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A Comparative Study On The Effects Of Various Protease Treatments Upon The Structure, Immunogenicity, And Infectivity Of The Elementary Bodies OF Chlamydia Trachomatis (Strains L2 And D) And Chlamydia Psittaci (Strain MN)

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A COMPARATIVE STUDY ON THE EFFECTS OF VARIOUS PROTEASE TREATMENTS UPON THE STRUCTURE, IMMUNOGENICITY, AND INFECTIVITY OF THE ELEMENTARY BODIES OF CHLAMYDIA TRACHOMATIS (STRAINS L2 AND D) AND CHLAMYDIA PSITTACI (STRAIN MN).

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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April 1, 1985
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April 1, 1985
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ABSTRACT

The effects of several proteases upon the structure, immunogenicity, and infectivity of the elementary bodies (EBs) of Chlamydia trachomatis (strains L2 and D) and of C. psittaci (strain MN) was studied. Four major proteases were studied and of these, thermolysin and proteinase K were extremely efficient in cleaving the chlamydial major outer membrane protein (MOMP) while trypsin and chymotrypsin demonstrated cleavage abilities to a more limited extent in all strains. Proteinase K had the added effect of cleaving the surface-exposed proteins on the chlamydial outer wall to the extent that monomeric MOMP was no longer detectable by immunoblot procedures with specific antisera and even antigenic fragments were absent.

The most interesting result noted was that while the MN strain was most resistant to cleavage by proteases in terms of structure, its infectivity was most drastically reduced by the treatment. The L2 strain, on the other hand, showed a subsequent increase in infectivity while the structure of its surface-exposed proteins was most drastically altered. It was also observed that trypsinized MN EBs had an increased ability to bind to eucaryotic cells while similarly treated L2 EBs had a decreased ability.

These results together indicated that protease treatment of chlamydial elementary bodies tended to alter infectivity by mainly affecting rates of internalization into human epithelial cells rather than the extent to which these same EBs could bind to and associate with the epithelial cells. This work helps to support previous results indicating that surface-exposed protease sensitive domains of
Chlamydial proteins may play a role in the initial interaction of chlamydiae with eucaryotic cells.
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<td>47</td>
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<td>CBB stain of PAGE for Experiment 13 (w/ 2-ME)</td>
<td>49</td>
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<tr>
<td>13</td>
<td>AgNO₃ (silver) stain of PAGE for Experiment 13 (w/ 2-ME)</td>
<td>50</td>
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<td>Autoradiogram corresponding to proteolysis done in Experiment 2</td>
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<td>19</td>
<td>Autoradiogram corresponding to proteolysis done in Experiment 3</td>
<td>58</td>
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<td>Autoradiogram corresponding to proteolysis done in Experiment 8</td>
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INTRODUCTION

Chlamydia are obligate, intracellular, procaryotes whose infectivity leads to a number of diverse diseases in man. Chlamydial infection causes major diseases in humans ranging from blindness to a host of sexually transmitted disorders.

Their life cycle is much more complex than the typical procaryotic cycle with it occurring in the phagosomal vesicles of eucaryotic cells. There are typically two distinct phases in the chlamydial developmental process, the first being a highly resistant and infective form termed the elementary body, and the second being the vegetative, non-infective reticulate body.

The elementary body transformation into the reticulate body is believed to be related to chemical or structural changes in the cell envelope. The chlamydial cell wall resembles that of gram-negative bacteria in that it possesses both an inner and outer membrane but differs in that it lacks demonstrable peptidoglycan in its outer wall.

The outer wall is rich in disulfide bonds and its structure is dominated by the presence of a major outer membrane protein (MOMP) which may compose up to 60% of the entire protein composition of the outer wall. This protein and the disulfide interactions are thought to be important structurally and functionally in the workings of the outer wall in chlamydial infectivity and immunogenicity. For this reason we have chosen to study the effects of various proteases and reducing agents upon chlamydial elementary bodies in vitro in an
attempt to better understand the modes of action of chlamydial infectivity in vivo.
LITERATURE REVIEW

Classification:

Chlamydia are obligatory intracellular procaryotes (7,29,40). These small bacteria are of interest today due to their medical and research values. The Chlamydia which were classified as viruses when first discovered are now recognized as procaryotic bacteria with a complex and peculiar life cycle (40).

A comparative listing is given in Table 1 which shows the justification for the classification of Chlamydia as bacteria (Table 1).

The chlamydiae are specifically classified as follows:

Kingdom: Procaryota  
Order: Chlamydiales  
Family: Chlamydiaceae  
Genus: Chlamydia  
Species: trachomatis and psittaci

Each species has a variety of serotypes or strains with C. trachomatis having fifteen serotypes and C. psittaci having six (40). Some experts (40) feel that the C. trachomatis species could be further divided into three distinct species as follows:

<table>
<thead>
<tr>
<th>Strains</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-1, L-2, L-3</td>
<td>LGV (lymphogranuloma venereum) species</td>
</tr>
<tr>
<td>A, B(a), B, C, D,</td>
<td></td>
</tr>
<tr>
<td>E, F, G, H, I, J, K</td>
<td>Conjunctivitis species</td>
</tr>
<tr>
<td>mouse pneumonitis</td>
<td>mouse pneumonitis species</td>
</tr>
</tbody>
</table>

The two species accepted at present are believed by most to have originated from a common ancestor based on the following observations: a) their complex and unique life cycle, b) their similiarity in size and shape, c) their virulence, metabolism, and
Table 1: Properties of chlamydiae, viruses, and bacteria (40).

<table>
<thead>
<tr>
<th></th>
<th>Chlamydia</th>
<th>Viruses</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td>Ca 350</td>
<td>15-350</td>
<td>300-3,000</td>
</tr>
<tr>
<td>Shape</td>
<td>Coccoid</td>
<td>Symmetrical</td>
<td>Varied</td>
</tr>
<tr>
<td>Obligatory intracellular parasites</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Complex cell wall</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Reproductive mode</td>
<td>Complicated cycle and fission</td>
<td>Eclipse-synthesis-assembly</td>
<td>Fission</td>
</tr>
<tr>
<td>Sensitivity to sulfonamides or antibiotics</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Metabolic enzymes</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Energy production</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
host-parasite interactions, and d) the presence of a genus-specific antigen (40). Additional factors exist for differentiation between the two species and these are given in Table 2.

Other experts deny the morphological and developmental similarities and claim that the two species aren't of common ancestry and instead are the result of convergent evolution. These scientists point to the fact that there is very little genetic relatedness between the two species (7,16,34).

**Growth cycle:**

The complex life cycle of the chlamydiae is dependent upon its ability to produce two forms; one infective and the other replicative. This unique life cycle helps facilitate its ubiquitous nature. The infective, extracellular form of Chlamydia is called the elementary body (EB) and the vegetative state is referred to as the reticulate body (RB). The entire metabolic stage of the Chlamydia occurs during the RB form within the phagosomes of its eucaryotic hosts (7,29,40).

Studies have shown that the chlamydiae infect a host by attacking the mucous membranes. The EBs initiate the contact which is dependent on heat-sensitive sites of the chlamydial outer membrane (11,12,28,29,31,40,45). These sites appear to be surface antigens and the ability to initiate contact relies on the multiplicity of inoculation (MOI) with a 1:1 ratio being optimal (22). Specific receptors on the host membranes are also necessary for infection (6,29,37,40).

Once the chlamydial EB attaches to the host membrane, it enters
Table 2: Comparison of *C. trachomatis* and *C. psittaci* (7).

<table>
<thead>
<tr>
<th>Species 1: <em>C. trachomatis</em></th>
<th>Species 2: <em>C. psittaci</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Replicates within cytoplasmic vesicles which have enough rigidity to displace the nucleus of the infected cells.</td>
<td>1. Replicates in cytoplasmic vesicles that spread throughout the cytoplasm of the host cell.</td>
</tr>
<tr>
<td>2. Iodine-staining carbohydrate (probably glycogen) is present in the vesicles at a defined stage in the agent's growth cycle.</td>
<td>2. Iodine-staining material not present in vesicles at all stages of the agent's growth cycle.</td>
</tr>
<tr>
<td>3. Guanine-plus-cytosine content of the agent's DNA (six strains) averaged 44.4%.</td>
<td>3. Guanine-plus-cytosine content of the DNA (10 strains) averaged 41.2%. Less than 10% homology exists between <em>C. trachomatis</em> DNA and <em>C. psittaci</em> DNA.</td>
</tr>
<tr>
<td>4. The organisms are parasites principally of humans in which they cause a variety of oculo-urogenital diseases (trachoma, inclusion conjunctivitis, LGV, urethritis, proctitis; occasionally associated with arthritis).</td>
<td>4. Parasites producing a systemic infection that have been detected in over 100 species of wild and domestic mammals. The diseases include psittacosis, ornithosis, pneumonitis, polyarthritis, enteritis, conjunctivitis, placentitis, and encephalitis.</td>
</tr>
<tr>
<td>5. Strains: all organisms causing trachoma in humans, inclusion conjunctivitis, LGV, and mouse pneumonitis.</td>
<td>5. Strains: Human pneumonitis (Borg) or psittacosis, meningopneumonitis (Cal 10), feline pneumonitis, ovine pneumonitis, 6BC (parakeet), bovine chlamydial abortion (EBA-59-795), ovine chlamydial abortion, psittacine origin, bovine encephalomyelitis, pigeon ornithosis, turkey ornithosis, sheep polyarthritis, and epizootic chlamydiosis.</td>
</tr>
</tbody>
</table>
the cell by preferential phagocytosis. The phagocytosis is preferential in that it occurs at a faster rate than the phagocytosis of other bacteria or inert particles (10,29). By a mechanism that isn’t fully understood, but is thought to involve chlamydial surface structure, the phagosomes containing Chlamydia are blocked from fusing with a lysozome (12,20,21,40). This prevents lysosomal fusion and thus eradication of the chlamydial pathogen.

Within six to eight hours following phagocytosis, the transformation of the infectious EB to the metabolically active RB occurs (27,29,40). The RB is much larger and more pleomorphic than the EB and multiplies to form a large population within the phagosome by binary fission. Many other distinguishing characteristics between the two forms can be found and are listed in Table 3.

At approximately 16-18 hours post-infection, the RBs that are actively synthesizing macromolecules peak in their protein production abilities. By this time, each initial RB has produced 200-1,000 progeny via binary fission (12). At this time, the RBs begin to condense and reorganize into the dense EB form on a 1:1 RB to EB ratio. When completely formed, the EBs initiate lysis of the host cells and begin anew another infectious cycle (7,29,40).

Properties:

The cell wall of the chlamydiae is of interest and is the major focus of many studies occurring today. The cell wall of Chlamydia resemble other Gram-negative (-) bacteria in that it possesses an inner and outer membrane in the wall (32). The chlamydial wall differs though in that it lacks peptidoglycan (2,24).
Table 3: Comparison between elementary and reticulate bodies (40).

<table>
<thead>
<tr>
<th>Property</th>
<th>Elementary body</th>
<th>Reticulate body</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>200-400 nm.</td>
<td>600-1,000 nm.</td>
</tr>
<tr>
<td>Rigid cell wall</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Extracellular stability</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Serotype-specific antigens</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Species-specific antigens exposed</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DNA nuceloid</td>
<td>nucleoid</td>
<td>dispersed</td>
</tr>
<tr>
<td>RNA/DNA ratio</td>
<td>&lt;1</td>
<td>3-4</td>
</tr>
<tr>
<td>Infective</td>
<td>+ (a)</td>
<td>-</td>
</tr>
<tr>
<td>Induce phagocytosis</td>
<td>+ (a)</td>
<td>-</td>
</tr>
<tr>
<td>Inhibit phagosome fusion</td>
<td>+ (a)</td>
<td>-</td>
</tr>
<tr>
<td>Toxicity</td>
<td>+ (a)</td>
<td>-</td>
</tr>
<tr>
<td>Metabolic activity</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Replication</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(a) Properties apparently blocked by specific antibody.
Another unique and important factor involving Chlamydia is the presence of a predominant single protein in the outer membrane of the cell wall (14,28,32,38,45). This protein is referred to as the major outer membrane protein (MOMP) and is exposed on the chlamydial surface. MOMP has been estimated to make up as much as 60% of the total composition of the outer membrane’s protein. MOMP’s molecular weight varies from 38,000-42,000 depending on the strain (14,18,28).

The roles of MOMP haven’t been fully defined but it is known to play a role in the structural stability of the cell wall and also functions as a porin or pore forming protein (5,29). Additionally, MOMP may also be important in surface antigenicity; studies are leading to the conclusion that MOMP may play a key role in Chlamydia-host interactions (18,28).

The various functions of MOMP are apparently regulated by disulfide bonds (5,28,38). These disulfide interactions may provide the cell structural stability in the absence of the peptidoglycans (5,29). This cross-linkage is important in resistance of the Chlamydia as well as being an integral component of the differentiation process from EB to RB (29). The elementary body has been shown to be more highly cross-linked and denser than its counterpart the reticulate body (18,28,29). This fact appears to be closely related to the EB’s ability to survive outside the host cell and thus be infective (7,29). The RB on the other hand, being less cross linked, is larger in size and also more labile outside of the host cell (7,28,29). The obviously important effects of the cross-linkage and the roles of MOMP have drawn well-warranted interest. Much study is being dedicated to this area with hopes that
many new aspects of these bacteria will become better understood.

One important aspect regarding chlamydial metabolism is that the RB is an energy parasite (40). Neither the RB, nor the metabolically inactive EB, have the necessary machinery and synthesis systems to facilitate the production of high energy bonds in the form of adenosine triphosphate. Instead, the RB uses the host cell's ATP for its own metabolism (40). Chlamydial infection does not block host cell protein synthesis at relatively low MOI (1:1); this is substantiated by the parasites use of the host's ATP (45).

Pathogenicity:

As mentioned, Chlamydia also have a tremendous impact in medicine (40). These bacteria hold claim as the leading cause of preventable blindness in the world as well as the greatest cause of non-gonococcal urethritis. Thus, they have great social and medical importance (39).

Being bacteria, Chlamydia are susceptible to antibiotics and such antimicrobial agents as the tetracyclines, erythromycins, and many broad spectrum antibiotics are effective and widely used in clinical treatment (40). A problem in clinical treatment however, is not the actual treatment itself, but instead the difficulty in diagnosis and detection due to insufficient equipment and knowledge in most clinical labs.

The Chlamydia are likely to be among the most ubiquitous parasites within the animal kingdom (40). Their natural hosts cover a very wide range including domestic mammals, man, many feral mammals, and all avian species (40). Animal models are used in
studying the progression of chlamydial infection and are responsible for many of the advances made (3).

The species *Chlamydia psittaci* was originally and predominantly found in avian species. It is believed to be infective to all avian species and can also infect man and other mammals. The diseases that may result from *C. psittaci* infections are severe pneumonia and systemic toxicity. The associated symptoms are fever, muscle aches, chills, and headaches.

The diseases of *Chlamydia trachomatis* are more natural, diverse, and widespread in the general human population. The diseases that they may cause in man are non-gonococcal urethritis, lymphogranuloma venereum, trachoma, inclusion conjunctivitis, cervicitis, epididymitis, salpingitis, proctitis, and pneumonia (26,39,40). Some of the diseases will be discussed in further detail.

The major ocular infection is conjunctivitis which causes scarring of the conjunctiva, causing the upper eyelid to turn inward and abrade the cornea, leading to blindness (39,40). Neonates are very susceptible to chlamydial infection when passing through an infected birth canal. Non-gonococcal urethritis (NGU) in the male genital tract is very common (39,40). NGU is sexually transmitted and can be diagnosed by eliminating gonorrhea as a causitive agent with subsequent treatment consisting of a tetracycline series. Often times however, the disease may come as a result of a combined infection by both gonorrheal and chlamydial agents and treatment must proceed accordingly. The female genital tract is also susceptible and most commonly involves the cervix, causing urethritis and proctitis (39,40). Additionally, chlamydial infections cause acute
salpingitis in females (39,40). Neonates are also adversely affected by chlamydial infections, not only through the occurrence of conjunctivitis, but also by pneumonia (40). Between 30%-50% of all pneumonia that occurs in the first six months of life is related to chlamydial infection (40).

Previous Experimentation:
The most prominent work in the area of protease treatment of Chlamydia, and its subsequent effect upon structure and infectivity, has been done by Hackstadt and Caldwell (28). They found that trypsin, chymotrypsin, and thermolysin all cleave MOMP extensively; enough to destroy its monomeric form. Trypsin and thermolysin also cleave MOMP to the extent that it was not detectable with monospecific polyclonal antibodies in immunoblotting procedures. Conversely, treatment with Staphylococcal V-8 protease did not appear to affect the structure of the chlamydial surface-exposed proteins. Interesting enough, they determined that infectivity toward human epithelial (HeLa) cells was not diminished by treatment with any of the enzymes.

Bose (9) and others (11,31,37) have also found that association or infectivity of EBs is not susceptible to treatment with a variety of proteases, including trypsin and chymotrypsin, although there was only minimal proteolytic cleavage observed. These studies have suggested that proteases can affect the structure of a number of proteins exposed on the surface of Chlamydia, but that this proteolysis doesn’t affect their infectivity for cell cultures.
MATERIALS AND METHODS

Organisms:

*Chlamydia trachomatis* organisms used were lymphogranuloma venereum (LGV) strain 434/Bu (serotype L2) and cervicitis strain UW-3/CX (serotype D). The *Chlamydia psittaci* organism used was meningopneumonitis (MN) strain Cal-10. They were obtained from the stock at the Rocky Mountain Laboratories in Hamilton, Montana. All strains were grown in HeLa 229 (human heteroploid cervical carcinoma) cells in 150-cm**2** polystyrene disposable tissue culture flasks (Corning Glass Works, Corning, N.Y.) as previously described (16).

Culture media consisted of Eagle’s minimum essential medium (MEM) (Auto Pow, Flow Laboratories, Inglewood, Calif.) supplemented with 10% heat inactivated fetal bovine serum (MEM-10), 10 ug/ml gentamicin, and 100 ug/ml vancomycin. The recipe for the preparation of MEM-10 was as follows:

**Minimum Essential Medium (MEM-10)**

H2O 720 ml.
Hank’s Balanced Salt Solution (HBSS) w/o sodium bicarbonate (10X) 100 ml.
(Gibco Laboratories, Grand Island, N.Y.)
Amino Acids (50X) 20 ml.
(Gibco Laboratories)
NaOH (0.2N) up to 15 ml.
-add until media becomes orange colored
Fetal Calf Serum 100 ml.
-inactivate at 56 C for 30 minutes
BME Vitamins (100X) 10 ml.
(Gibco Laboratories)
L-Glutamine (100X) 10 ml.
(Gibco Laboratories)
Gentamycin (1 mg/ml) in distilled H2O (D.H2O) 10 ml.
Vancomycin (10 mg/ml) in D, H2O 10 ml.
NaHCO3 (7.5%) 5 ml.
Strains L2-434 and MN were also grown in suspension cultures of L-929 cells (mouse heteroploid fibroblasts) as previously described (16) with supplemented MEM-10 culture media. The L-cell propagated organisms were used for the purification and isolation of the MOMP (39,500 dalton) protein to be used as a source of immunogen in the preparation of rabbit and mouse hyperimmune antisera.

Elementary bodies (EBs) of the L2 and D strains were purified from the HeLa 229 cell monolayers and those of the MN strain from the L-929 cells by centrifugation on discontinuous Renografin density gradients (E.R. Squibb & Sons, Princeton, N.J.) as previously described (1,2). Organisms were then maintained at -70 C (Revco Ultra Low; Revco Inc., Deerfield, MI.) until needed.

All of the above procedures were carried out at the Rocky Mountain Laboratories by Mr. Jim Simmons in the Laboratory of Microbial Structure and Function under the supervision of Dr. Harlan Caldwell.

Surface proteolysis of EBs:

Depending upon particular experimental protocol, varying amounts of purified EBs were pulled from the freezer (-70 C) and allowed to thaw at room temperature. Unless otherwise noted, 2 ml of strain L2 (8 x 10**8 IFU/ml), 2 ml of strain D (8.6 x 10**6 IFU/ml), and/or 4 ml of strain MN (4 x 10**8 IFU/ml) were used in each experiment in which that particular strain was studied. In early experiments, 25 ul of the thawed chlamydial sample of each strain was removed for protein concentration determination. The protein concentration was approximately 0.5 mg chlamydial protein per milliliter for each
strain. 14C-radiolabeled EBs were also used as indicated in certain experiments. All work with viable Chlamydia was done under an airated hood (Contamination Control, Inc., Lannsdale, P.A.). The general methodology of EB surface proteolysis was derived from similar work done by Hackstadt and Caldwell (28).

The thawed samples were placed in microfuge tubes (Eppendorf Microtesttubes, 1.5 ml; Brinkmann Instruments, Inc., Westbury, N.Y.) and pelleted at 8K for 8 min at either 0 C or at room temperature as noted in a microfuge (Microfuge 12; Beckman Instruments Inc., Fullerton, CA.). The supernatant was discarded and the pellets of each strain were combined and resuspended in 1 ml (1.2 ml when six proteases were used) of cold 10 mM sodium phosphate--15 mM NaCl, pH 7.4 (phosphate-buffered saline, PBS). The PBS was with or without 0.02% NaN3 (sodium azide, m.w. = 65 daltons; Sigma Chemical Co., St. Louis, MO.) as indicated. The suspensions were then vortexed (VWR Vortex Mixer; Scientific Industries, Inc., Bohemia, N.Y.) to dissociate the pellet.

Next, 200 ul aliquots of each sample were placed into five microfuge tubes and these were each pulsed with 25 ul of appropriate protease stock solutions at 15 min intervals over a 1 hr incubation period. Incubation was at 37 C with gentle mixing (Labquake Shaker; Labindustries, Berkeley, CA.). The various protease stock solutions were prepared as follows:

1. Control = 250 ul 10**-3 N HCl
2. Trypsin = 250 ul 10**-3 N HCl + 2.5 mg trypsin (bovine pancreas trypsin; Sigma)
3. Chymotrypsin = 250 ul 10**-3 N HCl + 2.5 mg chymotrypsin (bovine pancreas a-chymotrypsin; Sigma)
4. Thermolysin = 250 ul 10**-3 N HCl + 2.5 mg thermolysin (Type X thermolysin from Bacillus thermoprotolyticus; Sigma)
5. Proteinase K = 250 ul 10\*\*\*3 N HCl + 2.5 mg
proteinase K (Boehringer Mannheim Biochemicals,
Indianapolis, Ind.)
-protease stock solutions were maintained at 0 C

At 1 hr, 50 ul of 40 mM phenylmethylsulfonyl fluoride (PMSF, mw =
175 daltons; Sigma) (7 mg PMSF/ml 100% ethanol) (absolute ethyl
alcohol; AAPER Alcohol & Chemical Co., Louisville, KY) was added to
stop protease activity. The suspensions were then pelleted at 8K for
8 min at either 0 C or room temperature as noted in a microfuge. The
supernatants were discarded and the pellets were each resuspended in
1 ml of cold PBS and vortexed.

The samples were then washed to remove excess protease and
soluble proteolytic cleavage products from the solution. Washing was
accomplished by centrifugation three times at 10K for 8 min in a
microfuge at either 0 C or room temperature; each pellet being
resuspended in 1 ml of cold PBS and vortexed. The final pellet was
resuspended in 1 ml of cold 0.01 M sodium phosphate (pH 7.2)
containing 0.25 M sucrose and 5 mM L-glutamic acid (SPG) solution and
homogenized by vigorous vortexing. SPG was prepared as follows:

Sucrose-Phosphate-Glutamic acid (SPG)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>150 g.</td>
</tr>
<tr>
<td>0.2 M Na2HP04-dibasic</td>
<td>174 ml.</td>
</tr>
<tr>
<td>0.2 M NaH2PO4-monobasic</td>
<td>26 ml.</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>1.44 g.</td>
</tr>
<tr>
<td>D.H2O</td>
<td>2000 ml.</td>
</tr>
</tbody>
</table>

-adjust pH to 7.4 with 2 N NaOH if necessary
-sterilize 20 min. (autoclave)

A 100 ul aliquot was removed from each sample for determination
of inclusion-forming units (IFUs) and stored at -70 C. The remainder
was pelleted at 8K for 8 min in a microfuge at either 0 C or room
temperature and then resuspended in 50 ul Laemmli solubilizing buffer with or without 2-mercaptoethanol (2-ME; Sigma) as indicated. These samples were then subjected to PAGE as will be described to determine (and confirm) the extent of EB surface proteolysis.

This was the general protocol for surface proteolysis of EBs; each experiment, however, varied somewhat from the others in terms of temperatures, organisms, and the like. Below is a list of the particular specifications of each experiment:

**Experiment 1 - 1X enzyme, 2 hr.**
- a) 1 ml of L2 EBs and 2 ml of MN EBs were used
- b) 25 ul of each sample was pulled for protein concentration determination
- c) all centrifugations were done at room temperature
- d) PBS with NaN3 was used throughout
- e) protease stock solutions were one-tenth as concentrated; thus only 0.25 mg of each enzyme was added to the 250 ul 10**-3 N HCl
- f) incubation at 37 C was for 2 hr with 25 ul protease pulses every 30 min
- g) samples were washed by layering over 1 ml of 30% (vol/vol) Renografin and pelleting by centrifugation at 27K for 30 min at 5 C (Sorvall General Purpose RC-3 automatic refrigerated centrifuge)
- h) 200 ul of each suspension was pulled for infectivity determination

**Experiment 2 - 10X enzyme, 2 hr.**
- a) 1 ml of L2 EBs and 2 ml of MN EBs were used
- b) 25 ul of each sample was pulled for protein concentration determination
- c) all centrifugations were done at room temperature
- d) PBS with NaN3 was used throughout
- e) incubation at 37 C was for 2 hr with 25 ul protease pulses every 30 min

**Experiment 3 - 10X enzyme, 1 hr.**
- a) L2 and MN EBs were used
- b) all centrifugations were done at room temperature
- c) the original pellet was resuspended in 1.2 ml of PBS
- d) PBS with NaN3 was used throughout
- e) six protease stock solutions were used; the sixth being V-8 = 250 ul 10**-3 N HCl + 2.5 mg V-8 protease (V-8 protease from *Staphylococcus aureus*; Miles Laboratory, Naperville, Ill.)
Experiment 4 - post dithiothreitol (DTT) treatment
a) only L2 EBs were used
b) all centrifugations were done at room temperature
c) PBS with NaN3 was used throughout
d) 500 ul aliquots of the sample were placed into four microfuge tubes
e) two aliquots were pulsed with control stock solution and the other two with trypsin protease stock solution
f) one of each was washed and pelleted in straight PBS and the other in PBS + 10 mM DTT (Cleland's Reagent, m.w. = 154.3 daltons; Sigma)

Experiment 5 - thermolysin
a) 5 ml of L2 EBs were used
b) all centrifugations were done at room temperature
c) PBS with NaN3 was used throughout
d) the original sample was pelleted at 16K for 20 min and resuspended in 5 ml PBS
e) 1 ml aliquots of the sample were placed in five microfuge tubes
f) aliquots were pulsed with 50 ul of 30 mg thermolysin/ml 10**-3 N HCl protease stock solution
g) at 1 hr, 0.3 ml 40 mM PMSF was added to each aliquot

Experiment 6 - without NaN3
a) L2 and MN EBs were used
b) all centrifugations were done at room temperature
c) PBS without NaN3 was used throughout
d) the original pellets were each resuspended in 1.2 ml of PBS
e) 200 ul aliquots of each sample were placed into six microfuge tubes
f) the sixth 200 ul aliquot of each strain was pulsed with 25 ul of control stock solution and then incubated for 1 hr at 4 C

Experiment 7 - control IFU
a) 1 ml of L2 EBs were used
b) all centrifugations were done at room temperature
c) PBS without NaN3 was used throughout
d) four aliquots were pelleted; the fifth was stored at 4 C
e) the pellets were resuspended in 200 ul of either: 1) PBS, pH 7.2, 2) PBS, pH 7.4, 3) HBSS, or 4) SPG
f) 100 ul aliquots of each suspension was placed into two microfuge tubes
g) one aliquot of each suspension was pulsed with 25 ul of 10**-3 N HCl; the other with 25 ul of PBS, pH 7.4
h) the final washed pellets were each resuspended in 100 ul cold SPG and stored at -70 C for IFU
i) the refrigerated aliquot (not pulsed) was diluted 10**-1, 10**-2, and 10**-3 and frozen for IFU
Experiment 8 - pre DTT treatment
a) L2 and MN EBs were used
b) all centrifugations were done at 4 C
c) PBS without NaN3 was used throughout
d) the original pellets were resuspended in 1 ml PBS + 10 mM DTT and incubated at room temperature for 30 min
e) the suspensions were then converted to 20 mM iodoacetamide (mw = 184.9 daltons; Sigma) and incubated at 4 C for 30 min
f) the suspensions were washed and then pulsed and washed again like usual

Experiment 9 - blot against monoclonals
a) the samples prepared in Experiment 5 were used again

Experiment 10 - 4 C
a) L2 and MN EBs were used
b) all centrifugations were done at 4 C
c) PBS without NaN3 was used throughout

Experiment 11 - D strain
a) D strain EBs were used
b) all centrifugations were done at 4 C
c) PBS without NaN3 was used throughout

Experiment 12 - 14C labeled EBs
a) 2 ml 14C-L2 EBs and 2 ml 14C-MN EBs were used in addition to the control L2 and MN EBs
b) all centrifugations were done at 4 C
c) PBS without NaN3 was used throughout
d) the 14C-labeled organisms were pelleted and resuspended in 400 ul PBS
e) the 14C-labeled aliquots were pulsed only with control and trypsin protease stock solutions
f) of the final 14C-labeled SPG suspensions, 50 ul was pulled for IFU, 100 ul was pelleted and resuspended in Laemmli solubilizing buffer for PAGE, and 850 ul was frozen for an organism binding assay

Experiment 13 - repeat #10
a) L2 and MN EBs were used
b) all centrifugations were done at 4 C
c) PBS without NaN3 was used throughout

Experiment 14 - DTT/Antibody Binding
a) L2 EBs were used
b) all centrifugations were done at 4 C
c) PBS without NaN3 was used throughout
d) the original pellet was resuspended in 2 ml PBS
e) 500 ul aliquots were placed into four microfuge tubes
f) two aliquots were pulsed with control stock and the other two with trypsin protease stock
g) at 1 hr, one of each was converted to 10 mM DTT and all were incubated at 4 C for 30 min
h) at 1.5 hr, 50 ul of 40 mM PMSF was added to each
i) the samples were washed and resuspended in 0.5 ml PBS
j) from the final suspensions, 200 ul was pulled, pelleted, and resuspended in Laemmli solubilizing buffer for PAGE, the remaining 300 ul was diluted 1:7 in PBS with 0.1% bovine albumin and saved for an antibody binding assay

**Polyacrylamide gel electrophoresis (PAGE):**

The SDS-PAGE procedure of Laemmli for discontinuous gel systems (36) was used in a manner similar to that employed by Caldwell et al. (15) and Hackstadt et al. (29) for qualitative and preparative slab gel electrophoresis of chlamydial proteins. This protocol involved use of the tris(hydroxymethyl)aminomethane (Tris)-glycine system as described (36). The following stock solutions were prepared for implementation of SDS-PAGE:

1. Acrylamide:N,N’-Methylene-bis-acrylamide
   - (Acrylamide,BIS; Bio-Rad Laboratories, Richmond, CA)
   - respective ratio (wt/wt) was 30:0.8
   - mix 30 g Acryl. w/ 0.8 g BIS; QS to 100 ml w/ D. H2O
   - homogenize on rotary mixer plate with magnetic stir bar
   - vacuum filter through qualitative paper (Whatmann No.1)

2. 1.5 M Tris-HCl, pH 8.8
   - (Trizma Hydrochloride, Tris-hydroxymethyl-aminomethane) (Sigma)
   - mw = 157.6 daltons
   - (hydrochloric acid)
   - (J.T. Baker Chemical Co., Phillipsburg, N.J.)

3. 0.5 M Tris-HCl, pH 6.8

4. 10% (wt/vol) ammoniumpersulfate
   - (APS; Bio-Rad)
   - mw = 228.2 daltons

5. 10% (wt/vol) sodium-dodecyl-sulfate
   - (SDS; BDH Chemicals Ltd., Poole, England)
   - mw = 288.38 daltons
   - chemical formula: C(12)H(25)0SO(3)Na

6. 0.2 M ethylene-diamine-tetraacetic acid
   - (EDTA tetrasodium salt; Sigma)
   - mw = 380.2 daltons

Vertical discontinuous slab gels 15 cm wide x 11 cm tall were
prepared between cleaned glass plates (1.5 mm apart) sealed at the edges with spaghetti tubing and supported in the proper apparatus (double PAGE system; Bio-Rad). The final acrylamide concentration was 15% in the separating gels (except for that experiment noted in which it was 17.5%) and 5% in the upper stacking gels which were 1cm high. The separating and stacking gels were prepared in duplicate according to the following recipes:

### Separating Gel

- Acrylamide:BIS, 30:0.8
- 1.5 M Tris, pH 8.8
- 0.5 M Tris, pH 6.8
- TEMED
- D.H2O
- 10% APS

### Stacking Gel

- 30 ml.
- 15 ml.
- 5 ml.
- 0.02 ml.
- 15.3 ml.
- 0.1 ml.

*(TEMED; N,N,N',N' tetramethylethylenediamine; Bio-Rad)

-first mix water, acrylamide, and Tris; add TEMED and APS below surface of liquid when ready for use.

In those experiments noted, EDTA was also added to the gel mixtures (15% acrylamide) according to the following protocol:

### Separating Gel

- Acrylamide:BIS, 30:0.8
- 1.5 M Tris, pH 8.8
- 0.5 M Tris, pH 6.8
- TEMED
- D.H2O
- 10% APS
- 0.2 M EDTA

### Stacking Gel

- 30 ml.
- 12 ml.
- 1.88 ml.
- 0.03 ml.
- 16.8 ml.
- 0.6 ml.
- 0.6 ml.

The separating gels were mixed and poured and 20 minutes was allowed for polymerization before the stacking gels were added to the top. Five milliliters of water was also placed on top of each poured gel (separating and stacking) before polymerization to force the formation of even surfaces on the tops of the gels. This water was then removed after polymerization had occurred. Plastic combs, with
twenty fingers 5 mm. wide, were inserted into the stacking gels before polymerization occurred. This allowed for the presence of empty wells in the polymerized stacking gel into which samples could be placed for electrophoresis. After this, 45-50 minutes was allowed for the polymerization of the stacking gel before samples and electrophoretic (running) buffer was added to the apparatus.

Tris-glycine running buffer, pH 8.3, containing 0.1% SDS was prepared by combining 4.5 g Trizma base (Sigma), 21.5 g glycine (aminoacetic acid, aminoethanoic acid, glycocoll; mw = 75.1 daltons; Sigma), with 1.5 g SDS (BDH) and diluting to 1.5 liters with D.H2O. The mixture was then homogenized with a magnetic stir bar on a rotary mixer plate.

Before electrophoresis, samples were mixed with an equal volume of electrophoresis solubilizing buffer containing 0.1 M Tris-hydrochloride (pH 6.8), 2.5% (wt/vol) SDS, 5% 2-mercaptoethanol, 20% glycerol, and 0.001% bromophenol blue for tracking. The mixture was then heated to 100 C for 5-10 minutes. In certain experiments as noted, the 2-ME was replaced in the solubilization buffer with an equal volume of water. Solubilizing buffer was prepared in bulk as follows:

Solubilizing Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris, pH 6.8</td>
<td>10 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 ml</td>
</tr>
<tr>
<td>2-ME</td>
<td>1 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 ml</td>
</tr>
<tr>
<td>D.H2O</td>
<td>19 ml</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

Varying amounts of sample, dependent upon protein concentration, were placed into the wells in each gel lane and the upper and lower
buffer reservoirs were filled with running buffer. Unless otherwise noted, 20 ul/lane of sample was placed in those gels to be stained and 10 ul/lane in those to be immunoblotted. SDS-PAGE was carried out in a single dimension at a constant current of 25 mA per gel (500 V maximum) until the tracking dye reached the bottom edge of the gels (normally about 2 hours). A standard electrophoresis power supply (Instrumentation Specialties Co., Lincoln, Neb.) was utilized for this purpose.

The protein standards used for estimating chlamydial protein molecular weights were: phosphorylase b (94,000), bovine serum albumen (67,000), ovalbumen (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and a-lactalbumin (14,400) (low molecular weight markers (LMW); Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Other standards used for comparative purposes were: ovalbumin (43,000), a-chymotrypsinogen (25,700), b-lactoglobulin (18,400), lysozyme (14,300), bovine trypsin inhibitor (6,200), and insulin (a & b) (3,000) (Prestained Protein Low Molecular Weight Standards; Bethesda Research Laboratories, Gaithersburg, MD.).

Gel staining:

Following SDS-PAGE, the gels to be stained were immediately fixed for 30 min in 30% methanol-7% acetic acid (glacial acetic acid, 17 N; E.M. Science, Gibbstown, N.J.). After fixation, the gels were stained for 1-16 hr in 50% methanol-7% acetic acid containing .25% Coomassie blue (Brilliant Blue R; Sigma) and then destained in fixative solution supplemented with anion exchange resin beads (Dowex
1-X8 Anion Exchange Resin; Bio-Rad) for several hours. All incubations were at room temperature with gentle shaking. The gels were then equilibrated in D.H2O and photographed.

After Coomassie staining, the gels were silver stained in a manner similar to that as described (44) and as modified (33). They were fixed for 0.25-16 hr in a solution of 40% ethanol-5% acetic acid and then equilibrated for 15 min in D.H2O. Following equilibration, the gels were oxidized for 5 min in a glass pan with 0.7% periodic acid \((\text{H}(5)\text{I}_6)\), \(\text{mw} = 228\) daltons; Sigma) (1.4 g / 200 ml H2O) and then washed four times for 5 min each in D.H2O. Next, they were reduced for 15 min in a covered glass pan in a solution containing 2 ug/ml dithiothreitol and then washed three times for 5 seconds each in D.H2O.

Gels were then silver stained for 10 min in a glass pan followed with five-3 min D.H2O washes. Stain was made according to the following protocol:

Silver Stain

\[
\begin{align*}
50\% \text{ NaOH} &\quad .25 \text{ ml.} \\
\text{NH}(4)\text{OH} &\quad 2.0 \text{ ml.} \\
20\% \text{ AgNO}_3 &\quad 5.0 \text{ ml.} \\
\text{D.H2O} &\quad \text{QS to 200 ml.}
\end{align*}
\]

- add sodium and ammonium hydroxide to 150 ml D.H2O
- slowly add silver nitrate drop by drop with gentle stirring to avoid precipitation
- bring to volume with D.H2O and then mix well

The washed gels were then developed for 2-5 min in a solution containing 0.005% citric acid (Fisher Scientific Co., Fair Lawn, N.J.) and 0.018% formalin. Finally, they were washed again and equilibrated with D.H2O and then photographed. All incubations were at room temperature with gentle shaking.
Immunological reagents:

Preparation and specificity of the hyperimmune rabbit polyclonal antisera against the isolated sodium dodecyl sulfate (SDS)-denatured MOMP s and against intact viable L2 EBs was as previously described (16,18). The SDS-denatured MOMP s of Chlamydia serotypes L2, D, and MN were isolated as immunogens by preparatory SDS-polyacrylamide gel electrophoresis as described (18). Purification of the immunoglobulin G (IgG) fraction of the antisera was done by adsorption to Protein A-Sepharose CL-4B (Sigma) according to Goding (25) as described (19). The determination of protein concentration of isolated IgG by the Folin-phenol reagent method of Lowry using bovine serum albumen as a standard has been indicated (19).

The preparation, specificity, and purification of a line of monoclonal antibodies against surface antigens of the L2 serotype of C. trachomatis has been clearly delineated (13,42). Briefly this procedure involved preparation of a hybrid cell line by fusion of mouse myeloma cells (NS1) with L2 EB immunized spleen cells of BALB/C mice and the subsequent isolation of the L2 specific antisera.

All immunological reagents were prepared by personnel at the Rocky Mountain Laboratories under the supervision of Dr. Harlan Caldwell.

Radiolabeling:

The intrinsic radiolabeling of EBs with 14C-amino acids was as described (1,17) using a medium composed of MEM-10 supplemented with 1 µg/ml emetine hydrochloride (Sigma) and 1 uCi of 14C-amino acids
(specific activity, 1.88 mCi/mg; ICN, Chemical and Radioisotope Division, Irvine, CA.).

The monoclonal IgG antisera was radioiodinated using the Iodogen/chloroform procedure as indicated (19). This protocol involved the combination of 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, Ill.), chloroform, Na(125)I (ICN), and IgG in 1-dram vials. The specific activity of IgG radiolabeled by this procedure was between $3 \times 10^{5}$ and $5 \times 10^{5}$ cpm/ug.

Finally, Protein A (Pharmacia, Sweden) was radioiodinated using the chloramine-T (Sigma) protocol (19) of Hunter with a final specific activity of about 5 uCi/ug (4-5 x 10**6 cpm/10 ul).

Once again, all of the above radiolabeling procedures had been previously performed by personnel at the Rocky Mountain Laboratories prior to the initialization of our research work.

**Immunoblotting:**

The procedure of Towbin et al (43) and Bittner et al (8) as modified by Batteiger et al (4) for the electrophoretic transfer of proteins from polyacrylamide gels to diazobenzylloxymethyl cellulose on nitrocellulose paper (NCP) was similar to those methods previously described (13,19,29).

The following stock solutions were prepared for implementation of immunoblot procedures:

1. 0.5 M sodium phosphate buffer
   - sodium dibasic phosphate (anhydrous) 362.1 g.
   - Na(2)HPO(4), mw = 142 daltons (Sigma)
   - sodium monobasic phosphate (anhydrous) 62.0 g.
   - NaH(2)PO(4), mw = 138 daltons (Baker)
   - sodium azide 1.2 g.
2. 1.5 M NaCl sodium chloride
   -NaCl, mw = 58.5 daltons (Baker)
   D.H2O QS to 6 l.

3. 0.025 M sodium phosphate, pH 7.3 (Transfer Buffer)
   0.5 M sodium phosphate (stock) 150 ml.
   D.H2O QS to 3 l.
   -mix with magnetic stir bar on rotary mixer
   plate and degas under vacuum for 2 hours

4. phosphate buffer saline (PBS), pH 7.2
   -final concentration of 0.05 M sodium phosphate,
   0.15 M NaCl, and 0.02% NaN3
   0.5 M sodium phosphate (stock) 400 ml.
   1.5 M NaCl (stock) 400 ml.
   D.H2O QS to 4 l.

5. phosphate buffer saline-albumin (PBS-A), pH 7.2
   -final concentration of 3% bovine serum albumin
   Bovine Serum Albumin, Fraction V
   (Calbiochem-Behring Corp., La Jolla, CA) 6 g.
   PBS (stock) 200 ml.

6. phosphate buffer saline-Tween 20 (PBS-T), pH 7.2
   -final concentration of 0.05% Tween and .5mM EDTA
   polyoxyethylene sorbitan monolaurate
   (Tween-20; Sigma) 2ml.
   EDTA 7.6 g.
   PBS (stock) 4 l.

After SDS-PAGE was complete, the gel to be blotted was placed on
Whatman no. 1 filter paper that had been soaked in transfer buffer.
Any air bubbles between the NCP and gel were removed. The NCP (HAY
pore size 0.45 um; Millipore, Bedford, MA.) was evenly saturated with
transfer buffer and then placed onto the surface of the gel with all
air bubbles removed. Another sheet of soaked filter paper was placed
above the NCP and then all was sandwiched between two layers of
scouring pad (Scotch-Brite). This sandwich was then supported
between two porous plastic grids and the entire conglomerate was
placed into the immunoblot apparatus (Bio-Rad Trans Blot Cell, Model
160/1.6 Power Supply) with the gel nearest the cathode and NCP
nearest the anode. The apparatus was filled with degased transfer
buffer, a cooling coil and magnetic stir bar were added, and
electrophoretic transfer was carried out at 27 V (0.9-1.0 A) and 17 C for 2 h.

Following transfer, the sandwich was removed and the lanes and prestained molecular weight markers were marked on the NCP with a black sharpie pen before the gel was discarded. The NCP was then placed in 100 ml of PBS-A and incubated at room temperature for 15 min with gentle shaking (orbital shaker; Belco Glass Inc., Vineland, N.J.). The bovine serum albumin acted as a blocking agent to block unoccupied protein binding sites on the NCP. If the NCP was to have been stained with amido black, the albumin would have been omitted from the blocking buffer. Next, the NCP was washed twice in PBS-T at room temperature with gentle shaking for 10 min each. The Tween also acted as a blocking agent and it would have been omitted if glycolipid antigens were of interest because they bind nitrocellulose poorly in the presence of detergent.

The NCP was then placed into a heat sealable bag and incubated with antiserum or monoclonal antibody diluted 1:100 in PBS-A. Incubation was for 2-16 h at room temperature with gentle mixing (rocker platform; Belco). The NCP was washed three times for 10 min each with 100-200 ml PBS-T.

Once again the NCP was placed in a heat sealable bag and this time incubated with (125)I-labeled protein A diluted 1:300 in PBS-A (final specific activity 5x10**5 cpm/ml) (Gamma 4000 gamma counter; Beckman Instruments Inc., Fullerton, CA.). Incubation was for 2-16 h at room temperature with gentle mixing under a lead cover. The NCP was washed twice for 10 min each in PBS-T, then 6-8 times briefly with D.H2O, and then dried between sponge sheets for 1-2 h at 37 C
with aeration.

Finally, the NCP was subjected to autoradiography using Kodak X-ray film (X-Omat XAR-5 film; Eastman Kodak Co., Rochester, NY.) with a Lightening-Plus intensifying screen (Dupont Cronex Lightning Plus E.C.). Exposure was at -70 C for 0.5-18 hours in 8x10 Wafer Rigidform Cassettes (Halsey X-Ray Products, Inc., Brooklyn, NY). The film was developed using a Kodak X-Omat M-20 Processor.

Infectivity determination:

The general methodology as described by Furness et al. (23) for determining inclusion-forming units (IFUs) was used in a manner similar to that of Hackstadt et al. (29) and of Caldwell and Schacter (19) as modified. Inclusions were observed by an indirect immunofluorescence assay using polyclonal rabbit antiserum against species specific antigens of either C. trachomatis or psittaci and fluorescein isothiocyanate conjugated immunoglobulin G goat anti-rabbit serum (Cappel Scientific Division, Malvern, PA.). All assays were done in 24 chamber tissue culture chamber trays with a circular cover slip on the bottom of each well.

At 18-24 hr prior to the assay, each well was seeded with approximately 1.5 ml of 5 x 10**5 HeLa 229 cells in MEM-10. L-929 cells were used in one assay as indicated. The chamber trays were then incubated at 37 C and the cells were allowed to form into a monolayer.

Ten-fold dilutions (10**-1, 10**-2, and 10**-3) of the 100 ul protease treated samples saved for IFU were made by consecutive
additions of 900 ul of SPG and the subsequent removal of 100 ul aliquots. The MEM-10 was aspirated from the HeLa cell monolayers and they were infected in duplicate by adding 100 ul of each dilution of the chlamydial samples to each chamber. 100 ul of SPG was added to each well and the trays were incubated for 1 hr at 37 C.

The inoculum was removed and the infected monolayers were washed twice with 0.5 ml of SPG. They were then fed with 1 ml of pre-warmed MEM-10 and incubated for 40 hr at 37 C with 5% CO2. After incubation, the MEM-10 was aspirated out of each chamber and the monolayers were fixed in 0.5 ml of methanol for 30 min at room temperature. After fixation, the MeOH was removed and 1 ml of PBS (without NaN3, pH 7.4) was added to each well; the trays were stored at 4 C while the antibody solutions were prepared.

For infectivity determinations of the MN strain of C. psittaci, polyclonal rabbit antisera against whole MN EBs was diluted 1:250 in PBS. For all other strains, polyclonal rabbit antisera against the C. trachomatis 155 Kd species-specific antigen was diluted 1:20 in PBS. The PBS was removed from the monolayers and 200 ul of either of the above antiserum was respectively added to each well. The trays were incubated at 37 C with 5% CO2 for 1 hr after which the antiserum was removed and the monolayers washed three times with 0.5 ml of PBS for 10 min each.

Conjugated goat anti-rabbit IgG was diluted 1:80 in PBS and 100 ul of this dilution was added to each well. The trays were then incubated at 37 C for 0.5-1 hr followed by removal of the conjugate and three subsequent washes with 0.5 ml of PBS. The cover slips containing the washed monolayers were then removed from each well and
mounted in a drop of buffered glycerol (Buffered Glycerol Mounting Medium; Becton, Dickinson, & Co., Cockeysville, MD.) on glass slides.

The slides were observed under a Zeiss fluorescent microscope (W. Germany) and inclusions fluorescing yellow-green were counted. The IFU/ml was determined as follows:

1. count and sum inclusions observed in 15 random microscopic fields
2. divide total count by 15 to determine the average #/field
3. multiply the average by 744 (field area adjustment factor)
4. multiply by 5 (inoculation adjustment factor)
5. multiply by the log dilution of the inoculum to determine the final IFU/ml of each sample

Binding:

Two different types of binding assays were used in our experiment; the first was an organism binding assay to determine the amount of association of control or trypsinized 14C-chlamydiae to HeLa cells, the second was an antibody binding assay to determine the amount of association of species, subspecies, and strain antisera to control, trypsinized, and DTT treated chlamydiae.

The organism binding assay followed a protocol nearly identical to that of Hackstadt and Caldwell (28) in their modification of Soderlund and Kihstrom's procedure (41). The major modifications were that 100 usp units of heparin (Heparin Sodium Salt, 300 usp units; Sigma) were substituted for the 250 ug of proteinase K in the PBS added to one half of the wells and that the PBS was changed to 50 mM phosphate--150 mM saline, pH 7.2. The associated counts were again determined by liquid scintillation spectroscopy (Beckman LS 9000 Scintillation Counter; Beckman) as noted (28) and heparin
resistant and sensitive radioactivity was figured for both the control and trypsinized 14C-labeled chlamydiae.

The antibody binding assay utilized three different radioiodinated monoclonal antibodies developed by Caldwell (13): the first was L2I-45 which was species specific, the second was L2I-10 which was subspecies specific and recognized antigens from the same serogroup, and the third was L2I-5 which was strain specific and recognized elements from the same serotype. We incubated 0.1 ml. of the treated and washed EBs (which had been diluted 1:7 in PBS w/ 0.1% bovine serum albumin) in duplicate with the various monoclonal antibodies for 1 hr at 37 C on a rotary mixer. After incubation, the samples were pelleted at 8K for 8 min in a microfuge at room temperature and the supernatants were discarded. The pellets were washed twice with 500 ul of PBS with 0.1% bovine serum albumin and then counted on a gamma counter (Beckman 4000) to determine antibody association levels.

**Protein Concentration Determination:**

The basic procedure of Lowry using the Folin phenol reagent for protein measurement was utilized. Briefly this involved the preparation of a set of protein standards (1 mg/ml bovine serum albumin) diluted to various concentrations with 0.1 N NaOH and water, adding a set amount of PBS and dye reagent (Bio-Rad Protein Assay Dye Reagent Concentrate), measuring their optical density in a spectrophotometer at 595 nm, and then constructing a standard curve of protein concentration vs. OD-595.

The sample aliquots were mixed and solubilized at 70 C for 1 hr
with an equal volume of 0.1 N NaOH. They were then diluted in a similar fashion as the standards with 0.1 N NaOH, water, PBS, and dye reagent. Their optical densities were also recorded in the spectrophotometer. Protein concentrations were determined by comparison to the standard curve.
RESULTS

Effects of Protease Treatment on Chlamydial EB Structure:

A group of proteases, including trypsin, chymotrypsin, thermolysin, and proteinase K, were examined for their capacity to cleave EB surface-exposed proteins of the L2 and D strains of *Chlamydia trachomatis* and the MN strain of *C. psittaci*. A major emphasis of these protease cleavages was to observe the accompanying structural changes occurring within particular surface-exposed proteins, particularly the Major Outer Membrane Protein (MOMP). The elementary bodies were treated and subjected to the various proteases in the manner indicated in Material and Methods.

The proteolysis of the EBs was confirmed by PAGE analysis followed by either a Commassie brilliant blue (CBB) or silver (AgNO₃) stain or both. Structural alterations of particular proteins were noted in this manner.

Figures 1 and 2 are CBB and silver stains, respectively, of the protease treated EBs of the L2 and MN strains. In this instance, protease concentrations were only one-tenth those of all subsequent treatments (Experiment 1). These two figures demonstrate the fact that proteinase K serves to cleave most thoroughly the external proteins of both the L2 and MN strains. Trypsin, chymotrypsin, and thermolysin all cleave to a more limited extent as shown in the figures. Resultant MOMP structure, as compared to the control lane (no protease), is a good indicator of the degree of cleavage for a particular protease and is labeled on all figures.

The cleavage strength of these various proteases is best
FIGURE 1: CBB stain of PAGE for Experiment 1. Intact elementary bodies of *Chlamydia trachomatis* L2 strain and *C. psittaci* MN strain were subjected to 1 mg/ml protease for 2 hours at 37 C and then washed over a Renografin gradient before PAGE.

**LEGEND:**
- **L2** -- *Chlamydia trachomatis* L2 strain elementary bodies
- **MN** -- *Chlamydia psittaci* MN strain elementary bodies
- **CN** -- control (no protease)
- **TR** -- trypsin
- **CT** -- chymotrypsin
- **TH** -- thermolysin
- **PK** -- proteinase K

- Numbers on left boundary indicate molecular weights in daltons.
FIGURE 2: AgNO₃ (silver) stain of PAGE for Experiment 1. Intact elementary bodies of Chlamydia trachomatis L2 strain and C. psittaci MN strain were subjected to 1 mg/ml protease for 2 hours at 37°C and then washed over a Renografin gradient before PAGE.

LEGEND:

- L2 -- Chlamydia trachomatis L2 strain elementary bodies
- MN -- Chlamydia psittaci MN strain elementary bodies
- CN -- control (no protease)
- TR -- trypsin
- CT -- chymotrypsin
- TH -- thermolysin
- PK -- proteinase K

- Numbers on left boundary indicate molecular weights in daltons.
demonstrated in the MN lanes of Figure 1 which shows that proteinase K, trypsin, thermolysin, and chymotrypsin respectively have decreasing abilities to cleave chlamydial external proteins. In all cases, however, this low protease concentration does not allow for the complete destruction of MOMP in its monomeric form (note that MOMP is still faintly visible in the proteinase K lanes).

Various cleavage fragments are apparent in the lower section of the figures when the protease tracts are compared to the control tracts, especially in Figure 2 within the L2 strain. The origin of these fragments and the fate of MOMP will be better defined in a later subsection dealing with EB immunogenicity.

Figures 3 (CBB stain) and 4 (silver stain) correlate with Experiment 2 and show a much greater extent of cleavage due to the increased protease concentration during treatment. In comparing the L2 and MN strains it becomes obvious that the MN strain is much more resistant to all of the proteases tested, although cleavage still occurs to a significant degree, especially as noted in the proteinase K lanes. Once again, the cleavage fragments are seen in the lower regions of the protease lanes of the stained gels as compared to the control tracts. Also, clearing can be detected in the upper regions of the protease lanes indicating cleavage of the larger, higher molecular weight surface-exposed proteins of the chlamydial outer membrane.

The large fragment seen at about 32,800 K in the thermolysin tracts appeared to be an altered remnant of the actual thermolysin protease itself. This is documented by Figures 5, 6, 7, and 8 which are two dimensional electrophoresis patterns obtained for the
FIGURE 3: CBB stain of PAGE for Experiment 2. Intact elementary bodies of *Chlamydia trachomatis* L2 strain and *C. psittaci* MN strain were subjected to 10 mg/ml protease for 2 hours at 37°C and then washed by triple centrifugation before PAGE.

**LEGEND:**
- **L2** — *Chlamydia trachomatis* L2 strain elementary bodies
- **MN** — *Chlamydia psittaci* MN strain elementary bodies
- **CN** — control (no protease)
- **TR** — trypsin
- **CT** — chymotrypsin
- **TH** — thermolysin
- **PK** — proteinase K

- Numbers on left boundary indicate molecular weights in daltons.
FIGURE 4: AgNO₃ (silver) stain of PAGE for Experiment 2. Intact elementary bodies of Chlamydia trachomatis L2 strain and C. psittaci MN strain were subjected to 10 mg/ml protease for 2 hours at 37°C and then washed by triple centrifugation before PAGE.

LEGEND:
L2 — Chlamydia trachomatis L2 strain elementary bodies
MN — Chlamydia psittaci MN strain elementary bodies
CN — control (no protease)
TR — trypsin
CT — chymotrypsin
TH — thermolysin
PK — proteinase K

- numbers on left boundary indicate molecular weights in daltons.
FIGURE 5: Two dimensional electrophoresis pattern for control elementary bodies of Chlamydia trachomatis strain L2. The compounds were separated in the horizontal direction by chromatography and in the vertical direction by electrophoresis. Initial treatment was done with no protease (control) at 37 C for 1 hour and purification was by triple centrifugation.
FIGURE 6: Two dimensional electrophoresis pattern for thermolysed elementary bodies of *Chlamydia trachomatis* strain L2. The compounds were separated in the horizontal direction by chromatography and in the vertical direction by electrophoresis. Initial treatment was done with 10 mg/ml thermolysin at 37°C for 1 hour and purification was by triple centrifugation.
FIGURE 7: Two dimensional electrophoresis pattern for control elementary bodies of *Chlamydia psittaci* strain MN. The compounds were separated in the horizontal direction by chromatography and in the vertical direction by electrophoresis. Initial treatment was done with no protease (control) at 37°C for 1 hour and purification was by triple centrifugation.
FIGURE 8: Two dimensional electrophoresis pattern for thermolysed elementary bodies of *Chlamydia psittaci* strain MN. The compounds were separated in the horizontal direction by chromatography and in the vertical direction by electrophoresis. Initial treatment was done with 10 mg/ml thermolysin at 37°C for 1 hour and purification was by triple centrifugation.
separation of control and trypsinized MOMP of the L2 and MN strain. As can be seen, treatment with thermolysin produced similar patterns for both the L2 and MN strains, with each of them being distinct from the pattern obtained for the control group of the same strain. This indicated that the thermolysin protease itself may be binding to and migrating with a component of MOMP containing an antigenic determinant to which the antibody was specific in its attachment.

Figure 9, a silver stain correlating to Experiment 3, shows the results of running the enzymes themselves along with the control and protease treated L2 and MN elementary bodies. Also, staphylococcal V-8 protease was added to the battery and is shown to be an effective cleaving agent for the surface-exposed proteins of both strains. Aside from the addition of V-8 protease, the cleavage patterns and corresponding residual fragments are in agreement with the previously mentioned results.

In Experiment 11, an additional Chlamydia trachomatis strain (strain D) was subjected to the routine experimental protocol. Figures 10 and 11 show the CBB and silver stains of the resultant polyacrylamide gel electrophoresis for the control and protease treated EBs (treated with or without 2-ME as indicated). The results are similar to the L2 strain cleavage pattern with the exception that proteinase K was significantly less effective in its hydrolysis ability for D strain proteins as compared to those of the L2 strain. Also, trypsin and chymotrypsin appeared to show a diminished degree of cleavage potential as compared to previous results from the L2 strain.

The final study (comprising a combination of results from
FIGURE 9: AgNO$_3$ (silver) stain of PAGE for Experiment 3.
Intact elementary bodies of Chlamydia trachomatis L2 strain and C. psittaci MN strain were subjected to 10 mg/ml protease for 1 hour at 37 C and then washed by triple centrifugation before PAGE. Individual enzymes were also electrophoresed along with the treated EBs for comparison purposes.

LEGEND: L2 — Chlamydia trachomatis L2 strain elementary bodies
MN — Chlamydia psittaci MN strain elementary bodies
CN — control (no protease)
TR — trypsin
CT — chymotrypsin
TH — thermolysin
PK — proteinase K
V8 — staphylococcal V-8 protease

- numbers on left boundary indicate molecular weights in daltons
FIGURE 10: CBB stain of PAGE for Experiment 11. Intact elementary bodies of Chlamydia trachomatis D strain were subjected to 10 mg/ml protease for 2 hours at 37 C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer with and without 2-ME.

**LEGEND:**
- **D** — Chlamydia trachomatis D strain elementary bodies
- **+2ME** — suspended in buffer with 2-mercaptoethanol
- **-2ME** — suspended in buffer without 2-mercaptoethanol
- **CN** — control (no protease)
- **TR** — trypsin
- **CT** — chymotrypsin
- **TH** — thermolysin
- **PK** — proteinase K

- numbers on left boundary indicate molecular weights in daltons.
FIGURE 11: AgNO₃ (silver) stain of PAGE for Experiment 11.
Intact elementary bodies of Chlamydia trachomatis D strain were subjected to 10 mg/ml protease for 2 hours at 37°C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer with and without 2-ME.

LEGEND: D — Chlamydia trachomatis D strain elementary bodies
+2ME — suspended in buffer with 2-mercaptoethanol
-2ME — suspended in buffer without 2-mercaptoethanol

CN — control (no protease)
TR — trypsin
CT — chymotrypsin
TH — thermolysin
PK — proteinase K

- numbers on left boundary indicate molecular weights in daltons.
Experiments 11 and 13) was a comparison of the effects of the proteases in the presence and absence of 2-mercaptoethanol (2-ME). 2-ME is a reducing agent that functions by reducing disulfide bonds, thus freeing sulfhydryl groups (-SH) and causing protein chains bound by disulfides to be cleaved apart. The effects of 2-ME upon protease efficiency are shown by Figures 12-15 for the L2 strain of Chlamydia trachomatis and the MN strain of C. psittaci.

The experimentation verified the theoretical function of 2-ME as seen in the aforementioned figures. When 2-ME is added, monomeric MOMP is fragmented and absent to a greater degree than in the corresponding lanes in which 2-ME is absent. Figure 12 gives the most vivid representation as the reduction of monomeric MOMP is easily detectable in the 2-ME tracts. The other figures reiterate the reduction ability of 2-ME allowing for more efficient protease cleavage. The effects of the proteases are as expected and follow the same general pattern and degree of cleavage as noted in results from Experiment 1. The appearance of additional fragment bands verifies the added protease efficiency in the presence of 2-ME.

Effects of Protease Treatment on Chlamydial EB Immunogenicity:

After duplicate PAGE was performed for each sample as described in Materials and Methods, one gel was stained while the other was subjected to standard immunoblotting techniques. This allowed for a correlation to be made in each trial between extent of proteolysis of the surface-exposed chlamydial proteins, as determined by structural changes observed in the stained gel, and the EBs' ability to elicit a specific antibody reaction, as determined by autoradiogram patterns.
FIGURE 12: CBB stain of PAGE for Experiment 13. Intact elementary bodies of *Chlamydia trachomatis* L2 strain and *C. psittaci* MN strain were subjected to 10 mg/ml protease for 1 hour at 37 C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer with 2-mercaptoethanol.

**LEGEND:**
- L2 — *Chlamydia trachomatis* L2 strain elementary bodies
- MN — *Chlamydia psittaci* MN strain elementary bodies
- +2ME — suspended in buffer with 2-mercaptoethanol
- CN — control (no protease)
- TR — trypsin
- CT — chymotrypsin
- TH — thermolysin
- PK — proteinase K

- numbers on left boundary indicate molecular weights in daltons.
FIGURE 13: AgNO₃ (silver) stain of PAGE for Experiment 13. Intact elementary bodies of Chlamydia trachomatis L2 strain and C. psittaci MN strain were subjected to 10 mg/ml protease for 1 hour at 37 C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer with 2-mercaptoethanol.

LEGEND: L2 — Chlamydia trachomatis L2 strain elementary bodies
MN — Chlamydia psittaci MN strain elementary bodies
+2ME — suspended in buffer with 2-mercaptoethanol
CN — control (no protease)
TR — trypsin
CT — chymotrypsin
TH — thermolysin
PK — proteinase K
 Numbers on left boundary indicate molecular weights in daltons.
FIGURE 14: CBB stain of PAGE for Experiment 13. Intact elementary bodies of Chlamydia trachomatis L2 strain and C. psittaci MN strain were subjected to 10 mg/ml protease for 1 hour at 37 C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer without 2-mercaptoethanol.

LEGEND:
- L2 — Chlamydia trachomatis L2 strain elementary bodies
- MN — Chlamydia psittaci MN strain elementary bodies
- 2ME — suspended in buffer without 2-mercaptoethanol
- CN — control (no protease)
- TR — trypsin
- CT — chymotrypsin
- TH — thermolysin
- PK — proteinase K
- Numbers on left boundary indicate molecular weights in daltons.
FIGURE 15: AgNO₃ (silver) stain of PAGE for Experiment 13.
Intact elementary bodies of Chlamydia trachomatis L2 strain and C. psittaci MN strain were subjected to 10 mg/ml protease for 1 hour at 37 C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer without 2-mercaptoethanol.

LEGEND: L2 — Chlamydia trachomatis L2 strain elementary bodies
MN — Chlamydia psittaci MN strain elementary bodies
-2ME — suspended in buffer without 2-mercaptoethanol
CN — control (no protease)
TR — trypsin
CT — chymotrypsin
TH — thermolysin
PK — proteinase K
- numbers on left boundary indicate molecular weights in daltons.
observed following immunoblot procedures.

The specificities of the polyclonal and monoclonal antibodies used in the immunoblot procedures involving the L2 strain are indicated in Figure 16. Part 1 is an autoradiogram against intact elementary bodies of the various *Chlamydia trachomatis* strains in which polyclonal anti-L2 EB was used as the antisera in the immunoblot. Parts 2 and 3 are similar except that the antisera consisted of polyclonal anti-L2 EB MOMP for Part 2 and monoclonal anti-L2 EB MOMP for Part 3. The monoclonal antisera was used in subsequent immunoblots involving L2 EBs because of its great specificity for the MOMP protein while polyclonal anti-MN EB antisera was used for those immunoblots in which MN EBs were studied.

In Figure 17 (autoradiogram for Experiment 1), it can be seen that while the proteases cleaved the L2 surface-exposed proteins into smaller fragments that still possessed an antigenic determinant, opposite results occurred with protease treatment to the MN surface exposed proteins. This effect was namely that proteolysis did occur (as was noted in the stained gel) but that any resultant fragments were so small or so unrecognizable that the anti-MN EB antisera was unable to locate and specifically bind to these fragments, thus the clearing in the lanes below the level of monomeric MOMP.

The autoradiogram for Experiment 2 is illustrated in Figure 18 and it shows an almost total clearing of the L2 lanes by the protease treatments. The MN lanes in comparison still exhibit the presence of monomeric MOMP in all but the proteinase K lanes. This would indicate that the surface-exposed proteins of the MN strain are more resistant to protease cleavage than are the corresponding
FIGURE 16: Representation of the antigenic specificities of the various antisera used in the immunoblot protocol against different strains of chlamydial elementary bodies. Section 1 is the pattern obtained when the various strains are blotted against polyclonal anti-EB antisera. Section 2 involved blotting against polyclonal anti-MOMP antisera and Section 3 was blotted against monoclonal anti-MOMP antisera.

-The arrows on the left side of the figures indicate the location to which MOMP migrates in electrophoresis (39,500 K)
FIGURE 17: Autoradiogram corresponding to proteolysis done in Experiment 1. The chlamydial elementary bodies were subjected to 1 mg/ml protease for 2 hours at 37 C, washed over a Renografin gradient, electrophoresed by PAGE, and then immunoblotted. L2 organisms were blotted against monoclonal anti-MOMP antisera while MN organisms were blotted against polyclonal anti-EB antisera.

Legend: L2 -- Chlamydia trachomatis L2 strain elementary bodies
MN -- Chlamydia psittaci MN strain elementary bodies
CN -- control (no proteases)
TR -- trypsin
CT -- chymotrypsin
TH -- thermolysin
PK -- proteinase K

Numbers on left boundary indicate molecular weights in daltons.
FIGURE 18: Autoradiogram corresponding to proteolysis done in Experiment 2. The chlamydial elementary bodies were subjected to 10 mg/ml protease for 2 hours at 37°C, washed by triple centrifugation, electrophoresed by PAGE, and then immunoblotted. L2 organisms were blotted against monoclonal anti-MOMP antisera while MN organisms were blotted against polyclonal anti-EB antisera.

LEGEND:  
L2 -- *Chlamydia trachomatis* L2 strain elementary bodies  
MN -- *Chlamydia psittaci* MN strain elementary bodies  
CN -- control (no proteases)  
TR -- trypsin  
CT -- chymotrypsin  
TH -- thermolysin  
PK -- proteinase K  
- numbers on left boundary indicate molecular weights in daltons.
polypeptides in the L2 strain's outer wall. It is interesting to note, however, that even the control lane for the L2 strain shows a marked degree of breakdown over the previous autoradiogram. This was peculiar because the control groups were treated identical in both instances save for the method of purification following proteolysis (Renografin vs. triple centrifugation).

Figure 19 shows a similar autoradiogram except that in this instance the EBs were treated with a tenfold increase in enzyme concentration for a shorter time (<1 hr, corresponding to Experiment 3). As can be seen, protease treatment to both L2 and MN produced smaller fragments that were still recognizable by the antisera as being derived from MOMP. It is noticed that treatment by thermolysin and proteinase K had a much greater effect in the L2 strain in tending to destroy any fragments containing an antigenic determinant to which the antisera was specific in association. It appears that thermolysin cleaved the surface-exposed proteins of the MN strain quite efficiently but not to the extent that immunogenic fragments were no longer detectable. Additionally, it can be seen that the L2 control sample did undergo some spontaneous breakdown of MOMP even in the absence of proteases. Since the antisera was directed specifically against MOMP in the immunoblots of treated L2 samples, the fragments appearing below monomeric MOMP in the L2 control lane are necessarily spontaneous MOMP cleavage products formed as a result of mechanical, inorganic chemical, or thermal damage due to the standard experimental protocol.

Figure 20 is the autoradiogram corresponding to the immunoblot done with Experiment 8 in which the elementary bodies were subjected
FIGURE 19: Autoradiogram corresponding to proteolysis done in Experiment 3. The chlamydial elementary bodies were subjected to 10 mg/ml protease for 1 hour at 37 C, washed by triple centrifugation, electrophoresed by PAGE, and then immunoblotted. L2 organisms were blotted against monoclonal anti-MOMP antisera while MN organisms were blotted against polyclonal anti-EB antisera.

LEGEND: L2 -- Chlamydia trachomatis L2 strain elementary bodies
MN -- Chlamydia psittaci MN strain elementary bodies
CN -- control (no proteases)
TR -- trypsin
CT -- chymotrypsin
TH -- thermolysin
PK -- proteinase K
V8 -- staphylococcal V-8 protease

- numbers on left boundary indicate molecular weights in daltons.

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FIGURE 20: Autoradiogram corresponding to proteolysis done in Experiment 8. The chlamydial elementary bodies were reduced by 10 mM DTT for 30 minutes, subjected to 10 mg/ml protease for 1 hour at 37 C, washed by triple centrifugation, electrophoresed by PAGE, and then immunoblotted. L2 organisms were blotted against monoclonal anti-MOMP antisera while MN organisms were blotted against polyclonal anti-EB antisera.

LEGEND: L2 — Chlamydia trachomatis L2 strain elementary bodies
MN — Chlamydia psittaci MN strain elementary bodies
CN — control (no proteases)
TR — trypsin
CT — chymotrypsin
TH — thermolysin
PK — proteinase K
V8 — staphylococcal V-8 protease

- numbers on left boundary indicate molecular weights in daltons.
to DTT treatment before proteolysis. As can be seen, thermolysin and proteinase K cleaved L2 surface proteins very efficiently, so much so that neither monomeric MOMP nor immunogenic fragments were no longer detectable. This indicates that DTT may be working to expose susceptible bonds within MOMP that are hidden in unreduced EBs, thus allowing for the more complete fragmentation of the major protein. Other proteases appeared to have results consistent with previous trials in cleavage efficacy against the L2 proteins as well as all treatments against MN proteins.

The autoradiogram done following the protease treatment of the D strain (Experiment 11) is given in Figure 21. Although the exposure turned out to give a broad, blurry picture in which the individual lanes and bands aren't easily distinguished, it is still possible to see that treatment with thermolysin and proteinase K again produced the most efficient protein cleavage for this strain like the others. There were few resultant fragments following thermolysin or proteinase K treatment containing any sort of antigenic determinant for which the antisera was specific in its association.

Figures 22 and 23 are the autoradiograms obtained from Experiment 13 in which L2 and MN EBs were treated with protease and then suspended in Laemmli buffer with and without 2-mercaptoethanol. As can be seen, treatment with 2-ME in both the L2 and MN strains had a profound effect upon the subsequent immunogenicity of the proteins and their specific association with the particular antisera used. In both instances, 2-ME treatment tended to allow the higher molecular weight proteins to be more completely cleaved by trypsin and chymotrypsin and this resulted in the appearance of several lower
FIGURE 21: Autoradiogram corresponding to proteolysis done in Experiment 11. The chlamydial elementary bodies were subjected to 10 mg/ml protease for 1 hour at 37 C, washed by triple centrifugation, electrophoresed by PAGE, and then immunoblotted. D organisms were blotted against polyclonal anti-EB antisera.

**LEGEND:**
- D -- *Chlamydia trachomatis* D strain elementary bodies
- CN -- control (no proteases)
- TR -- trypsin
- CT -- chymotrypsin
- TH -- thermolysin
- PK -- proteinase K

Numbers on left boundary indicate molecular weights in daltons.
FIGURE 22: Autoradiogram corresponding to proteolysis done in Experiment 13. The chlamydial elementary bodies were subjected to 10 mg/ml protease for 1 hour at 37 C, washed by triple centrifugation, suspended in Laemmli buffer with or without 2-ME, electrophoresed by PAGE, and then immunoblotted. L2 organisms were blotted against monoclonal anti-MOMP antisera.

LEGEND: L2 -- Chlamydia trachomatis L2 strain elementary bodies +2ME -- suspended in buffer with 2-mercaptoethanol -2ME -- suspended in buffer without 2-mercaptoethanol CN -- control (no proteases) TR -- trypsin CT -- chymotrypsin TH -- thermolysin PK -- proteinase K

- numbers on left boundary indicate molecular weights in daltons.
FIGURE 23: Autoradiogram corresponding to proteolysis done in Experiment 13. The chlamydial elementary bodies were subjected to 10 mg/ml protease for 1 hour at 37 C, washed by triple centrifugation, suspended in Laemmli buffer with or without 2-ME, electrophoresed by PAGE, and then immunoblotted. MN organisms were blotted against polyclonal anti-EB antisera.

![Image of autoradiogram]

**LEGEND:**
- MN: Chlamydia psittaci MN strain elementary bodies
- +2ME: Suspended in buffer with 2-mercaptoethanol
- -2ME: Suspended in buffer without 2-mercaptoethanol
- CN: Control (no proteases)
- TR: Trypsin
- CT: Chymotrypsin
- TH: Thermolysin
- PK: Proteinase K

- Numbers on left boundary indicate molecular weights in daltons.
molecular weight fragments with demonstrable antigenicity. The same effect was produced with thermolysin treatment for the MN strain only. Also, for both strains, treatment with proteinase K in the absence of 2-ME resulted in a total clearing of the lane while the presence of 2-ME saved the MOMP in its monomeric form. This is notable because 2-ME generally had the effect of allowing more of the surface-exposed proteins to be solubilized and enter into the polyacrylamide gel where they could be separated by electrophoresis (note the heavy presence of unsolubilized proteins at the top of each lane in the samples not treated with 2-ME).

Effects of Protease Treatment on Chlamydial EB Infectivity:

As described in Materials and Methods, a variety of proteases were utilized to determine their effect upon the structure and subsequent resultant infectivity of several strains of chlamydial organisms in the elementary body form. These were the L2 and D strains of Chlamydia trachomatis and the MN strain of C. psittaci.

The inclusion-forming units (as a basis for quantification of infectivity) were determined in several separate, independently run experiments for each of these strains and proteases. The original infectivities of each of the EB stock solutions prior to protease treatment were:

- L2 strain = 8.00 x 10**8 IFU/ml
- D strain = 8.60 x 10**6 IFU/ml
- MN strain = 4.00 x 10**8 IFU/ml

A complete listing of all infectivity results, including determinations for individual trials and an overall average for each strain, is given in Tables 4, 5, and 6. Except for the second trial
TABLE 4: Infectivity determination results for protease treated elementary bodies of the L2 strain of *Chlamydia trachomatis*.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>TRYPsin</th>
<th>CHYMOTRYPSIN</th>
<th>THERMOLYSIN</th>
<th>PROTEINASE K</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRAIN L2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRIAL #1: IFU/ml.a</td>
<td>9.1 X 10**6</td>
<td>8.62 X 10**6</td>
<td>1.45 X 10**7</td>
<td>1.18 X 10**7</td>
<td>------------</td>
</tr>
<tr>
<td>% controlb</td>
<td>100</td>
<td>94.6</td>
<td>159.2</td>
<td>129.5</td>
<td>-----</td>
</tr>
<tr>
<td>TRIAL #2: IFU/ml.</td>
<td>8.20 X 10**5</td>
<td>1.31 X 10**7</td>
<td>5.80 X 10**6</td>
<td>6.30 X 10**6</td>
<td>2.40 X 10**3</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>1597.6</td>
<td>707.3</td>
<td>768.3</td>
<td>0.29</td>
</tr>
<tr>
<td>TRIAL #3: IFU/ml.</td>
<td>1.02 X 10**7</td>
<td>3.80 X 10**6</td>
<td>8.90 X 10**6</td>
<td>8.43 X 10**6</td>
<td>2.48 X 10**5</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>37</td>
<td>87.3</td>
<td>82.6</td>
<td>2.4</td>
</tr>
<tr>
<td>MEAN: IFU/ml.</td>
<td>6.71 X 10**6</td>
<td>8.51 X 10**6</td>
<td>9.73 X 10**6</td>
<td>8.84 X 10**6</td>
<td>8.35 X 10**4</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>126.8</td>
<td>145.0</td>
<td>131.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

a Inclusion Forming Units (IFUs) were determined as described in Materials and Methods.
b % control = IFU per ml / IFU per ml for control group of the same trial.
TABLE 5: Infectivity determination results for protease treated elementary bodies of the MN strain of *Chlamydia psittaci*.

<table>
<thead>
<tr>
<th>STRAIN MN</th>
<th>CONTROL</th>
<th>TRYPSIN</th>
<th>CHYMOTRYPSIN</th>
<th>THERMOLYSIN</th>
<th>PROTEINASE K</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIAL #1:</td>
<td>IFU/ml</td>
<td>1.20 X 10**7</td>
<td>5.33 X 10**6</td>
<td>2.55 X 10**6</td>
<td>7.19 X 10**6</td>
</tr>
<tr>
<td>% control</td>
<td></td>
<td>100</td>
<td>44.4</td>
<td>21.3</td>
<td>59.9</td>
</tr>
<tr>
<td>TRIAL #2:</td>
<td>IFU/ml</td>
<td>6.50 X 10**6</td>
<td>6.70 X 10**4</td>
<td>6.30 X 10**4</td>
<td>4.60 X 10**4</td>
</tr>
<tr>
<td>% control</td>
<td></td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>TRIAL #3:</td>
<td>IFU/ml</td>
<td>1.25 X 10**7</td>
<td>8.92 X 10**6</td>
<td>1.25 X 10**7</td>
<td>1.02 X 10**7</td>
</tr>
<tr>
<td>% control</td>
<td></td>
<td>100</td>
<td>71.4</td>
<td>100</td>
<td>81.6</td>
</tr>
<tr>
<td>TRIAL #4:</td>
<td>IFU/ml</td>
<td>9.50 X 10**4</td>
<td>5.20 X 10**4</td>
<td>3.20 X 10**4</td>
<td>4.30 X 10**4</td>
</tr>
<tr>
<td>% control</td>
<td></td>
<td>100</td>
<td>54.7</td>
<td>33.7</td>
<td>45.3</td>
</tr>
<tr>
<td>MEAN:</td>
<td>IFU/ml</td>
<td>7.77 X 10**6</td>
<td>3.59 X 10**6</td>
<td>3.79 X 10**6</td>
<td>4.37 X 10**6</td>
</tr>
<tr>
<td>% control</td>
<td></td>
<td>100</td>
<td>46.2</td>
<td>48.8</td>
<td>56.2</td>
</tr>
</tbody>
</table>

---

*a* Inclusion Forming Units (IFUs) were determined as described in Materials and Methods.

*b* % control = IFU per ml / IFU per ml for control group of the same trial.
TABLE 6: Infectivity determination results for protease treated elementary bodies of the D strain of Chlamydia trachomatis.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>TRYPSIN</th>
<th>CHYMOTRYPSIN</th>
<th>THERMOLYSIN</th>
<th>PROTEINASE K</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRAIN D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRIAL #1: IFU/ml</td>
<td>7.94 X 10^6</td>
<td>1.12 X 10^4</td>
<td>9.42 X 10^6</td>
<td>1.62 X 10^7</td>
<td>5.21 X 10^6</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>0.2</td>
<td>118.6</td>
<td>204.0</td>
<td>65.6</td>
</tr>
<tr>
<td>TRIAL #2: IFU/ml</td>
<td>1.35 X 10^6</td>
<td>----------</td>
<td>7.94 X 10^5</td>
<td>1.07 X 10^6</td>
<td>----------</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>----------</td>
<td>58.8</td>
<td>79.3</td>
<td>---</td>
</tr>
<tr>
<td>MEAN: IFU/ml</td>
<td>4.65 X 10^6</td>
<td>----------</td>
<td>5.11 X 10^6</td>
<td>8.64 X 10^6</td>
<td>----------</td>
</tr>
<tr>
<td>% control</td>
<td>100</td>
<td>----------</td>
<td>109.0</td>
<td>186.7</td>
<td>---</td>
</tr>
</tbody>
</table>

- Inclusion Forming Units (IFUs) were determined as described in Materials and Methods using HeLa cell hosts.
- % control = IFU per ml / IFU per ml for control group of the same trial.
- Infectivity determination was as described except that L-929 cells were used as host cells in place of the HeLa cells.
on the D strain in which L-929 cells were used, all other infectivity determinations were conducted on HeLa cell monolayers.

As can be seen, the individual proteases had different effects on infectivity from strain to strain. In the L2 strain of Chlamydia trachomatis, trypsin, chymotrypsin, and thermolysin generally enhanced infectivity while proteinase K constantly lowered the EBs' ability to infect the epithelial cells. In the D strain, treatment with trypsin or proteinase K alone tended to lower infectivity while the opposite effect was observed for chymotrypsin and thermolysin. Finally, in the MN strain, all four enzyme treatments produced a marked decrease in noticeable infectivity (except for one instance, that being chymotrypsin treatment in the third trial in which infectivity was not altered). In all cases, the resultant infectivity of the control groups for each trial and strain were lower than those of the original stock solutions.

Figures 24-28 are photographs of various inclusions formed by control L2 and MN EBs in the HeLa cell monolayers during particular infectivity determination studies. The inclusions (bright spots in the dark background) were visualized as indicated in Materials and Methods through an indirect immunofluorescence assay. These photos are representative of the general appearance of both L2 and MN inclusions formed by control, as well as proteolysed, EBs throughout the experiments. Such inclusions formed the basis for the determination of IFUs referred to above.

Finally, Table 7 contains the results from the organism binding assay in which radioactively labeled L2 and MN EBs (control and trypsinized) were allowed to associate with HeLa cells as explained.
FIGURE 24: Photograph of inclusions formed by control L2 elementary bodies in HeLa cells. Inclusions were visualized by indirect immunofluorescence with fluorescein conjugated antisera. Organisms were diluted to $10^{12}$ before infectivity determination. This photograph was taken with a 60 second exposure at a 16X magnification.
FIGURE 25: Photograph of inclusions formed by control L2 elementary bodies in HeLa cells. Inclusions were visualized by indirect immunofluorescence with fluorescein conjugated antisera. Organisms were diluted to $10^{-2}$ before infectivity determination. This photograph was taken with a 60 second exposure at a 40X magnification.
FIGURE 26: Photograph of inclusions formed by control L2 elementary bodies in HeLa cells. Inclusions were visualized by indirect immunofluorescence with fluorescein conjugated antisera. Organisms were diluted to 10**-2 before infectivity determination. This photograph was taken with a 60 second exposure at a 100X magnification.
FIGURE 27: Photograph of inclusions formed by control MN elementary bodies in HeLa cells. Inclusions were visualized by indirect immunofluorescence with fluorescein conjugated antisera. Organisms were diluted to $10^{-2}$ before infectivity determination. This photograph was taken with a 60 second exposure at a 16X magnification.
FIGURE 28: Photograph of inclusions formed by control MN elementary bodies in HeLa cells. Inclusions were visualized by indirect immunofluorescence with fluorescin conjugated antisera. Organisms were diluted to $10^{-2}$ before infectivity determination. This photograph was taken with a 60 second exposure at a 40X magnification.
TABLE 7: Binding of control and trypsinized 14C-labeled L2 and MN chlamydial elementary bodies to HeLa cells expressed in counts per minute (CPM).\(^a\)

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>L2 CONTROL</th>
<th>WITHOUT</th>
<th>HEPARIN</th>
<th>MEAN CPM</th>
<th>S. ERROR</th>
<th>PERCENT BOUND</th>
<th>MEAN PROTEIN</th>
<th>S. ERROR</th>
<th>PERCENT PROTEIN</th>
<th>ADJUSTED CPM</th>
<th>ADJUSTED PERCENT BOUND</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1663.83</td>
<td>60.97</td>
<td>100</td>
<td>480.57</td>
<td>42</td>
<td>100</td>
<td>1663.83</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CONTROL</td>
<td>374.67</td>
<td>5.3</td>
<td>22.52</td>
<td>448.65</td>
<td>39.67</td>
<td>93.36</td>
<td>401.32</td>
<td>24.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRYPsin</td>
<td>701</td>
<td>84.19</td>
<td>42.13</td>
<td>421.1</td>
<td>67.83</td>
<td>87.63</td>
<td>799.95</td>
<td>48.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRYPsin</td>
<td>214.67</td>
<td>12.29</td>
<td>12.9</td>
<td>441.54</td>
<td>26.47</td>
<td>91.88</td>
<td>233.64</td>
<td>14.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STRAIN</td>
<td>MN CONTROL</td>
<td>WITHOUT</td>
<td>HEPARIN</td>
<td>84.5</td>
<td>3.04</td>
<td>100</td>
<td>501.78</td>
<td>103.58</td>
<td>100</td>
<td>84.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CONTROL</td>
<td>61.67</td>
<td>3.33</td>
<td>72.98</td>
<td>464.83</td>
<td>21.09</td>
<td>92.64</td>
<td>66.57</td>
<td>78.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRYPsin</td>
<td>97.17</td>
<td>9.22</td>
<td>114.99</td>
<td>533.51</td>
<td>54.39</td>
<td>106.33</td>
<td>91.39</td>
<td>108.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRYPsin</td>
<td>74</td>
<td>16.09</td>
<td>87.57</td>
<td>471.01</td>
<td>52.52</td>
<td>93.88</td>
<td>78.82</td>
<td>93.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) associated counts per minute determined by liquid scintillation spectroscopy.
\(^b\) mean CPM was counts taken directly from the spectroscope reading.
\(^c\) percent bound = mean CPM / mean CPM for control without heparin.
\(^d\) micrograms of protein determined by the Lowry method.
\(^e\) adjusted CPM = mean CPM / percent protein (decimal form).
\(^f\) adjusted percent bound = adjusted CPM / adjusted CPM for control without heparin.
in the Materials and Methods section. The purpose of this experiment was to determine the exact cause of the respective increase or decrease in infectivity for the protease treated L2 and MN EBs. Was it due to an increased (decreased) ability of the EBs to associate and bind with epithelial cells or was it due to an increase (decrease) in the rates of internalization of the EBs by the epithelial cells once they had become bound? As can be seen from the table, trypsinized L2 elementary bodies demonstrated a lowered ability to bind to epithelial cells as compared to the corresponding control samples. Also, trypsinized MN EBs showed a greater ability to bind to HeLa epithelial cells than did the corresponding control samples. These observations appeared to be in opposition to the results obtained from the infectivity determination studies. Namely, the strain with increased infectivity showed a lowered binding ability and vice versa.

Effect of Chlamydial Protein Reduction by DTT on Proteolysis Results:

Dithiothreitol (DTT) is a chemical agent that reduces sulfhydryl groups (-SH). It is believed that the various proteases lack a higher efficiency in cleaving chlamydial surface exposed proteins because of the presence of numerous disulfide bonds (S-S) within the polypeptides themselves.

The L2 strain of Chlamydia trachomatis and the MN strain of C. psittaci were treated with 10 mM DTT prior to the normal protease battery. A CBB stained SDS-PAGE of the L2 and MN strains is pictured in Figure 29. If this figure is compared to the prior PAGE figures lacking the additional DTT treatment, additional cleavage of MOMP is
FIGURE 29: CBB stain of PAGE for Experiment 8. Intact elementary bodies of *Chlamydia trachomatis* L2 strain and *C. psittaci* MN strain were subjected to 10 mg/ml protease for 1 hour at 37 C after being reduced by 10 mM DTT for 30 minutes. The samples were then washed by triple centrifugation before PAGE.

LEGEND:  
L2 — *Chlamydia trachomatis* L2 strain elementary bodies  
MN — *Chlamydia psittaci* MN strain elementary bodies  
CN — control (no protease)  
TR — trypsin  
CT — chymotrypsin  
TH — thermolysin  
PK — proteinase K  
- numbers on left boundary indicate molecular weights in daltons.
apparent. The control tracks serve as a good index for the amount of monomeric MOMP normally present. Both strains show an increased degree of cleavage following reduction, with MN still being much more resistant to cleavage than the L2 strain. The lower molecular weight regions of the gels show additional fragments because of the reduction by DTT which split the sulfhydryl groups and allowed for more complete dissociation of the polypeptides.

The L2 strain was further tested with various combinations of trypsin, with or without 2-ME, and DTT; the idea being that DTT would increase the amount of disulfide bond reduction begun by trypsinization of MOMP. Figures 30 and 31 are photographs of CBB and AgNO(3) stained gels corresponding to Experiment 14. Combinations of DTT and trypsin show increased reduction and cleavage efficiency over either treatment separately. This is apparent by observing MOMP present at its normal 39.5K location in the DTT lanes and its absence in the combination and trypsin lanes. Also, the combination lanes show a greater aggregation of protein concentrated at the bottom of the lanes than do the trypsin lanes.

The combination of DTT and trypsin is thought to function by having each reduce some bonds that the other can’t and thereby further opening up the protein for increased cleavage. As a result, no major differences are seen between the particular sequence in which DTT and trypsin are applied.

The lanes that were run in the absence of 2-ME show a reduced degree of cleavage. This is obviously exemplified by the predominant fragment apparent at 32,500 K in Figure 31. Thus, the role 2-ME plays in reduction is also a significant factor in extent of
FIGURE 30: CBB stain of PAGE for Experiment 14. Intact elementary bodies of *Chlamydia trachomatis* L2 strain were subjected to various combinations of 10 mg/ml trypsin and 10 mM DTT for 1.5 hours at 37 °C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer with and without 2-ME.

LEGEND: L2 -- *Chlamydia trachomatis* L2 strain elementary bodies
+2ME -- suspended in buffer with 2-mercaptoethanol
-2ME -- suspended in buffer without 2-mercaptoethanol

CN -- control (no protease or DTT)
TR -- trypsin treatment alone
DT -- DTT treatment alone
T/D -- trypsin treatment followed by reduction with DTT
D/T -- DTT treatment followed by proteolysis with trypsin

- numbers on left boundary indicate molecular weights in daltons.
FIGURE 31: AgNO₃ (silver) stain of PAGE for Experiment 14.
Intact elementary bodies of Chlamydia trachomatis L2 strain were subjected to various combinations of 10 mg/ml trypsin and 10 mM DTT for 1.5 hours at 37°C and then washed by triple centrifugation before PAGE. Final samples were suspended in Laemmli buffer with and without 2-ME.

LEGEND:  L2 -- Chlamydia trachomatis L2 strain elementary bodies  
+2ME -- suspended in buffer with 2-mercaptoethanol  
-2ME -- suspended in buffer without 2-mercaptoethanol  
CN -- control (no protease or DTT)  
TR -- trypsin treatment alone  
DT -- DTT treatment alone  
T/D -- trypsin treatment followed by reduction with DTT  
D/T -- DTT treatment followed by proteolysis with trypsin  
- numbers on left boundary indicate molecular weights in daltons.  

79
proteolysis.

Protein Determination:

As stated in the Materials and Methods section, the chlamydial samples used in experimentation were obtained from stock cultures prepared at the Rocky Mountain Laboratories. This necessitated analyzing the chlamydial samples for protein concentration so that comparable quantities of L2, D, and MN protein was utilized in the various protease experiments.

The results obtained by standard protein concentration determination methods as described previously are indicated in Figure 32. A "best fit line" was determined by the least squares method and the results show that the L2 strain stock had a protein concentration of 0.606 ug/ml while the MN strain stock had a concentration of 0.401 ug/ml. These values were taken into consideration when determining the amount of the various strain stocks to be used for particular protease experiments. This allowed for similar protein amounts to be used between the various strains so that the results of other experiments would have a basis for comparison purposes.
FIGURE 32: Graph of least squares line for chlamydial stock protein concentration determination (chlamydial data points on standard Bio-Rad protein concentration curve).

<table>
<thead>
<tr>
<th>Bio-Rad protein concentration (ug/ml)</th>
<th>Optical density (595 nm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>.1</td>
</tr>
<tr>
<td>0.2</td>
<td>.2</td>
</tr>
<tr>
<td>0.3</td>
<td>.3</td>
</tr>
<tr>
<td>0.4</td>
<td>.4</td>
</tr>
<tr>
<td>0.5</td>
<td>.5</td>
</tr>
<tr>
<td>0.6</td>
<td>.6</td>
</tr>
<tr>
<td>0.7</td>
<td>.7</td>
</tr>
<tr>
<td>0.8</td>
<td>.8</td>
</tr>
<tr>
<td>0.9</td>
<td>.9</td>
</tr>
</tbody>
</table>

Average protein concentrations: L2 strain = 0.606 ug/ml  
MN strain = 0.401 ug/ml
DISCUSSION

It is suggested by the data we have obtained that chlamydial EBs are susceptible to cleavage by proteolytic agents and that the surface-exposed proteins are the components of the outer wall that undergo cleavage, with the most visible alteration occurring in the MOMP protein (39,500 K). The studies done demonstrate the fact that the MN strain of C. psittaci is much more resistant to cleavage than the L2 strain of C. trachomatis. This increased resistance of the MN surface-exposed proteins is attributed to the presence of a higher degree of cross linkages, most likely disulfide bonds.

Of the proteases used, proteinase K consistently served as the strongest cleaving agent, drastically reducing the surface-exposed proteins to lower molecular weight fragments, many of which were not detectable by standard gel staining or immunoblotting techniques. Thermolysin, trypsin, and chymotrypsin followed respectively in cleavage potential.

Some of the cleaved fragments are obviously components of the original MOMP protein because they react with anti-MOMP antibodies in immunoblotting protocols. As was the case in PAGE procedures, the MN strain was much more resistant to cleavage and MOMP was often found in the monomeric state as opposed to the multimeric L2 MOMP.

A seemingly contradictory discovery was made at this point. Infectivity studies show that the highly cleaved L2 strain actually had an increased infectivity potential after proteolysis as compared to the controls. Byrne and Moulder also saw an increase in specific activity (infectivity) after protease treatment (10). Conversely,
the MN strain which was most resistant to cleavage in its surface-exposed proteins, showed a decreased infectivity level in comparison to the uncleaved control group.

There are several hypothesized, albeit unproven, explanations for this observation. The results tend to rule out the role of MOMP and other surface-exposed proteins in initial chlamydial-host interactions, and thus these proteins don’t appear to be the prime agents involved in infectivity. It is possible, however, that protease resistant surface-exposed proteins play a part in the host-bacterial interaction. Another hypothetical suggestion speaks to the fact that proteolytic cleavage by proteases may be necessary for infectivity in that it may work to expose active sites needed for association or internalization by the L2 strain. The MN strain either requires a stronger proteolytic agent to expose the active sights or it may function in infectivity through an entirely different manner. Since the MN strain is resistant to cleavage structurally, although proteolysis decreases infectivity, the surface-exposed proteins, including MOMP, may play a more major role in infectivity than they do in the L2 strain.

Our studies also address the issue that disulfide bonds are of considerable importance for the stability of the surface-exposed proteins. When these disulfides are specifically broken with dithiothreitol (DTT), an increased susceptibility to the normal battery of proteases is observed. It is commonly believed that the disulfides serve as a major structural retention factor and are also important in the differentiation of the resistant, extracellular EB to the metabolically active, intracellular RB. The EB is much more
dense and compact and this may impart the greater resistance and infectivity. These EB functions are greatly facilitated by the predominance of the disulfide linkages.

Thus, it seems that the MN strain and the L2 strains differ in an important characteristic, namely method of infectivity, that is as yet to be proven or understood. They show a high degree of relatedness, however, in that their life cycles are identical and they have similar structural and functional properties.

The fact that they have different susceptibilities to the battery of proteases and that their infectivities are affected in different directions by protease treatment refutes total compatability between the two strains (and species). The mode of the surface-exposed proteins in the infection process also undoubtedly varies significantly from one strain to the other. Our studies tend to indicate that the MN strain relies more heavily of the naturally exposed surface-protein determinants for infectivity as cleavage with the proteases is shown to decrease infectivity drastically. Converely, the L2 strain appears to depend less extensively upon the naturally exposed protein regions and more so upon those segments exposed by the protease treatments as supported by their increased infectivity following proteolysis.

Further studies into the similarities and differences in the structure, composition, and activity of the two species will inevitably make these original findings and their significance more understandable and clear. Continuing studies will lead to the discovery of a point in the chlamydial life cycle where specific treatments can be most efficiently applied to inhibit the growth
and/or infectious nature of these bacteria. It will be interesting to note what the final outcome is of our discovery concerning the inverse relationship, in both the L2 and MN strains, between extent of proteolysis to the surface-exposed proteins and subsequent infectivity potential of the elementary body. The fact that these relationships are in different directions in the two species may lead to some interesting discoveries regarding methods of association and internalization of the EBs in initiation of infectivity in eucaryotic cells.


