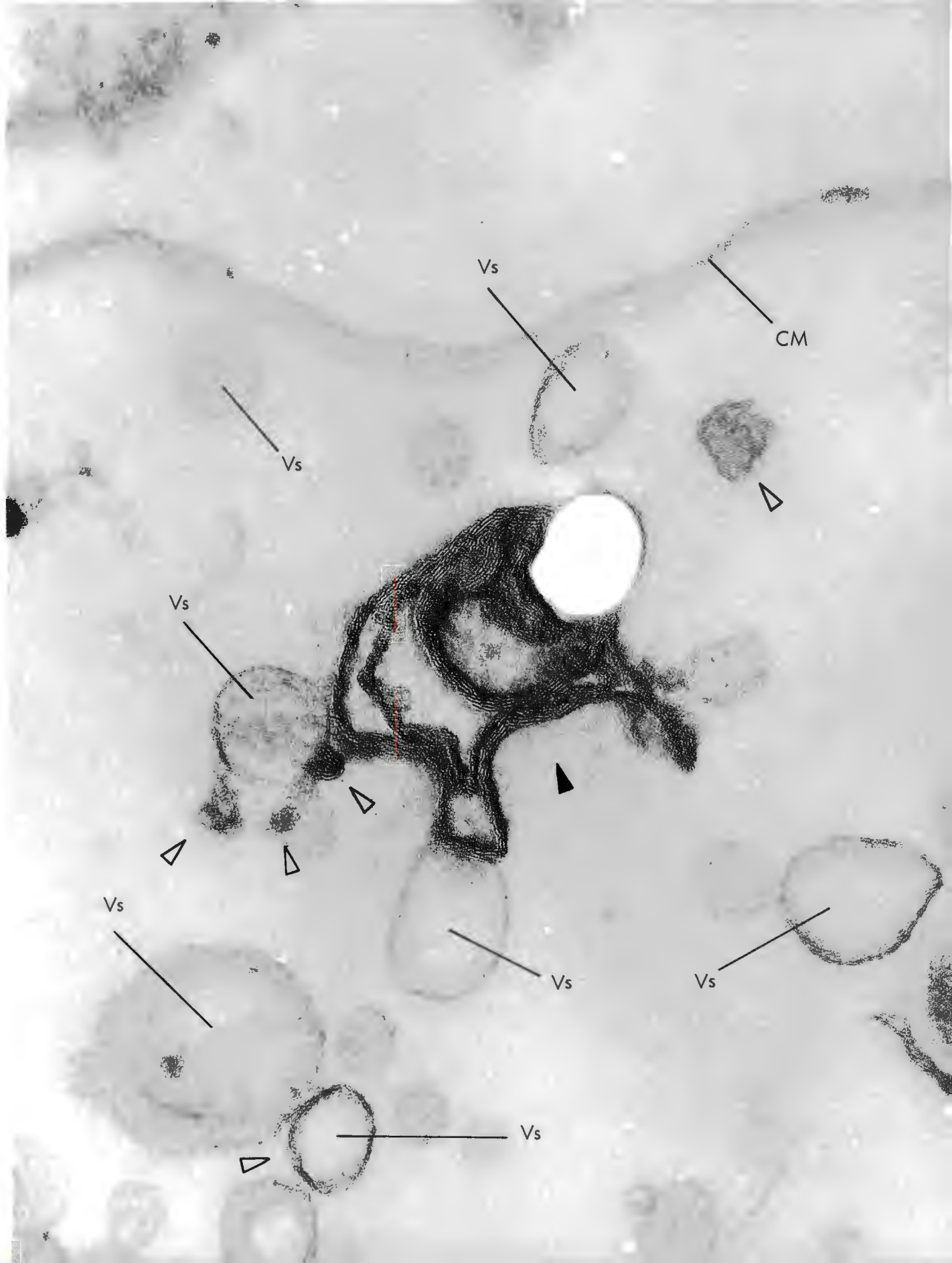


PLATE 47

Section of part of an L-form cell. A number of membrane-bound vesicles are shown at various stages of conversion to multiple membrane of the vesicular type (▷). The large whorled structure (▶) could have been derived from a vesicle. Electron micrograph X 78,660, printed through a density mask.



Vs

CM

Vs

Vs

Vs

Vs

Vs

Vs

PLATE 48

Typical field seen in Epon-embedded sections of glycine spheroplasts. Note the highly vacuolated state of the cytoplasm of these cells. One cell has a large bud-like structure (▷) and another has a bud and three small blebs (▶). Electron micrograph X 7,100.

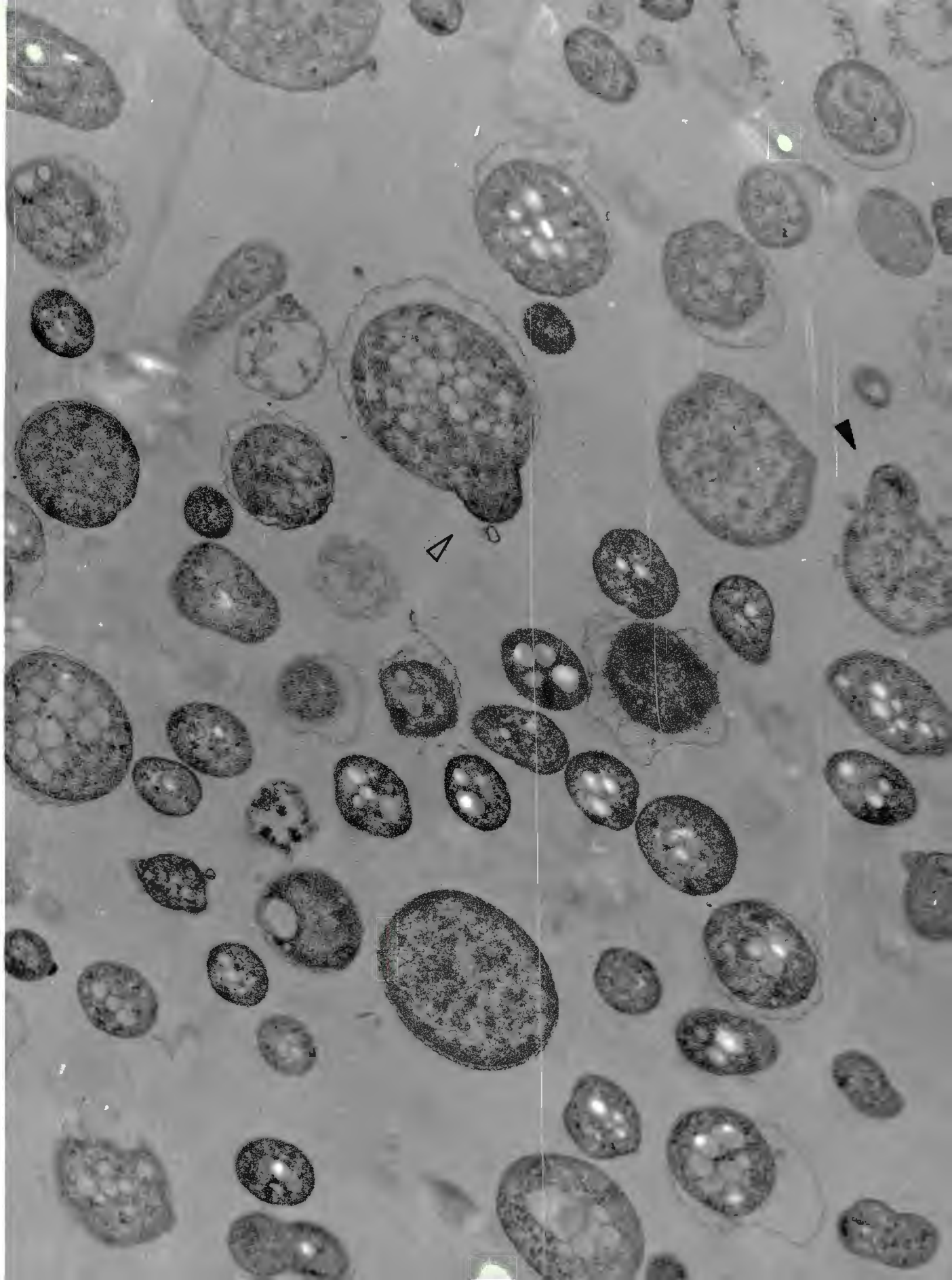


PLATE 49

Section of glycine spheroplast showing blebbing of the cell wall (▷).  
Note the highly vacuolated cytoplasm. Electron micrograph X 78,660.

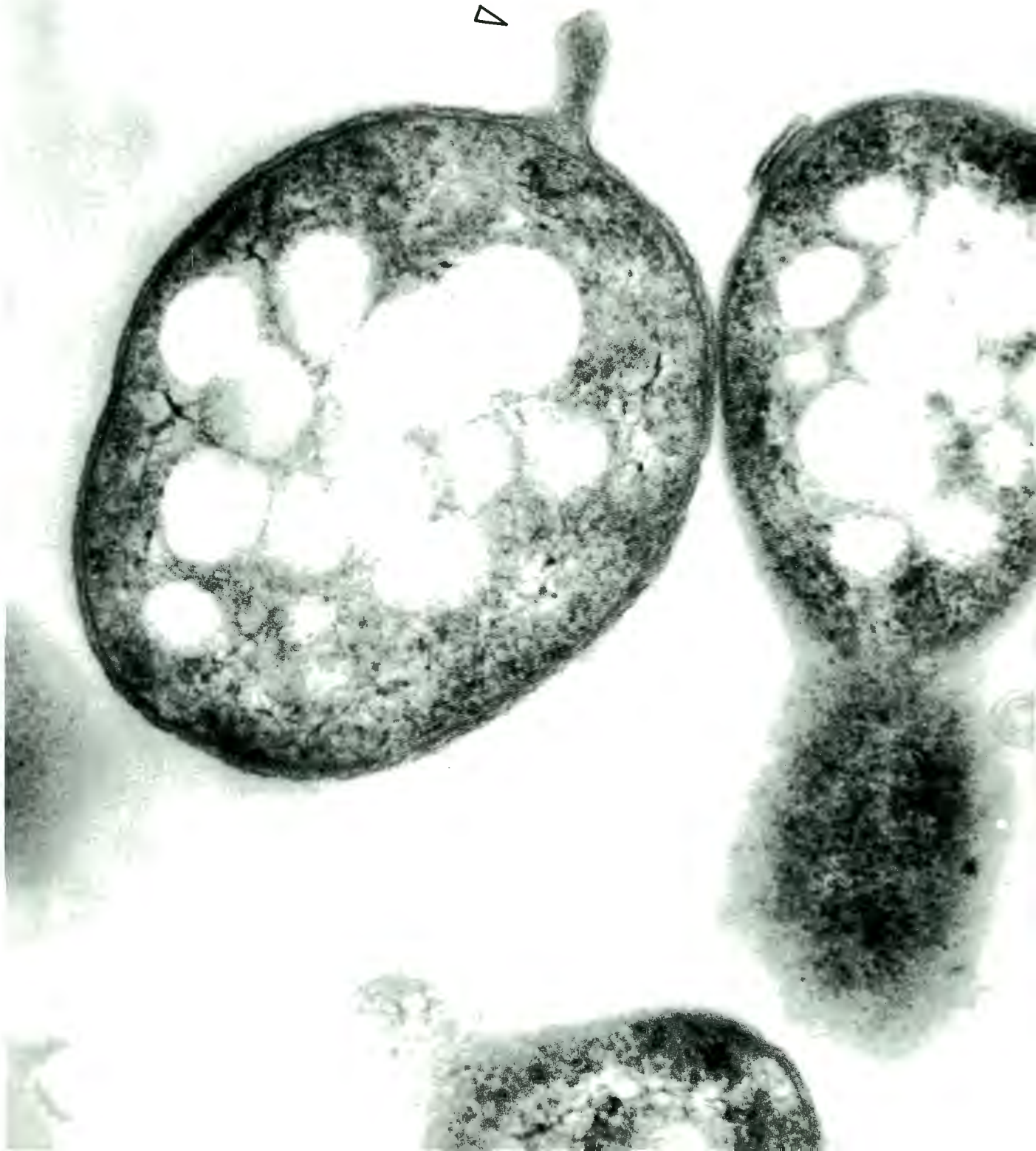
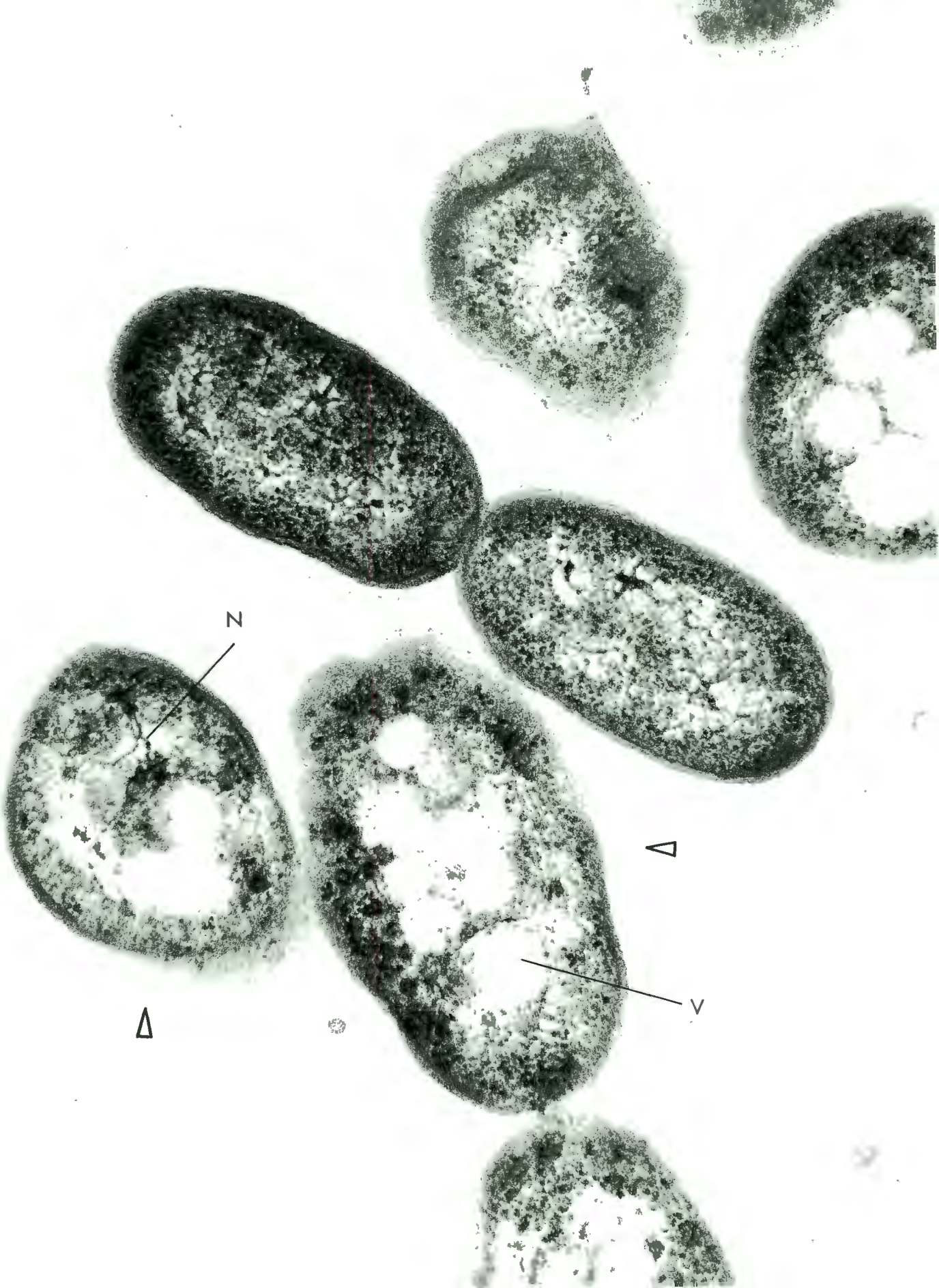


PLATE 50

Section of cells from glycine culture. Two normal bacilli are seen. The two cells (▷) below appear to be at an early stage of spheroplast formation. Electron micrograph X 57,660.



## KEY TO SYMBOLS ON PLATES

Bl	bleb
CM	cytoplasmic membrane
CW	cell wall
Fd	fold
Fg	flagellum
Lc	lysed cell
Mm	multiple membrane
N	DNA plasm
Pt	protoplast
Rb	ribosome (s)
Sc	spherical cell
Sp	spherule (s)
Tt	tetrad
V	vacuole
Vs	vesicle
Wf	wall fragment (s)