ULTRASTRUCTURAL CHARACTERIZATION OF POLYMORPHIC CHLAMYDIAL PARTICLES OF AN EGG-PASSAGED *C. PSITTACI* 6BC STRAIN NONCYTOTOXIC FOR CULTURED MAMMALIAN CELLS

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ABSTRACT

ULTRASTRUCTURAL CHARACTERIZATION OF POLYMORPHIC CHLAMYDIAL PARTICLES OF AN EGG-PASSAGED C. PSITTACI 6BC STRAIN NONCYTOTOXIC FOR CULTURED MAMMALIAN CELLS

A strain of C. psittaci 6BC, passaged in the yolk sac of embryonated chicken eggs, has been examined by means of electron microscopy. The chlamydial bodies present in thin sections of a semipurified preparation of this agent have been characterized, with special emphasis on the ultrastructure of their surface layers.

Three morphologically distinct forms were found: large, fragile reticulate bodies, irregular in shape and of moderate density, enclosed by two double track structures; and two types of small, rigid elementary bodies, one enclosed by two double track structures (Type II), the other by only one double track (Type I).

The two types of elementary bodies were found in samples of C. psittaci 6BC fixed by two methods, one designed to preserve spatial relationships, the other to improve contrast. The difference in the buoyant densities of the two types was sufficient to allow their separation by discontinuous sucrose density gradients. It was therefore concluded that the two forms of elementary bodies are distinct entities.

The ultrastructure of the surface layers of neither type I nor type II elementary bodies, nor reticulate bodies, was strictly comparable to the ultrastructure of the surface layers of a Gram-negative bacterium. The reticulate bodies and type II elementary bodies had two double track structures, as do Gram-negative bacteria, but the area between the two double tracks was uneven in width and often

contained cytoplasmic elements. The type I elementary bodies had only one double track structure, beneath which was a clear zone of variable width.

In internal organization as well, the three chlamyidal forms differed from Gram-negative bacteria. The reticulate bodies were characterized by an internal fibrous net surrounded by a variable amount of cytoplasm; and both the type I and type II elementary bodies had a central or eccentric dense nucleoid surrounded by a less dense, granular cytoplasm.

The success of this type of analysis of a chlamydial strain non-cytopathogenic for cultured mammalian cells lends hope that a similar analysis of a strain causing cytopathic effects may be carried out. One could then determine whether differences in the cytopathogenic properties of chlamydial strains can be related to the ultrastructure of the surface layers of their component forms.

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INTRODUCTION

The pathogenic potential of the psittacosis agent for humans was recognized by the end of the ninteenth century and received much attention during outbreaks of disease in different parts of the world in 1929 and 1930 (Meyer, 1964). Despite the application of public health measures chlamydial outbreaks have continued to occur in man and animals in many countries (Storz, 1971).

Endemic human chlamydial infections have been recognized in the Canadian Arctic for many years (J.C. Wilt et al, 1959; Hildes et al. 1958; Hildes et al, 1965; Wyman et al, 1969) and several reservoirs of Chlamydiae in birds and animals have been identified in the Canadian North (Spalantin et al. 1966; P.C. Wilt. 1971; P.C. Wilt et al, 1972). Careful clinical and epidemiological studies performed in Manitoba have clearly demonstrated the pathogenic potential of these agents in terms of duration and severity of disease in human (J.C. Wilt et al. 1971).

The interest of several workers in this department has expanded from applied research as described above, to the exploration of the fundamental properties of chlamydial agents.

We have in a series of studies approached the mechanisms of the pathogenicity of the 6BC strains of psittacosis from the aspect of the host cell response. Comparative studies of two 6BC strains, one L-cell "adapted" and the other egg "adapted", revealed that the former induced cytopathic interactions in cultured mammalian cells, while the latter was noncytopathic for the same host cells. It has further been shown that lysosomes play an important role in the mechanism of the "toxic" effect of certain 6BC strains upon cultured mammalian macrophages and L-cells (Kordová et al. 1971; Kordová and Wilt, 1971; Kordová et al.

1972a, 1972b).

That the chlamydial agent acquires or loses its pathogenicity when passaged in cultured mammalian cells as compared to the yolk sac of embryonated chicken eggs has been demonstrated by several authors (Mitsui et al. 1967; Reeve and Taverne, 1967). However, how and why the agent does so is not known. A similar phenomenon known as "phase variation" has long been recognized in Coxiella burneti (Stoker and Fiset, 1956), another member of the order Rickettsiales (family Rickettsiae). In this organism, the shift from the animal-maintained "smooth" strain to the egg-grown "nonsmooth" strain is accompanied by changes in virulence, antigenicity, immunogenicity and density (Ormsbee, 1969). Some authors have described a variant of C. burneti occurring in tissue culture cells, which has a filamentous capsule-like layer associated with its surface (Rosenberg and Kordová, 1962; Burton et al. 1971). Encapsulated coxiellae have not as yet been described in infected yolk sac.

Chemical similarities between chlamydial and rickettsial envelopes, and those of Gram-negative bacteria have been found (Allison and Perkins, 1960; Perkins and Allison, 1963), and it has been suggested that the envelopes of all three are structurally similar as well (Higashi, 1968). Since it is known that in some bacteria changes in pathogenic, morphologic and antigenic properties may be due to a change in the organisms surface layers (Braun, 1965), it is possible that such a mechanism for altering pathogenicity may also operate in Chlamydia.

With these considerations in mind, it was decided to examine the chlamydial particles in a sample of C. psittaci 6BC which had been passaged in yolk sac and was noncytopathic for tissue culture cells,

with a view to later comparing the ultrastructure of their surface layers to that of particles from an L-cell passaged strain of 6BC which does produce CPE in tissue culture cells.

This work represents an integrated part of a project involving investigation of some of the mechanisms and factors influencing the pathogenicity of Rickettsiales and the pathogen-host cell interactions (J.C. Wilt and N. Kordová).

The examination and isolation of the particles present in a semi-purified preparation of C. psittaci 6BC, yolk sac passaged, was carried out in the Department of Medical Microbiology, The University of Manitoba, Winnipeg, Manitoba and in the Department of Biology, The University of Calgary, Calgary, Alberta, under the conjoint supervision of Dr. N. Kordová and Dr. J.W. Costerton.

HISTORICAL REVIEW

A. TAXONOMIC CLASSIFICATION

The nature and classification of the agents of psittacosis has been a source of disagreement among microbiologists from the time of the first rigorous studies of them by Bedson and Bland (1932, 1934). The controversy has centred around the finding that, although the Chlamydia are small, host dependent parasites, (suggestive of a virus-like nature) they apparently undergo a unique developmental cycle, first described by Bedson and Bland in 1932. A schematic representation of the proposed developmental cycle is presented in Figure I. It is from a recent monograph "Chlamydia and Chlamydia-induced Diseases", by Storz (1971).

The salient feature of the conceptualized unique life cycle of Chlamydia is the alternation of two developmental forms: the rigid, infectious small particle (elementary body), and the nonrigid, noninfectious large particle (reticulate body). The mechanisms for the reversion from elementary body to reticulate body and back to elementary body are not known, and have been the subject of many speculations (Moulder, 1966; Christofferson and Manire, 1969; Tribby, 1970).

This proposed unique developmental cycle did not meet with universal acceptance. Pollard's group in a series of reports (Pollard et al, 1960; Pollard and Starr, 1962; and Pollard and Tanami, 1962) described a virus-like developmental cycle, characterized by the occurrence of an eclipse period during which there was disappearance of the infecting morphological entity and its antigen, followed by the appearance of cytoplasmic inclusions containing RNA, then cytoplasmic inclusion containing DNA, then protein, and finally mature chlamydial particles. Chlamydial agents were at times considered as large viruses (Moulder, 1962).

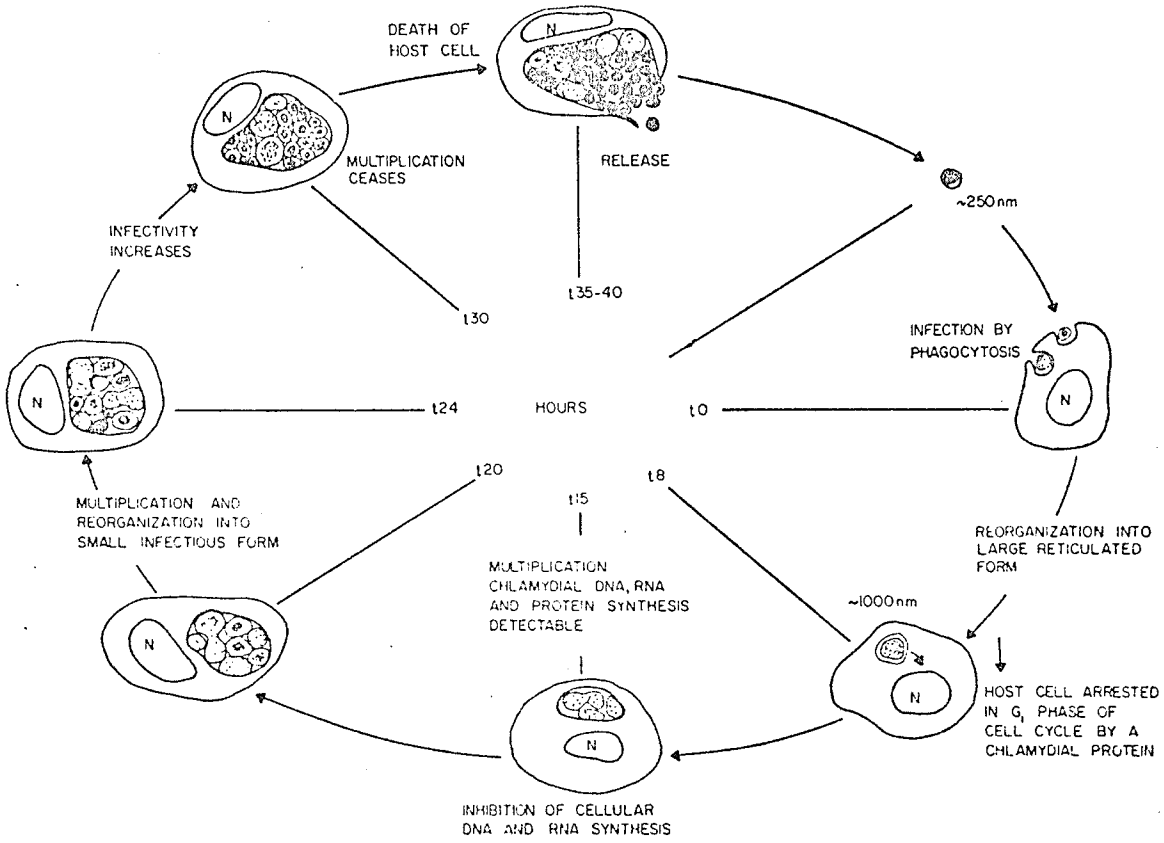


FIGURE I

THE PROPOSED DEVELOPMENTAL CYCLE OF CHLAMYDIA

In a review article in 1958, Bedson suggested that the agent of psittacosis was a new kind of infectious entity, not immediately identifiable as either virus or bacterium (Bedson, 1958).

Lwoff, in 1957, presented a set of criteria by which he rigorously defined a virus. Moulder (1962, 1964, 1967) applied these criteria to Chlamydia, and concluded that they are not viruses. He therefore assumed that they are bacteria. Since the Chlamydia were known to be sensitive to some of the same cell wall poisons (in particular, penicillin and D-cycloserine) as Gram-negative bacteria (Moulder, 1964), Moulder proposed that Chlamydia are small, host dependent Gram-negative bacteria.

It is now generally accepted that Chlamydia are host dependent Gram-negative bacteria which undergo a unique developmental cycle. These organisms are at present classified under the order Rickettsiales (Breed, Murray and Smith, 1957), genus Chlamydia, but it has recently been suggested that they be classified under their own order, Chlamydiales, since their developmental cycle is so unique (Storz and Page, 1971).

B. PURIFICATION AND CHEMICAL COMPOSITION OF CHLAMYDIA

a) Introduction

Attempts to purify Chlamydiae have for the most part met with mixed success due to the extreme range in properties exhibited by the diverse chlamydial life forms, and the efficacy of examining a highly purified sample has been questioned (Weiss and Dressler, 1962). Highly purified and separated preparations give no indication of the range of variability within a strain's population, and may exclude biologically significant parts of the original inoculum. Also, it is known that the fragile reticulate bodies of Chlamydiae readily leak out much of their RNA (Weiss and Dressler, 1962) and it is therefore possible that puri-

fication could alter the chemical composition of the chlamydial particles somewhat.

With the reservation in mind that the purification procedures themselves may have changed the properties of Chlamydiae, a review of the literature concerning their properties and chemical composition is presented here.

b) Properties and chemical composition of a mixture of reticulate and elementary bodies and their envelopes

Of necessity early studies of the chemical composition of Chlamydiae employed preparations which had been freed to some extent from host material, but which contained a mixture of reticulate and elementary bodies. Jenkin (1960) investigated the composition of egg-passaged meningopneumonitis particles, and their envelopes, as isolated from them by treatment of whole particles with detergent and enzymes; Perkins and Allison (1963) applied the same techniques to a variety of chlamydial agents to determine whether or not muramic acid and diaminopimelic acid are present in their envelopes. Table 1 is a summary of the chemical content of a mixture of elementary and reticulate bodies, and isolated envelopes of a meningopneumonitis strain.

As can be seen from the table, mixed populations of chlamydial strains are characterized by an extremely high lipid content, two or three times as high as the lipid content of most Gram-negative bacteria (Kates, 1964). This value varies with the host system, as does the actual makeup of the lipid (Makino et al, 1970).

The presence of both RNA and DNA in the particles has been used as evidence for the exclusion of Chlamydia from the viruses (Moulder, 1964). The traces of muramic acid found by Perkins and Allison (1963)

TABLE 1*

CHEMICAL CONTENT OF A MIXTURE OF ELEMENTARY AND RETICULATE BODIES OF MENINGOPNEUMONITIS, AND OF THEIR ISOLATED ENVELOPES

Constituent	Per Cent Dry Weight	
	Intact Chlamydiae	Cell Wall
α-Amino acid nitrogen	5.3	5.2
Total nitrogen	6.5	7.2
Total protein	33.1	32.5
Hexosamine	0.62	0.44
Reducing sugar	1.05	1.14
Total carbohydrate	1.67	1.58
Muramic acid	trace	trace
Ribonucleic acid	2-7	trace
Deoxyribonucleic acid	3.4	trace
Total nucleic acid	5.62	0.4
Cholesterol	0	0
Phospholipid	7.5	1.5
Lipid (unknown composition)	35-40	50 or less
Acid soluble fraction	7-10	?

* From: Storz, 1971

have been cited as evidence of their bacterial nature.

c) Properties and chemical composition of isolated reticulate and elementary bodies and their envelopes

To date, purification of elementary and reticulate bodies has been successfully carried out by Tamura's group (Tamura and Higashi, 1963; Tamura et al. 1967) on the L-cell passaged Cal 10 strain of meningopneumonitis. These workers removed the reticulate bodies from suspensions of meningopneumonitis by treatment of the agent with DNase, RNase and trypsin, and by sonication; then purified the remaining elementary particles by differential and density gradient centrifugation. Reticulate bodies were isolated from infected L-cells at 18 hours post infection, at which time the number of elementary bodies in the cells was limited. Tamura's group (Tamura and Manire, 1967; Manire and Tamura, 1967) isolated envelopes from the isolated elementary and reticulate bodies, and determined their chemical composition. Tables 2 and 3 compare the meningopneumonitis elementary and reticulate bodies, and their envelopes, as reported by Tamura and coworkers, and others (Litwin, 1959).

Isolated elementary bodies and reticulate bodies differ in size, lability, organization of their chromosomal material, and relative amounts of RNA and DNA. The lability of the reticulate body has been attributed by Matsumoto and Manire (1970) to a lack of the rigid factor in the envelope of these bodies. It has been proposed that the reticulate bodies are the intracellular, dividing form of the organism, and as such require large amounts of RNA, and a membrane or envelope of exceptional permeability to allow passage of needed macromolecules into the growing particles (Matsumoto and Manire, 1970). Elementary bodies on the other hand are thought of as extracellular forms requiring protection from the

TABLE 2

COMPARISON OF ISOLATED RETICULATE AND ELEMENTARY BODIES

Property		Elementary	Reticulate
diameter		0.2μ - 0.3μ	0.5 - 1.0μ
infectivity		yes	no
DNA distribution		dense nucleoid	dispersed
RNA/DNA ratio		1:1	4:1
sensitivity to:	freezing (-70°C)	no	yes
	sonication (10 kc, 5')	no	yes
	lysozyme	no	yes
	sodium dodecyl sulphate	no	yes
	trypsin	no	yes
	mercaptoethanol	no	yes

TABLE 3

PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF THE ENVELOPES OF ELEMENTARY AND RETICULATE BODIES OF THE CAL 10 STRAIN OF MENINGOPNEUMONITIS

Property	Elementary body	Reticulate body
appearance (air dried)	folded, rigid	thin, flattened
buoyant density, CsCl	1.295	1.395
amino acids	all common ones	no cystine or methionine
muramic acid	no	?
hexosamines	yes	?
protein	70-75%	?
phospholipid	3% of total in Ebs	7% of total in Rbs
Diaminopimelic acid	no	no

*Tabulated from: Manire, 1966; Tamura and Manire, 1967; Manire and Tamura, 1967

environment by a tough envelope (Matsumoto and Manire, 1970).

The envelopes of meningopneumonitis elementary bodies and reticulate bodies are significantly different, as can be seen from Table 3. Elementary bodies have a rigid envelope containing all the common amino acids, as well as traces of hexosamines and phospholipids. Reticulate body envelopes lack cystine and methionine, two amino acids capable of forming disulphide bridges which might impart some rigidity to the structure.

The inability to demonstrate muramic acid in purified reticulate or elementary body envelopes is interesting, since this finding contradicts the results of earlier workers using mixed populations (see above), and does not correlate well with the suggested bacterial nature of Chlamydia.

C. THE ULTRASTRUCTURE AND MORPHOLOGY OF CHLAMYDIA

a) Introduction

In approaching a review of the electron microscopic studies of chlamydial morphology and ultrastructure, consideration must be given to the fact that the agent of psittacosis has been considered, at times, as a large virus (Moulder, 1962) and that several workers have reported a virus-like replication process for a number of psittacosis strains (Pollard and Starr, 1962; Mitsui et al. 1964). This has, of course, influenced the terminology used in interpretation of the polymorphic chlamydial populations, and especially their surface structure, which has been variously referred to as a bilaminar envelope (Armstrong et al. 1963), a trilayered membrane (cell wall) (Anderson et al. 1965), and a double membrane (Erlandson and Allen, 1964). The confusing descriptions of the ultrastructure of chlamydial particles are also understandable

when one considers the inappropriate fixation methods used in early electron-microscopic studies.

Summarized below are the descriptions of Chlamydia as observed by different workers, and also the little that is known about their surface layers. There is general agreement that all chlamydial strains have a similar morphology by light and electron microscopy; therefore data referring to the ultrastructure of C. trachomatis as well as to C. psittaci have been included in this review.

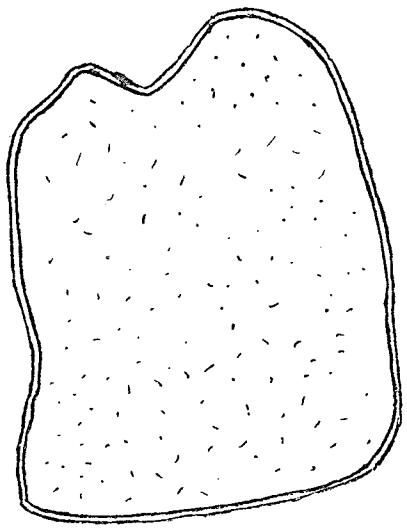
b) Morphology and ultrastructure of Chlamydia as it is now generally envisaged

Four morphologically distinct forms of Chlamydiae have been described in infected cells: giant or polygonal bodies, reticulate bodies, intermediate bodies and elementary bodies. They are diagrammatically represented in Figure II.

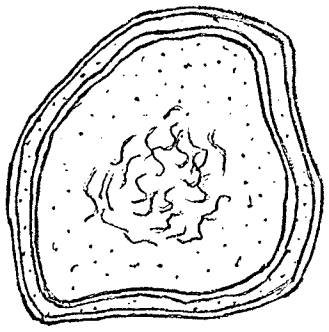
Giant bodies of C. trachomatis in chick embryo yolk sac were described by Mitsui et al. (1964). These bodies were polymorphic, with diameters ranging from 1-5μ. Within the particles was seen a reticulated substance of moderate density, which consisted of two components: double stranded fine filaments, and fine granules 100-150 A in diameter, which resembled ribosomes. Anderson and coworkers (1965) found the same polymorphy and internal structure in giant bodies of C. psittaci in monkey kidney cells. They considered the fine filaments to be DNA; the granules to be ribosomes.

Reticulate bodies, also referred to as "initial" or "large" bodies (Anderson et al. 1965; Matsubara and Mitsui, 1968) were described by Anderson and coworkers as spherical bodies, 0.5 - 1.0μ in diameter, with an internal structure like that of the giant bodies, but having more prominent DNA strands forming a distinct central "nuclear" area.

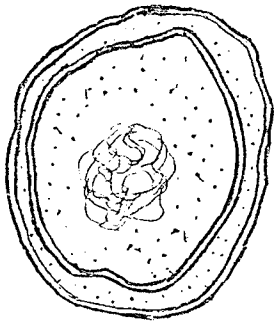
Giant Body
1-2μ



Reticulate Body
0.5-1μ



Intermediate Body
0.5μ



Elementary Body
0.20-0.35μ

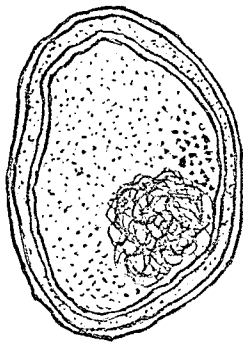


FIGURE II

CHLAMYDIAL FORMS

Lépinay et al.(1970) described reticulate bodies as having a less dense central area in which were visible filaments 20 A wide, organized in an irregular network or web, and assumed to be DNA; at the periphery of this central zone was a more dense area which contained particles 150 A in diameter.

Intermediate bodies, as observed by Lépinay et al. (1970) were smaller than reticulate bodies, being 0.5μ in diameter, and had a dense central condensation, from which fine strands or filaments occasionally extruded. The cytoplasm was comparable in consistency to that of the reticulate bodies. This description is similar to that given by Anderson et al.(1965).

Recent workers (Lépinay et al. 1970) have described elementary bodies as spheres, 0.30 - 0.35μ in diameter, which contain an eccentric, dense mass (the nucleoid) surrounded by a dense cytoplasm containing granules 150 A in size (ribosomes). The star-shaped elementary bodies, observed frequently in the past (Mitsui et al. 1964), and elementary bodies in which the internal material has retracted from the outer surface layers (Anderson et al, 1965), are fixation artifacts due, in most cases to use of permanganate or osmium tetroxide as fixatives.

c) Exceptions to the general picture

The four morphological forms described above do not appear invariably in all infections by Chlamydiae. Mitsui et al. (1967) have reported that giant bodies appear much less frequently in HeLa cells infected with the T'ang strain of trachoma, than in yolk sac infected with the same agent. Infections in which the host cell, be it HeLa cell (Matsubara and Mitsui, 1968) or L-cell (Matsumoto and Manire, 1970), has been treated with penicillin do not give rise to elementary bodies;

the forms observed are enlarged abnormal reticulate bodies and intermediate bodies.

d) Surface layers of the chlamydial forms

The surface layers of giant bodies have not yet been resolved. Anderson and coworkers (1965) described a "tri-layered membrane (cell wall) and a second, ill-defined membrane just beneath the outer membrane" in these bodies. Their electron micrographs, however, are not of sufficient clarity to support such a description. Mitsui et al. (1964) suggested that giant bodies are surrounded by a double membrane, each membrane showing a bimolecular leaflet. Their micrographs do support the conclusion that the envelope of giant bodies is double in nature. No recent work, using modern fixation techniques, has been done to clarify the structure of the surface layers of giant bodies.

The reticulate bodies are bound by a structure which was described as a double membrane by Mitsui et al (1964) and as a bilaminar envelope reminiscent of a cell wall and cytoplasmic membrane by Armstrong et al. (1963) and Armstrong and Reed (1964). Anderson et al. (1965) suggested that, like giant bodies, the reticulate bodies "seemed to be bound by a trilayered membrane (cell wall) and a second ill-defined membrane just beneath the outer membrane". Erlandson and Allen (1964) found "whorl"-like and complex internal membranes continuous with the outer unit membrane of reticulate bodies; they considered these internal structures to be analogous to the mesosomes of bacteria. The confusion evident from these diverse descriptions of the same entity is a result of the poor quality of the micrographs on which the descriptions were based.

The surface layers of reticulate and intermediate bodies have been resolved in a recent work by Lépinay et al. (1970), who described them as

consisting of two concentric unit membranes, separated by a clear space approximately 100 A in width. The micrographs accompanying their description do clearly show two unit membranes around the reticulate and intermediate bodies; however, the space between the two unit membranes is not of constant width and seems in places to contain cytoplasmic elements.

With respect to elementary bodies, Erlandson and Allen (1964) described a single unit membrane around these particles, while Anderson et al. (1965) postulated that the inner second trilayered membrane in elementary bodies cannot be seen readily because it has retracted along the internal nucleoid. Lépinay et al. (1970, 1971) found that a single unit membrane could be resolved in preparations prefixed with glutaraldehyde, then fixed with osmium tetroxide; when the elementary bodies were fixed by the procedure of Ryter and Kellenberger (1958) a second unit membrane became visible, although it did not follow the outer unit membrane closely.

Manire (1966) and Matsumoto and Manire (1970) prepared purified elementary body and reticulate body cell walls. Electron micrographs of purified elementary body cell walls shadow cast or negatively stained with phosphotungstic acid revealed a geometric array of hexagonal structures on one side of the wall. Reticulate body envelopes showed only traces of these regular structures. As pointed out by Manire, such a pattern is reminiscent of the cell wall of Spirillum serpens (Murray, 1963), a Gram-negative bacterium. Matsumoto and Manire succeeded in isolating subunits of the hexagonal array, and found them to be 20 nm in diameter. Matsumoto and Manire suggested that the subunits are the rigid components of elementary body walls; lack of the subunits would

account for the fragility of reticulate bodies.

Lépinay et al.(1971) used the method of Seligmann and Thiery to demonstrate a layer of polysaccharide in the surface of whole elementary bodies, beneath the outer unit membrane. This layer was much thinner or completely absent from reticulate and intermediate bodies. The location of this layer corresponded to the location of the rigid mucopeptide layer in some Gram-negative bacteria (Murray et al. 1965) and may, therefore, be the layer which confers rigidity on the elementary bodies. The exact nature of the polysaccharide was not determined. It remains to be seen whether this layer has the hexagonal structures seen by Matsumoto and Manire (1970).

MATERIAL AND METHODS

A. THE AGENT

a) Introduction

The yolk sac of embryonated chicken eggs represents the most favorable growth medium for Chlamydiae. The majority of the chlamydial isolates and laboratory strains can be maintained only in the yolk sac of eggs; those strains which have been adapted to growth in tissue culture cells are few in number and all mutliply extensively in yolk sac (Storz, 1971). An egg-passaged chlamydial strain was therefore chosen for these preliminary studies.

b) Passage history and biological activity

The 6BC strain of C. psittaci was originally obtained as a lyophilized homogenate of infected yolk sac, from the American Type Culture Collection (ATCC). On receipt, the agent had been passaged in the yolk sac of chick embryos (CE) 54 times; its $CELD_{50}$ was $10^{6.3}$/0.03 ml.

The lyophilized homogenate was resuspended in Eagle's Minimum Essential Medium (MEM) (Flow Laboratories, Inc. Rockville, Maryland), containing 0.1% $NaHCO_3$, 200 ug of streptomycin sulfate per ml and 10% heat-inactivated calf serum (Grand Island Biological Co. N.Y.).

Infectivity of the resuspended homogenate was titrated in this laboratory in several host systems. Its tissue culture (TC) ID_{50} was 5×10^9/ml in chick embryo yolk sac entodermal cells in vitro, and 5×10^8/ml in L-cells (clone 929). In mouse peritoneal macrophages in vitro the $TCID_{50}$ was $5 \times 10^{8.5}$/ml. Except for the appearance of typical stained chlamydial forms in the cells, no cytopathic changes were observed in any of the infected mammalian cells.

c) Preparation of semipurified chlamydial suspension

The lyophilized homogenate of C. psittaci 6BC obtained from ATCC was found by electron microscopy to contain very few recognizable chlamydial forms, in spite of its high infectivity. It was therefore passaged in embryonated chicken eggs to increase the number of intact chlamydial particles present.

Procedure

An amount of 0.25 ml of a 10^{-3} dilution of C. psittaci 6BC, resuspended in MEM as described above, was injected into the yolk sac of five day old embryonated chicken eggs. The eggs were incubated at 37°C, humidity 86%; 5 days after infection 10% of the embryos had died. The eggs were removed from the incubator and left overnight at 4°C. They were then opened and the yolk sacs collected in sterile petri dishes. Smears were made of each yolk sac; these were stained by the method of Giménez (1964) and May-Grunwald-Giemsa to verify the extent of infection. Random samples of yolk sacs were tested for freedom from bacterial and mycoplasmal contamination. The yolk sacs were homogenized by shaking them with glass beads in a small amount of MEM. The suspension was decanted and spun at low speed (800 rpm for 8 minutes) to remove tissue debris; the supernatant was centrifuged at 15,000 x g for 1 hour. The upper layer of lipid was removed with sterile swabs, the supernatant was decanted and frozen; and the pellet was resuspended in MEM and subjected to three runs of alternating high and low speed centrifugation. The final pellet was again checked for chlamydial particles, using the Giménez stain. The pellet was stored as a thick suspension in MEM, at -70°C until use.

B. EXAMINATION OF THE PHYSICAL PROPERTIES OF THE CHLAMYDIAL PARTICLES PRESENT IN THE SEMIPURIFIED SUSPENSION OF THE AGENT BY DISCONTINUOUS SUCROSE DENSITY GRADIENT CENTRIFUGATION

a) Introduction

Several workers have approached the study of the ultrastructure of highly purified and isolated chlamydial particles by removing completely either the reticulate bodies (Tamura and Higashi, 1963) or the elementary bodies (Tamura et al. 1967). We have examined first the entire population of our whole chlamydial strain, and have attempted further to separate and characterize all morphological forms present in the inoculum.

b) Density gradient centrifugation

Since it has been shown that centrifugation in sucrose gradients makes possible finer separation of particles of slightly different sedimentation properties than does centrifugation in other salts (Luria and Darnell, 1967), sucrose has been employed as the gradient material in this study.

Procedure

Discontinuous sucrose density gradients were prepared by the underlay technique, using 1.4 ml of 30% sucrose, 1.4 ml of 45% sucrose, and 1.6 ml of 69% sucrose in 5.5 ml cellulose nitrate tubes. The sucrose solutions were prepared in 0.033 M tris buffer, pH 7.4, and autoclaved at 15 psi for 15 minutes. The cellulose nitrate tubes were sterilized under ultraviolet light for one hour.

After preparation the gradients were left at $4^{o}C$ overnight; they were then loaded with 0.5 ml of sample, balanced with paraffin oil, and centrifuged at 35,000 rpm for 22 hours in the SW-65 rotor in a Spinco L2-65B centrifuge at $4^{o}C$. After centrifugation, a picture of the banding pattern was taken, the bands were collected with a needle and syringe from the top of the gradient.

and samples of each band were checked in an Abbe refractometer to determine the concentration of sucrose at which each band occurred. The bands were then diluted with MEM, and centrifuged for one hour at 45,000 rpm in an SW-65 rotor. The resulting pellets were stored at -70°C, or fixed in situ for electron microscopy.

C. ELECTRON MICROSCOPY

a) Introduction

Concentrated samples of the semipurified whole inoculum as well as its separated bands, were fixed and embedded for thin sectioning. In addition the original lyophilized egg passaged 6BC strain received from ATCC, and a sample of uninfected yolk sac, were examined. The procedure used was previously described (Forsberg et al, 1970), and was designed to give optimum preservation of the material for ultrastructural studies.

b) Processing of samples prefixed with glutaraldehyde

A thick suspension of each sample in MEM was prefixed in situ with fresh 1% glutaraldehyde (Ladd Research Industries, under noble gas) in phosphate buffer, pH 7.0, for 30 to 60 minutes at room temperature. Each sample was then enrobed in 4% Difco Bacto agar at 42-45°C (Murray et al, 1965). The resulting cores of agar were fixed with 5% glutaraldehyde in phosphate buffer for 2 hours at room temperature, then washed 5 times, 20 minutes per wash, with phosphate buffer. The cores were post-fixed in a 2% osmium tetroxide (Scientific and Industrial Chemicals Corp., New York) solution in acetate-veronal buffer (Ryter and Kellenberger 1958b), containing 0.16% tryptone broth and 7 mM $MgCl_2$. The sample was fixed for 2 hours at room temperature, then washed 5 times as above in acetate-veronal buffer. After fixation the cores were stained with 1% aqueous uranyl acetate (Fisher Scientific Co.) for 1 hour and washed

with 5 changes of acetate-veronal buffer as above, then dehydrated through an acetone-water series. The acetone was replaced with 2 changes of propylene oxide, after which the cores were infiltrated with Vestopal W (Polysciences) plus activator plus initiator. The activator was 0.5% cobalt naphthenate (Polysciences); the initiator was 1.0% tertiary butyl perbenzoate (Polysciences). The infiltrated cores were embedded in fresh Vestopal W and the plastic was polymerized at 65°C for 60 hours.

c) Processing of samples fixed with osmium

In the interests of obtaining better contrast, a sample of the whole, semipurified 6BC inoculum was fixed by the method of Ryter and Kellenberger (1958a). The material was fixed in 2% osmium tetroxide in acetate-veronal buffer, pH 6.4, containing 0.16% tryptone broth and 7 mM $MgCl_2$, for 2 hours at room temperature. It was then enrobed in agar and processed as above.

d) Thin sectioning and staining

The thin sectioning and electron microscopy, as well as the preparation of pictures, were performed at the Department of Biology, University of Calgary, Calgary, Alberta.

Blocks were trimmed with a razor blade, and sectioned on an LKB ultratome III, using glass knives. Sections were collected on naked 400 mesh copper girds, then stained for 30 minutes with freshly prepared aqueous 1% uranyl acetate, pH 4.7 - 4.8, washed on a swirling water bath, and stained for 15 minutes with lead citrate, rinsed with 1/100 N NaOH, and washed thoroughly. After drying the copper grids were backed with carbon for 2-3 seconds.

The sections were examined in an AEI EM 801 electron microscope.

RESULTS

A. CHLAMYDIAL PARTICLES PRESENT IN WHOLE, SEMIPURIFED YOLK SAC PREPARATIONS OF C. PSITTACI 6BC

a) Introduction

Semipurified inocula, prefixed with glutaraldehyde, and fixed with osmium, were each found to contain two morphologically distinct types of elementary bodies, as well as a smaller number of reticulate bodies.

b) Elementary bodies

Two morphologically distinct forms of particles having the size, shape and internal organization of elementary bodies were present in semipurified inocula prefixed with glutaraldehyde, and fixed by the method of Ryter and Kellenberger.

The two forms were readily distinguishable from one another by the presence or absence of a second double track structure internal to the enclosing double track which was common to all particles. Elementary bodies hereafter referred to as type II are those which possessed two double track structures; elementary bodies referred to as type I evidenced only one double track.

(i) Glutaraldehyde - prefixed elementary bodies

Type II

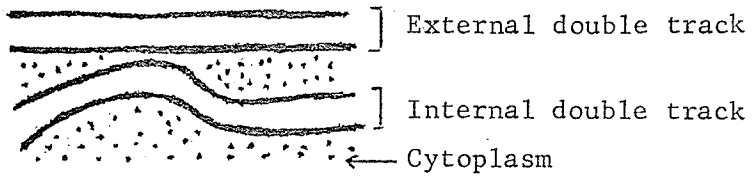
Particles of this type conformed to the general description of elementary bodies as given by Lépinay et al.(1970). The average size was 0.25μ by 0.40μ, with a mean diameter of 0.32μ. The oval shape was probably a result of the compression of a spherical body upon sectioning, since the long axis of the particles was invariable oriented in one direction.

Each elementary body possessed a central or eccentric dense core,

from which tufts or filaments often protruded (Figures 2, 3). The core was surrounded by a granular cytoplasm of moderate and variable density; in general the cytoplasm was more dense toward the edge of the particle.

The elementary bodies were enclosed by two double track structures, both of unit membrane size (7.5 - 8.5 nm in width) when in phase.

The space between the double tracks was highly variable. Most frequently they were closely apposed (Figures 2 and 4), and lacked completely the periplasmic space which separates the cell wall and plasma membrane of Gram-negative bacteria. Along some areas of the surface layers, the inner double track had moved away from the external one (Figures 3 and 4), and was separated from it by material having the density and granularity of the cytoplasm. Separation of the internal double track did not result in any irregularity of shape in the elementary body, which suggests that the inner double track is not responsible for maintenance of shape. Below is a schematic representation of the surface morphology of type II elementary bodies.



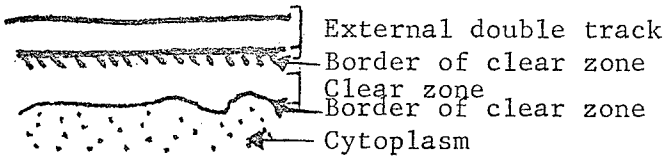
Type I

In size and gross appearance, type I elementary bodies resembled type II. They averaged 0.25μ by 0.36μ in size, and had a mean diameter of 0.30μ.

Internally, type I elementary bodies were characterized by a dense, granular cytoplasm in which a more darkly stained core or nucleoid could

occasionally be seen (Figures 5 and 8). Fibres emanating from the nucleoid were rarely distinguishable against the dark background of the cytoplasm.

Of great interest is the surface layers of these particles. All were enclosed by an external double track structure, beneath which was an electron translucent zone of varying width. This clear zone was bordered by a thin, dark layer adjacent to the inner track of the external double track: in some elementary bodies, the inner track and the thin, dark border were separated in places (Figure 5), but for the most part they were conjoined (Figures 6, 7 and 8), giving the impression of an overly thick inner track. The cytoplasmic border of the clear zone was a thin electron dense thread, much thinner than the dark layers of the double track (Figure 7). A graphic representation of the surface morphology of type I elementary bodies is given below:



The clear zone was readily distinguishable from a true double track structure by its delicate electron dense borders, which were much thinner than the corresponding layers in a double track; and by its width, which as well as varying considerably from particle to particle, and within a given elementary body, was usually greater than the width of a double track. Where it is out of phase, the clear zone was capable of mimicking a second double track (Figures 5 and 8); the true picture, however, was revealed by a closer examination of the rest of the particle.

Ruptures in the surface layers of type I elementary bodies, as shown

in Figure 8, occurred rather frequently. It is likely that these breaks occurred during sectioning, since they were all located in the same quadrant of the particle, with respect to the longitudinal axis. Such ruptures were not observed in type II elementary bodies; this is a further indication that the two types of elementary body may have dissimilar surface layers.

(ii) Osmium tetroxide-fixed elementary bodies

The differences between type I and type II elementary bodies were readily demonstrable also in material fixed by the method of Ryter and Kellenberger. This is in contrast to the report of Lépinay et al (1970) who reported that all elementary bodies have 2 double tracks, and that osmium fixation will reveal the inner double track if it is not visible in glutaraldehyde-prefixed material.

Type II

In the osmium-fixed material, type II elementary bodies manifested surface irregularities. The particles were roughly ellipsoid with a surface wrinkled to a greater or lesser extent; in rare instances (Figure 16), the particles resembled the wrinkled pea or star-shaped elementary bodies described by early workers (Mitsui et al, 1964). Their average size, 0.24μx 0.39μ, compared favorably with the size of their glutaraldehyde-prefixed counterparts, but the size range observed was much broader.

As would be expected in osmium-fixed material the dense core or nucleoid, eccentric in the majority of particles, was quite prominent (Figure 9). DNA-like fibres were extant in those particles having a less dense cytoplasm (Figure 11). The cytoplasm was granular, and of an even constituency throughout the particle, with the exception of the area around the nucleoid where it was rarified (Figure 11).

The increased contrast in this preparation enhanced recognition of the presence of two double tracks surrounding these type II elementary bodies. However, poor preservation of spatial relations (e.g. wrinkling and folding of the membranes as in Figures 9 and 10), a result of the fixative used, made it difficult to find elementary bodies in which the two double tracks were both in phase for most of their length. An exception is shown in Figure 11; in the majority of particles the double tracks faded in and out of phase, and only rarely was the inner double track seen to be parallel to and closely associated with the outer double track. Osmium fixation has been valuable in confirming the presence of two double tracks in these particles, but it was not of advantage in elucidating the spatial relationships between the surface layers.

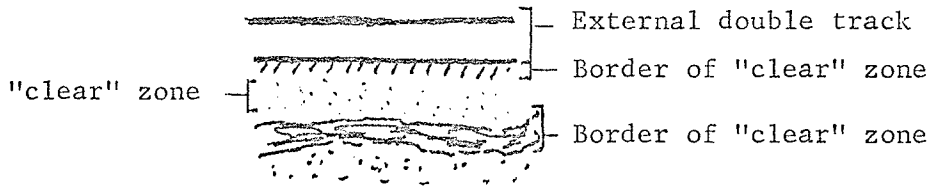
Type I

Type I elementary bodies appeared to be more resistant to the deleterious effects of fixation and cutting than type II. Their shape was more regular than that of osmium fixed type I elementary bodies; ruffles or wrinkles at the surface of the particles were less frequently observed. With respect to size, the type II particles averaged 0.26 x 0.36μ, approximately the same as type I particles prefixed with glutaraldehyde.

A prominent eccentric nucleoid was present in all particles; it was seated in a granular cytoplasm of moderate density. DNA-like fibres were easily recognized in these particles (Figures 12 and 13), a result of the increased contrast produced by osmium fixation. Granules in the cytoplasm were numerous; and the density of the cytoplasm was less than that of glutaraldehyde-prefixed type I elementary bodies.

Examination of a large number of type I elementary bodies fixed by

osmium revealed no hint of a second inner double track in these particles, even though the "clear zone" underlying the outer double track was no longer as sharply defined as in glutaraldehyde-prefixed type I elementary bodies. The structure of the surface layers of the type I particles appeared to be as follows:



The border between the clear zone and the outer double track, and the clear zone and the cytoplasm, were not the slender threads seen in glutaraldehyde-prefixed preparations; width and density of the borders varied greatly within each particle (Figures 12 and 14).

The most obvious difference between glutaraldehyde-prefixed and osmium-fixed type I elementary bodies was loss of resolution of the clear zone in osium-fixed particles. This is not unexpected since it is known that the reduced osmium produced by fixation does not always bind at the reaction site. Movement of the reduced osmium after fixation or during fixation was probably also responsible for the variation in the width of the borders of the clear zone.

Of greatest importance is the fact that, in spite of the increased contrast given by the use of osmium as a fixative, a second double track could not be detected in the type I elementary bodies.

Rare forms

Two forms of elementary bodies, distinct from those described above, and not observed in our glutaraldehyde-prefixed material, were found to occur, although very rarely, in osmium-fixed preparations. The first

was the extremely wrinkled particle referred to above (Figure 16); its classification as type II is somewhat arbitrary, considering the state of its surface layers. This type of particle is most probably a fixation artifact, but the action of lysosomal enzymes which are present in yolk sac (De Duve, 1959) cannot be ruled out.

The second rare form of elementary body, illustrated in Figure 15, was enclosed by a double track of unit membrane size, but had neither a second double track nor a clear zone; it could not be classed as type I or type II. In some areas the cytoplasm extended to the outer double track, a marked departure from the norm observed in all other elementary bodies. It is possible that this form is also a fixation artifact, since it occurred rarely, and was not seen in our glutaraldehyde-prefixed preparations.

(iii) Elementary bodies enclosed in vesicles

In both glutaraldehyde-prefixed, and osmium-fixed samples of the whole semipurified inoculum, type I and type II elementary bodies were occasionally observed in membrane-bound vesicles (Figure 18, glutaraldehyde-prefixed; Figure 20, osmium-fixed). The number of membranes around the vesicle was highly variable; the membranes were generally separated from the elementary body (which was, as far as could be determined, of normal configuration) by a clear space. A partially tangential section of an enclosed elementary body from the glutaraldehyde-prefixed material is shown in Figure 18. The elementary body in Figure 20 is more readily recognized as such.

c) Reticulate bodies

The reticulate bodies found in glutaraldehyde-prefixed, and osmium-fixed semipurified whole inocula were similar in appearance, and agreed

in general morphology with those described by previous workers.

(i) Glutaraldehyde-prefixed reticulate bodies

These reticulate bodies were enclosed by two double tracks, each 7.5 - 8.5 nm in width, which apparently did not confer structural rigidity on the reticulate bodies, since they appeared in a variety of configurations. An average size was difficult to determine, since the particles were so irregular in shape, but the average diameter was approximately 0.55μ. The enclosing double tracks were frequently separated from one another and, in contrast to the situation with elementary bodies, the outer double track of the reticulate body deviated considerably from its path (Figure 17), as did the inner double track.

Cytoplasm was sparse; it often occurred at the periphery of the reticulate body, and occasionally between the double tracks. The inner portion of the reticulate body was occupied by a network of fine filaments, reminiscent of DNA strands (Figure 17).

(ii) Osmium-fixed reticulate bodies

Reticulate bodies were infrequently observed in osmium-fixed preparations, and were less well preserved than those prefixed with glutaraldehyde. Two double tracks surrounded the reticulate bodies, but were often incomplete (Figure 19). Irregularity of shape was marked, and the contents of the reticulate body had often leaked out. Cytoplasm was very sparse (Figure 19). One can only conclude that osmium is not a favorable fixative for reticulate bodies.

B. COMPOSITION OF CHLAMYDIAL POPULATIONS SEPARATED FROM WHOLE, SEMIPURIFIED INOCULUM BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

Three dense zones were found in the sucrose density gradients after centrifugation (Figure 1): the meniscus, made up of membranes; a thin band at a sucrose concentration of 31.1%, containing type 1 elementary bodies; and a thicker, more flocculent band at 40.9% sucrose, containing type II elementary bodies, and reticulate bodies.

(a) Meniscus

No Chlamydiae-like particles were found in thin sections of the material in the meniscus. Scanning large areas revealed only a large amount of membranous material, arranged into membrane-bound vesicles and myelin-like figures. A representative area of the meniscus is shown in Figure 21.

(b) Contents of the thin band

The particles found in this band consisted almost entirely of type I elementary bodies: the ultrastructure of their surface layers was marked by the presence of only one double track, having beneath it an electron translucent zone.

With a few exceptions (Figure 26) these isolated type I elementary bodies were regular in shape. In size, shape and density, they resembled rather closely the elementary bodies of type I observed in whole semipurified preparations. The differences which were seen such as their more spherical shape and slightly larger size, and the irregularity of the clear zone, may have been due to the high concentration of sucrose to which the particles were exposed in the sucrose density gradient.

All particles had a readily identifiable clear zone (see figures 22 and 23, for example) with sharply defined borders (Figures 22 and 24).

In some cases the clear zone apparently had moved into the cytoplasm away from the external double track (Figure 25); it was nevertheless still easily differentiated from a second double track.

Ten to fifteen percent of the elementary bodies in this band were enclosed in membrane-bound vesicles (Figures 23 and 24). The elementary body was usually separated from the vesicular membrane by some distance, and debris or small vesicles were often present in the vacuole along with the elementary body (Figure 23).

One particle was found in this band which had the surface layers characteristic of a type II elementary body; it too was enclosed in a membrane-bound vesicle (Figure 27). As well as having two double tracks, this particle differed from type I elementary bodies in the band in the rarification of its cytoplasm and the prominence of its dense eccentric nucleoid. The finding of such a particle indicates that the separation of type I and type II elementary bodies may not have been absolute; a mixed population was apparently present in the thin band, although the type I elementary bodies were in a large majority.

(c) Contents of the thick band

The thick band also contained a mixed population of chlamydial particles, in this case, however, made up of reticulate bodies and type II elementary bodies.

The reticulate bodies, illustrated in Figures 34 and 35, were as described in the semipurified preparation: large in size, irregular in shape, bounded by two double tracks, having sparse peripheral cytoplasm and a net of DNA-like fibres. They were relatively few in number.

The morphology of the elementary bodies in this band was typical

of the type II elementary bodies observed in whole, semipurified preparations. They were enclosed by two double tracks, and had a prominet nucleoid in a granular cytoplasm of moderate density (Figures 28 and 31). Fibres were occasionally visible (Figures 30 and 34).

Of interest is the inner double track of the isolated type II particles which, in some cases, was almost completely separated from the outer double track by cytoplasm (Figures 28 and 33). In other particles, it remained closely apposed to the outer double track for much of its length (Figures 32 and 34). "Wandering" of the inner double track was observed in type II elementary bodies in whole semipurified preparations, as mentioned above, but not to the extent seen in the isolated elementary bodies. It may be that exposure of the particles to sucrose altered the internal consistency somewhat, since the isolated elementary bodies were more round and slightly larger than elementary bodies in the whole semipurified preparation.

One elementary body deviating from the above description was found (Figure 29). Its darkly stained cytoplasm was similar to that in type I elementary bodies, but it was enclosed by two concentric double tracks. This particle was also noteworthy for the excessive wrinkling at its surface, a phenomenon not seen in other elementary bodies in this band.

C. UNINFECTED YOLK SAC

As a control, uninfected yolk sac was "semipurified" in the same manner as the infected yolk sac, prefixed with glutaraldehyde, and embedded. As can be seen in Figure 36, no Chlamydiae-like particles were present. The material consisted of membranes, myelin figures, and cell debris.

D. LYOPHILIZED INFECTED YOLK SAC

As mentioned earlier, the commercial preparation of C. psittaci 6BC which consisted of a lyophilized homogenate of infected yolk sac, contained very few particles resembling any of the chlamydial forms, in spite of its high titre ($TCID_{50}$ of 5×10^8 in L-cells) at that dilution. Only two elementary body-like particles, one of which is shown in Figure 37, were found in thin sections of this preparation. Small masses of DNA-like fibres enclosed in one or more membranes (Figure 38) occurred frequently. A more rare occurrence were large masses of such fibres, surrounded by debris and tentatively enclosed by a complex membrane system (Figure 39).

A very rough approximation of the number of particles present in this inoculum, based on the number seen in a thin section, gives a value of 12,000 particles per ml. This value can hardly account for a $TCID_{50}$ of 5×10^8/ml of this material.

PLATE I

Banding pattern of the whole, semipurified C. psittaci 6BC from yolk sac in sucrose density gradients. Material occurred at the meniscus, at a sucrose concentration of 31.1% (thin band) and 40.9% (thick band). Figure I. This is a representative picture of the banding pattern found in four experiments.

PLATE I

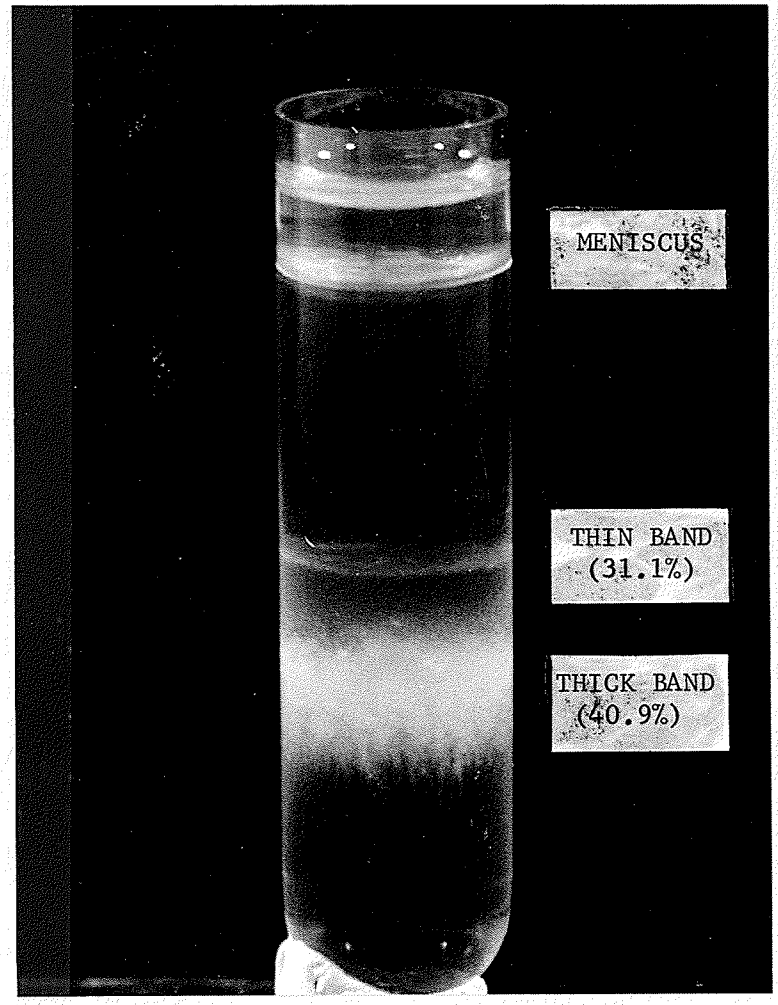


PLATE II

Electron micrographs of semipurified samples of C. psittaci 6BC from yolk sac; prefixed with glutaraldehyde. Figures 2-8.

(2) Type II elementary body, having a dense nucleoid (n) and two closely apposed double track structures. x 100,000.

(3) Type II elementary body, having a dense nucleoid with protruding fibres (f). Note the separation of the two double track structures in places (arrows). x 100,000.

(4) Higher magnification of a type II elementary body. The two double tracks are closely apposed in some areas, and separated in other (arrows). x 160,000.

(5) Type I elementary body. Note the absence of an internal double track in this, as well as in the other type I elementary bodies. In its place is a clear zone (z) of varying width. The border of the clear zone is partially separated from the inner track of the double track (arrow). x 100,000.

(6) Type I elementary body. The clear zone is visible over only part of its length; in some areas it resembles a double track (arrow). x 100,000.

(7) Higher magnification of a type I elementary body, having a clear zone extending the entire circumference of the particle. The thickened inner track (t) of the double track is visible in places (arrow), as is the thin cytoplasmic border (b) of the clear zone (arrow). x 160,000.

(8) Two type I elementary bodies. Note that they are oriented in the same direction with respect to their longitudinal axis. Both have dark nucleoids (n). The clear zone in some areas of both resembles a second double track (arrows). Note the rupture (r) of the surface layers of the particle on the left. x 62,500.

The bar on this and all subsequent micrographs represents 0.1 μ.

PLATE II

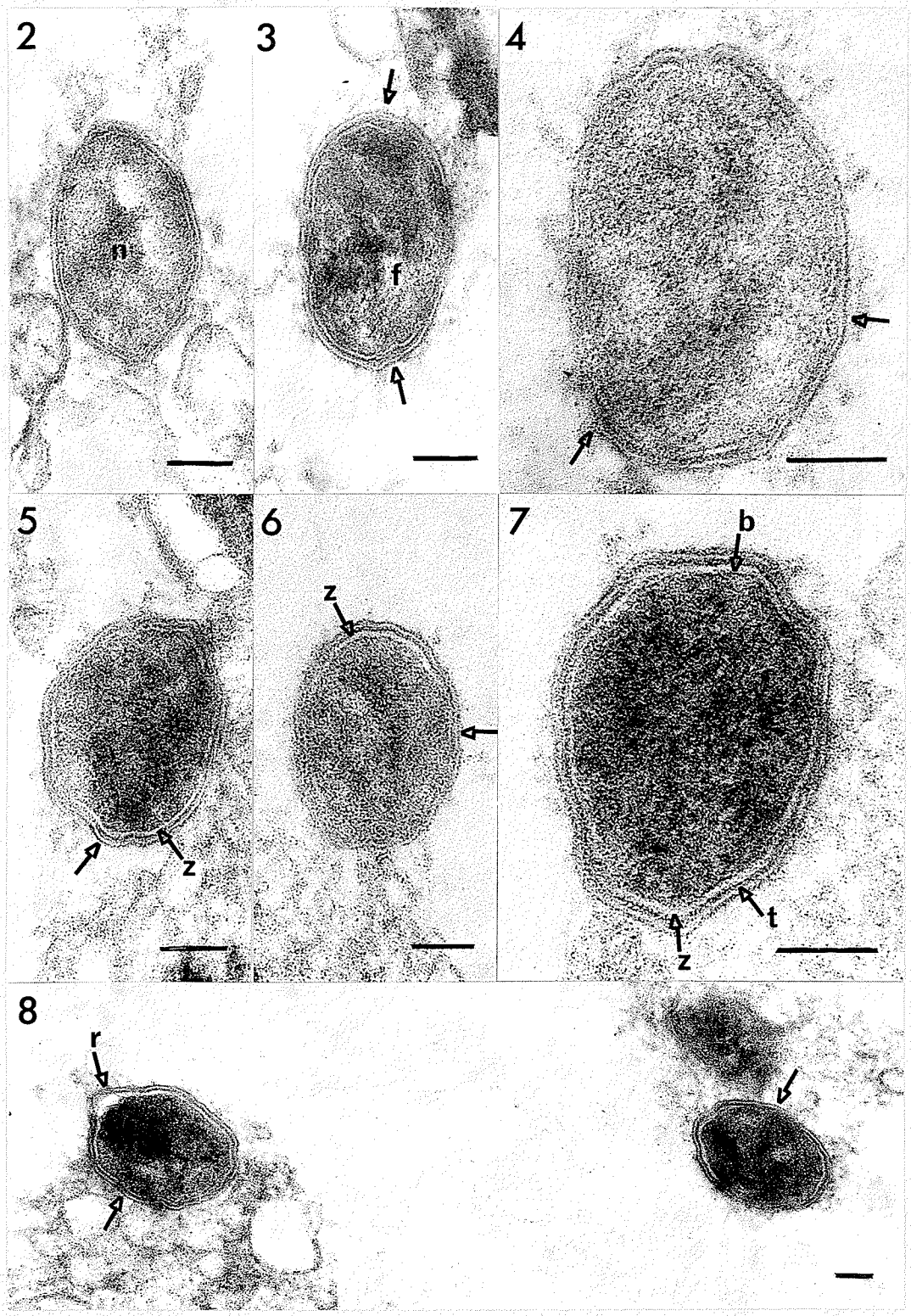


PLATE III

Electron micrographs of a semipurified sample of C. psittaci 6BC from yolk sac, fixed by the method of Ryter and Kellenberger. Figures 9-16.

(9) Type II elementary body, having two double track structures visible in some areas (arrow). Note the wrinkling of the particle. The nucleoid is well defined. x 100,000.

(10) Type II elementary body, having two widely separated double tracks, the inner one of which is quite irregular (arrow). This particle is also misshapen. x 100,000.

(11) Higher magnification of a type II elementary body, in which the two double tracks are more readily seen (arrow). The nucleoid is prominent, and is bordered by an area of rarefied cytoplasm in which tufts or fibres (f) are visible. x 160,000.

(12) Type I elementary body. Note the presence of only one double track structure, beneath which is a grey zone (z) bordered by an irregular dark layer (b) along the cytoplasm. x 100,000.

(13) Type I elementary body, in which the inner track of the double track is thickened (arrow). The clear zone is indistinct. The nucleoid is dark, with fibres (f) emanating from it. x 100,000.

(14) Higher magnification of a type I elementary body, showing the distinct nucleoid, and the presence of a greyish zone (z) under the external double track. x 160,000.

(15) A rare form of elementary body, having cytoplasm next to the enclosing double track (arrow) without an intervening internal double track or clear zone. x 100,000.

(16) Another rare form of elementary body, having the wrinkled surface layers typical of poorly fixed particles. Note the retraction of the cytoplasm from the outer surface layers (arrow). x 100,000.

PLATE III

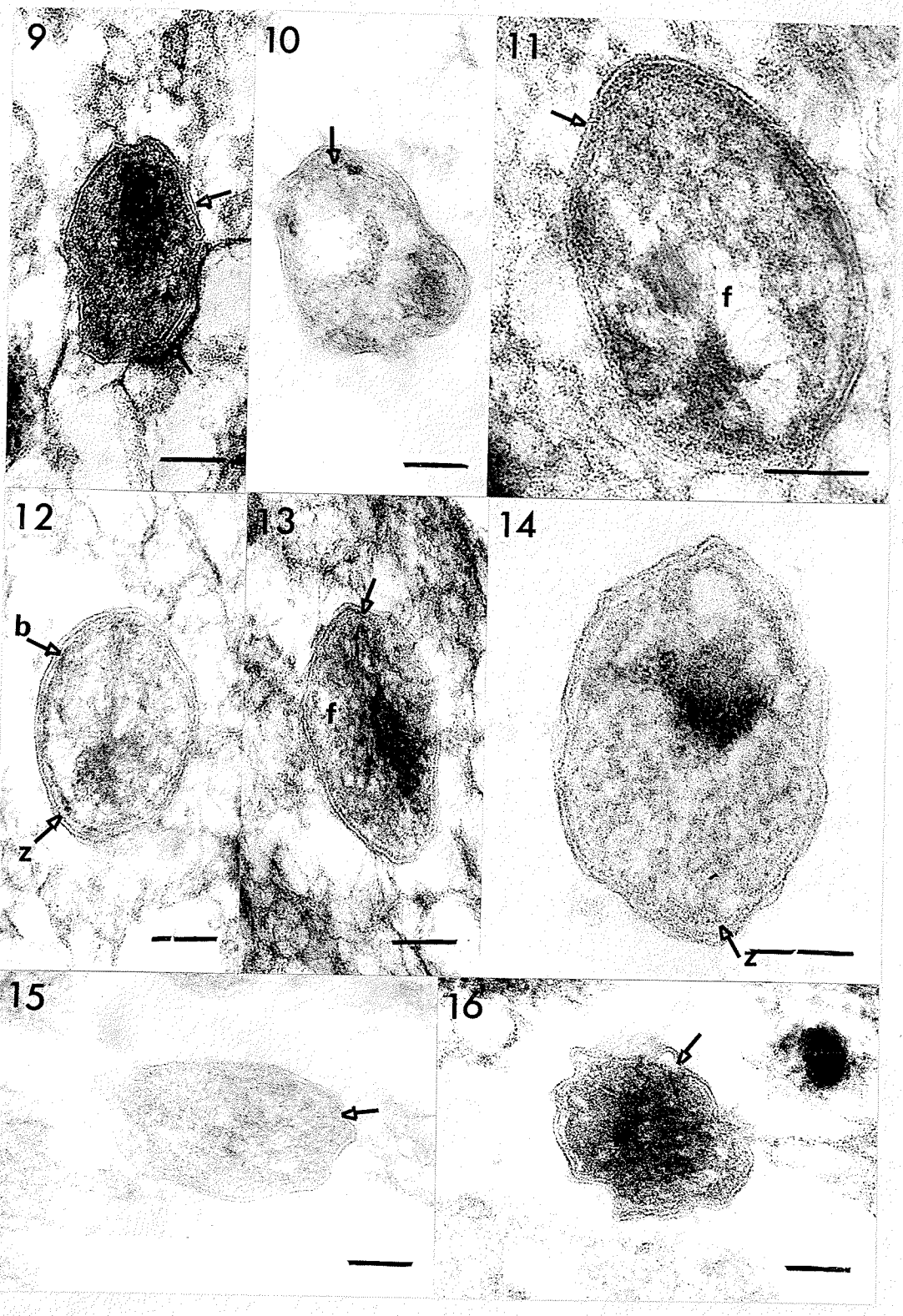


PLATE IV

Figures 17-20

(17) Electron micrograph of the glutaraldehyde-prefixed yolk sac preparation of C. psittaci 6BC, showing a reticulate body enclosed by two double track structures, the inner one of which wanders (arrow). Cytoplasm is sparse. DNA-like fibres (f) are evident in the centre of the particle. Note the irregular shape of the reticulate body. x 100,000.

(18) Electron micrograph of the glutaraldehyde-prefixed yolk sac preparation of C. psittaci 6BC, showing a partial tangential section through an elementary body which is within a multimembraned structure (m). x 100,000.

(19) Electron micrograph of the yolk sac preparation of C. psittaci 6BC fixed by the method of Ryter and Kellenberger, showing a reticulate body (RB) and an elementary body (EB). Note the poor preservation of the reticulate body; the two double tracks around it are widely separated, and enclosed a very sparse cytoplasm. The elementary body also has two double track structures. x 100,000.

(20) Electron micrograph of the yolk sac preparation of C. psittaci 6BC fixed by the method of Ryter and Kellenberger showing an elementary body (EB) enclosed in a membrane-bound (m) vacuole. x 100,000.

PLATE IV

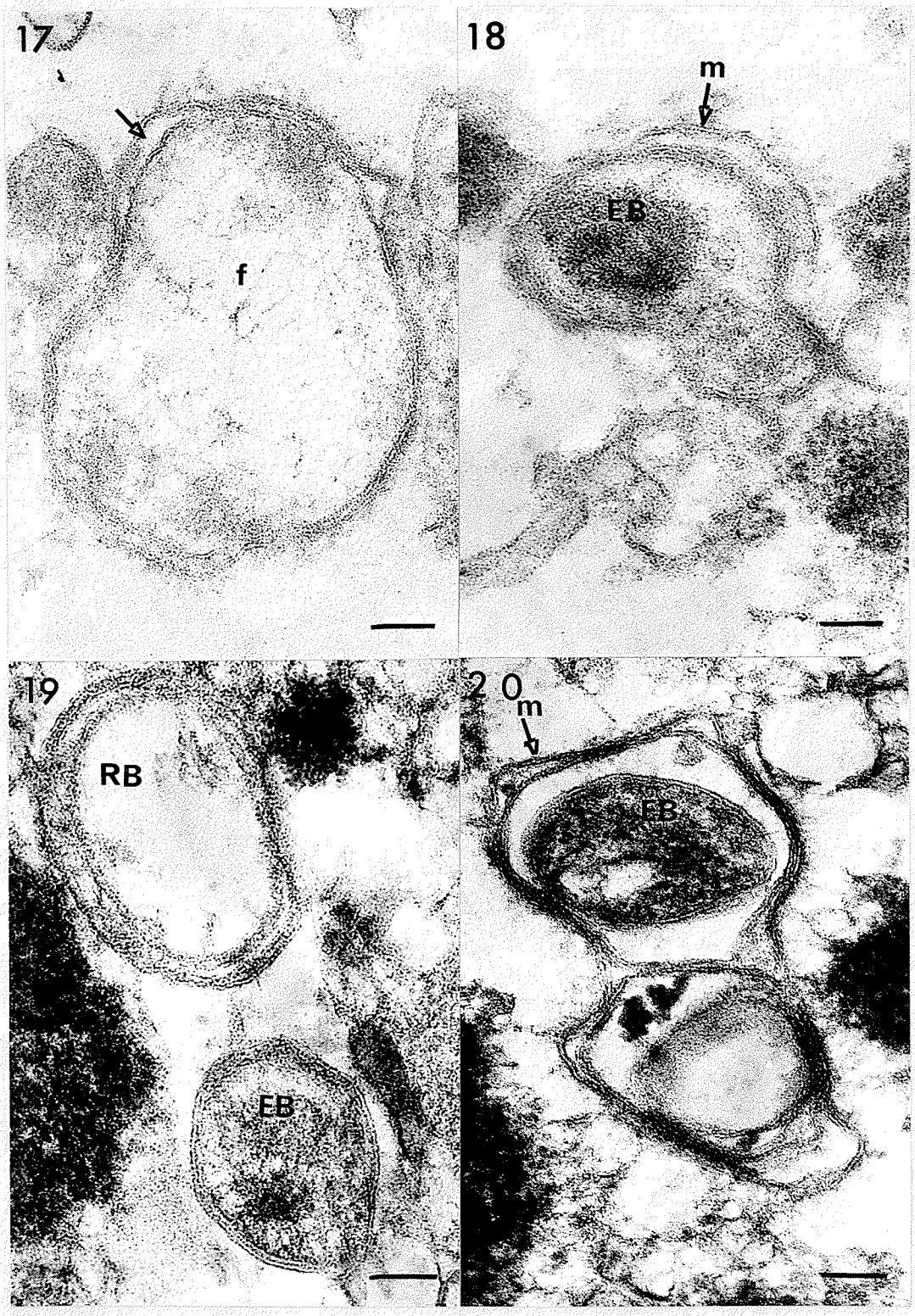


PLATE V

Electron micrograph of a sample from the meniscus of the sucrose density gradient after centrifugation. Note the absence of chlamydia-like particles, and the abundance of membranous material (m). Figure 21. x 48,000.

This, and all succeeding preparations, were prefixed with glutaraldehyde.

PLATE V

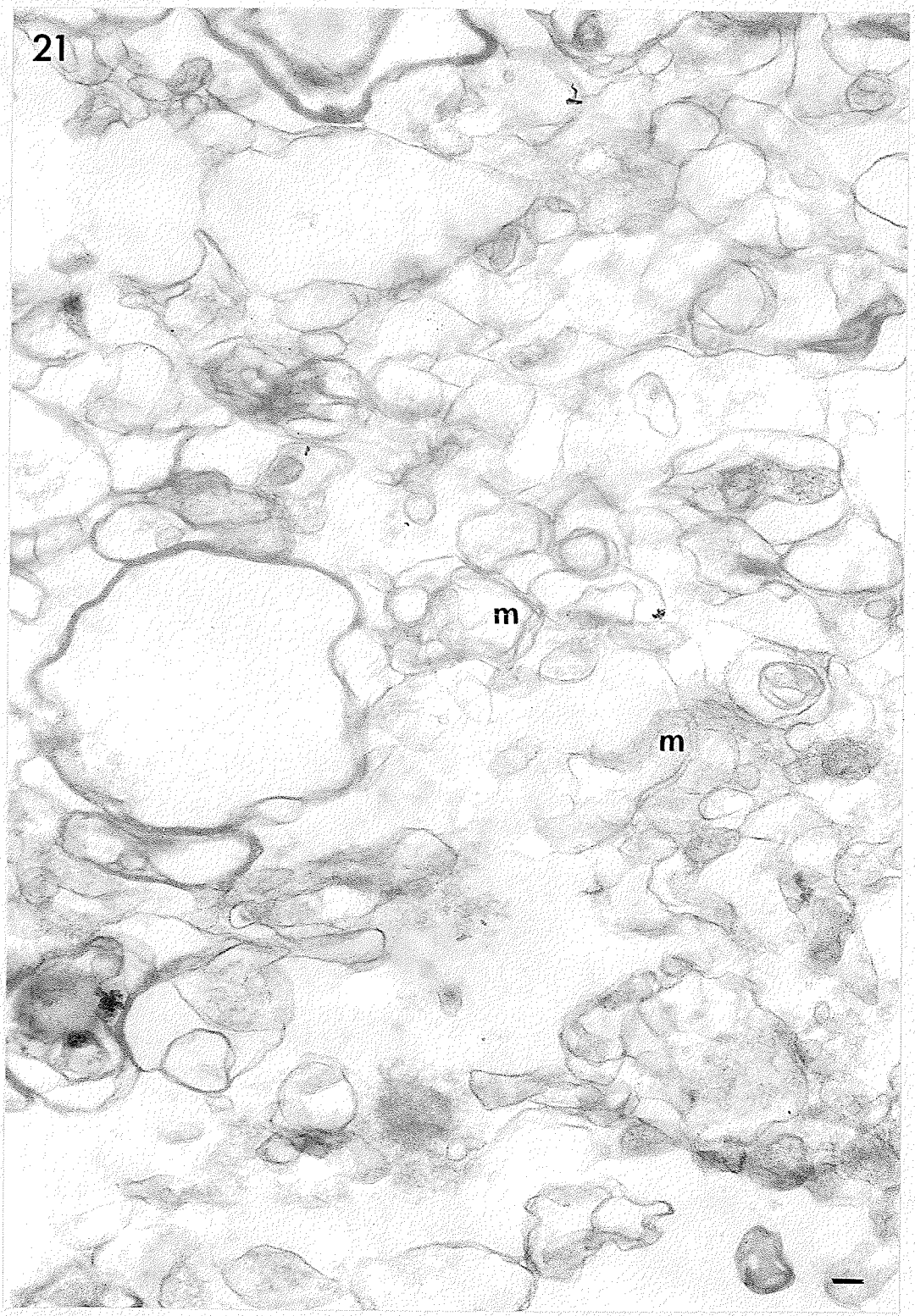


PLATE VI

Electron micrographs of the material in the density gradient after centrifugation, occurring at a sucrose concentration of 31.1%. Figures 22-27.

(22) Type I elementary body with a clear zone (z) extending into the cytoplasm. Note the sharp cytoplasmic border (b) of the clear zone (arrow), and the absence of a second double track. x 100,000.

(23) Type I elementary body contained in a membrane-bound vesicle, along with cell debris. The clear zone in this particle is not visible over its entire length. x 100,000.

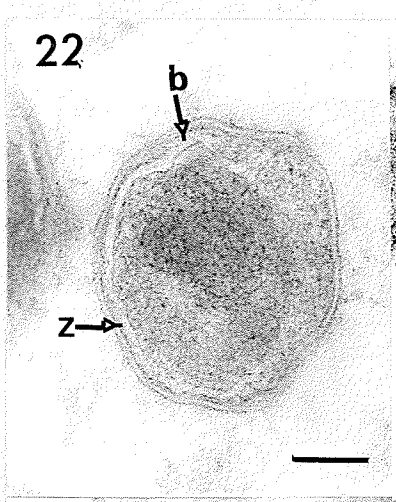
(24) Type I elementary body, also contained in a vesicle. In this particle the clear zone makes a sharp bend into the cytoplasm (arrow). The inner track (t) of the double track is thickened (arrow). x 100,000.

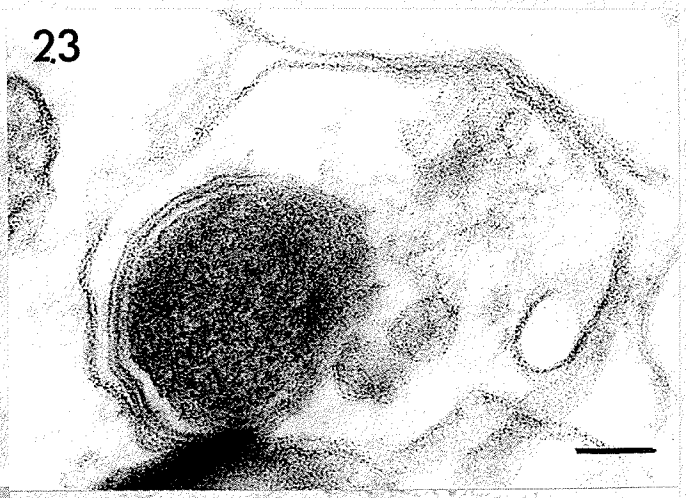
(25) Higher magnification of a type I elementary body. Note the movement of the clear zone (z) away from the enclosing double track. The clear zone is not continuous. x 220,500.

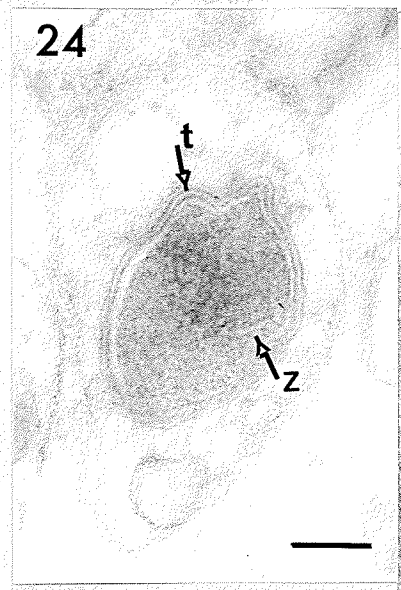
(26) Type I elementary body. One of the few found in this band with marked irregularities in the surface layers. The clear zone is not visible throughout its entire length. x 100,000.

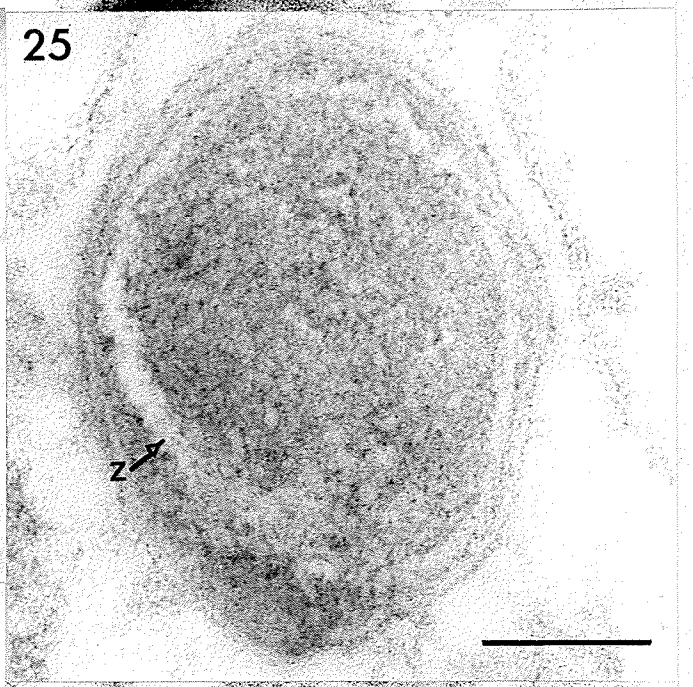
(27) Type II elementary body, the only one found in this band. It is also in a membrane-bound vesicle. Note the presence of two double track structures around it, the inner one of which wanders (arrow). The nucleoid (n) is prominent. x 100,000.

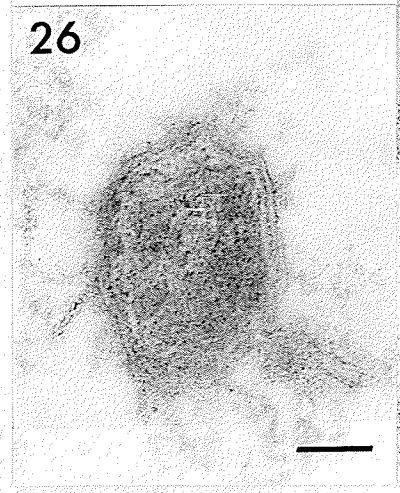
PLATE VI











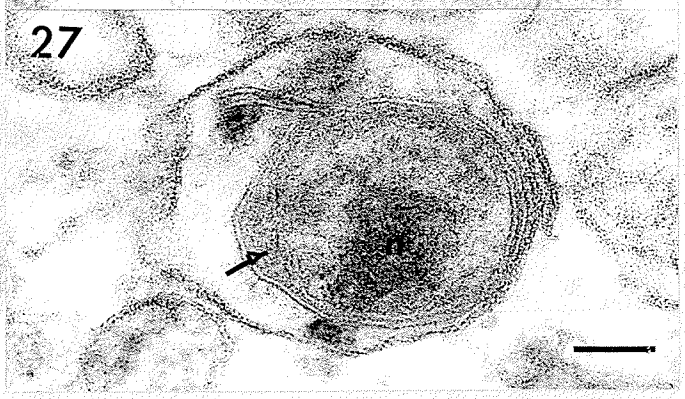


PLATE VII

Electron micrographs of the particles present in the band occurring at a sucrose concentration of 40.9%. Figures 28-33.

(28) Type II elementary body having a typical dense, eccentric nucleoid (n), and two double track structures. Note the irregular path followed by the inner double track as it circles the nucleoid (arrow). x 100,000.

(29) Type II elementary body, atypical with respect to its overall density and the wrinkling of its surface layers. Two double track structures are, however, present (arrow). x 100,000.

(30) Higher magnification of a type II elementary body, having a fibrous (f), dense nucleoid (n), and two double track structures (arrow). x 160,000.

(31) Type II elementary body with a dense nucleoid (n). The inner double track (arrow) is out of phase for much of its length. x 100,000.

(32) Type II elementary body. The two double track structures are seen to be conjoined where they are in phase (arrow). x 100,000.

(33) Higher magnification of a type II elementary body, having a dense nucleoid with fibres (f) around it, and two double track structures which are separated from one another in some areas by cytoplasm (arrow). x 160,000.

PLATE VII

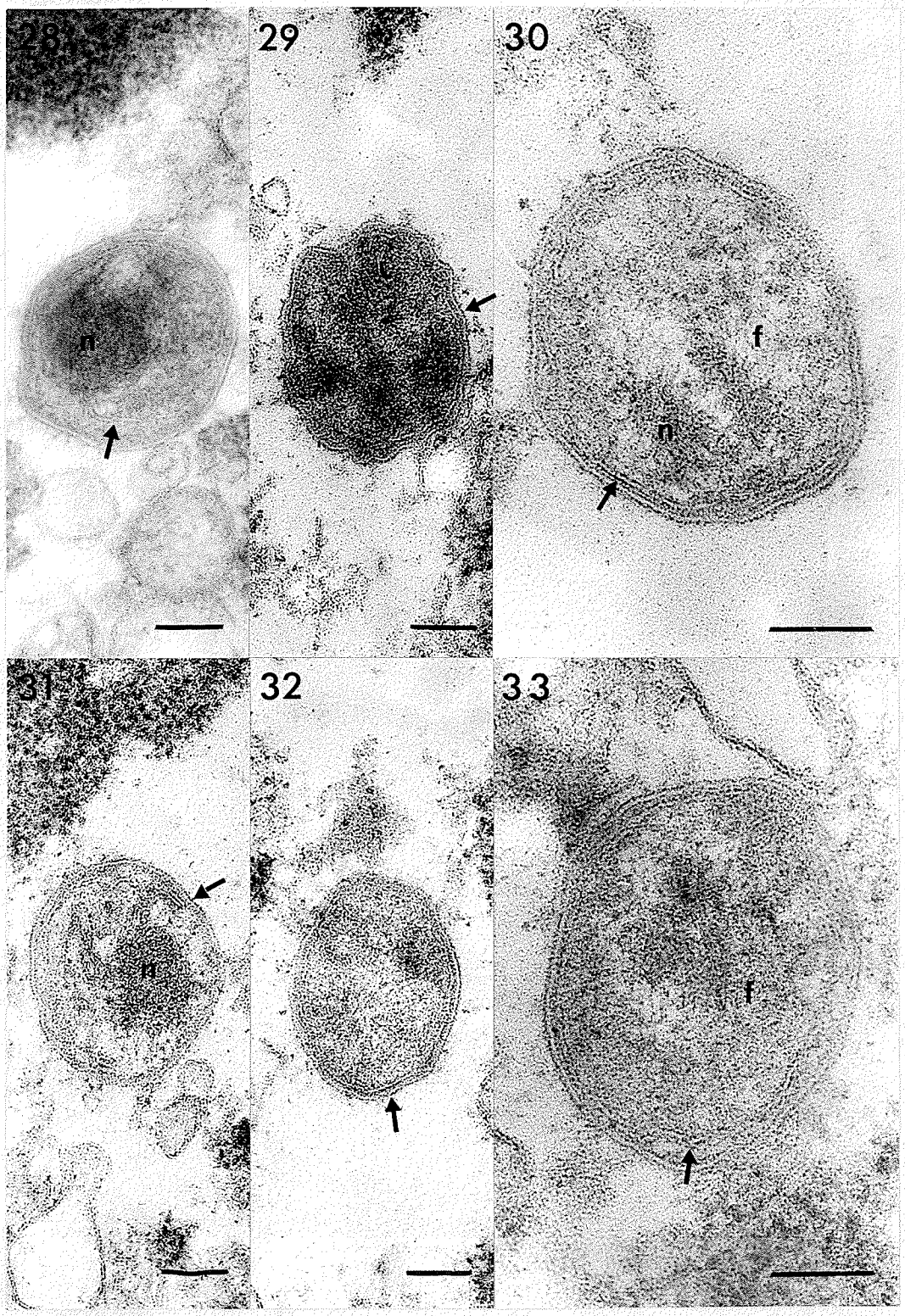


PLATE VIII

Further electron micrographs of particles from the band occurring at a sucrose concentration of 40.9%. Figures 34 and 35.

(34) A reticulate body and a type II elementary body. Note the swirling fibres (f) around the nucleoid of the elementary body (EB), and the close apposition of its two double tracks (arrow). The reticulate body (RB) is irregular in shape, and has apparently been deformed by the elementary body. Note the presence of two double track structures where the surface layers of the reticulate body are in phase (arrow), and the scarcity of cytoplasm. The interior of the reticulate body is occupied by a net of DNA-like fibres (f). x 100,000.

(35) A second reticulate body (RB), in which the interior fibrous net (f) is more apparent. Note the whorl in the membrane (w). This particle is also irregular in shape. The second double track is separated from the outer in places (arrow). x 100,000.

PLATE VIII

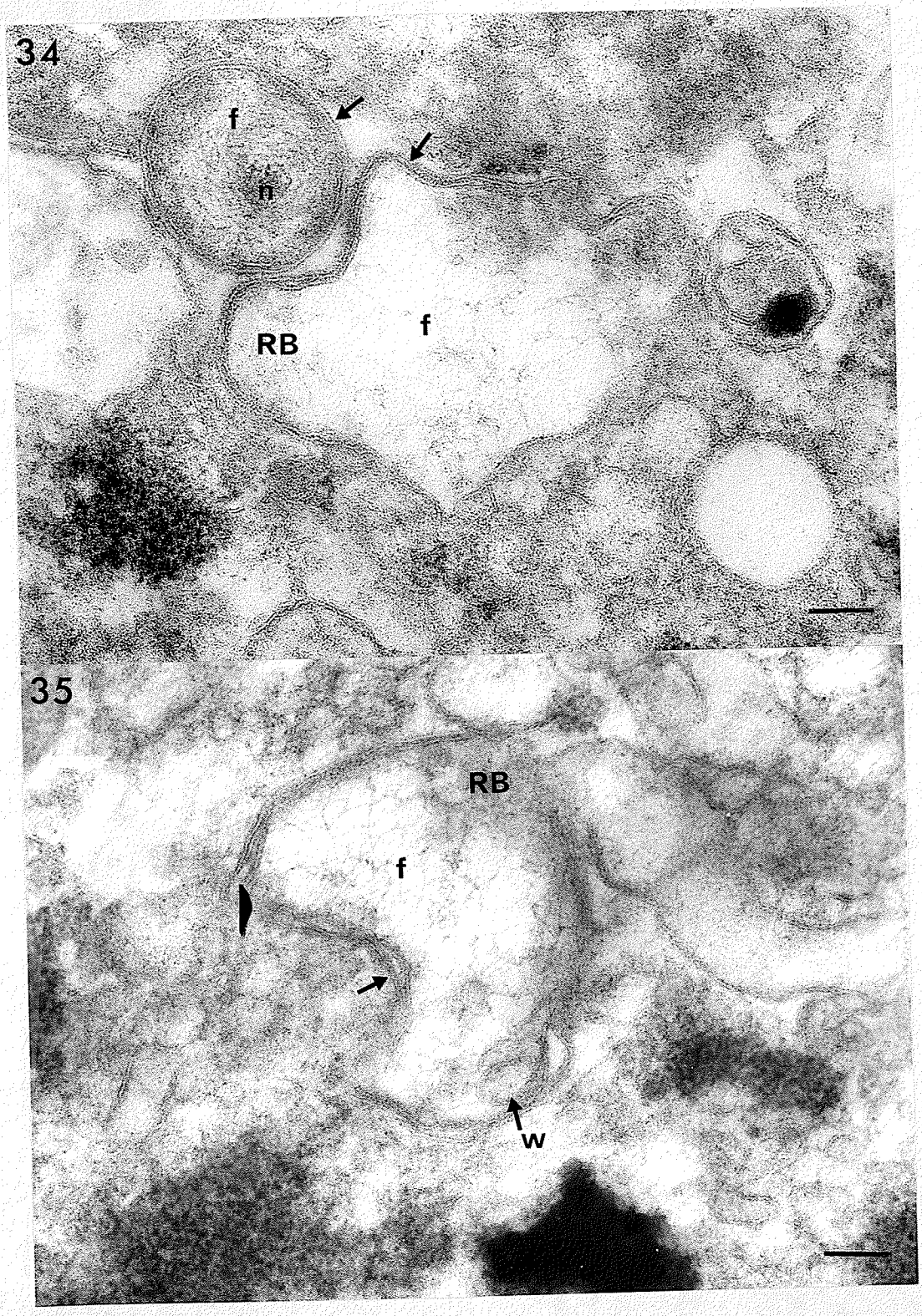


PLATE IX

A low magnification electron micrograph of a representative area of uninfected chick embryo yolk sac which had been subjected to the same "semi purification" procedure as had the infected yolk sac. Note the absence of chlamydia-like particles (Figure 36). x 30,000.

PLATE IX

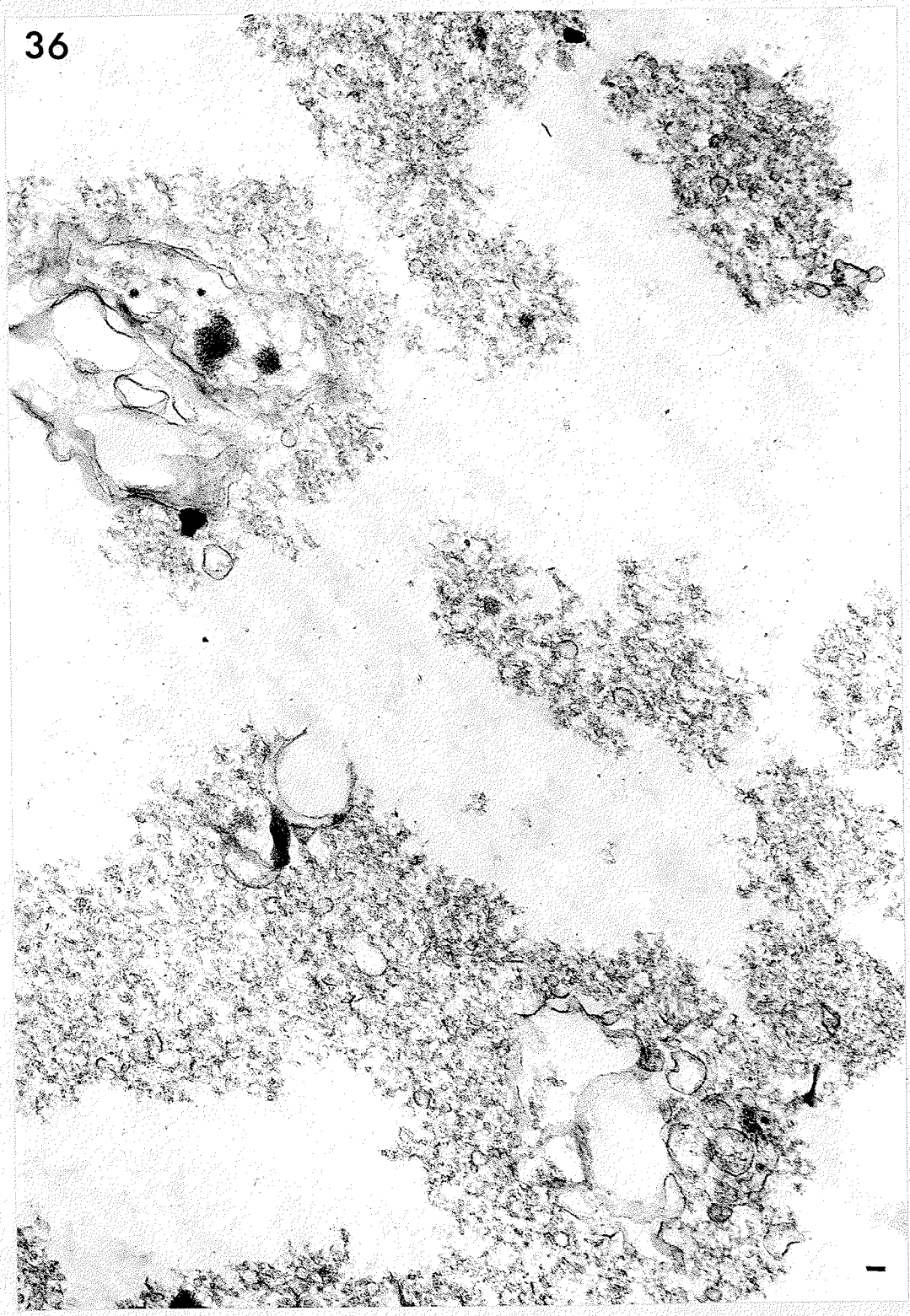


PLATE X

Electron micrographs of the lyophilized preparation of C. psittaci 6BC, prefixed with glutaraldehyde. Figures 37 and 38.

(37) A low magnification micrograph of an area containing one of only two particles found which resembled elementary bodies. This particle (arrow) appears to have only one double track structure. x 60,000.

(38) An area of lyophilized yolk sac containing a membrane-bound concentration of fibrous material (f). x 60,000.

PLATE X

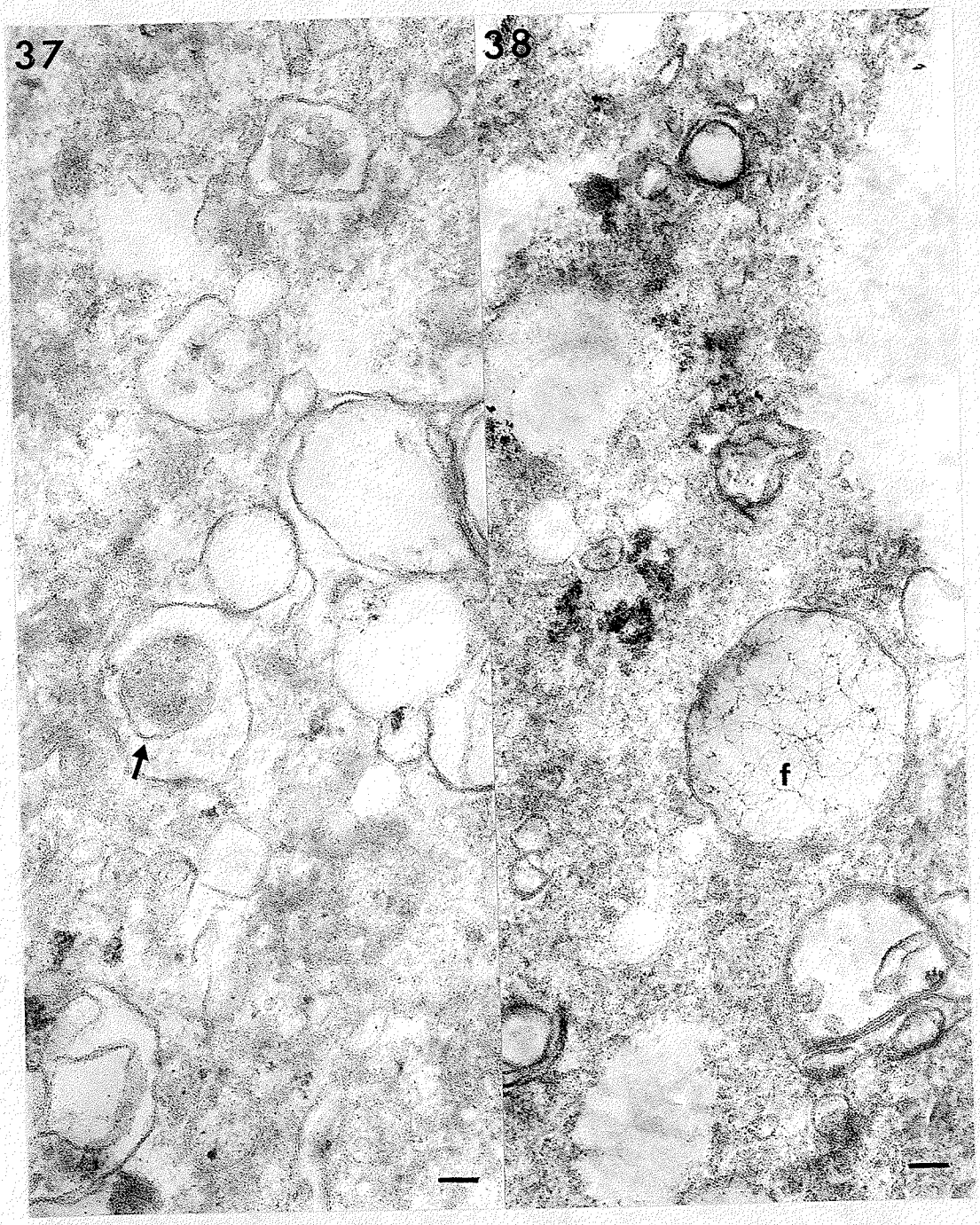
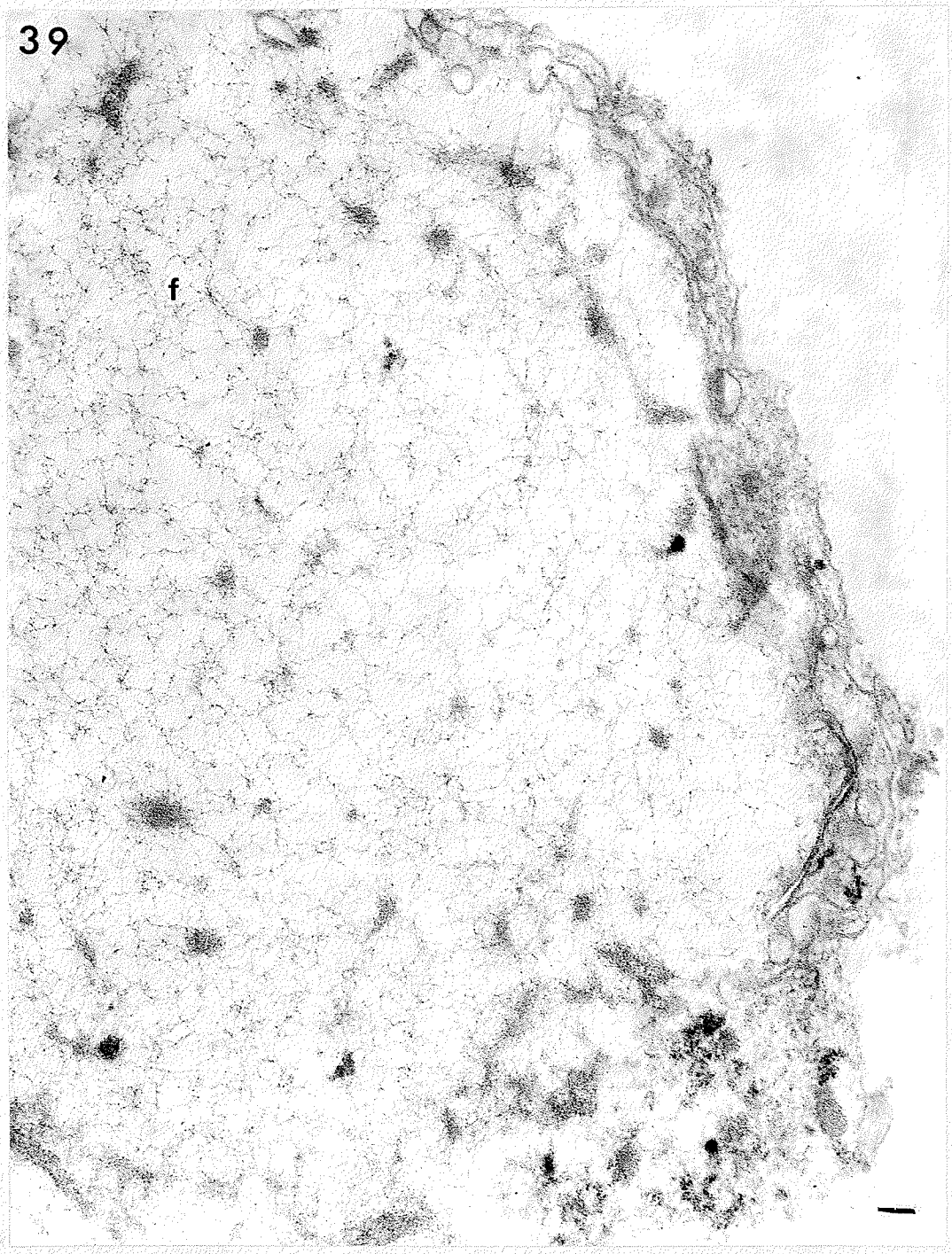


PLATE XI

Electron micrograph of a very large area of fibrous material (f), from the lyophilized yolk sac preparation of C. psittaci 6BC. Figure 39 x 50,000.

PLATE XI



DISCUSSION

A) Introduction

There are two major differences between these results and those of previous workers:

1) two forms of elementary bodies have been identified and characterized, as opposed to only one previously; and

2) the ultrastructure of the surface layers of both forms of elementary bodies, as well as the reticulate body, does not entirely agree with that of Gram-negative bacteria, a finding which has in the past been attributed to improper fixation.

B) Verification of the presence of two morphologically distinct forms of elementary bodies from the yolk sac of infected chick embryos

When preparing a semipurified sample of a polymorphic organism such as C. psittaci, which has a population of very rigid and very fragile particles, it is necessary to use purification procedures which minimize the risk of excluding or destroying any of the morphological forms. For this reason, the infected yolk sacs were homogenized with glass beads, and the chlamydial particles separated from cell debris by high and low speed centrifugation. Neither enzyme treatments nor ultrasonication have been employed. Since this procedure apparently has not fragmented a goodly number of the fragile reticulate bodies (Figure 34), it is unlikely to have altered the more rigid elementary bodies. It appears therefore improbable that the type I elementary body, which differs so markedly from the classical description of an elementary body in the structure of its surface layers, is a purification artifact.

The demonstration of type I and type II elementary bodies in both glutaraldehyde-prefixed, and osmium-fixed semipurified 6BC preparations is especially important. It has been proposed by several authors (Anderson et al, 1965; Lépinay et al, 1970), that a second double track is present in all elementary bodies, but may require the heightened contrast produced by osmium fixation to resolve it. In this case, osmium fixation has not resolved a second double track in the type I elementary body. It would seem, then, that the type I and type II elementary bodies are distinct entities.

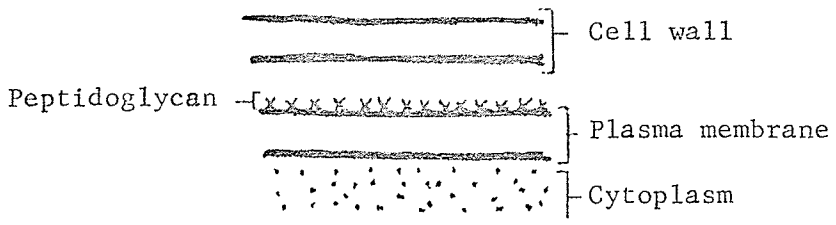
The fact that the type I and type II elementary bodies observed in semipurified preparations can be separated from one another by sucrose density gradient centrifugation is a further indication that the two forms are distinct entities. Although the separation was apparently not complete, the small amount of type II elementary bodies contaminating the type I band may be due to mixing during collection of the bands. This appears quite possible, since the two types of elementary bodies have similar densities, and were harvested from closely located bands in the density gradients. Further separation, as of the reticulate bodies from the type II elementary bodies, will be attempted with different sucrose density gradients, as well as with other gradient materials such as CsCl and Renografin.

C) The ultrastructure of type I and type II elementary bodies and reticulate bodies, compared to that of Gram-negative bacteria

a) Introduction

The presence of two double track structures in Gram-negative bacteria is universal, with the exception of altered life forms such as

L-forms (Hijman, 1969). A schematic representation of the surface structure of a Gram-negative bacterium is given below:



b) Chlamydial forms having two double track structures

Examination of the surface structure of type II elementary bodies and of reticulate bodies reveals a superficial resemblance to the above schematic representation. Two double track structures are present in both type II elementary bodies and reticulate bodies. However, the distance between the two double tracks varies dramatically in both forms; that is, in the same particle they may be inseparable at one point, and separated by a good deal of cytoplasm at another. In other words, an even periplasmic space (Mitchell, 1961) does not exist. There is either no space at all between the two double tracks of type II elementary bodies and reticulate bodies, or it is occupied by granular cytoplasm.

Such a structural difference raises the possibility of a functional difference in the two double tracks of Chlamydiae and those of Gram-negative bacteria. In bacteria, the outer double track is the cell wall; it is, as far as is known, a protective device which, under appropriate conditions, the organism can do without (Hijmans, 1969). The inner double track is the plasma membrane or osmotic barrier of the bacterium; if it is breached, the organism cannot survive.

The inner double track of the type II elementary bodies and reticulate

bodies, however does not seem to serve as a plasma membrane, since cytoplasmic elements are often found on both sides of it, and since it is sometimes discontinuous. The internal constituents are enclosed by the outer double track, not the inner one; and it appears that the outer double track of Chlamydiae is the permeability barrier of the organism.

The outer double track, or a thin layer just beneath it, must also be responsible for maintaining the rigidity of type II elementary bodies; the wandering inner double track could not fulfill such a function. In bacteria, rigidity is maintained by the peptidoglycan layer which may lie along the plasma membrane (Forsberg, Costerton and MacLeod, 1970), or along the inside of the cell wall (Murray et al, 1965). The presence of such a layer has been postulated in elementary bodies, but has never been clearly demonstrated. The data on muramic acid, present in bacterial peptidoglycan, is conflicting, and cannot resolve the question of whether or not peptidoglycan is present in chlamydial envelopes. If present, it is probably a very thin layer under the external double track of type II elementary bodies, since in places the outer and inner double tracks are very closely aposed to each other with very little space between them for a thicker layer of peptidoglycan.

c) Chlamydial particles with one double track structure

The surface structures in type I elementary bodies do not appear to be analagous to the cell wall and plasma membrane of Gram-negative bacteria. These particles have only one double track structure, which has beneath it an electron translucent zone, as compared to the two double track structures of Gram-negative bacteria.

The functions of the plasma membrane and cell wall are apparently

carried out by this clear zone and the outer double track. If the clear zone is actually a thick deposition of carbohydrate material (Lépinay et al. 1971) it is conceivable that no material passes in and out of the particle. In other words, the particle is a spore-like life form, and is not metabolically active. The occurrence of bacterial spores is of course well known, but they do not in general resemble in ultrastructure the type I elementary body. They contain much protein, and are not enclosed by a double track.

Those bacterial life forms which are enclosed by only one double track structure (spheroplasts, protoplasts, and L-forms) do not have an underlying clear zone such as is found in the type I elementary body. They are usually very fragile (Hijmans, 1969), a property apparently not shared by type I elementary bodies.

d) Internal organization of chlamydia and Gram-negative bacteria

When fixed by the method of Ryter and Kellenberger, the Gram-negative bacterium exhibits a mass of relatively fine DNA fibres, often arranged in parallel, curved bundles towards the centre of the particle, and surrounded by a granular cytoplasm (Ryter and Kellenberger, 1958).

Reticulate bodies also have a central net of fibres, but they are not arranged in bundles; rather the fibres are interwoven with considerable space between them. The nucleoid of elementary bodies is a compact mass, from which a few fibres may protrude. Very rarely (Figure 34) is a particle seen with the central fibres arranged in parallel bundles.

The similarities in internal organization between chlamydial particles and Gram-negative bacteria are limited to the presence of ribosomes in

the cytoplasm, and to the lack of a nuclear membrane.

D) The Ultrastructure of Chlamydiae, as compared to that of Rickettsiae

a) Introduction

Since the morphology of the chlamydial particles as described in this study does not truly resemble that of Gram-negative bacteria, one is left with the question of where Chlamydiae do fit. The answer would seem to be with the Rickettsiae, a group of obligate intracellular parasites which share many properties with Chlamydiae, and are currently classified with them in Bergey's manual under the taxon Rickettsiales. In recent years, with the advent of improved electron microscopic techniques, the morphological similarities between the two groups of organisms have been stressed by several authors.

b) Overall structural similarities

Large pleomorphic forms of Rickettsiae akin to the reticulate bodies of Chlamydiae were described by Rosenberg and Kordová in 1960 and by Anderson and coworkers in 1965. More recently, rickettsial particles similar to the elementary, reticulate and intermediate bodies of chlamydiae have been demonstrated in hemocytes infected with R. melolonthae by Devauchelle et al. (1971).

Wiebe and coworkers (1972) have described two morphologically distinct forms of C. burneti (phase I) separated by density gradient centrifugation. Both these forms, which are morphologically similar to elementary and reticulate bodies in Chlamydiae, respectively, are infectious. The observations of Wiebe et al. (1972) are in agreement with earlier findings as well as with the recent findings of Nermut et al (1968) who also described two forms of Coxiella burneti.

c) Structure of the surface layers of Chlamydiae and Rickettsiae

With respect to surface layers, the data about Rickettsiae are conflicting, as are those of Chlamydiae. Bird and coworkers (1967) observed two double tracks in particles of R. prowazeki; Nermut et al. (1968) found only one double track in particles of C. burneti, but postulated that a second double track (the plasma membrane) was present but not resolved. Burton et al. (1971) also described only one double track in C. burneti particles. However, in some small, dense forms a capsule-like material has been observed when the agent was grown in L-cells. In a more recent work, Nermut et al. (1971) suggest that the surface layers of Coxiella burneti Phase I resemble in some aspects those of Gram-positive bacteria.

The micrographs of Wiebe et al. (1972) demonstrate two double track structures in the large forms of C. burneti; it is interesting to note that the inner double track has in places moved away from the outer double track and is separated from it by cytoplasm-like material. This is in agreement with our findings with respect to the inner double track of type II elementary bodies and reticulate bodies of C. psittaci.

The surface structure of the small, dense forms of Rickettsiae is unclear at present, but it does not appear to be typical of that of a Gram-negative bacterium (see, for example, the micrographs of Devauchelle et al. 1971, and Wiebe et al.1972).

Morphologically, it would appear that the Chlamydiae and Rickettsiae resemble each other more closely than they do Gram-negative bacteria.

E) Speculations

a) Possible significance of the two types of elementary bodies

The type I elementary body has not previously been described. How

might one account for its presence in a chlamydial preparation?

If one accepts the hypothesis that Chlamydia multiply by a unique developmental cycle, then it is no problem to fit in another life form such as the type I elementary body. One could assume that it arises via a reorganization of the type II elementary body, which in turn arises from reorganization of a reticulate body. There is the question of how a type II particle loses a double track and replaces it with an electron translucent layer, but this difficulty is not insurmountable - presumably a deposition of the electron translucent mateeral could occur in the plasma membrane, distorting the membrane's appearance. Why such a phenomenon should occur is another question. Since type I elementary bodies seem to be more resistant to the rigors of electron microscopy (e.g., fixation and cutting) than type II particles, it may be speculated that type I elementary bodies represent a spore-like stage in the developmental cycle.

An alternative to this hypothesis is the possibility of host-mediated alteration of the life forms of Chlamydiae. It has been suggested by several workers (Nermut et al. 1968) that the nonrigid forms of Chlamydiae and Rickettsiae are degenerate particles which have been attacked by host enzymes. The unusual behavior of the second double track of type II elementary bodies might also be due to enzyme activity on the part of the host. In view of the recent findings concerning the involvement of lysosomes in chlamydial infections (Kordová, Wilt and Sadiq, 1971; Kordová, Poffenroth and Wilt, 1972a, 1972b), this possibility should be taken into consideration.

Although not generally accepted today, a virus-like mode of reproduction by both Chlamydiae (Pollard and Starr, 1962) and Rickettsiae

(Kordová, 1959, 1971) has been proposed. If further investigation should verify this type of reproduction, one might speculate about many different possible immature, mature, empty etc, chlamydial particles, about variants in strains and genetic and nongenetic interactions between chlamydial particles and their host cells.

b) Importance of the host system in determining the chlamydial population present after infection.

Chick embryo yolk sac is the most favorable medium for the propagation of Chlamydiae. Strains grown in yolk sac however, are for the most part noncytopathic with respect to mammalian cells in vitro. Such is the case with the yolk sac passaged 6BC strain used in this study; it can infected cultured L-cells, but the original inoculum will not produce a cytopathic effect in them (Kordová, Poffenroth and Wilt, 1972). A 6BC strain which has been adapted to and maintained in cultured L-cells is capable of producing a cytopathic effect in the L-cells, and in mouse peritoneal macrophages in vitro (Kordová et al, 1971; Kordová and Wilt 1972). Could one expect the same chlamydial forms to occur in egg-passaged and L-cell passaged 6BC strains, which have such different biological properties?

Studies of Coxiella burneti strains passaged in yolk sac and in cultured mammalian cells have revealed differences in virulence, antigenicity, immunogenicity and density (Ormsbee, 1969) as well as differences in the cell types of this rickettsial agent present after infection of these two hosts. Particles having a distinct filamentous capsule-like layer were observed in mammalian cells infected with "smooth" Coxiella burneti (Rosenberg and Kordová, 1962; Burton et al. 1971). In this related organism then, the differences in biological

properties of yolk sac and tissue culture cell passaged strains have to some extent been reflected in the ultrastructure of the particles themselves.

It is therefore possible that examination of the cytopathic L-cell maintained 6BC strain will reveal differences between its constituents, and the constituents of the non-cytopathic yolk sac passaged 6BC strain. Such an examination will be of value in determining if the toxicity of a chlamydial preparation can be reflected in the morphology of its particles, especially their surface layers.

f) Conclusion

This study has been a preliminary step in the long term investigation of the pathogenicity of chlamydial strains. Having determined the structure and content of the different particles of a noncytopathic egg-grown 6BC strain, the next step will be to examine in a similar manner, a cytopathic L-cell grown 6BC strain.

It will also be necessary to further purify the chlamydial forms isolated from the yolk sac passaged 6BC preparation, and determine their antigenic, immunogenic, pathogenic and other properties. It will be interesting to see how these properties relate to the ultrastructure of the surface layers of the three particles described in this report.

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