ABSTRACT

ULTRASTRUCTURAL AND HISTOCHEMICAL STUDIES OF MYCOPLASMA INFECTED TURKEY SINUS EPITHELIAL CELLS

by Grant Wayne Boam

Four-week-old turkey poults were injected into the infraorbital sinuses with 0.2 ml. broth culture of *Mycoplasma gallisepticum* S-6 strain. Infected and control birds were killed each week for 6 weeks.

The epithelial lining cells of the infraorbital sinuses were stained to demonstrate the activity of selected enzymes from the hydrolytic, proteolytic, and oxidative groups. Only enzymes characteristic of basic energy-generating pathways were found--succinic acid, isocitric acid, lactic acid, and malic acid dehydrogenase. Enzyme activity did not increase or decrease with infection, epithelial cell hyperplasia, or increase in host age from 5 to 10 weeks.

The mycoplasma-infected cells were also examined with an electron microscope. The organisms were found in large numbers during the first week of infection but were fewer in number by the sixth week. The infection was uncomplicated by secondary invaders during the first week but, by the sixth week, bacteria and fungal spores had appeared. Mycoplasmas were lying between cells and within cells, but few were free in the exudate. The nuclei were only minimally affected; they were apparently pressed toward the base of the cell. The mitochondria and ribosomal structures of infected cells disappeared from the cytoplasm. The cilia and microvilli were lost from the surface of heavily infected cells but not from adjacent uninfected cells.

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By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

The avian infraorbital sinuses are shallow cavities lined by pseudostratified, ciliated, columnar epithelium with abundant goblet cells. The epithelial cells of the mucous membrane rest on a thin basement membrane and lamina propria. Healthy sinuses are empty except for small amounts of mucus to moisten the surface.

Because of their anatomical location, the main stream of air does not pass over these epithelial surfaces during respiration. Since the surface lining cells do not actively participate in gas exchange, it seems reasonable to assume that the cellular metabolic activity would be little more than enough to maintain the biolotical integrity of the mucous membrane. With *Mycoplasma gallisepticum* infection in turkeys, however, extensive changes occur in the sinuses and lining membranes. The sinus fills with mucous exudate, the epithelial cells undergo hypertrophy and hyperplasia, and mononuclear inflammatory cells accumulate in the submucosa. There is little evidence of necrosis in the early stages.

The body of research work on this disease, its clinical appearance, histopathology, and treatment is extensive. The cultural, cellular and subcellular characteristics of the organism have also been widely studied.

The objectives of this study were to: (1) describe the pathogenesis of mycoplasma infection in the turkey sinus epithelial cell by use of the electron microscope, and (2) correlate the progress of the disease

with changes, if any, in the enzymatic composition of the cells. It is known that the stresses on cellular systems will cause induction of enzymes not normally produced by those cells, or toxins may cause destruction of the cell's capabilities to produce enzymes needed for metabolism.

These studies were conducted using young susceptible turkey poults with experimentally produced infections.

LITERATURE REVIEW

Avian Mycoplasmosis

History. A disease in turkeys similar to that which is now called chronic respiratory disease (CRD) or infectious turkey sinusitis (ITS) was first described by M'Fadyean (1893). Dodd (1905) made a more detailed description of a similar disease in 7 turkeys from one farm with epizootic pneumoenteritis. The first accurate description of CRD in the United States was made by Tyzzer (1926). He also suggested the use of argyrol (4% silver nitrate) injections into the sinus as a treatment.

Delaplane *et al.* (1933) reported a high incidence of a rapidly spreading respiratory disease in turkeys of the northeastern United States. It was not until 1938, however, that ITS was identified by Dickinson and Hinshaw as a separate disease distinguishing it from vitamin A deficiency and fowl coryza.

There are conflicting reports in the literature that the entire syndrome of avian upper respiratory disease was caused by a bacterium. Eliot and Lewis (1934) reported a "hemophilic" bacterium which is probably the same organism isolated by Page *et al.* (1963) and considered to be the causative agent of true fowl coryza.

Nelson (1935) described coccobacilliform bodies associated with sinus infections of turkeys. The next year he cultured a filterable organism in egg embryos and tissue culture (Nelson, 1936). This was

probably the same organism now known to cause CRD in turkeys and chickens described by Van Roekel and Gray (1957).

Chronic respiratory disease was named and described by Delaplane and Stuart in 1943. They suggested that the agent was viral-like in its characteristics.

Groupé *et al.* (1948, 1949) isolated the "turkey sinusitis organism" from chick embryos. Delaplane (1948, 1949) described the lesions produced in the chick embryos which he attributed to a virus. This work was verified and expanded by Chute (1953, 1954). He described hemorrhages of the skin of the embryo and of the amnionic and yolk sac membranes. The embryo was stunted in size and had enlargements of the joints of the legs, wings, and articulations of the mandible.

In 1952, Van Roekel *et al.* used all the cultural procedures, except the tissue culture method of Nelson, to study CRD. A pure culture of organisms from ITS was serially passaged in chicken eggs. When yolk cultures were injected into chicken sinuses a condition similar to sinusitis in turkeys was produced. Markaham and Wong (1952) succeeded in isolating pleuropneumonia-like organisms (PPLO) from exudates of chickens and turkeys with CRD using serum-enriched cell-free medium.

By 1960 it was well established that the organism causing CRD and ITS was the same (Van Roekel and Olesiuk, 1953; Osborn and Pomeroy, 1958; Adler, 1960a). Freundt (1960) classified the mycoplasma on the basis of morphology and species while Edward and Kanarck (1960) named the causative organism of ITS, *M. gallisepticum*. The most common strain used in research is the S-6 mutant of Zander (1961). Yoder and Hofstad (1964) reviewed the characterization of mycoplasma found in the avian species.

Mycoplasma gallisepticum is often found in pure culture in the sinuses; however, complex infection with multiple microbial agents has

been reported (Biddle and Cover, 1957; Adler *et al.*, 1962; Blake, 1962; Glantz, 1962).

Culture. After the initial cell-free culture of *M. gallisepticum* by Markaham and Wong (1952), many reports of successful propagation of organisms on artificial media began to appear in the literature (Grumbles *et al.*, 1953; Adler *et al.*, 1954; Hofstad and Doerr, 1956; Taylor and Fabricant, 1957, 1958; Adler and Berg, 1960). The organisms cultivated on artificial media were used for serologic studies and early investigations for vaccine production (Yamamoto and Adler, 1958; Hall, 1962). They were used for biochemical and metabolic studies, and also to study growth and reproduction (Ruys *et al.*, 1967; Razin *et al.*, 1967b).

Hayflick and Stinebring (1960) and Hayflick (1965) reported the growth and appearance of mycoplasma in tissue culture. The mycoplasma species infecting cell cultures have often led to misinterpretation of data if their presence was unknown.

Dmochowski *et al.* (1967) described cultural characteristics on cell-free media and pleomorphism of these organisms by electron microscopic examination.

Serology. Jungherr *et al.* (1953) and Fahey *et al.* (1954) first used serological studies to diagnose CRD. Gianforte *et al.* (1955) and Moore *et al.* (1960) are only 2 of the many research groups that have studied the development of the disease in infected birds with serum assays. The Salsbury Laboratories* have developed a commercially available plate test antigen for mycoplasma screening.

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*Salsbury Laboratories, Charles City, Iowa.

Many workers both in the laboratory and in field trials (Fabricant and Levine, 1953; Olsen *et al.*, 1964; Olsen *et al.*, 1962; McMartin and Adler, 1961; Adler *et al.*, 1960; Adler, 1960b; Domermuth, 1962; Domermuth, 1957; Olesiuk and Van Roekel, 1960) have demonstrated an immune response after infection with *M. gallisepticum*. From this work it is evident that the strain of organisms used, the number of organisms, and the route of inoculation were all important factors in quantitating the response. Adler *et al.* (1960) suggested the experimental infection of young poults to build a life-long immunity. Luginbuhl *et al.* (1967) studied the advantages and disadvantages of infecting young birds. He reported vaccine efficacy, carrier state, passive antibody transfer to the young bird and persistence of antibody, and concluded that limited use of vaccination was advisable.

Transmission. Experimentally the disease can be produced by exposing young animals to the mycoplasma by unnatural routes, such as swabbing the trachea, intramuscular injection, and others (Jerstad *et al.*, 1950). It is generally believed that natural transmissions of the organisms are airborne, which may be influenced by climatic conditions (Fahey and Crawley, 1955). Transmission of the mycoplasma through the egg is possible but inconsistent (Jerstad *et al.*, 1959a,b). Abbot (1960) and Kumar (1963) have made additional reports of egg transmission and have stressed the economic importance of this mode of transmission in preventing eradication. Several methods of killing the organism in the egg have been suggested (Mataney *et al.*, 1955). Hoyt *et al.* (1952) used sulfamethazine, crystal violet and antibiotics.

Morowitz and Maniloff (1967) described the life cycle of the organism in and out of the host animal. Osborn and Pomeroy (1958) list turkeys,

chickens, ducks, pheasants, guinea fowl, pigeons, partridges, peacocks, and cotton rats as host species of *M. gallisepticum* with Swiss mice and Norway rats refractory. Madden *et al.* (1967) isolated *M. gallisepticum* from a Bob-White quail.

Electron microscopy. The electron microscopic reports on *M. gallisepticum* describe the organism and its structure (White *et al.*, 1954; Morowitz *et al.*, 1962; Dmochowski *et al.*, 1967; Maniloff *et al.*, 1965), its colonial growth (Shifrine *et al.*, 1962) and the structures of the organism as they relate to the function (Maniloff and Morowitz, 1967; Razin, 1967a). Reagan (1953) compared subcellular characteristics of 4 strains of CRD agents. A general electron microscopic survey of mycoplasma species was made by Domermuth *et al.* (1964). Edwards and Fogh (1960) studied the fine structure of mycoplasma in tissue culture and Chu and Horne (1967) compared several mycoplasma species with similar appearing viruses.

Histochemistry. Pollack *et al.* (1965a,b) described a staining procedure to localize enzymes in the mycoplasma organism. Rottems and Razin (1966) localized adenosine triphosphatase activity in the mycoplasma membrane by a colorimetric method. Adenosine triphosphatase participates in the utilization of energy to transport nutrients from the media across the membrane. Munkres and Wachtel (1967) localized acid and alkaline phosphatase inside the organism associated with the metabolism of phospholipids.

Pathology. The changes in tissues associated with the invasion of mycoplasma were described before the causative agent was isolated. An extensive, detailed report describing the pathogenesis of ITS by light microscopy was published by Jungherr (1948). He also described the air sacculitis

and pneumonia which often accompany the sinusitis. Hitchner (1949) described the histopathology in birds of different ages and duration of infection. Barber (1962) traced the progressive development of lymphofollicular nodules first described by Nelson (1935) and assumed by Jungherr (1949) to be pathognomonic for ITS. Cordy and Adler (1957) isolated mycoplasmas from the brains of turkeys and believed them to be the cause of encephalitis.

Treatment and control. Many antibiotics have been employed in eggs and birds to treat CRD. Aureomycin (Lecce and Sperling, 1955), chloromycetin, streptomyin, tetracycline (Domermuth, 1958) and terramycin (Lecce and Sperling, 1955; Yamamoto and Adler, 1956) were found to be effective. Benton and Cover (1958) and Domermuth (1958) found nitrofuran compounds effective. Sulfonamides have been found effective only in the egg (Wong and James, 1953). Gale *et al.* (1967) found tylosin effective against combinations of infections. The addition of sodium sulfate to the water as a drug potentiator increases the blood level of chlortetracycline. Gale and Baughn (1964) found that this increase in the blood level did in fact increase the therapeutic effectiveness of chlortetracycline against experimental mycoplasma infections. The first effective treatment for CRD was 4% silver nitrate described by Tyzzer (1926) when he first recognized the disease in the United States.

Development of Electron Microscopy

The construction of the first crude light microscope in the 16th century revealed new and unexplored worlds. Although these developments opened a vast field of exploration to the scientist, the factor restricting the study of the ultrastructure of cells and tissues was the fundamental nature of light, which imposes a physical limit to the resolution

This problem was partially alleviated in the 20th century by the development of workable electron microscopes.

The biologist has now been given visual access to worlds between the cellular and molecular levels. This new vision spans 3 orders of magnitude (10^{-4} to 10^{-7} cm) which, when explored, must be interpreted and correlated with findings of light microscopy, gross examination of the entire organism, and biochemistry.

The light microscope reached the peak of its development more than 2 centuries after its invention; however, the electron microscope reached a comparable level in the short span of 2 decades. In the past 50 years it has been possible to examine and evaluate a vast cellular and histological realm by light microscope. It will take a far greater time to record and interpret the information made visible on the ultrastructural level.

Theoretical. Principles of physics used in today's electron microscopes were understood in the latter part of the 19th century. However, it was not until de Broglie's theory (1924) that moving electrons can be assigned very short wave lengths and Busch (1926) demonstrated that suitably shaped magnetic or electrostatic fields could be used as true lenses for an electron beam, that satisfactory progress was made in constructing a functional electron microscope.

Ruedenberg applied for the first patents on an electron microscope in which the principles of physics and electronics known at that time were incorporated. As it turned out, the instrument was more descriptive than functional, but the evolution of electron microscopy had begun (Wischnitzer, 1962).

Beuche and Johnson (1932) built an electrostatic instrument which was designed to produce an enlargement of electron emitting source.

The prototype microscope incorporating the principles currently used in design was built by Knoll and Ruska (1932). Their instrument utilized an electron transmission gun which produced electron photomicrographs of an enlarged illuminated specimen (Ruska, 1932).

Driest and Muller (1935), using Ruska's instrument, first demonstrated resolving powers greater than the light microscope. Von Borries and Rusaka (1939) constructed an electron microscope, capable of resolving 100 Å, which was built and marketed on a commercial basis in 1939. Sophistication of the instrument was rapid. Von Ardenne (1944) achieved resolving powers of 12-15 Å and Hillier (1946) demonstrated 10 Å resolving power. Hillier and Ramberg (1947) developed the compensating objective that improved image quality by eliminating astigmatism in imperfect magnetic lenses.

In the span of 20 years the science of electron microscopy has been refined to a point that today's microscopes possess resolution capabilities which border on theoretical limits.

Staining

Uranyl acetate. Watson (1958) was the first investigator to explore the use of uranyl acetate as an electron stain. It was at first considered a stain with little specificity because proteins will stain fairly intensely with uranyl acetate but cytomembranes do not stain as well. It does not produce the vivid pictures that lead does.

Huxley and Zubay (1961) have demonstrated that DNA binds a specific amount of uranyl acetate (approximately equivalent to its own dry weight). Marinozzi and Gautier (1962) also emphasized the specific binding of

uranyl acetate with nucleoprotein, the exact character of the reaction depending in part upon the fixation employed. It is now generally agreed that uranyl acetate is a specific DNA nucleoprotein stain.

Lead. An interpretation of alkaline lead differential staining was made by Marinozzi (1963). He concluded that lead reacts with reduced compounds such as osmium which may be present in the sections. Lead is bound specifically to RNA in the section by other reactions. Thus, after osmium tetroxide fixation, alkaline lead compounds act as a general stain, whereas after formalin fixation they become RNA specific stains.

Daems and Persijn (1963) have postulated at least 3 mechanisms of attachment of lead to reactive groups. First, lead is attached to cytomembranes; this reaction requires the presence of negatively charged reduced osmium on the polar groups of the phosphatides. Second, staining of glycogen is based on the chelation of lead by hydroxyl groups of carbohydrates. Finally, nucleoprotein staining has a preference for RNA.

The combination of uranyl acetate and lead hydroxide is commonly used today to stain aldehyde-fixed tissues.

Histochemistry of Turkey Sinus

There is a vast amount of work published on the histochemical reaction of various tissues and cells. Pearse (1958) described some of the applications of histochemistry in cellular pathology. There is no literature known to this author concerning the enzyme histochemistry of the normal or infected turkey sinus. In an attempt to anticipate what could be expected to happen in the turkey sinus because of mycoplasma infection, similar investigative work in mammals was reviewed.

In the presence of an infection with *Mycobacterium paratuberculosis*, Merkel *et al.* (1968) reported an increased activity of alkaline

phosphatase in the tissue cells, while lesions and bacillary products caused an increase in acid phosphatase and esterase activity in the macrophages.

The enzymatic response in tumors may be dependent on size of growth as shown by Loeb (1965), who studied the intensities of 4 enzyme-catalyzed reactions in 56 neoplastic tissues of the dog. He found that intensities of reactions in neoplastic tissue were in fairly good agreement with enzyme-catalyzed reactions in their tissues of origin. In addition, intensities of the 4 enzyme-catalyzed reactions were highest in small tumors and lowest in large tumors. The mean value of tissue lactic dehydrogenase activity was significantly higher in malignant tumors than in benign tumors. This was thought to be from an increased synthesis of the enzyme by the tumor. A second explanation not mentioned by Loeb cannot be ruled out, which is that storage of the enzyme in malignant tissues may be enhanced as a result of autolytic changes found in neoplastic tissues. A high activity of tumor tissue phosphohexose isomerase correlated closely with the property of invasiveness of tumors. Alkaline phosphatase was higher in osteogenic sarcomas than in other neoplasms.

Activities of 5 lysosomal enzymes were investigated in 4- to 6-week-old normal lambs and lambs suffering from white muscle disease which results in tissue necrosis (Desai, 1966). In all instances, enzyme activity was higher in dystrophic muscle than in normal muscle with the increase being 35-fold for aryl sulphatase, 5-fold for B-galactosidase and cathepsin, and 2-fold for acid phosphatase.

Hydrolytic enzymes. This group of enzymes includes esterases that catalyze the cleavage of phosphoric acid esters.

Alkaline phosphatase. Phosphate esters, such as glycerophosphate, glucose-1-phosphate, creatinine phosphate, and the nucleotide of adenosine triphosphate, are split by alkaline phosphate activity (Gomori, 1949; Feigin and Wolfe, 1955; Friedenwald and Gryler, 1958). Alkaline phosphatases hydrolyze most orthophosphomonoesters at an optimum pH of 9.0 to 9.6.

Many authors feel alkaline phosphatase is composed of several enzymes which are active upon closely related substrates with optimal activity at alkaline pH (Emmel, 1950; Burgos *et al.*, 1954, 1955). The function of both acid and alkaline phosphate is the dephosphorylation necessary for absorption, transport and metabolic control. They may be active in some ester synthesis and maintenance of intracellular inorganic phosphate sufficient for osteogenesis (Morton, 1961). There are many reports of alkaline phosphatase localized in epithelial lining cells of mammals (Shuitka, 1960; Padukula *et al.*, 1961; Pearse and Riecken, 1967; Maronpot and Whitehair, 1967; Thake, 1968).

Acid phosphatase. There are several enzymes in this group. Phosphomonoesterase II and nonspecific acid phosphatase hydrolyze several phosphoric acid esters at an optimum pH of 5.0 (Barka, 1960; Barka, 1961). Acid phosphatases are widely distributed in animal tissues. Gutman (1938, 1940, 1941) extensively studied the prostatic acid phosphatase and proposed possible functions. Until recently, however, histochemists have tended to overlook the importance of this group of enzymes. Fennell (1966) reported a marked increase in acid phosphatase in chicken cells undergoing necrosis from tissue graft rejection.

Burstone (1961) developed a cytochemical method which couples alpha-naphthal Azo dye to demonstrate hydrolysis of phosphoamides. Improvements of this method are widely used today.

Proteolytic enzymes. Proteolytic enzymes have 2 major subgroups, the proteases and the peptidases. The protease subgroup hydrolyzes peptide bonds between specific amino acid combinations in the protein chain. Peptidases hydrolyze only the terminal bonds of the protein chain. They are specific for either -n- or -c- terminal bondings.

Leucine aminopeptidase. This enzyme is a peptidase (exopeptidase) which requires an n- terminal free α-amino acid group for its action. The aminopeptidase acts most rapdily on terminal leucine residues but also liberates all other amino acids found in proteins, although its release of certain residues is very slow.

The enzyme is widely distributed in animal cells that have a rapid protein turnover (Ticktin and Trujillo, 1966). It is specifically located at the level of the microvilli in the small intestine of man (Pearse and Riecken, 1967). Nachlas *et al.* (1957a) and Nachlas *et al.* (1962) described a tissue localization staining procedure that is still widely used. He also suggested the use of small intestine as a control.

Oxidative enzymes

The dehydrogenases. Most of the dehydrogenase enzymes commonly studied are in the tricarboxylic acid cycle. This is the anaerobic part of the coupled respiration of the cell. Products of glycolysis progressively dehydrogenated with carbon fragments going into other pathways for nutrient synthesis and energy are generated. The hydrogen, in a reduced form, is removed through a series of cytochrome enzymes. It is then reacted with oxygen to form water and in the process more energy (ATP) is formed. Pasteur recognized that glycolysis was linked to respiration of the cell in his early biochemical work.

Succinic acid dehydrogenase. This enzyme catalyzes the conversion of succinate to fumerate in the tricarboxylic acid cycle (Krebs cycle). The hydrogen ion is transported to the reduced flavine adenine dinucleotide as its first step in the electron transport system. Nachlas *et al.* (1957b) reported high activity of many tissues in the mammal, including skeletal muscle. Succinic acid dehydrogenase is not a soluble enzyme and is always found associated with mitochondria.

Isocitric acid dehydrogenase. This is a tricarboxylic acid cycle enzyme that catalyzes the reaction of isocitric acid to alpha ketoglutaric acid through the rapid intermediate compound oxalosuccinic acid. There are 2 products of this reaction. Carbon dioxide is lost from the carbon chain and hydrogen ion is given up to the electron transport system with the yield of one ATP. Hess *et al.* (1958) described the localization procedure with skeletal muscle as the control tissue.

Malic acid dehydrogenase. The reaction, malic acid oxidation to oxaloacetic acid in the presence of malic acid dehydrogenase and DPN, is found in the mitochondria. This enzyme is insoluble and has never been isolated free of the mitochondria. Nachlas (1957) reported a localization procedure using kidney as a control tissue. Pearse and MacPherson (1958) reported general activity in mammalian tissue cells with high activity in the kidney.

Lactic acid dehydrogenase. Henderson (1965) suggested that the lactic acid dehydrogenase may regulate the reaction between oxidized and reduced diphosphopyridine nucleotide. Isoenzyme LDH5 may react with DPNH to convert pyruvate to lactate and DPN whereas LDH1 interacts with DPN to convert lactate to pyruvate and DPNH. Nachlas (1957) localized

the enzyme in many tissues of the body. He used smooth muscle for a control tissue. It yields a constant reaction.

Alcohol dehydrogenase. This enzyme is widely distributed in both animals and plants. In a review of the optic system Pirie (1958) describes the function of an alcohol dehydrogenase in utilization of vitamin A in the visual system.

By Hess' procedure (Hess, 1958) concentrations of alcohol dehydrogenase can be localized in epithelial cells.

Glutamic acid dehydrogenase. This enzyme catalyzes the combination alpha-ketoglutaric plus hydrogen plus ammonia ion in presence of DPNH to form L-glutamate. Thus glutamic acid, an amino acid, is formed from a product of the TCA cycle. Glutamic acid dehydrogenase is almost universally distributed in animal tissues. Steroids will cause the dissociation of glutamic acid dehydrogenase and may limit its activity in the body (Yielding and Tompkins, 1962).

Glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase catalyzes the first reaction in the pentase monophosphate shunt--glucose 6 phosphate to 6-phosphogluconolactone in the presence of TPN. Because the equilibrium constant for this reaction is large, it has often been used to generate TPNH (Green, 1960). This enzyme is localized by the procedure of Hess (1958) in many tissues.

Esterases

Myristrol cholinesterase. This enzyme catalyzes the breakdown of myristrol choline at neuromuscular junctions. Myristrol choline is the specific substrate used to study the cholinesterase distribution in

nerve tissues of dogs (Hard and Peterson, 1950). The myristrol method was first developed by Gomori (1948). Holmstedt (1957) modified and refined the procedure. Duffy *et al.* (1967) described a procedure to demonstrate cholinesterase adaptable for both cytochemistry and electron microscopy.

MATERIALS AND METHODS

Electron Microscopy

Experimental animals. Day-old, white broad-breasted male turkey poults were obtained from a certified mycoplasma-free flock. The first 2 weeks the turkeys were housed in electric battery brooders, then transferred to the floor. The birds were divided into experimental and control groups and housed in separate isolation rooms, where feed and water were available *ad libitum*. Movement of other animals was minimal in the building, and special care was taken in handling the birds to prevent infection.

At 4 weeks of age, the birds in the experimental group were inoculated into the infraorbital sinus with 0.2 ml. of a newly resuspended broth culture of lyophilized *Mycoplasma gallisepticum* (S-6 culture #1299 gl(P1), obtained from Dr. Yamamoto, Davis, California). An excess of birds was inoculated to assure adequate numbers of infected birds at each sampling period.

One week through 6 weeks after the inoculation date, 2 clinically affected turkeys and 2 control turkeys were killed each week (Figures 1 and 2). Mucus was withdrawn from each infected bird and cultured on agar-base media and in broth (see Appendix A) to confirm that mycoplasma was the causative agent of the sinusitis. Just prior to killing the turkeys, each sinus was injected with 4% neutral (pH 7.2) filtered solution of glutaraldehyde. Immediately after the head was removed, each outer sinus wall (Figure 3), including the skin and mucous membrane, was

dissected free, pinned flat to paraffin in a petri dish with the mucous membrane uppermost, and flooded with a quantity of glutaraldehyde sufficient to cover the tissue. After 4 hours of fixation the glutaraldehyde was decanted and replaced by Sorensen's buffer (pH 7.2), which was changed 6 times in the next 2 hours. The tissues thus preserved were stored in Sorensen's buffer for a maximum of 1 month (see Appendix B).

The tissues were prepared for embedding by blotting dry with a paper towel and positioned under a dissecting microscope. With an oil-free, single-edge razor blade the skin and mucous membrane were separated through the loose connective tissue of the submucosa of the sinus (Figure 4). The membrane was divided into halves and a 3 mm. strip was sliced off. This was diced into 2 x 3 mm. sections. These pieces were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epon 812* using rubber flat molds (see Appendix C).

The heat-cured blocks were mounted in a flat embedding chuck** (vise-type) and trimmed by hand with a razor blade according to Sorvall's technique (Thin Sectioning, p. 61) to a 1-mm.-wide trapezoid-shaped block face.

Microtomy. The sections were cut on a Sorvall MT-2 Porter-Blum ultramicrotome. This instrument utilizes a double pivot cantilever arm to determine the advance. The specimen, which is mounted in a vise-type holder, passes over the knife edge and the section is floated on water. The speed of the cantilever arm does not determine the section thickness.

Glass knives were used to cut the sections. These knives were prepared fresh each day. They were broken on an LKB 7800B Knifemaker from precut strips of LKB glass.***

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*Ladd Research Industries, Inc., Burlington, Vermont.

**Sorvall Corporation, Norwich, Conn.

***LKB-Produkter Ab., Stockholm, Sweden.

As a preliminary screening of blocks to assure proper orientation of tissue, "thick" sections (approx. 2 μ) were cut and stained (see Appendix D). The blocks, on which cross sections of the

Ultra thin sections were cut according to the procedure in the "thin section" guide (Sorvall). The sections were floated on acetone-water in a boat around the glass knife made of black plastic electrician's tape.* Silver to gray sections were selected which were between 60 and 90 mμ in thickness. A color scale chart, "continuous interference color and thickness scale for thin sections" (Sorvall), was used to make the thickness determinations. The thin sections were picked up on the dull side of a 400-mesh copper grid (LKB). The grid was then dried on filter paper and stored in LKB grid boxes under vacuum.

Staining. Reynolds (1963) lead citrate was the primary stain with uranyl acetate as a counter stain (Spink, 1968) (see Appendix D).

The grids were floated tissue-side down on drops of lead citrate stain in porcelain depression dishes. After 15 minutes they were blotted dry on filter paper and transferred to drops of fresh, saturated uranyl acetate solution. After a 1-hour staining period, the grids were washed with distilled water and a jet of 0.04 N NaOH, dried, then stored under vacuum.

Microscopy. The tissues were examined under an RCA EMU-4 electron microscope.** This instrument has a resolving capability of 8 Å and a useful magnification power of 1,400-200,000 times. With the "selected area

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*The 3-M Company, Minneapolis, Minn.

**RCA Scientific Instruments Engineering, Camden, N.J.

defraction" feature of the instrument, the grids were scanned at a 1:1 ratio to locate the tissue sections.

The instrument is capable of 50 and 100 Kv. operation of the gun assembly. Fifty Kilovolts were most suited for this work. The condenser lens system located just under the gun assembly controls the intensity of electron beam on the specimen. The specimen chamber is designed for minimum temperature variation and minimum contamination of the column. Objective lenses are coil-heated and water-cooled for constant operating temperature. The projector system, which includes the intermediate and projector lenses, forms the final image. The image is projected on a fluorescent viewing screen. Laminated lead glass shields the operator from x-ray exposure.

Photography. Kodak Electron Image plates 3-1/4 x 4 inches were used. The EMU-4 Electron Microscope has a capacity of 6 racks of 3 plates each.

A beam intensity meter and an automatic exposure timer are built into the instrument. Two exposures were made of each field at 2 and 4 seconds with a beam intensity of 0.5 electron speed.

After exposure the plates were removed from the instrument and processed according to Kodak procedures (see Appendix E).

Printing. The 3-1/4 x 4-inch plate negatives were printed using a tungsten lamp enlarger. An Ilfoprinter* was used to make a scanning proof of each plate. The final prints were produced by the pan method using Dektol** developer, Kodak stop bath--with indicator (10 seconds), Kodak fixer (10 minutes), and Kodak Photoflo in the wash (20 minutes). The prints were dried on a drum print dryer.

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*Ilford Company, Essex, England.

**Kodak, Rochester, New York.

Histochemistry

Experimental animals. Day-old broad-breasted white male turkeys were divided into 2 groups and housed in separate heated isolation rooms with feed and water available at all times. *Mycoplasma gallisepticum*, S-6 Strain (courtesy of Dr. Yamamoto, University of California, Davis, California), was injected into the sinuses of experimental turkeys at 4 weeks of age. The other group was held as controls. One week later and for 5 successive weeks, 2 infected and 2 control birds were killed each week and the sinus membranes were stained for activity of 11 selected enzymes.

Experimental design. The entire experimental procedure was repeated twice. This involved 2 different groups of birds, but all birds were the same age for any given point in the experiment and all birds were from the same mycoplasma-free parent stock. Enough birds were infected to assure that infected birds would be available each week. Only birds with swollen sinuses were selected and at necropsy the sinus cavities were examined for mucous exudate and cultures were taken to confirm mycoplasma infection. *Mycoplasma gallisepticum* was recovered in pure culture from each bird.

Tissue sections and histochemical techniques. The sinus wall, including skin and mucous membrane, was removed and immediately frozen on steel specimen holders that were partially submerged in acetone and dry ice. The frozen tissues were then held at -30 C. in the storage chamber of a Pearse cold microtome (cryostat), Type H.*

Sections were cut at 12 μ, transferred to coverslips and incubated for 30 minutes. The staining procedures were started less than 2 hours

*South London Electrical Equipment Co., London.

after the turkeys were killed. Procedures for localization of 11 different enzymes which can be grouped according to the type of reaction catalyzed were applied to individual sections. Localization of alkaline (AP) and acid phosphatase (acid P) were determined by the procedure of Burstone (1961), leucine aminopeptidase (LAP) by the method of Nachlas *et al.* (1957a, 1962), succinic acid dehydrogenase (succinic DH), malic acid dehydrogenase (MDH), and lactic acid dehydrogenase (LDH) by the method of Nachlas *et al.* (1957b), isocitric acid dehydrogenase (ICDH), alcohol dehydrogenase (ADH), glutamic acid dehydrogenase (GIDH), and glucose-6-phosphate dehydrogenase (G6PDH) according to Hess *et al.* (1958), and myristrol cholinesterase (Che) according to Gomori (1949). Intensity of enzyme staining for a positive reaction was given a value of 1+ rather than a graded score because the intensity of enzyme reaction under these conditions appeared maximal in both control and experimental tissues (Table 1).

Controls. Sinus tissue was taken from uninfected control turkeys of the same age as the infected turkeys for simultaneous staining. In addition, a tissue known to have a high concentration of each enzyme was taken from a control bird and stained simultaneously as a positive control for the histochemical procedure as well as for comparison with the infected sinus tissue.

These positive control tissues were: liver for G6PDH, Che, GIDH, and ADH; kidney for MDH and AP; skeletal muscle for succinic DH and ICDH; small intestine for LAP; smooth muscle for LDH; and thymus for acid P. A final control was the incubation of a section of uninfected sinus without the specific substrates (Table 1).

Table 1. Enzyme, reaction catalyzed, control tissues and references for eleven enzymes

Enzyme	Reaction Catalyzed	Control Tissues	References
Alkaline phosphatase	Nonspecific phosphomonoesterase pH 9	Kidney	Burstone, 1961
Acid phosphatase	Nonspecific phosphomonoesterase pH 5	Thymus	Burstone, 1961
Leucine aminopeptidase	Hydrolysis of N-terminal peptide bonds	Small intestine	Nachlas *et al.*, 1957a Nachlas *et al.*, 1962
Succinic acid dehydrogenase	Succinic acid ⇄ furraric acid	Skeletal muscle	Nachlas *et al.*, 1957b
Isocitric acid dehydrogenase	Isocitric acid ⇄ α-ketoglutaric acid	Skeletal muscle	Hess *et al.*, 1958
Malic acid dehydrogenase	Malic acid ⇄ oxalocetic	Kidney	Nachlas *et al.*, 1957b
Lactic acid dehydrogenase	Pyruvic acid ⇄ lactic acid	Smooth muscle	Nachlas *et al.*, 1957b
Alcohol dehydrogenase	Acetaldehyde ⇄ alcohol or alcohol $+O_2$	Liver	Hess *et al.*, 1958
Glutamic acid dehydrogenase	α-ketoglutaric acid ⇄ glutamic acid	Liver	Hess *et al.*, 1958
Glucose-6-phosphate dehydrogenase	Glucose-6-phosphate ⇄ phosphoglucono lactone	Liver	Hess *et al.*, 1958
Myristrol cholinesterase	Hydrolysis of fatty acids	Liver	Gomori, 1948

RESULTS

Electron Microscopy

Sections from each week, 1 through 6, were examined. The change in the pattern of infection each week was not significantly different, so the weeks, 2 through 5, have been deleted from the study and a comparison of Weeks 1 and 6 will be reported.

The electron microscope examination of sinus tissue after 1 week of infection revealed mycoplasma organisms packed between and within the cells (Figures 5 and 6). Even though organisms were closely packed, they were not distorted from pressure. They appeared as round bodies, each with a dense dark central structure surrounded by a narrow lighter zone containing granular particles. Granule-filled spaces and clear spaces with no organisms were also seen (Figure 7). There was slight variation in size among the organisms but no pleomorphism.

Intracellular populations of mycoplasmas varied from large numbers, densely packed (Figure 6) to as few as a single organism in some cells (Figure 8). Mycoplasmas were located in the cytoplasm but never in the nucleus. They appeared as deep in the cell as the level of the nucleus but never below it (Figures 8 and 9). The nuclei of infected cells were intact, and cells remained attached to the basement membrane (Figure 9).

Many ciliated epithelial cells also had microvilli (Figures 10 and 13). In heavily infected cells both of these structures were reduced in number or appeared to be absent (Figures 11 and 12). In lightly infected cells (Figure 8) these structures remained unchanged. In infected cells

which had lost most of their microvilli, the microvilli that remained appeared as short rounded projections. Any uninfected cells that had both fully developed cilia and microvilli appeared normal even though they were adjacent to infected cells (Figures 12 and 18).

Internal cellular structures, other than the nucleus, were not readily visible in cells that were filled with organisms (Figure 6). In cells containing few organisms (Figure 8) and in uninfected cells, the internal structures were only mildly affected. In Figure 11 there is some swelling of the mitochondria. There also was some increased granularity of the cytoplasm in some cells from infected sinuses but the cells and intracellular structures appeared to be morphologically normal (Figures 12 and 14).

The contour of the epithelial surfaces in infected sinuses was irregular (Figures 5, 11 and 14) compared with the even, symmetrical surfaces of membranes from control birds (Figure 13). Occasionally, however, uninfected cells lying between 2 infected cells were compressed and bulged slightly above the surface (Figure 18).

In parasitized cells that were undergoing degeneration, only the distal part of the cell seemed to be affected. This segment of the cell and its contents were sloughing away from the base of the cell (Figure 15). In spite of these degenerative changes, the nuclei appeared intact and the cytoplasm between the nucleus and basement membrane was normal in appearance. There was an occasional cell that had some of the early stages of necrosis (Figure 15).

Some exudate was lying on the free surface of the cells (Figure 15). This was probably mucus and cellular debris. Mycoplasmas were seen in the exudate but not in the large numbers found between or within cells.

At the sixth week, few mycoplasmas were seen but many bacteria (*Pseudomonas sp.*) as well as fungal spores had appeared. In some cells at this stage, regardless of the infectious agent, the microvilli were reduced in number and shortened, and the contour of the membrane surface was still irregular (Figure 14). At this time, however, some cells appeared to be regenerating in spite of the infection. Mycoplasma organisms were not found in the cells, and relatively few were seen in the exudate that filled the sinuses. In some areas there appeared to be a healed surface covered by healthy-appearing ciliated cells (Figure 17).

Histochemistry

Clinically, slight swelling of the sinuses was noticeable 96 hours after inoculation (Figures 1 and 2). After 1 week sinusitis was apparent, and after 6 weeks the sinuses were enlarged, soft, and fluctuating; and mucous exudate appeared in the nostrils if they were pressed. Mycoplasmas were readily cultured from the exudate.

Four of the 11 enzymes investigated were present in sufficient concentrations in the epithelial cells to be localized by the staining methods used. These 4 (succinic DH, ICDH, MDH and LDH), all members of the dehydrogenase group, are illustrated in Figures 19, 20, 21 and 22. Furthermore, the intensities of the enzyme-catalyzed reactions were essentially the same in the epithelial cells of both experimental and control birds. The 7 other enzymes were absent or in concentrations insufficient to permit their localization following 30-minute incubation in substrate solution.

Mycoplasma infection did not cause the appearance of any enzymes not found in the control cells; neither did it cause any to disappear. Thus, in the presence of infection, there was no apparent change in the

qualitative composition of the cellular enzyme components. In the third and sixth weeks, slight succinic DH and MDH activities were found outside of the cell at the free surface.

In the fifth week of infection in a 9-week-old bird, AP was found in the submucosa. It appeared as a diffuse reddish-stained substance evenly distributed in the tissue, and numerous localized areas of AP activity were seen in the capillary walls. Alkaline phosphatase had not been noted in these locations before.

All of the tissues selected for controls because of known high levels of given enzyme activity were positive in these procedures (Table 2).

Figure 1. Front view of a turkey infected with *Mycoplasma gallisepticum* S-6. Swollen sinus and nasal discharge are clinical signs of infectious turkey sinusitis.

Figure 2. Side view of a turkey infected with *Mycoplasma gallisepticum* S-6.

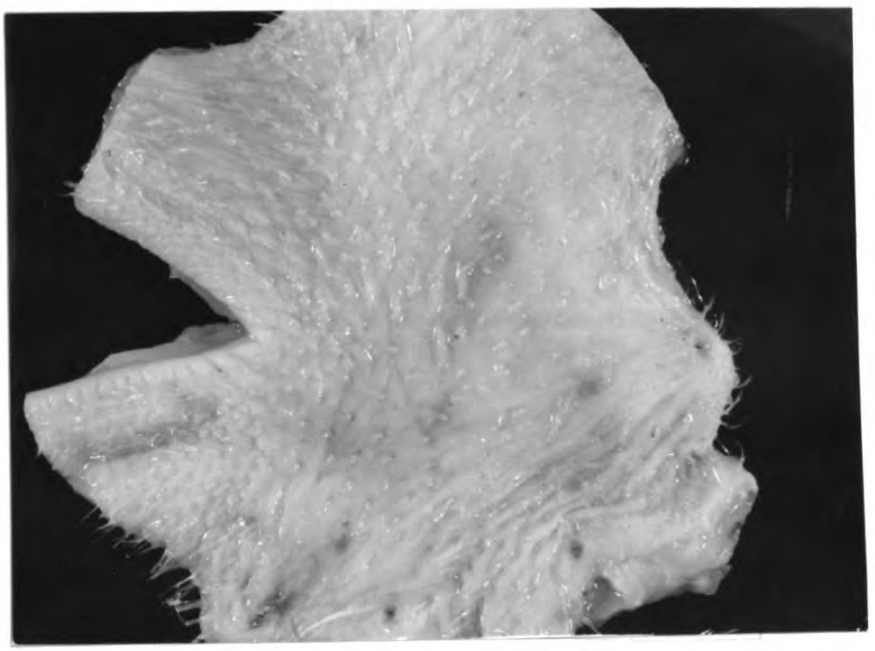

Figure 3. Outside surface of sinus skin covering.

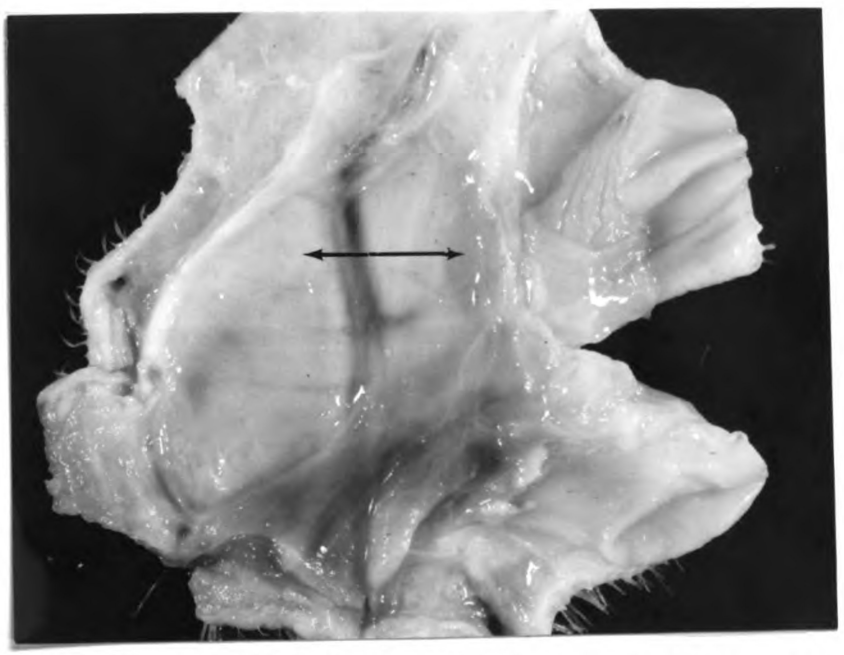

Figure 4. Inside surface of sinus and mucous membrane lining. Arrow indicates area sampled for electron microscopy.

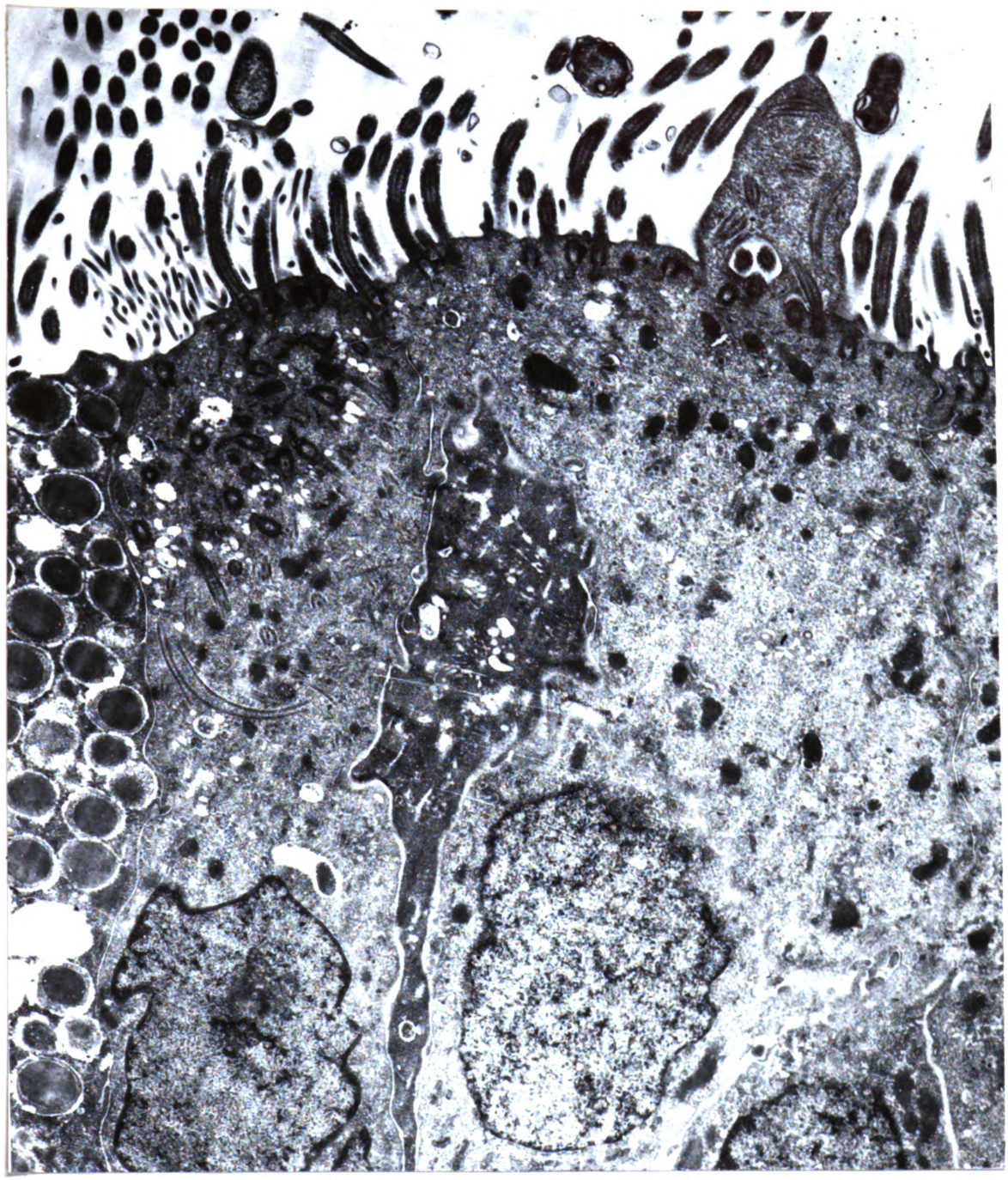

Figure 5. Infected turkey--first week. *Mycoplasma galliseptiocum* packed between cells (arrow). x 6,800.

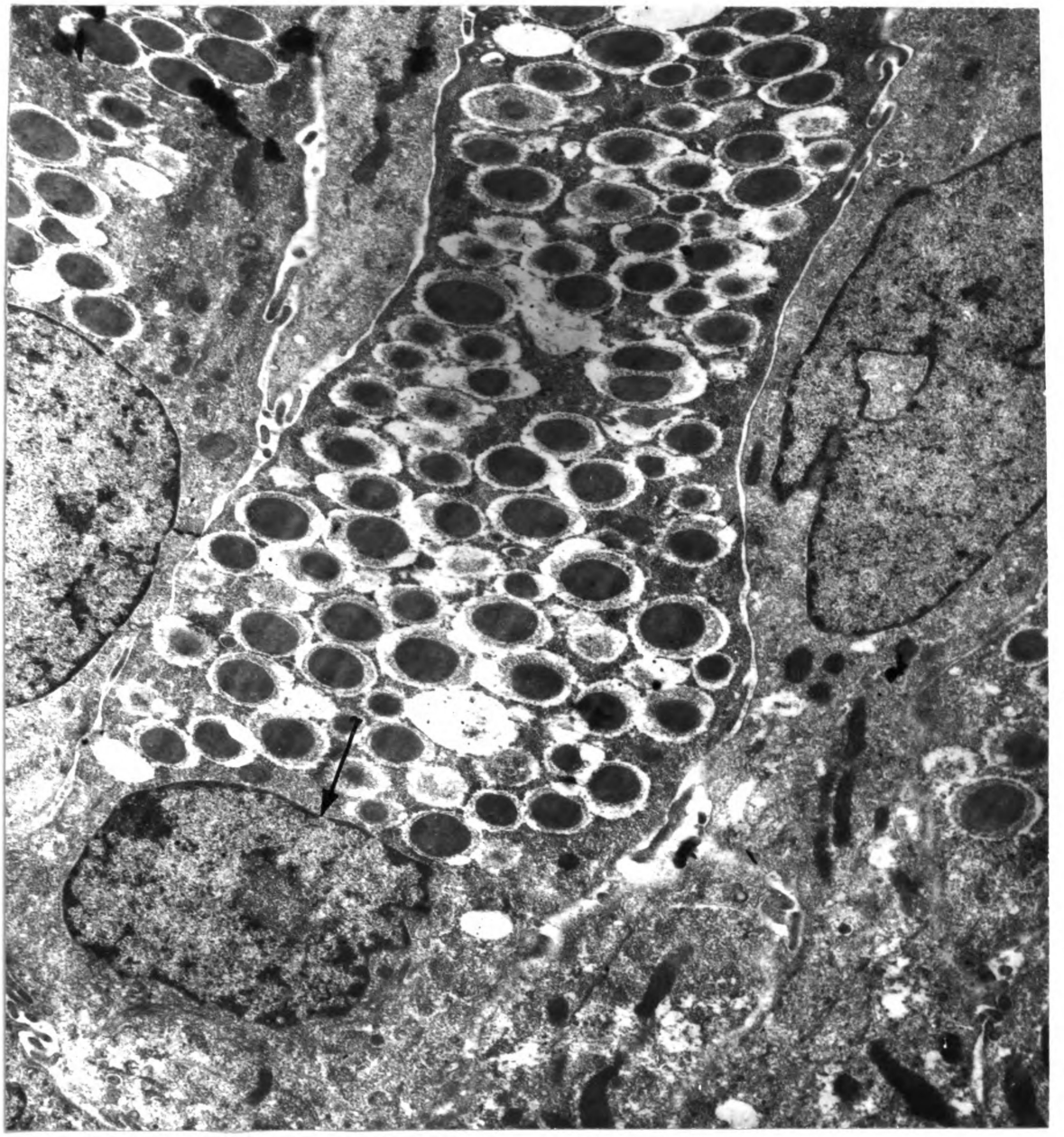

Figure 6. Infected turkey--first week. Mycoplasma filling the cytoplasmic space of the epithelial cell with loss of fine structure. The nuclei are depressed toward the cell base (arrows). x 13,600.

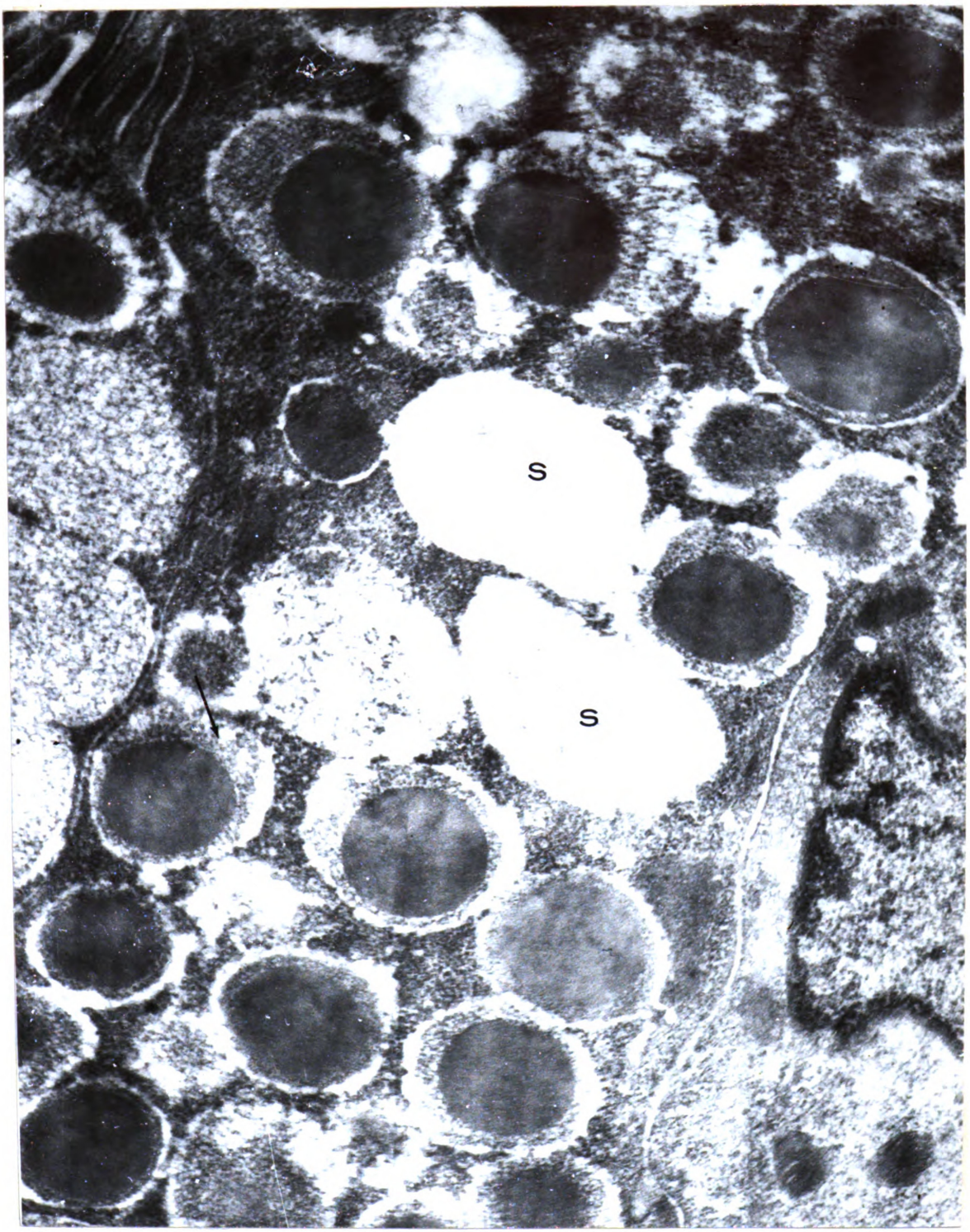

Figure 7. Infected turkey--first week. The organisms appear as closely packed, round, structureless bodies surrounded by a narrow zone of granular particles (arrow). Organisms have disappeared from clear spaces (s). There was a slight variation in size among the organisms but no pleomorphism. x 20,400.

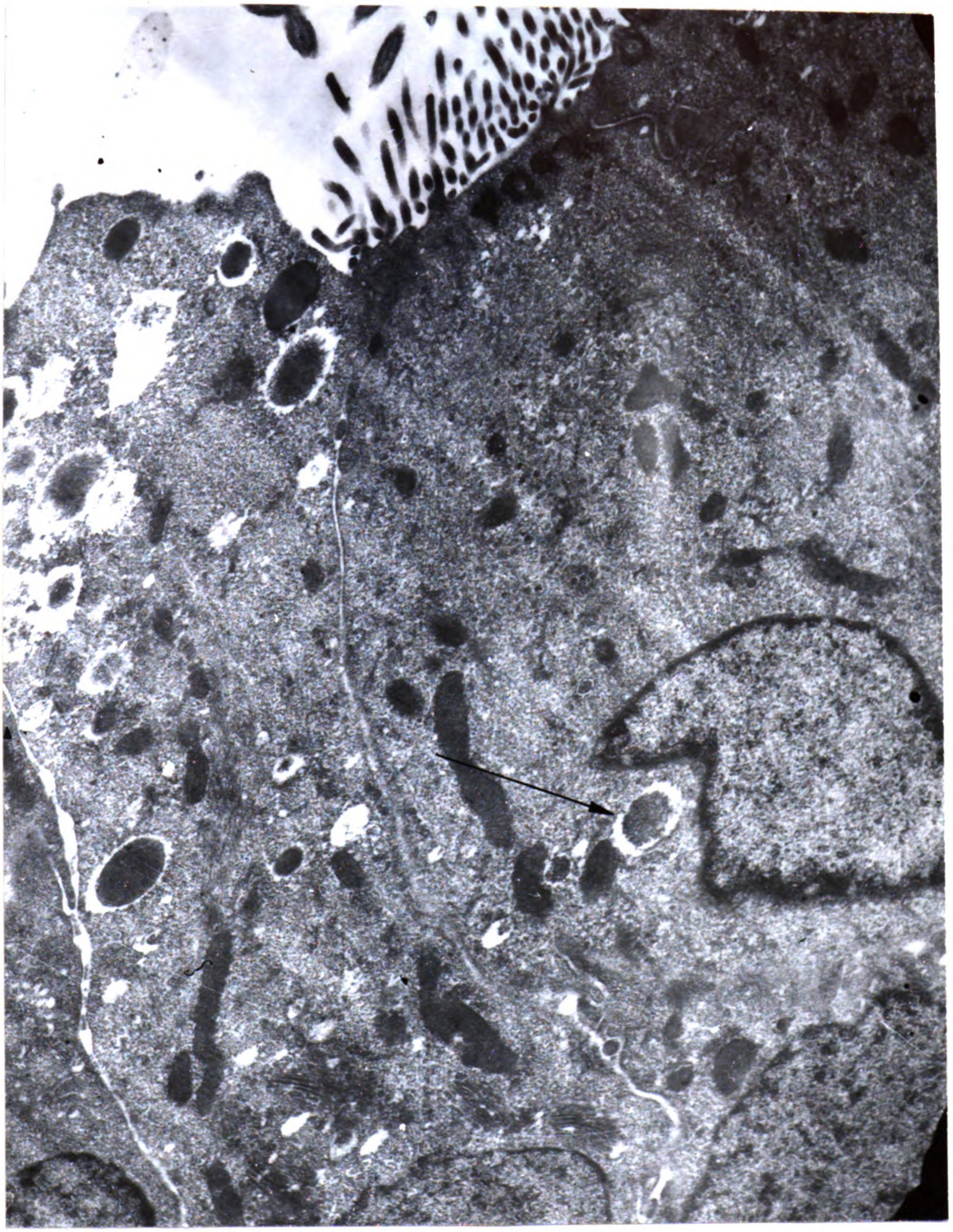

Figure 8. Infected turkey--first week. A single organism at the level of the nucleus (arrow). The cell retained its fine structure and cilia. An adjacent cell is more severely infected and has lost surface and cytoplasmic structures. x 9,520.

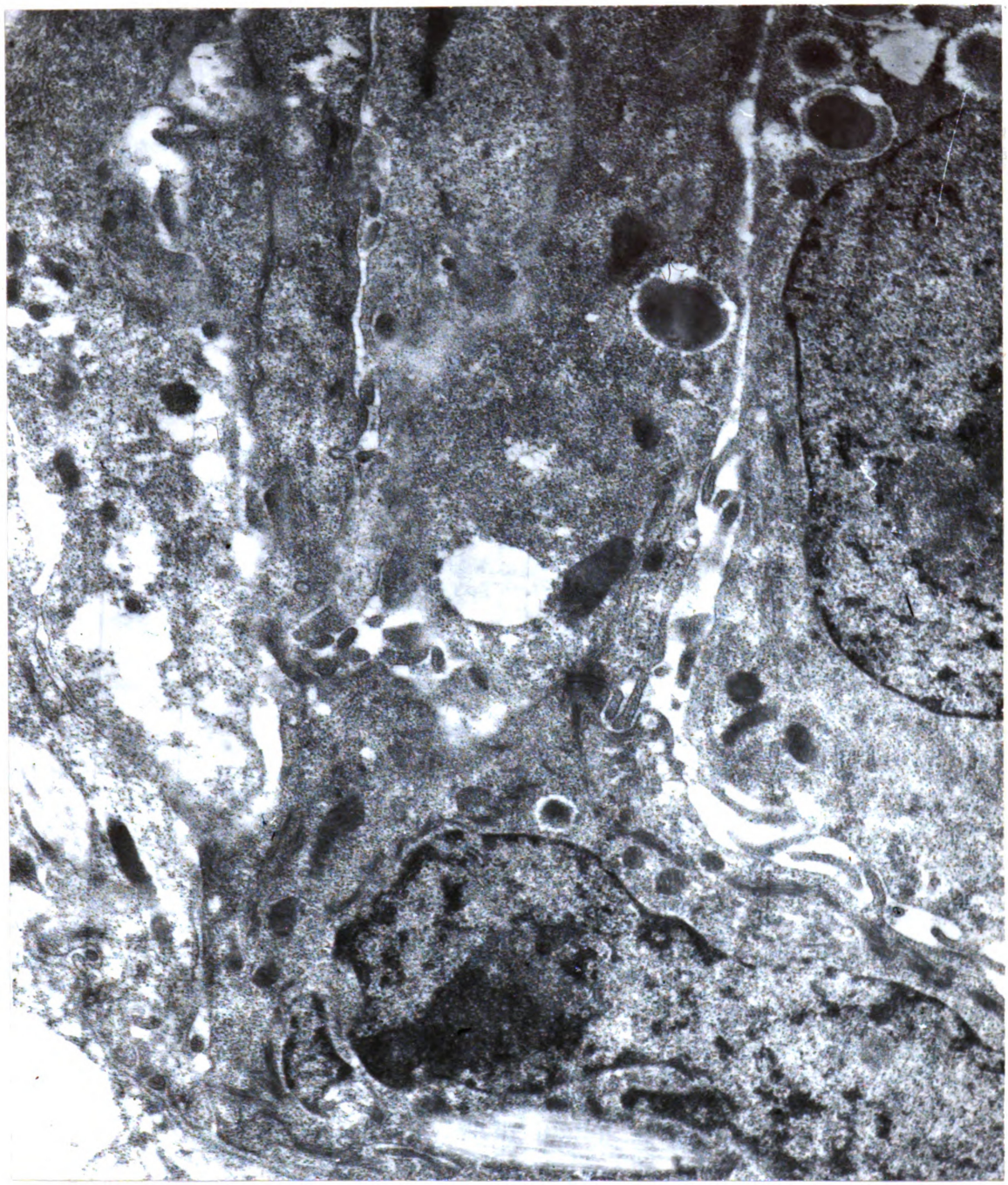

Figure 9. Infected turkey--first week. Basal portion of the cells at the attachment to the basement membrane appear normal. x 4740.

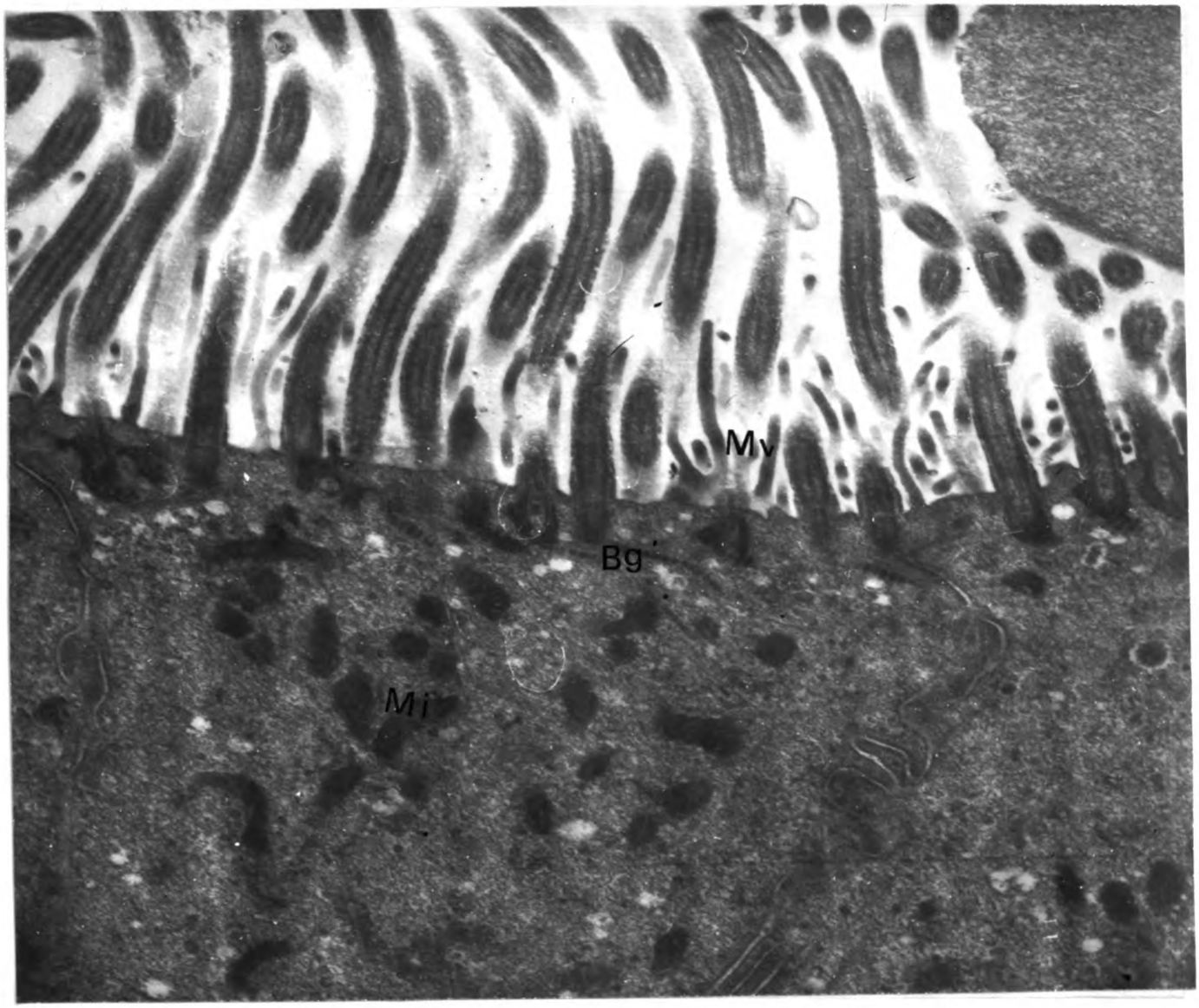

Figure 10. Infected turkey--first week. There is no evidence of *Mycoplasma galliseptiçum* infection in this section. Normal mitochondria (Mi), basal granule of the cilia (Bg), and microvilli projection (Mv) are seen in this cell. x 6800.

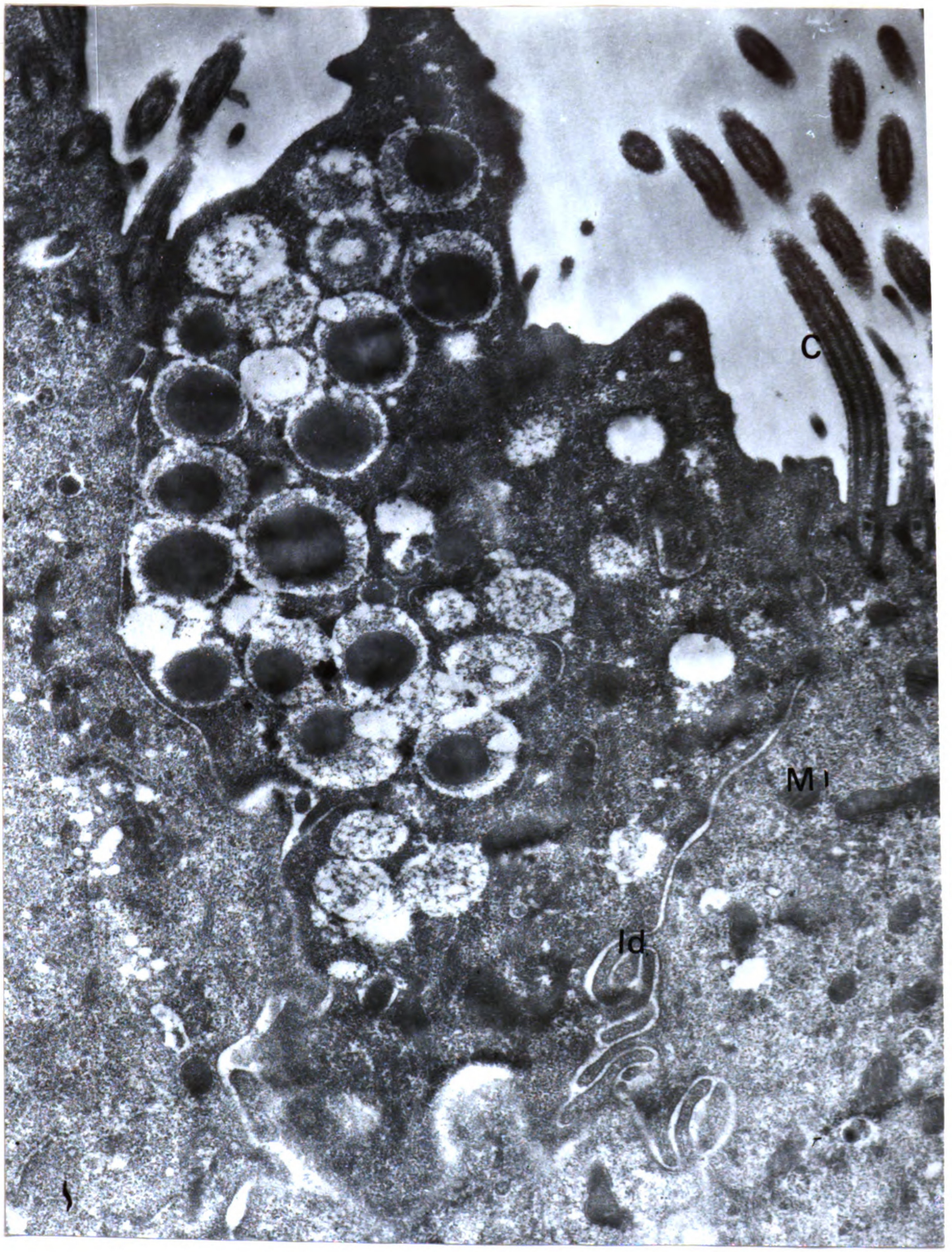

Figure 11. Infected turkey--first week. Infected cell (center) between 2 normal cells. Mitochondria (Mi) and cilia (C) are seen in the normal cells but not in the infected cell. The cellular interdigitations appear normal (Id). x 13,600.

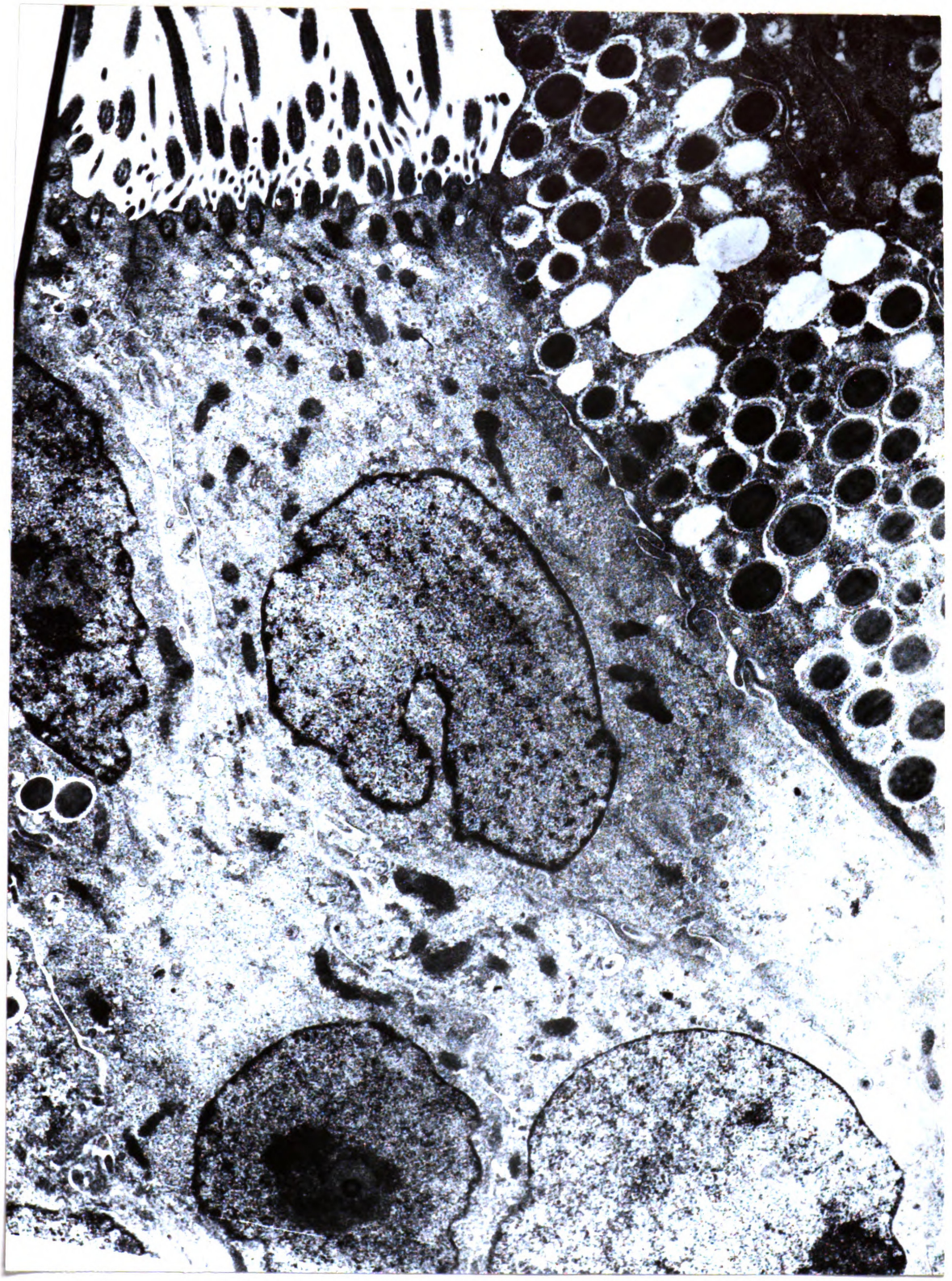

Figure 12. Infected turkey--first week. Loss of cilia and microvilli from infected cells. Normal appearing fine structure in unaffected cell. x 6800.

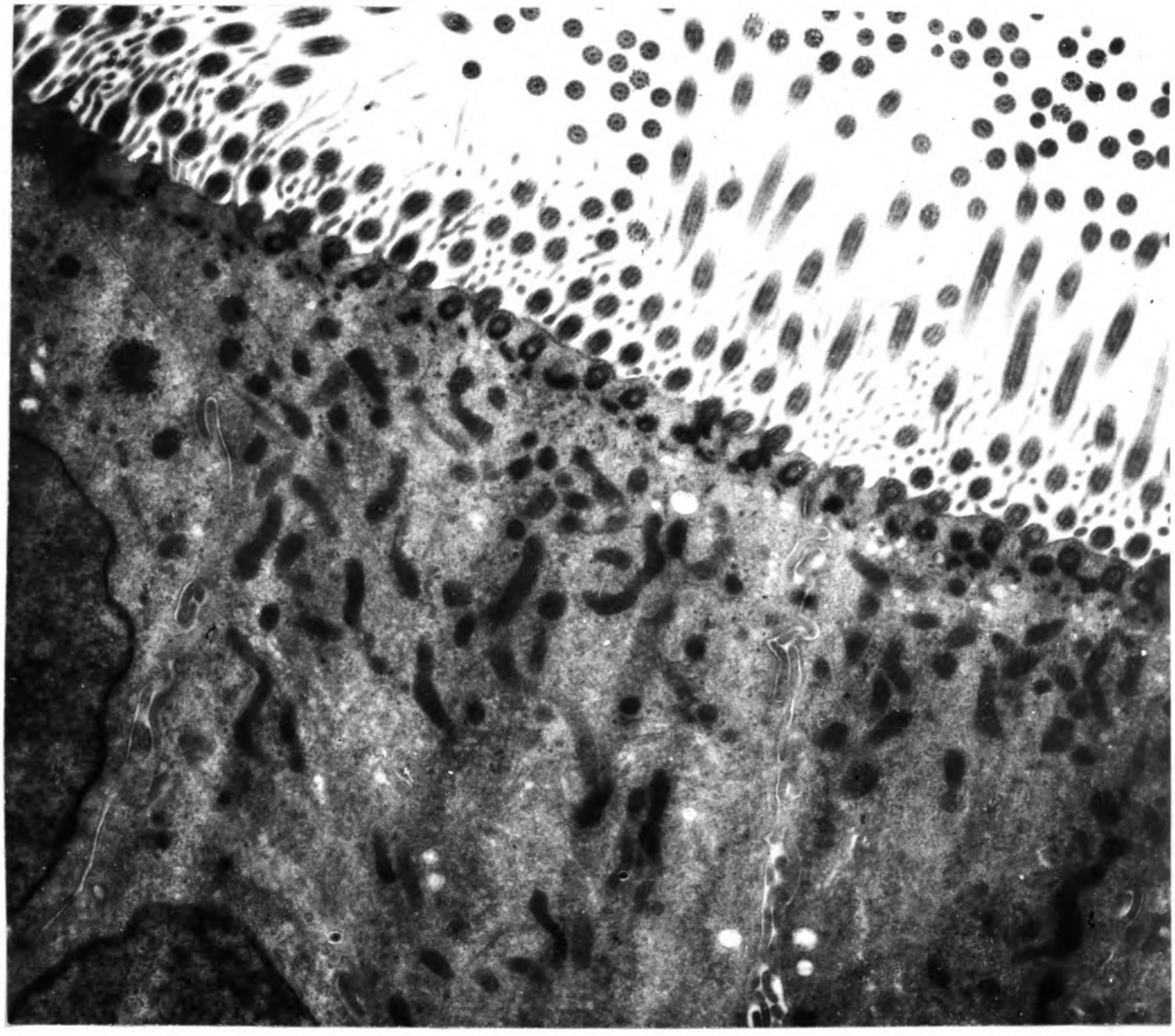

Figure 13. Control turkey--first week. Even contour of the surface cells. There are normal appearing fine structures with the nuclei at different levels in the cells. x 6800.

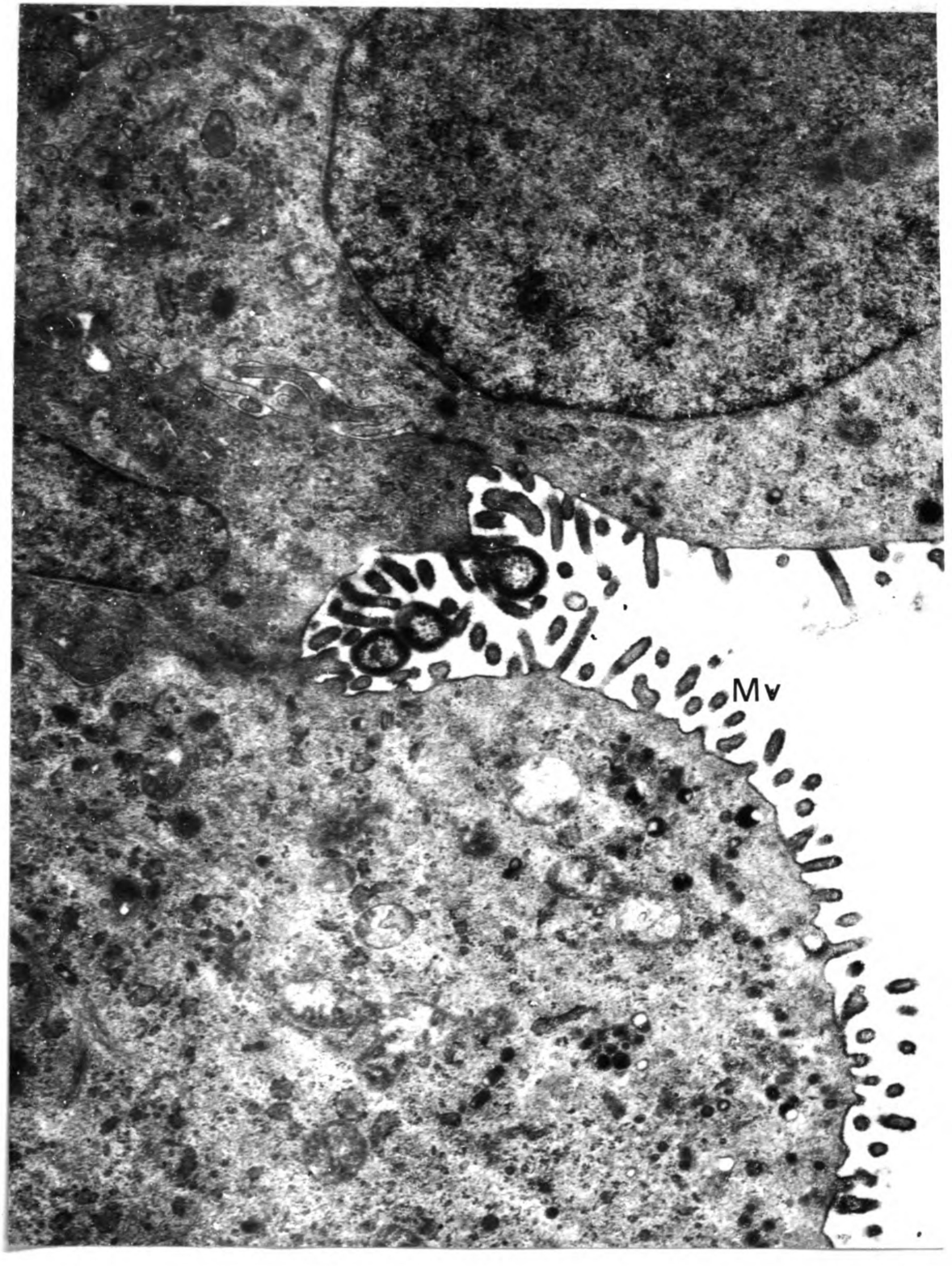

Figure 14. Infected turkey--sixth week. Contour of the cells is irregular with the loss of cilia and shortened microvilli (Mv). x 9520.

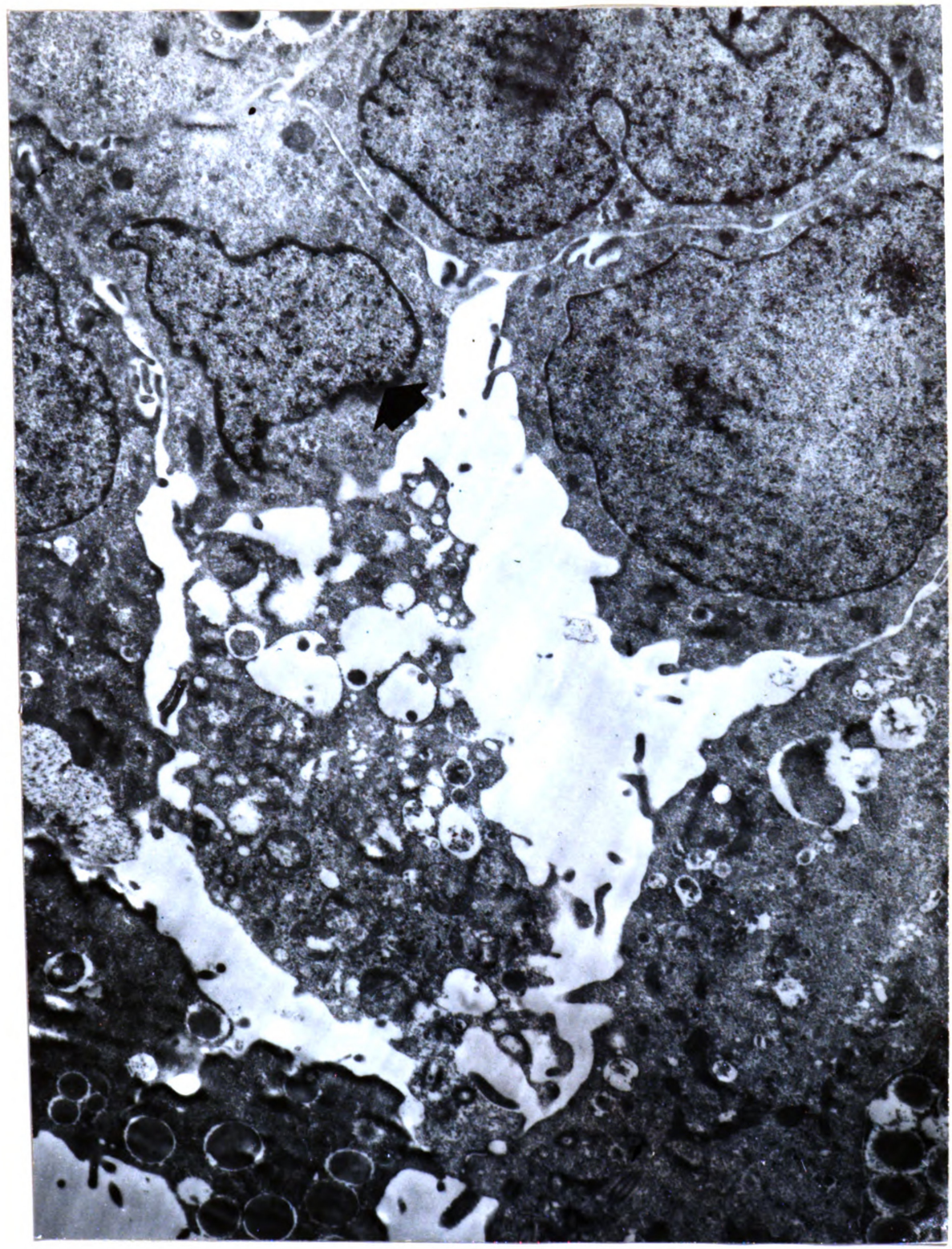

Figure 15. Infected turkey--first week. Most nuclei appear normal; however, some show signs of early necrosis (arrow). There is degeneration of the cytoplasm of the cells. x 6800.

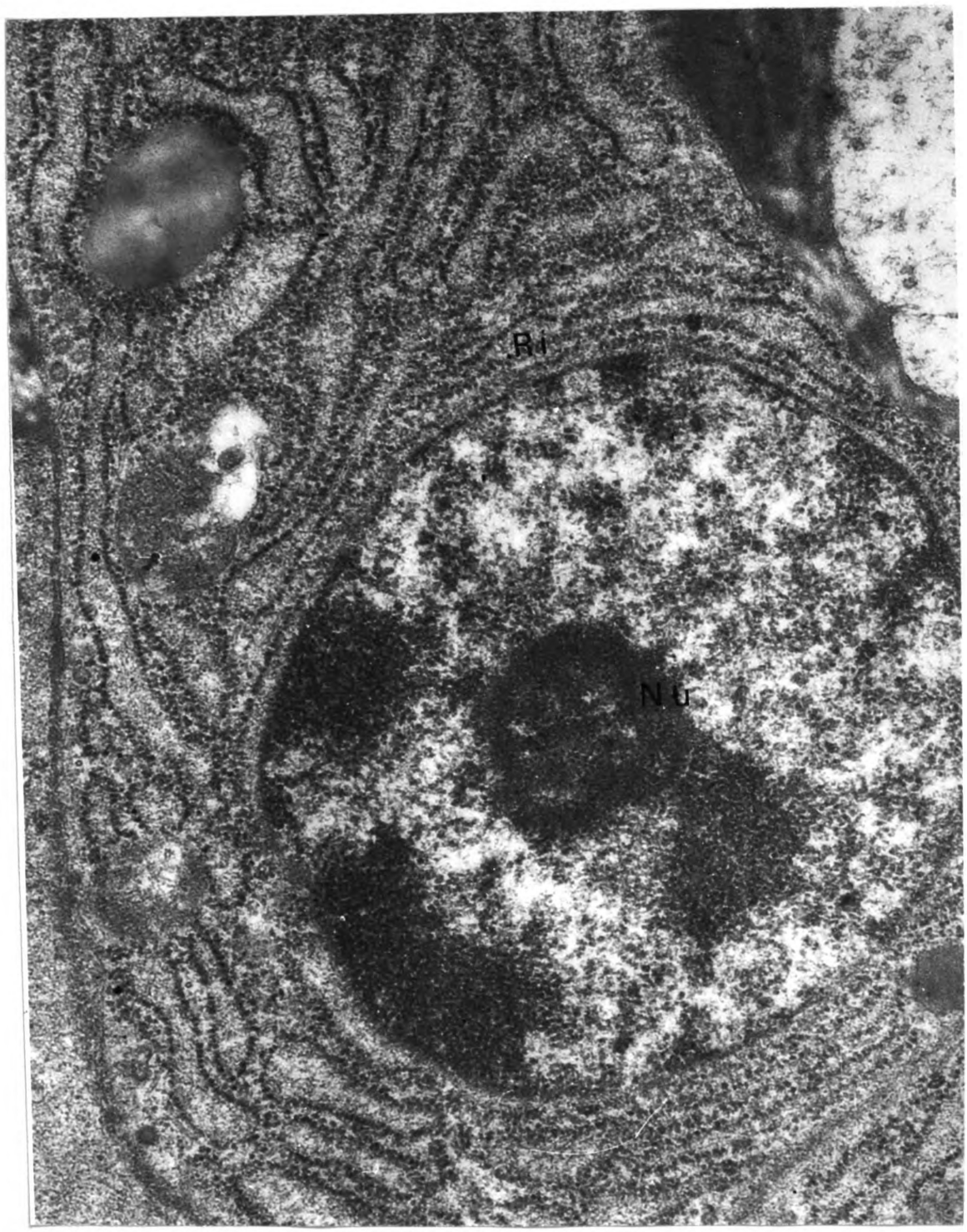

Figure 16. Infected turkey--sixth week. This cell has increased ribosomes (Ri) and nucleolus (Nu) which indicate active protein synthesis. x 20,400.

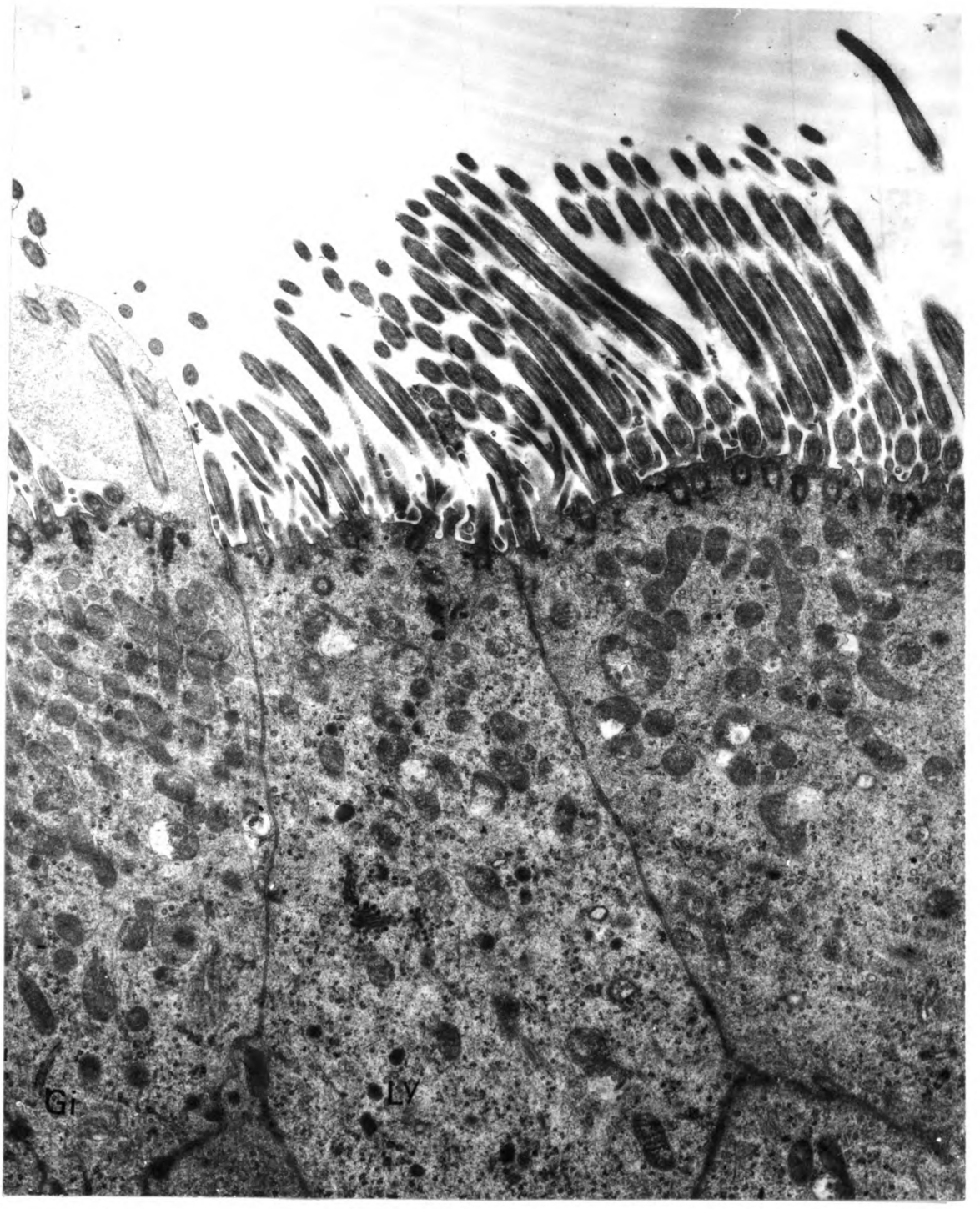

Figure 17. Infected turkey--sixth week. Increased size and number of mitochondria indicate higher cellular metabolic rate through the tricarboxylic acid cycle. Lysosomes (Ly) and Golgi apparatus (Gi) are also seen. x 6400.

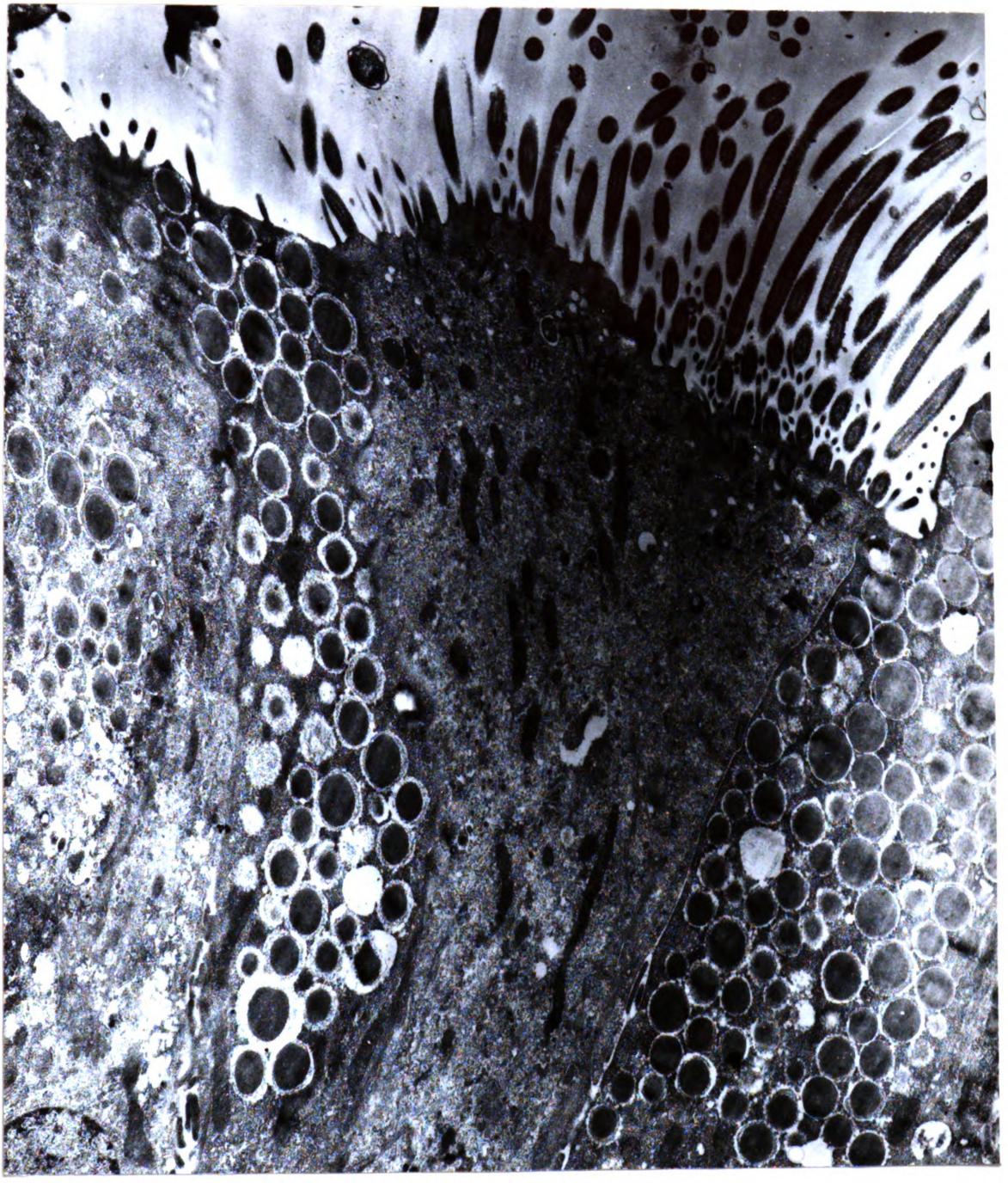

Figure 18. Infected turkey--first week. Normal cell is compressed between 2 infected cells. x 6800.

Figure 19. Succinic acid dehydrogenase. The enzyme is localized in the free surface of the cell. x 550.

Figure 20. Isocitric acid dehydrogenase. Enzyme activity distributed throughout the cytoplasm. x 550.

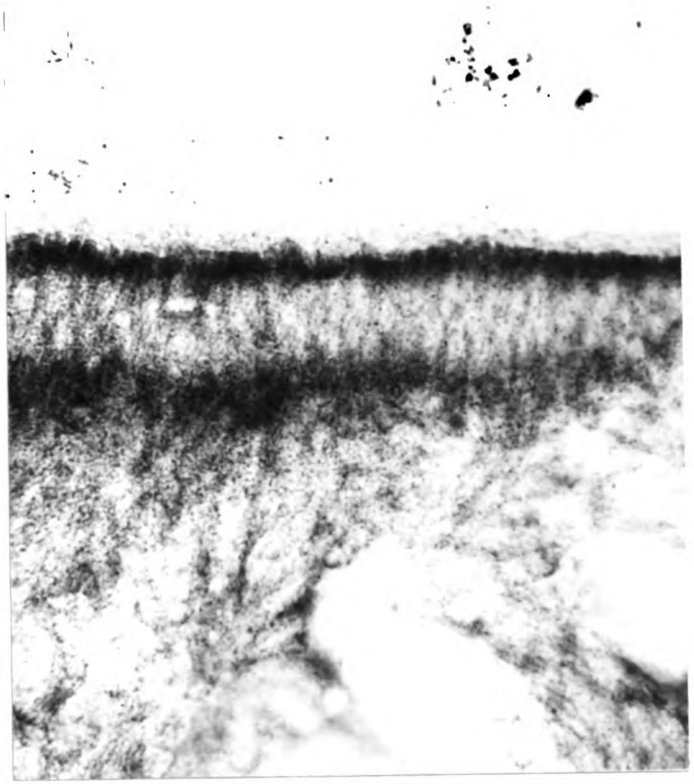

Figure 19

Figure 20

Figure 21. Malic acid dehydrogenase. The enzyme is concentrated in the free surface of the epithelial cell.

Figure 22. Lactic acid dehydrogenase. The enzyme activity is evenly distributed throughout the cytoplasm.

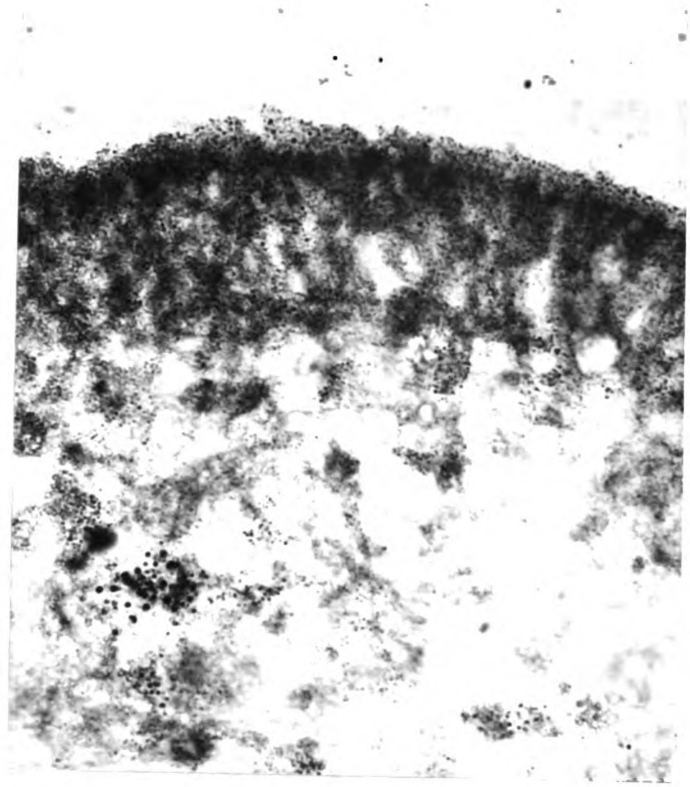

Figure 21

Figure 22

Table 2. Enzyme activity of control and mycoplasma-infected epithelial cells in turkey infraorbital sinus

Age (wk.)	Wk. of Infection	AP		Acid P		CHE		LAP		Succinic DH		ICDH		MDH		LDH		ADH		GlDH		G6PDH	
		C*	I*	C	I	C	I	C	I	C	I	C	I	C	I	C	I	C	I	C	I	C	I
5	1	-	-	0	-	0	-	-	-	+	+	0	+	0	+	+	+	-	-	0	-	-	-
6	2	-	-	-	-	-	-	-	-	+	+	0	+	0	+	+	+	-	-	0	-	0	-
7	3	-	-	-	-	9	-	-	-	+	+	0	+	+	+	+	+	-	-	0	-	-	-
8	4	-	-	0	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
9	5	-	-	-	-	-	-	-	-	0	+	+	+	0	-	+	0	-	-	-	-	-	-
10	6	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-

*C - control sinus

*I - infected sinus

\- = no activity

\+ = activity present

0 = unsuitable section

DISCUSSION

Electron Microscopy

Most of the mycoplasmas, after 1 week of infection, were situated either between the epithelial cells or within them. Very few were lying free in the lumen or trapped in the exudate. Since some cells were filled with organisms or spaces between cells were packed, it must be that a characteristic of this strain is to colonize in certain areas rather than being distributed diffusely throughout the lumen and tissue.

The morphologic appearance of the mycoplasmas in these tissues resembled the forms that have been seen in human tissue. The large, round, dense body is the mycoplasma, and the granular particles that surround and cling to the organism are probably only proteinaceous material. The light space is probably a vacuole in which the organism lies (Figure 7). The mycoplasmas reported by Dmochowski *et al.* (1967) and Hummler and Armstrong (1967) were also in vacuoles in the cells.

The granule-filled spaces devoid of owganisms and the empty clear spaces were probably where organisms had undergone degeneration or had dropped out during sectioning.

Mycoplasmas did not appear flattened or distorted, in spite of their densely packed arrangement between and within cells. Since these organisms occupy space, it must be that the cell changes shape to accommodate the organisms.

One week after infection, even with large numbers of organisms present, only a few were undergoing degeneration. This may not be an accurate

observation, however, since markedly degenerate organisms might not be readily recognized and only those with some form remaining would be seen.

Since numerous organisms were packed into the cells, it was difficult to evaluate the fine structure of the cytoplasm. Figure 7 shows no evidence of mitochondria admixed with the mycoplasma. The mitochondria in some of the cells adjacent to infected cells appear to be unaltered in size, number or structure, while others (Figure 15) show structural changes consistent with increase in the tricarboxylic acid cycle enzymes. As stated in the section on histochemistry, however, we measured no increase of TCA cycle enzymes by our methods.

Bacteria were not part of the population of the sinus the first week. At the sixth week, however, bacteria and fungal spores were present but fewer mycoplasmas were seen. Microvilli on infected cell surfaces were decreased in number and were shorter than similar structures on normal cells. The shorter microvilli on cells after 6 weeks of infection may indicate early recovery and regeneration of these structures rather than their continuing degeneration. It appears that cilia and microvilli are affected by any type of infection, since, at 6 weeks, with few mycoplasmas remaining, these structures were still not normal on some cells.

The loss of surface structures in the presence of infection is not unique with these cells. Microvilli on jejunal epithelium were also fewer in number and shorter than normal in the presence of bacterial or viral infections. In germfree baby pigs infected with the virus of transmissible gastroenteritis, the villi of the jejunum were lost for a time (Trapp *et al.*, 1966). During the infection, their position was indicated only by a low, broad stump with wide spacing between these bases. Thake (1968) also reported that microvilli on epithelial cell surfaces in pigs

infected with the same virus were markedly reduced in number and were much shorter than normal.

In germfree baby pigs given virulent *Escherichia coli* organisms orally, intercellular spaces were widened, microvilli on the epithelial cells were shortened and reduced in number, the terminal web was vacuolate, cytoplasm was pressed to the periphery of the cells by large vacuoles, and cells became degenerate. Bacteria invaded the cells and were present in the cytoplasm (Drees, 1969).

In mycoplasma infection only the distal part of some cells was degenerating but it appeared that, unless further degeneration occurred, the cells would readily recover if the infection were removed. This is suggested by the fact that infected cells remained attached to the basement membrane and the nuclei were generally intact. This limited peripheral degeneration of cells is in agreement with observations that necrosis of the cells in the early stages of infection is minimal (Jungherr, 1949) and with the absence of acid phosphatase in the infected cells. There were occasional cells (Figure 15) with wrinkled nuclear membranes and loss of rough endoplasmic reticulum. These may be examples of early necrosis as described by Sandritter and Wartman (1967). There was no other evidence of necrosis seen such as karyorrhexis or karyolysis.

Bacteria in the sinus of these birds 6 weeks after infection confirms the work of Groupé *et al.* (1948). Perhaps some of the profuse production of mucus in sinusitis may be caused by secondary infection rather than solely from *M. gallisepticum*, since they were less numerous after 6 weeks of infection than were bacteria and fungal spores.

The decreased population of organisms after 6 weeks of infection suggests that some resistance may have developed in the host. This decrease of organisms in later stages of infection and the appearance

of bacteria and fungal spores have also been reported by other workers (Lutsky and Organich, 1966; Organich *et al.*, 1966; White *et al.*, 1954). Also, healing of cells apparently progresses rapidly at this time, as indicated by the increase in rough endoplasmic reticulum for protein synthesis (Figure 16). The mitochondria of recovered cells (Figure 17) are increased in size and number. This also indicates an increase in cellular metabolic activity through the TCA cycle enzymes. There are also more lysosomes seen in the healed cells, which indicates increased hydrolytic enzyme activity.

It would be expected that with such increase in intercellular enzymatic activity, some changes in the histochemistry would have been seen.

Superficial structures were lost from cells which were parasitized (Figure 11) but not from adjacent cells that were uninfected (Figure 12). The loss of these structures was probably caused by changes in the host cell rather than by direct effect of the organism on these structures. If the host cells were not parasitized, these structures remained intact.

Many investigators report that a characteristic of mycoplasmas is pleomorphism as well as variation in size (Chu and Horne, 1967; Dmochowski, 1967; Freundt, 1960; Hayflick, 1968; Hummler and Armstrong, 1967; Reagan *et al.*, 1967; White *et al.*, 1954). Most of these workers studied organisms that were grown in broth, chick embryos, or tissue culture, except for those in human tissues. The organisms observed in this study were consistent in shape and structure. The only variation was in size, but this may have been caused by crowding, a difference in the age of the organism in its growth phase, or by sectioning. There were no filamentous forms or other unusual shapes.

Histochemistry

In the study of *Mycoplasma gallisepticum* infection of the turkey sinus epithelial cells, the assumption must be made that each of the animals on the experiment will respond to the microorganism in a similar fashion. Variation in the response between the 2 biological systems was minimized by selecting host animals from inbred lines and using a mycoplasma strain of proven genetic stability in the animal.

The absence of any known unique function of these epithelial cells suggests biochemical activity of no more significance than maintaining the integrity of the membrane. Under normal conditions little more energy is required by these cells than that which can be derived from the tricarboxylic acid cycle (TCA). Succinic DH, ICDH and MDH, which were found to be active in these cells, are members of the TCA cycle which is localized in the mitochondria (Green, 1960). Mitochondria are found in most cells, and the TCA cycle enzymes function to aid cells in meeting their basal energy requirements. Moreover, the presence of ICDH indicates that these cells are using this cycle as their energy source (Henderson, 1965).

The absence of G6PDH, the first reaction in the pentose shunt, suggests that the cells are not utilizing this pathway to obtain energy. Lactic dehydrogenase, the fourth enzyme, is active in the reversible reaction of pyruvic acid and lactic acid (Lehninger and Wadkins, 1962). Henderson (1965) suggests that the activity of this latter enzyme may regulate the ratio between oxidized and reduced diphosphopyridine nucleotide.

The appearance of succinic DH and MDH granules outside the cell suggests that the integrity of the cell membrane was interrupted by the infection, allowing the mitochondria to pass out of the cell. These 2

enzymes are not soluble and have never been prepared mitochondria-free. Pearse (1960) found that succinic acid and malic acid dehydrogenases were the most reliable from the histochemical point of view for demonstration of TCA cycle activity. The appearance of AP at this stage of infection probably indicates the invasion of macrophages which carry this enzyme in the lysosomes (Hirsch, 1961). The localized AP activity in the capillary walls probably indicated the location of migrating heterophils.

The absence of acid P indicates that necrosis and cellular degeneration were absent or only minimal at this stage of the infection but acid P could be expected to appear under more severe conditions (Weber, 1961; Fennell, 1966). Since no measurable acid P was found, this is consistent with Jungherr's report (Jungherr, 1949) and the electron microscopic observations that necrosis is minimal in mycoplasma sinusitis.

The consistency in enzyme activity of control cells at 5 weeks of age and at 10 weeks indicates that these enzymes are necessary for intracellular function regardless of the age of the host and do not increase in number or disappear with changes in age. The intensities of enzyme reactions were unaltered despite the possible increase in energy demands that might be expected because of infection. Mitosis, which must occur when cellular hyperplasia is found, apparently did not affect the enzyme activity.

SUMMARY

The epithelial lining cells of infraorbital sinuses from turkey poults infected with *Mycoplasma gallisepticum* (S-6) were stained to demonstrate the activity of selected enzymes from the hydrolytic, proteolytic, and oxidative groups. Only enzymes characteristic of basic energy-generating pathways were found--succinic acid, isocitric acid, lactic acid, and malic acid dehydrogenase. Enzyme activity did not increase or decrease with infection, epithelial cell hyperplasia, or increase in host age from 5 to 10 weeks.

The mycoplasma-infected cells were also examined with an electron microscope. The organisms were found in large numbers during the first week of infection but were fewer in number by the sixth week. The infection was uncomplicated by secondary invaders during the first week, but by the sixth week bacteria and fungal spores had appeared. Mycoplasmas were lying between cells and within cells, but relatively few were free in the exudate. Cellular degeneration was limited to the distal part of the cell. The nuclei and bases of the cells were not affected. Such cells would probably recover if the infection were removed. Cilia and microvilli were lost from heavily infected cells but not from adjacent uninfected cells.

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APPENDICES

APPENDIX A

MYCOPLASMA CULTURE MEDIA[†]

1. Difco* PPLO broth or agar base (without crystal violet).
2. Yeast autolysate, 1%.**

   The pH is usually adjusted to 7.2-7.5 prior to autoclaving for 15 minutes at 121 C.
3. Before using add 10% sterile equine serum.
4. Penicillin (500 units/ml.) and thallium acetate (1:4,000 final conc.) added as abacterial inhibitors.

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*Difco Company, Detroit, Mich.

**Formula for yeast autolysate:

A. Dissolve 50 grams of fresh bakers yeast in 100 ml. of 0.2 molar KH_2PO_4 at 80-85 C. for 20 minutes.

B. Filter through several thicknesses of gauze.

C. Centrifuge and put the supernatant through a Seitz filter into a sterile container.

[†]Courtesy of Dr. R. Yamamoto, Davis, California.

APPENDIX B

FIXATION

Glutaraldehyde 4% (Sabatini *et al.*, 1963)

Stock buffer salt solution:

Monosodium (mono basic) sodium phosphate 2.26%

Stock alkali solution:

Sodium hydroxide.....2.52%

Working solution:

Monosodium phosphate solution.....83 ml.

Sodium hydroxide solution.....17 ml.

25% glutaraldehyde.....18 ml.

Fix tissue in working solution for 4 hours, decant and change to Soerensen's* buffer pH 7.4. Change Soerensen's buffer 6 times in the next 2 hours and store in the last change.

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*Soerensen's Phosphate Buffer pH 7.2 (Culling, 1963)

Stock solutions:

M/15 Sodium phosphate dibasic
(Na_2HPO_4, prepared by dissolving 9.465 g. of the salt in distilled water and making up to 1 liter in a measuring flask.

M/15 Potassium acid phosphate
(KH_2PO_4, prepared by dissolving 9.07 gm. in distilled water and making up to 1 liter.

Working solution:

To prepare 100 ml. M/15 buffer solution pH 7.2, mix 72 ml. M/15 sodium phosphate dibasic and 18 ml. M/15 potassium acid phosphate.

Osmium tetroxide (Palade, 1952)

Stock buffer solution:

Sodium veronal (barbital).....14.7 gm.

Sodium acetate..... 9.7 gm.

Distilled water.....500 ml.

Stock osmium tetroxide solution:

Osmium tetroxide.....2 gm./100 ml. water

Working fixative:

Buffer..... 5 ml.

0.1 N HCl..... 5 ml.

Distilled water..... 2.5 ml.

2% osmic tetroxide solution.....12.5 ml.

Follow procedure according to Luft, Appendix C.

APPENDIX C

EMBEDDING

INSTRUCTIONS FOR EMBEDDING TISSUE

IN EPON 812

BASED ON LUFT'S PROCEDURE

Resin Formulations

The weight per epoxide equivalent (W.P.E.) of epoxy resins can vary from batch to batch. Manufacturing specifications for Epon 812 list a W.P.E. range from 140-160. This variation is about 13% and is a probable cause for poor sectioning characteristics of a block and the production of nonreproducible embeddings. To obtain reproducible blocks for sectioning we recommend that formulations be made on a weight basis using epoxy resins analyzed for W.P.E. and anhydrides analyzed for purity [Ladd Research Industries, Inc., analyzes the epoxy resins and anhydrides which they market. The results of these analyses are printed on the labels affixed to each bottle of resin or anhydride.] On a weight basis formulations are expressed in parts of reactants per 100 parts of epoxy resin (p.h.r.). The amount of anhydride to be incorporated is calculated as follows:

$$\text{P.h.r. anhydride} = \frac{100}{\text{WPE}} \times \text{anhydride equivalent} \times \text{ratio (anhydride-epoxy)}$$

where:

(a) 100 = grams of epoxy resin;
(b) W.P.E. (epoxy equivalent) = weight of eposy resin containing one equivalent weight of epoxide;
(c) anhydride equivalent = molecular weight of anhydride;
(d) ratio (anhydride-epoxy) = ratio of equivalent of anhydride to epoxide equivalent

In this procedure the anhydride epoxy ratio is 0.7:1 (equivalent of anhydride to equivalent of epoxy resin).

Mixture A

When the W.P.E. of Epon 812 is 150, weigh out 100g. of DDSA in an 8oz. bottle. Add 80g. Epon 812 on top of the DDSA. Cap and shake vigorously to ensure homogeneity.

Mixture B

When the W.P.E. of Epon 812 is 150, weigh out 84g. NMA in an 8oz. bottle. Add 100g. Epon 812 on top of the NMA. Cap and shake vigorously to ensure homogeneity.

The weights of anhydrides were obtained by the following calculations:

$$\text{P.h.r. DDSA} = \frac{80}{L50} \times 226 \times 0.7 = 99.3 \text{ DDSA}$$

$$\text{P.h.r. NMA} = \frac{100}{150} \times 178 \times 0.7 = 83.07 \text{ NMA}$$

Whenever the W.P.E. shown on the Epon 812 label is not 150 use Table I to determine the quantities of DDSA and NMA required to make Mixtures A and B.

Table I

Epon 812 W.P.E.	Mixture A* DDSA Wt. in grams	Mixture B** NMA Wt. in grams
140	106	89
141-142	105	88
143-144	103	87
145-146	102	86
147	101	85
148-149	100	84
150-151	99	83
152-153	98	82
154	97	81
155-156	96	80
157-158	95	79
159-160	94	78

Mixtures A and B can be used up to six months or longer if refrigerated. Accelerator can be added to Mixtures A and B if the solutions are stored at -30° C or lower. In this case the components can be used for up to six months. Immediately after use, however, the components should be refrigerated again. Before use, the refrigerated mixtures should be warmed to room temperature before opening. This will eliminate water condensing in the resin which cause holes and soft blocks. Anhydrides are hygroscopic

*For DDSA use 80g. of Epon 812.

**For NMA use 100g. Epon 812. Both mixtures A and B were designed to fit 8 oz. bottles.

and pick up moisture from the atmosphere. Water can cause slow hydrolysis of the anhydrides to free acids. Both trapped water and free acid can give rise to soft blocks for sectioning. After using anhydrides or Mixtures A and B jet Freon (Blast Off, Ladd Cat. #402) into the bottles to exclude air. All embedding components should be capped immediately after use.

Complete Resin Mixture

Hardness of the blocks for sectioning depends upon the ratio of Mixtures A and B in the Complete Resin Mixture. Mixture B alone gives very hard blocks, whereas Mixture A above gives soft blocks. The ratios of A and B and their respective hardness values were assessed by Dr. Luft and given in his paper. These ratios have been converted to a weight basis which closely approximates the volume basis. The values are shown in Table II.

Table II

Mixture A Wt.in grams	Mixture B Wt.in grams	Total Wt. in grams	Accelerator DMP-30ml.	Equivalent Hardness
10	0	10	0.14	100% N-butyl Methacrylate
7	3	10	0.14	10-15% Methyl Methacrylate in N-butyl
5	5	10	0.14	15-20% Methyl Methacrylate in N-butyl
3	7	10	0.14	20-30% Methyl Methacrylate in N-butyl
0	10	10	0.14	30-50% Methyl Methacrylate in N-butyl

Add 0.14 ml. of DMP-30 using tuberculin syringe.

Many failures in obtaining good blocks are due to inadequate mixing. Stire the complete resin mixture for at least five minutes after adding the accelerator.

DMP-30 is toxic and can cause sensitization to amines. Cleanliness, careful handling and adequate ventilation should be observed. The DMP-30 bottle should be capped immediately after use to avoid inhaling the vapors.

A. Fixation

(1) Osmium Tetroxide

We recommend fixation of tissue pieces no more than a few mm. per side, in the standard Palade fixative - buffered osmium tetroxide. The latter should be made from 2% stock osmium tetroxide solution with pH of 7.4.

Procedure

(a) Place tissue in container with buffered osmium tetroxide at approximately 0° C for 5 minutes.

(b) Remove container holding tissue from ice bath so that tissue can equilibrate at room temperature over a period of 30-60 minutes.

(2) Glutaraldehyde

Glutaraldehyde is a dialdehyde O=CH-CH_2-CH_2- CH_2- CH=O which reacts with active hydrogen groups. In tissues, glutaraldehyde reacts with amino, imino and polyhydroxyl alcohols. These reactions form cross-links or inter-molecular bridges which bind the ultracellular structure to give artifact-free images. Furthermore, this dialdehyde fixative preserves enzyme activity at levels high enough to allow histochemical procedures after days or weeks of storage in cold buffer.

A specially purified glutaraldehyde concentrate is now available (Ladd Cat. #252).

The electron microscopist is referred to papers of Sabatini, Tormey, Powell, and Ledbetter. See References.

B. Dehydration

After rinsing out the fixative in distilled water, dehydrate in a graded series of ethyl alcohol as follows:

1. 50% Ethyl Alcohol - 15 mins.
2. 75% Ethyl Alcohol - 15 mins.
3. 95% Ethyl Alcohol - 15 mins.
4. Absolute Alcohol - 15 mins.
5. Absolute Alcohol - 10 mins.

Tissue is not moved from one bath to another, instead the solution is poured out slowly, from the vial, allowing the tissue to remain behind.

C. Infiltration Procedure

1. Leave material in bath 1 of propylene oxide - 15 mins.
2. Leave material in bath 2 of propylene oxide - 15 mins.
3. Complete Resin - Propylene oxide 1:1 = 60 mins. with occasional stirring (see heading Complete Resin Mixture)

Propylene oxide is used as a clearing agent and also appears to have some fixation properties. Since it is miscible with both alcohol and resin it allows the tissue to be thoroughly impregnated by the resin. It is easily handled with a 10ml. syringe equipped with a long needle. Propylene oxide is volatile and somewhat toxic thus adequate ventilation and careful handling should be observed. Spills or waste can be washed down with water.

D. Trimming

The tissue is now taken out of the vial with a pipette and carefully dropped onto a wooden tongue depressor with a small amount of liquid surrounding each piece. It is then cut with a clean, sharp razor blade to the size that will be encapsulated.

E. Encapsulation

Small Tissues

Tissue is transferred on the end of a needle on wooden applicator stick to the top of a complete resin filled capsule. During settling, most of the solvent diluted resin surrounding the tissue will be removed.

Large Tissues

Before transferring large tissue pieces to the capsule allow it to settle through 5-10ml. of complete resin. The two settling steps provide a more thorough removal of solvent diluted resin.

We recommend the use of BEEM Capsules.

They produce pre-trimmed blocks, permit orientation of the specimen and require no pre-drying. To facilitate the orientation of the tissue in the capsule, a BEEM Capsule Holder is used on top of a light table or viewer. This illuminates the capsule from base to top very well.

F. Cure

The resin can be cured overnight at 60° C or preferably as follows:

(a) Incubation overnight at 35° C
(b) Incubation next day at 45° C
(c) Incubation next night at 60° C

The blocks are ready for sectioning after removal from the oven and on reaching room temperature.

Plastisolve X-3 (Ladd Cat. #275) is an effective solvent for polymerized epoxies and polyester resin.

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APPENDIX D

STAINING

Staining thick epoxy resin sections (Richardson, 1960)

Richardson *et al.* (1960) described one method which has been found to be particularly valuable for epoxy-embedded sections from 0.5 to 1.0 μ in thickness. The embedding material is not removed in this method. The sections, dried down on a glass slide, are first treated by flooding the slide with a puddle of fresh 1% periodic acid in water for 5 minutes. They are then rinsed for about a minute in water and blotted dry with lens tissue. The sections are then flooded with a 1:1 mixture of 1% azure blue in water and 1% methylene blue in 1% borax solution (pH adjusted to about 4.5) and the slide is carefully heated to steaming on a hot plate for 3 to 5 minutes. The two 1% stain solutions can be made and stored separately, but should be mixed together directly on the slide at the time the staining is done. After the sections are stained, heat to dry state and then rinse thoroughly with water, blot dry, and mount in any convenient mounting medium.

Reynolds lead citrate stain (Reynolds, 1963)

Place in a 50 ml. volumetric flask:

lead nitrate	$Pb(NO_3)$	1.33 gm.
sodium citrate	$Na_3(C_6H_5O_7) \cdot H_2O$	1.76 gm.
freshly boiled and cooled distilled water		30 ml.

Shake intermittently and vigorously for 30 minutes. A heavy, white precipitate will form. Add 8.0 ml. of N NaOH, dilute to 50 ml. with the boiled and cooled distilled water, and mix by repeated inversion until the precipitate is dissolved. The pH should be about 12. The stain is then ready for use and will keep for several months if kept stoppered. Discard the stain when a precipitate appears in the flask, or when excessive contamination is found on a series of stained grids.

Procedure:

(1) Place a drop of staining solution on a clear wax or plastic plate.

(2) Place a drop of distilled water nearby on the plate, and immerse the grid to be stained in the water drop for about 1 minute. If a wax plate is used, the edge of the grid can be pressed slightly into the wax so that the grid will stand upright. The water-soak wets the surface of the section, reducing the amount of contamination at the interface between the section and the staining solution.

(3) Using forceps, transfer the grid from the water drop to the staining solution drop without drying the grid.

(4) Leave the grid in the stain for 3-10 minutes depending on how much staining is desired. In general, thinner sections should be stained longer.

(5) When staining is complete, remove the grid from the drop of stain and immediately rinse it in a jet of water from a wash bottle. (Some workers precede this with a jet of 0.02 N NaOH, but this does not always seem to be necessary.) Aim the jet at both sides of the grid, and be sure to wash out any staining solution between the tips or points of the forceps.

(6) Dry the grid by placing a piece of lens tissue between the tips or points of the forceps and advancing it toward the grid. This approach prevents washing the fluid between the tips or points of the forceps over the grid. The grid is ready for use when dry.

(7) Use each drop of water and stain for only one grid.

(8) Store grids in vacuum dessicator jar.

Uranyl acetate stain

Put a few crystals of uranyl acetate in a 10 ml. volumetric flask and add water. Shake for 20 to 30 minutes or allow to dissolve overnight.

Float grids, section side down, on a drop of saturated uranyl acetate solution. Staining will require 30 minutes to several hours.

Prepare fresh saturated uranyl acetate solution before each use (Pease, 1964).

APPENDIX E

KODAK ELECTRON IMAGE PLATE DEVELOPMENT*

Dark Room Handling

Use a Kodak Safelight Filter, Wratten Series 1 (red), in a suitable safelight lamp with a 15-watt bulb. The safelight lamp should be kept at least 4 feet from the plates. Lint-free cotton gloves, such as Kodak Cotton Gloves (available in three sizes through your Kodak dealer), are recommended when plates are handled, to protect the hand and the plates.

Particles of dust and paper fiber may be generated by the sharp edges of photographic plates as a result of plate movement in the package during shipment and handling. These particles must be removed before the plates are exposed in order to prevent formation of dust spots and pinholes. Brushing the plate sharply on the darkroom workbench is generally effective.

Exposure and Development Conditions

Electron Speed 50 kv to 100 kv.....0.6

Detective Quantum Efficiency.....0.5

Development Aim.....Intermediate speed and grain

Kodak HRP Developer: 68 F (20C) with Nitrogen-Burst Agitation at 8-second Intervals:

For 1 liter working solution: HRP (1:4), plus 1 gram Kodak anti-fog No. 1.-----Develop 3 minutes.

For 1 gallon working solution: HRP (1:4), plus 3.8 grams Kodak anti-fog No. 1.-----Develop 3 minutes.

Rack-in Tank Development Without Nitrogen-Burst Agitation

Develop plates at 68 F. (20 C). Use development conditions such as those shown in the table on page 1. Proceed as follows.

Start the timer. Smoothly and carefully lower the plates as a unit into the developer. Immediately tap the plates sharply two or three times on the upper edge of the tank to dislodge any air bubbles clinging to the emulsion. Check the spacing of the plates to make certain that they are at least 0.5 inch apart.

Allow the plates to remain undisturbed in the developer for about 20 seconds. Then, quickly but smoothly, lift them clear of the solution. Keeping the plane of the plates perpendicular to the surface of the bench or sink, rotate them one quarter turn, either clockwise or counter-clockwise, and back. Immediately reimmerse the plates, and again lift them clear of the solution, rotating them one quarter turn in the opposite direction. It is important that the angle of rotation should not be appreciably less than 90°. Quickly reimmerse the plates in the solution and check their spacing as before, to make certain that they are at least 0.5 inch apart. The entire cycle of lifting and reimmersion should be as rapid as possible without interfering with smooth operation. It should be completed in about 5 to 7 seconds. *Repeat the cycle several times a minute.* When development is complete, lift the plates from the developer, drain them for 1 or 2 seconds, and transfer them to the rinse tank.

Rinse plates in running water for 1.5 minutes at 65 to 70 F. (18 to 21 C.). Agitate the plates continuously by lifting and reimmersing them in the rinse tank. Do not use conventional stop bath solutions because they may produce a mottled appearance on plates used for electron exposures. Remove the plates to the fixing tank.

Fix plates for 8 to 10 minutes at 65 to 70 F. (18 to 21 C.) with Kodak fixer or Kodak Fixing Bath F-5. Agitate plates frequently during fixing.

Wash plates 20 to 30 minutes in running water. To minimize drying marks, treat the plates in Kodak Photo-Flo Solution after washing, or wipe surfaces carefully with a soft viscose sponge.

Rapid Wash.--Kodak Hypo Clearing Agent can be used after fixing to reduce washing time and to conserve water. First remove excess fixer (hypo) by rinsing the plates in water for 30 seconds; then bathe them in Kodak Hypo Clearing Agent solution for 1 to 2 minutes with moderate agitation. Wash for 5 minutes in a water flow sufficient to give at least one complete change of water during the 5-minute wash period.

Dry in a dust-free place.

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*Kodak Pamphlet No. P-116.

VITA

The author was born in Salt Lake City, Utah, on January 31, 1941. He received his primary and secondary education in Ft. Collins, Colorado, and Salt Lake City, Utah. After graduation from high school in 1959, he continued his education at Utah State University, Logan, Utah.

In January, 1961, he was called by the Church of Jesus Christ of Latter-day Saints to serve a full time mission in South Germany. Fall term, 1963, he returned to Utah State, where he completed a B.S. degree in bacteriology in 1966.

In 1966 he accepted a Federal appointment with the United States Department of Agriculture, Agriculture Research Service, Animal Disease and Parasite Division, Logan, Utah.

He entered the College of Veterinary Medicine, Michigan State University, spring term 1967. In September, 1968, he was admitted to graduate school, Michigan State University. He received his D.V.M. degree in December, 1969, and continued in the Master's degree program.

The author married Sharon Hintze in November, 1963, and they have three children: Karine 5, Bryan 2, and Jan 1.