JOURNAL OF BACTERIOLOGY, July 1982, p. 420–428
0021-9193/82/070420-09$02.00/0

Vol. 151, No. 1

Chlamydia trachomatis Has Penicillin-Binding Proteins but Not Detectable Muramic Acid

ALAN G. BARBOUR,* KEN-ICHI AMANO, TED HACKSTADT, LINDA PERRY, AND HARLAN D. CALDWELL

National Institute of Allergy and Infectious Diseases, Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, Hamilton, Montana 59840

Received 21 December 1981/Accepted 17 March 1982

Chlamydia trachomatis LGV-434 was grown in HeLa 229 cells. Benzylpenicillin completely inhibited the formation of infectious elementary bodies (EBs) at a concentration of 19 pmol/ml or higher and produced abnormally large reticulate bodies (RBs) in the inclusions at 30 pmol/ml or higher. The possible targets for penicillin in *C. trachomatis* were three penicillin-binding proteins (PBPs) which were identified in the Sarkosyl-soluble fractions of both RBs and EBs. The apparent subunit molecular weights were 88,000 (PBP 1), 61,000 (PBP 2), and 36,000 (PBP 3). The 50% binding concentrations of [^{3}H]penicillin for PBPs 1 to 3 in EBs and RBs were between 7 and 70 pmol/ml. Such high susceptibility to penicillin was shown by an organism that did not have detectable muramic acid (<0.02% by weight) in preparations of either whole cells or sodium dodecyl sulfate-insoluble residues.

Chlamydiae are obligate intracellular bacteria that have a genome one-eighth that of *Escherichia coli* and three times that of bacteriophage T4 (22). Chlamydiae multiply in vacuoles in the cytoplasm of host cells by a unique developmental cycle characterized by change of the infectious, but nonreplicative, elementary body (EB) to the replicative, but noninfectious, reticulate body (RB) (33). After initiating the infection, the EB converts to the RB which then multiplies by binary fission for approximately 18 to 24 h. At that time, they reorganize without another division into EBs. The smaller EB has a cell envelope that is more rigid and more stable extracellularly than that of the larger RB (33). Both developmental forms have, like gram-negative bacteria, an inner and outer membrane. The nature of the space between the two membranes, which in gram-negative bacteria is occupied by peptidoglycan, has not been completely defined. Manire and Matsumoto found a regularly arrayed layer in EBs, but not in RBs (25, 27, 29). However, the reports of Garrett et al. (13) and Manire and Tamura (26, 39) of an absence of muramic acid in these organisms suggest that this or any other layer is not murein.

This apparent major difference from eubacteria in cell wall constituents is not accompanied by an absence in chlamydiae of susceptibility to penicillin. Although chlamydial infections are not generally treated with beta-lactam antibiotics, studies of both laboratory animals and tissue culture systems have shown that chlamydiae are inhibited in their growth by penicillin (10, 17, 19, 24, 28, 35, 40, 41, 43). The mechanisms of these actions are not known, however. In addition, most of the detailed studies of the effect of penicillin on chlamydiae have used *Chlamydia psittaci*. *C. psittaci* resembles *C. trachomatis* in morphology and its developmental cycle, but these two species share only 11% DNA homology (23, 44).

Penicillin seemed to us, therefore, to be a useful tool for studying both the developmental cycle of *Chlamydia* spp. and the nature of their unique cell envelope. Our studies to date of *C. trachomatis* have shown that EB and RB cells of this strain have three proteins that bind penicillin. RB cells stop dividing and do not convert to EBs in the presence of very low concentrations of penicillin. We have, in addition, confirmed that muramic acid is undetectable in these organisms.

MATERIALS AND METHODS

Organism and growth conditions. *C. trachomatis* strain LGV-434 (L2 serotype) was used. Chlamydiae were grown in monolayers of HeLa 229 cells in Eagle minimal essential salts medium supplemented with 10% fetal calf serum (MEM-10) as described previously (7).

Purification of chlamydiae. EBs were purified by centrifugation in discontinuous Renografin gradients (7). RBs were obtained by collecting the band at the interface of the 40%–44% discontinuous Renografin gradient used for EB purification. This material was mixed with an equal volume of 10 mM sodium phosphate–250 mM surcrose–5 mM L-glutamate–150 mM sodium chloride buffer (SPGS). This suspension was

centrifuged at 30,000 × *g* for 20 min at 4°C. The pellet was resuspended in SPGS and layered over a 30-ml 10 to 60% linear Renografin gradient. Gradients were centrifuged at 43,000 × *g* for 1 h. RBs, which banded in the 1.17- to 1.19-g/ml portion of the gradient, were collected and diluted with an equal volume of SPGS. The RBs were centrifuged at 30,000 × *g* for 20 min, resuspended in SPGS, and adjusted to give a protein concentration determined by the Bradford method (4) of approximately 0.5 mg/ml. The purity of EB and RB preparations was assessed by electron microscopy as described previously (7).

Inclusion-forming units (IFUs) were determined for EB preparations by the method of Furness et al. (12). Briefly, HeLa cells grown in Lab-Tek tissue culture chamber slides (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.) were infected in duplicate with 10-fold dilutions of chlamydial suspensions. The number of IFUs per milliliter was determined by counting inclusions in Giemsa-stained preparations of the slide. Dilutions resulting in 10 to 20 inclusions per field at a magnification of 450× were counted. A minimum of 30 microscopic fields for each duplicate dilution was used for calculating IFUs.

Chemicals. [^{3}H]benzylpenicillin, ethylpiperidinium salt ([^{3}H]penicillin), with a specific activity of 31 Ci/mmol, was the generous gift of E. Stapley and P. Cassidy (Merck & Co., Inc., Rahway, N.J.). [^{14}C]benzylpenicillin (30 mCi/mmol), [*U*-^{14}C]sucrose (435 mCi/mmol), and (hydroxy[^{14}C]methyl)inulin (5 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. Tritiated water (8,100 Ci/g) was purchased from New England Nuclear Corp., Boston, Mass. The potassium salt of benzylpenicillin (penicillin) was obtained from Eli Lilly & Co., Indianapolis, Ind. A picomole of benzylpenicillin is 0.0004 μg. Muramic acid and diaminopimelic acid (DAP) were purchased from Sigma Chemical Co., St. Louis, Mo.

HeLa cell volume determination. We calculated HeLa cell volumes from the distribution of inulin, sucrose, and water using a modification of the method of Stock et al. (38). HeLa cells grown under the conditions described above were suspended in Hanks balanced salt solution, pH 7.4, over a concentration range of 3.5×10^6 to 3.5×10^7 cells per ml. The suspending solution contained tritiated water (30 μCi) and either [*U*-^{14}C]sucrose (2.5 μM) or (hydroxy[^{14}C]methyl)inulin (25 μM). After incubation of the cells for 15 min at 21°C, the cells were pelleted in a Beckman microfuge. Fifty microliters of the supernatant was taken for liquid scintillation counting (Beckman LS-9000). The remainder of the supernatant was removed, and the pellet was transferred to a scintillation vial for counting. The volume occupied by the labeled solute in the pellet (Vs) in milliliters was obtained from the formula Vs = (0.05) (Cp/Cs), where Cs was the disintegrations per minute in the sample of supernatant and Cp was the disintegrations per minute associated with the pellet. The estimated intracellular volume was the difference between the volumes (Vs) occupied by water and by sucrose or inulin.

Uptake of labeled penicillin by HeLa cells. Subconfluent monolayers of cells, which had been planted 24 h previously, were grown in MEM-10 and 50 μM [^{14}C]benzylpenicillin in six-well plastic culture plates (Linbro Division, Flow Laboratories). After 5 or 23 h, the medium was removed by aspiration, and the cells were rapidly washed with several volumes of 140 mM NaCl. The cells were solubilized with 0.1 N NaOH at 56°C for 30 min. After neutralizing the solution with 1.0 N acetic acid, radioactivities of the samples were measured by liquid scintillation counting. Determinations were in triplicate. The protein concentrations of the solubilized cells were determined by the Bradford method (4). Comparison of these values with a standard curve of protein concentration versus cell count gave the estimated cell count for each sample.

Penicillin susceptibility. HeLa cells were grown in culture chamber slides without antibiotics. Cells were planted at 7×10^4 cells per chamber. After formation of the monolayer (approximately 6 h), the medium was removed and replaced with medium containing various concentrations of penicillin. After 18 h of incubation at 37°C, the medium was removed, and the cells were infected with 0.2 ml of EB inoculum containing approximately 1 IFU per HeLa cell. After 90 min of incubation at 37°C, the inoculum was removed, and the cells were washed twice with Hanks balanced salt solution. Monolayers were refed with MEM-10 containing the same concentration of penicillin. After 42 h of incubation, monolayers were fixed with absolute methanol for 10 min and Giemsa stained. Duplicate cultures were fixed with osmium tetroxide (OsO_4) fumes by placing monolayers 2 cm above a 1% OsO_4 solution for 20 min. Cultures were examined by phase-contrast microscopy (Zeiss photomicroscope 3). Fifty cellular inclusions were examined at each penicillin concentration. The minimum inhibitory concentration (MIC) by morphological criteria was defined as the lowest concentration at which all of the inclusions examined appeared either empty or contained only very few large particles.

We also estimated the MIC by the criteria of inhibition of infectivity. Cells were incubated with penicillin as described above except that 75-cm plastic flasks (Corning Glass Works, Corning, N.Y.) were used in place of chamber slides. Chlamydiae were added at an IFU-to-cell ratio of 1 and absorbed to cells for 90 min at 37°C. Monolayers were washed twice with Hanks balanced salt solution and fed with MEM-10 containing the same concentration of penicillin. After 48 h of incubation, the cultures were harvested separately, and the IFUs recovered from each culture were determined. The MIC by the infectivity assay was the lowest concentration of antibiotic that resulted in 100% inhibition of infectivity, i.e., no detectable IFUs.

Binding of labeled penicillin to EBs and RBs. The binding method was a modification of that used by Spratt (37). Samples (150 μl each) of the suspensions of purified EBs or RBs in SPGS at protein concentrations of approximately 0.5 mg/ml were mixed with 15 μl of labeled penicillin in serial twofold dilutions. After 20 min of incubation of the whole cells with labeled penicillin at 35°C, 5 μl of unlabeled penicillin (100 mg/ml) was added, followed by 15 μl of 20% sodium lauroyl sarcosinate (Sarkosyl)–1.5 mM EDTA. The mixture was incubated at 35°C for 40 min. The Sarkosyl-insoluble fraction was removed by centrifugation at 128,000 × *g* for 30 min in a Beckman airfuge. A 100-μl portion of the supernatant, which contained predominantly inner membrane proteins (7), was added to 50 μl of gel sample buffer. To study binding to the outer membrane (7), the Sarkosyl-insoluble fraction

was reextracted with 150 μl of 2% Sarkosyl–1.5 mM EDTA, pelleted by centrifugation at 128,000 × *g* for 30 min, and suspended in 100 μl of distilled water. This suspension was added to 50 μl of gel sample buffer. To examine binding of [^{3}H]penicillin to membranes of lysed chlamydiae, we added 15 μl of 10% Triton X-100 to 150 μl of RB suspension. This was incubated at 35°C for 15 min before addition of [^{3}H]penicillin and extraction with Sarkosyl.

Gel electrophoresis and detection of PBPs. Penicillin-binding proteins (PBPs) were identified in the Sarkosyl-soluble fraction of whole and lysed cells by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography using methods described previously (2). The exposure time for [^{3}H]penicillin fluorograms on presensitized Kodak X-Omat AR film at −76°C was 14 days. The amount of binding of [^{3}H]penicillin to PBPs was estimated by examining the fluorographic images with a Joyce-Loebl microdensitometer. Integration of peaks was performed with the aid of a Zeiss MOP 3 image analyzer. The criteria for saturation of, and 50% binding to, PBPs were those previously reported (2).

Amino acid and muramic acid analysis. The amino acid and muramic acid contents of whole EBs and RBs were compared with those of *Neisseria gonorrhoeae* strain JS1 and *E. coli* strain K802 (obtained from L. W. Mayer, Rocky Mountain Laboratories, Hamilton, Mont.). Purified EBs and RBs were suspended in 0.5% Formalin overnight, pelleted, washed with water, and lyophilized. The *N. gonorrhoeae* and *E. coli* strains were grown to late log phase in GCH broth (2) and Mueller-Hinton broth, respectively. The cells were harvested (8,000 × *g* for 20 min) and resuspended in phosphate-buffered saline with 0.5% Formalin overnight. The fixed cells were washed with water and lyophilized. Each sample of fixed whole cells was hydrolyzed in 6 N HCl at 100°C for 15 h in sealed tubes. The samples were dried with a rotary evaporator and applied to a Beckman 118CL amino acid analyzer. Amino acid analysis was also performed on the SDS-insoluble residue of cells of the three organisms. Formalinized cells were boiled in 2% SDS for 5 min and then centrifuged at 100,000 × *g* for 1 h. The insoluble pellet was reextracted with boiling 2% SDS for 5 min and recentrifuged. The residue was dried, weighed, and prepared for amino acid analysis.

RESULTS

Purification of chlamydiae. RBs and EBs were separated and purified by density gradient centrifugation. In Fig. 1 are electron photomicrographs of thin sections of the purified RBs and EBs. The RB cells are characterized by a diffuse matrix and absence of a dense core (nucleoid). EB cells are smaller and electron dense.

HeLa cell volume determination and uptake of labeled penicillin. Interpretation of in vitro susceptibility tests performed in tissue culture required prior estimation of the concentration of penicillin that the chlamydiae were exposed to inside the HeLa cells. Consequently, we determined the mean cell volume of HeLa cells, the cell counts in the samples, and the amount of [^{14}C]benzylpenicillin associated with the cell fraction. The cell volume was calculated using the distributions of labeled sucrose, inulin, and water in the cells. The mean cell volume (±standard error) of HeLa 229 cells in MEM-10 by this method was 3,550 (±161) μm^3.

Preliminary studies had shown a linear relationship between the amount of labeled penicillin added to a HeLa cell culture and the amount associated with the cells over a concentration range of 0.5 to 50 nmol/ml. Addition of unlabeled penicillin (500 nmol/ml) to each sample did not alter this relationship. When we incubated HeLa cells with 50 nmol of [^{14}C]benzylpenicillin per ml (1.5 μCi/ml), the radioactivity associated with the washed cells was 1,424 (±30) cpm at 0 h (i.e., immediately washing the cells after the addition of penicillin), 2,375 (±168) cpm at 5 h, and 4,764 (±223) at 23 h. These values corresponded to 22, 57, and 93 pmol of labeled penicillin, respectively. The mean total cell volumes were 1.9, 2.1, and 1.5 μl for the 0-, 5-, and 23-h samples. We assumed that the zero time value did not represent intracellular penicillin and, therefore, subtracted this "background" concentration from the 5- and 23-h values. The corrected intracellular concentrations were 15 and 52 nmol/ml, respectively.

Penicillin susceptibility. In Fig. 2 are light photomicrographs of Giemsa-stained preparations (Fig. 2A and B) and phase-contrast photomicrographs of osmium tetroxide-fixed preparations (Fig. 2C and D) of infected HeLa cells. Frames A and C of Fig. 2 show untreated cells. The intracellular vacuoles contain numerous small, dense EBs. In contrast, infected HeLa cells exposed to 30 pmol of penicillin per ml for 42 h have only vacuoles that appear either empty or to contain only a few very large structures (Fig. 2B and D). The MIC by morphological criteria was 30 pmol/ml. At 13 pmol/ml, a partial effect was seen; at least 20% of the vacuoles were abnormal.

Figure 3A shows the reduction of infectivity at various concentrations of penicillin. The MIC by this criterium was 19 pmol/ml; there were no detectable IFUs. From the graph it can be seen that the penicillin concentration that would be expected to reduce the IFUs by 50% is approximately 4 pmol/ml.

We determined the time period during which penicillin could be added to cultures after initiation of infection and still inhibit production of IFUs by that culture. Figure 3B shows the results of this experiment. The penicillin concentration was 60 pmol/ml. Penicillin completely inhibited formation of IFUs, i.e., EBs, for up to 14 h. Thereafter, the effect waned. However, even at 55 h the treated culture had fewer IFUs than the parallel untreated culture.

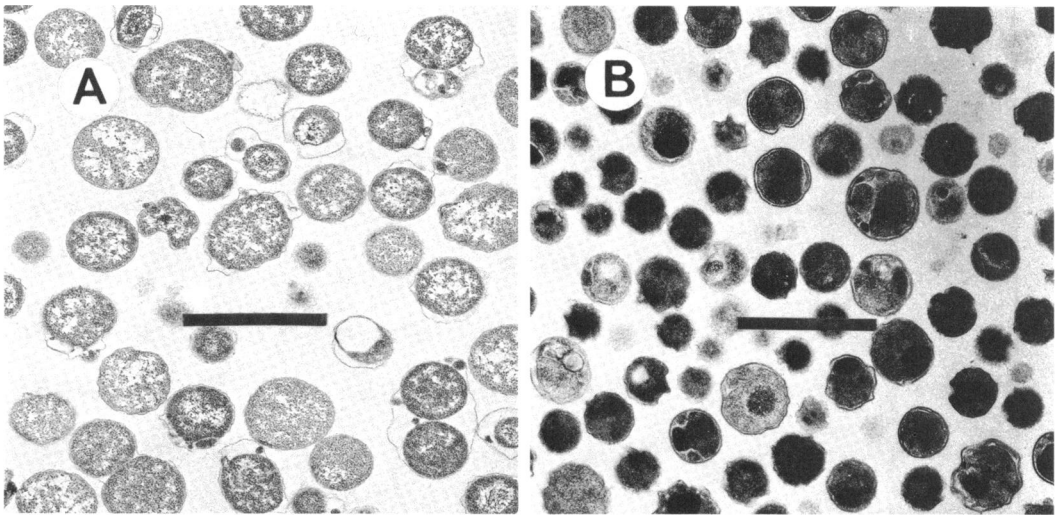

FIG. 1. Electron microscope photomicrographs of thin sections of purified *C. trachomatis* LGV-434 RBs (A) and EBs (B). Bar, 1 μm.

We also incubated purified EBs with 3, 30, 310, and 3,100 pmol of penicillin per ml in SPGS for 15 min at 37°C. The penicillin was removed by centrifuging and washing with buffer. The EBs were then planted on HeLa cells at an IFU-to-cell ratio of 1. After 48 h of incubation, the number of IFUs was determined, and the chamber slides were Giemsa stained. A parallel, untreated culture was also examined in this way. There were no atypical vacuoles noted, and there was no reduction in IFUs at any concentration of penicillin. Thus, any penicillin which bound to EBs did not affect infectivity or transition to RBs.

Binding of labeled penicillin to PBPs. Both EBs and RBs had three PBPs associated with the Sarkosyl-soluble fraction of whole cells (Fig. 4). The apparent subunit molecular weights, when assessed by addition of molecular weight standards to the gel, were 88,000 (PBP 1), 61,000 (PBP 2), and 36,000 (PBP 3). Figure 5A compares the binding of the same concentration of [^{3}H]penicillin to PBPs in EBs and RBs. Integration of the area under each of the three peaks showed that EBs bound to their PBPs only 33% as much penicillin as RBs did at saturating concentrations of penicillin (50 pmol/ml) and approximately equivalent masses of cells as estimated by protein concentration. However, as can be seen in Table 1, the 50% binding concentrations for both forms were essentially the same.

The PBPs identified with [^{14}C]benzylpenicillin were the same in number and apparent molecular weight as those identified with [^{3}H]penicillin (data not shown). The three PBPs were also present when RBs were first lysed with Triton X-100 before incubation with labeled penicillin (Fig. 5B). There were no detectable PBPs in the Sarkosyl-insoluble fraction of cells or in cells that had been first boiled for 5 min, solubilized in 1% SDS, or incubated with 10 μmol of unlabeled penicillin per ml before incubation with [^{3}H]penicillin.

The percentages of total radioactive penicillin bound to individual PBPs at a concentration of 50 pmol/ml were estimated by densitometry. In RBs, 19, 20, and 61% bound to PBPs 1, 2, and 3, respectively. In EBs, the corresponding values were 26, 15, and 59%.

Amino acid and muramic acid analysis. Table 2 shows the results of amino acid and muramic acid analyses. When muramic acid or DAP was applied to the amino acid analyzer, the minimum amount of either of the acids that could be reliably detected was 0.1 μg. Samples 500 μg in weight were hydrolyzed and analyzed. Therefore, the muramic acid and DAP contents of either RB or EB cells were less than 0.02%. Although, as expected, the proportions of muramic acid and DAP increased in the SDS-insoluble fractions of *E. coli* and *N. gonorrhoeae*, these two substances still were not detected in *C. trachomatis*. *C. trachomatis* was also remarkable for the low content of total amino acids in the residue.

We thought that chlamydiae might have an autolytic system which could result in breakdown of peptidoglycan and loss of its constituents during the isolation and purification of chlamydiae. To assess this, chlamydiae-infected and noninfected HeLa cells were suspended in

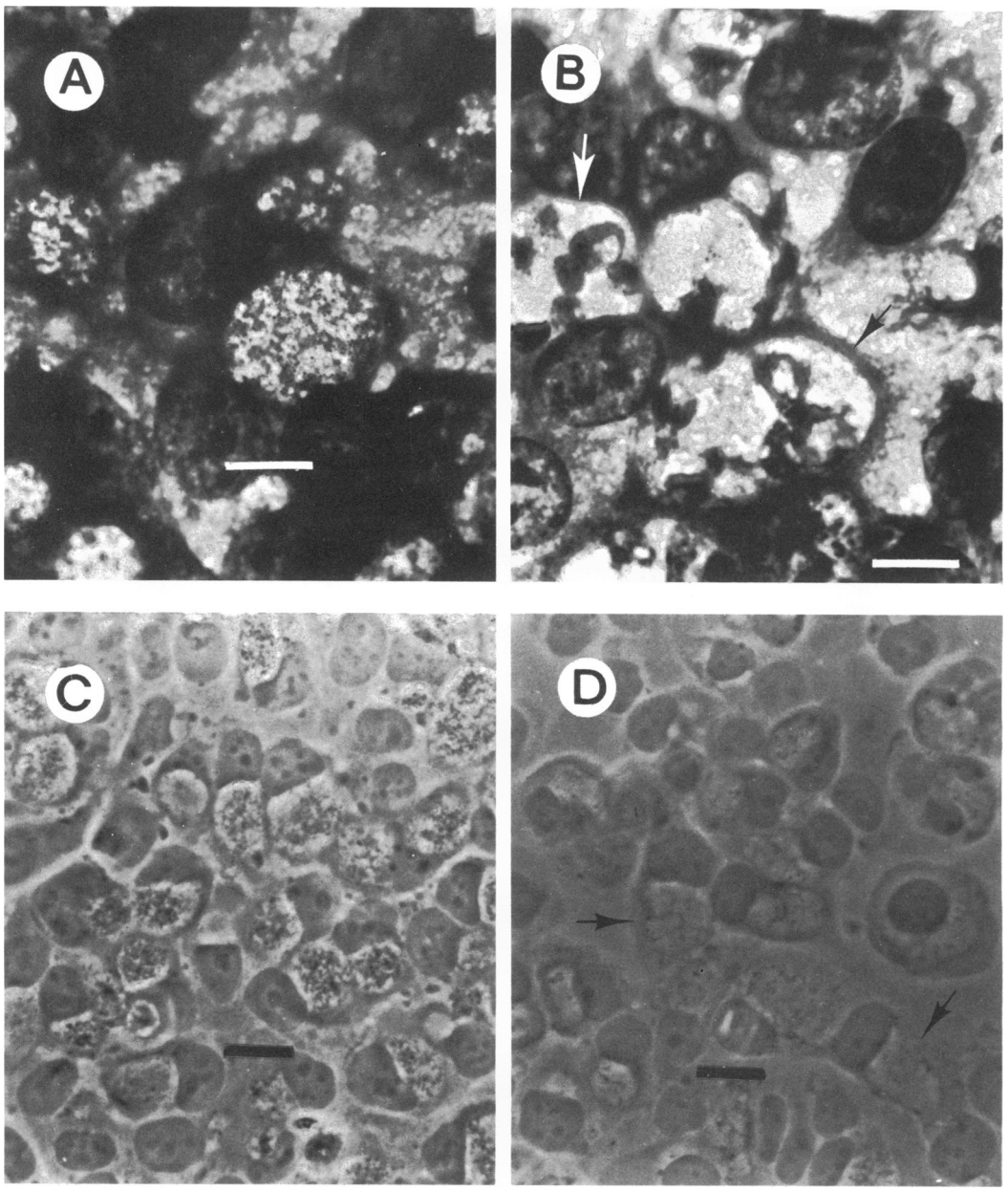

FIG. 2. Light photomicrographs of Giemsa-stained preparations (A and B) and phase photomicrographs of osmium tetroxide-fixed preparations (C and D) of *C. trachomatis*-infected HeLa 229 cells. Frames A and C show untreated cells, and frames B and D show infected cells exposed to 30 pmol of penicillin per ml for 42 h. Arrows indicate abnormally large RBs in the vacuoles of treated cells. Bar, 10 μm.

boiling 2% SDS following brief sonication and centrifugation (100,000 × *g* for 30 min at 4°C). Amino acid analysis of the twice-extracted, SDS-insoluble residues did not reveal muramic acid or DAP in either infected HeLa cells or, as expected, in noninfected cells. These substances were detected, however, in a positive control, HeLa cells to which *E. coli* K802 had been added before SDS extraction (data not shown).

DISCUSSION

Hamre and Rake first demonstrated that penicillin could protect embryonated eggs from infection with *C. trachomatis* and *C. psittaci* (17).

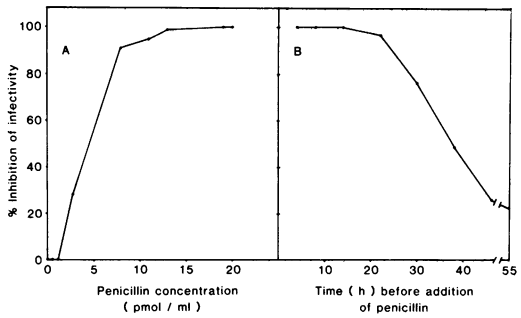

FIG. 3. (A) Reduction of infectivity of *C. trachomatis* in HeLa cells by penicillin. HeLa cells were preincubated with penicillin at various concentrations for 18 h. Chlamydiae were added at an IFU-to-cell ratio of 1. After 48 h, the IFUs recovered from each culture were determined. (B) Inhibition of infectivity of *C. trachomatis* in HeLa cells by penicillin added to the culture at various intervals after initiation of infection. The concentration of antibiotic was 60 pmol/ml. Chlamydiae were added at an IFU-to-cell ratio of 1 at 0 h. IFUs were determined 62 h later.

Other investigators have since confirmed the efficacy of penicillin in inhibiting division of chlamydiae in both yolk sac cultures (10, 35, 43) and tissue cultures (19, 24, 28, 40, 41).

We estimated the uptake of penicillin by HeLa cells before attempting interpretation of the results of susceptibility experiments. Eagle reported the uptake by HeLa cells of approximately 80% of ^{35}S-labeled penicillin in the media (8). In our studies, we found that the intracellular concentration of [^{14}C]benzylpenicillin was 30 and 104% of the extracellular penicillin concentration after 5 and 23 h, respectively. The uptake of penicillin, at least by HeLa cells after 5 h or more, appears to be substantial. The true MIC values, i.e., the minimal inhibitory concentrations inside the HeLa cells, were, therefore, probably no less than 50% of the extracellular MIC values listed above.

The data indicated that *C. trachomatis* LGV-434 in HeLa cells was susceptible to very low concentrations of penicillin. Complete inhibition of infectivity was seen at 19 pmol/ml. Partial inhibition of the production of IFUs occurred at a fourfold-greater dilution. The MIC by morphological criteria was approximately the same as the MIC by the criteria of inhibition of infectivity. The former assay demonstrated inhibition of division, but apparently not of growth, of individual RBs; only very large forms were seen. In the latter assay we assessed production of the infectious EBs. The sensitivity of the assay for IFUs was such that it would have likely detected unconverted EBs persisting from the original inoculum. It appears then that penicillin did not block conversion of EBs to RBs. We have confirmed, then, other reports of the two aspects of the action of penicillin on chlamydiae, i.e., inhibition of RB division and of conversion of RBs to EBs (24, 28, 40). However, we could not discern whether the two effects could be dissociated. It is possible that RBs could divide yet be blocked in conversion to EBs.

Tamura and Manire had shown that the effect of penicillin on formation of IFUs of *C. psittaci* waned with increasing intervals of time between initiation of infection of tissue culture cells and addition of penicillin (39). The same phenomenon occurred in *C. trachomatis*. After 14 h, the inhibitory effect of penicillin decreased. This time period corresponds to the number of hours before the first appearance of EBs in vacuoles (33). The failure of presumably bound penicillin to alter the infectivity of EBs once the antibiotic had been removed from the media also showed the lack of discernible effect of penicillin on EBs. However, this resistance is probably attributable to the nonreplicative nature of EBs. Eagle and Musselman demonstrated that prolonged incubation of penicillin with *Staphylococcus aureus* cells in stationary phase did not affect subsequent division of the cells once penicillin was removed and growth medium was reintroduced (9).

C. trachomatis had three PBPs in both the RBs and EBs. The absence of these PBPs in preparations of cells that had been first boiled, solubilized with SDS, or incubated with excess

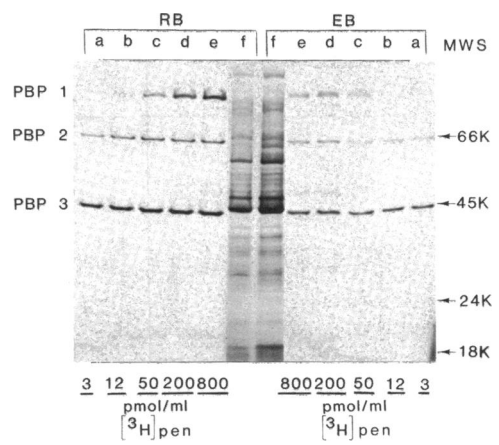

FIG. 4. Fluorograms of PBPs of *C. trachomatis* RBs and EBs. Electrophoresis was from top to bottom. Tracks a to e show fluorographic images of PBPs 1 to 3 at various concentrations of [^{3}H]penicillin. The f tracks contain Coomassie blue-stained proteins in whole cell preparations of RBs and EBs. The molecular weight standards (MWS; obtained from Sigma Chemical Co.) were bovine serum albumin (66K), ovalbumin (45K), trypsinogen (24K), and β-lactoglobulin (18K).

unlabeled penicillin suggested that binding to these proteins was specific and dependent on the integrity of native tertiary structure. Binding to PBPs did occur in the presence of the nonionic detergent, Triton X-100. PBPs were not detected in the Sarkosyl-insoluble fractions of either RBs or EBs. Caldwell et al. have shown that this fraction predominantly consists of the outer membrane (7). Chlamydial PBPs appear then to be associated with the cytoplasmic membrane or cytoplasm.

When the mass of cells in preparations of EBs and RBs was equivalent in protein content, there was approximately three times as much total bound [^{3}H]penicillin in RBs as in EBs. On the other hand, the 50% binding concentrations were very close in both cell types. This is consistent with the presence of fewer accessible PBPs in the EB preparations than in the RB preparation, if the affinities of the PBPs were equivalent in both cell types.

The 50% binding concentrations of [^{3}H]penicillin to the PBPs were within a factor of 5 of the MICs. This suggests that the PBPs in *C. trachomatis* are functional and not vestigial. An effect of penicillin at its MIC on chlamydiae that is independent of binding to the three PBPs described here does not appear to be likely if the action of penicillin on chlamydiae is similar to its action on *E. coli*. At least three of the seven PBPs of *E. coli* are critical targets of penicillin; i.e., binding to one of these PBPs leads to cessation of cell division or to lysis of the cells (37).

Comparison of PBPs of *C. trachomatis* with PBPs of other bacteria also shows that the number of PBPs, their approximate molecular weight, and the percentages of total bound penicillin for each of the three PBPs are similar in *C. trachomatis* and *N. gonorrhoeae* (2, 14). These two organisms also share similar cell shapes and ecological niches and as much apparent DNA homology as apparently exists between *C. psittaci* and *C. trachomatis* (23, 44). *N. gonorrhoeae* differs from *C. trachomatis* in having a genome approximately twice the size of *C. trachomatis*, in being capable of extracellular growth, and in having detectable muramic acid in its cell envelope. The lack of muramic acid in *C. trachomatis*, as well as in *C. psittaci* (13, 26, 39), also distinguishes chlamydiae from all other eubacteria (11, 45). Archaebacteria have been separated from eubacteria primarily on the basis of rRNA relatedness, but archaebacteria are also notable for their lack of muramic acid (11, 45). To our knowledge, the rRNA of any chlamydial species has not been studied in regard to its relatedness to other bacteria. However, from antibiotic studies of *C. trachomatis* which show susceptibility of these organisms to chloramphenicol (1, 41), it appears that the translation apparatus in chlamydial cells is eubacterial, and not archaebacterial, in nature (45). The penicillin susceptibility of chlamydiae also contrasts with the lack of susceptibility to beta-lactam antibiotics shown by such archaebacteria as *Halobacterium* spp. and *Methanococcus* spp. (21, 30). Chlamydiae are also susceptible to cycloserine; this effect is antagonized by D-alanine (31).

Thus, chlamydiae are probably eubacteria, but the absence of detectable muramic acid makes this genus unique. It is possible that there is a small amount of muramic acid in the chlamydial cells as the reports of Jenkin (20) and Perkins and Allison (32) suggested. However, such a small amount of muramic acid, if it existed in a polymeric form with another aminosugar, would not be capable of covering the chlamydial cell. *E. coli* probably has only a monolayer of peptidoglycan surrounding the cy-

TABLE 1. [^{3}H]penicillin concentrations required to give 50% binding of individual PBPs in RBs and EBs of *C. trachomatis*

PBP	Mol wt[a] (10^3)	Concn (pmol/ml) of [^{3}H]penicillin required to give 50% binding in:	
		RB	EB
1	88	70 (±10)[b]	33 (±9)
2	61	15 (±12)	11 (±9)
3	36	8 (±3)	7 (±5)

[a] Apparent subunit molecular weight (see text).
[b] Mean value from three determinations ± standard error.

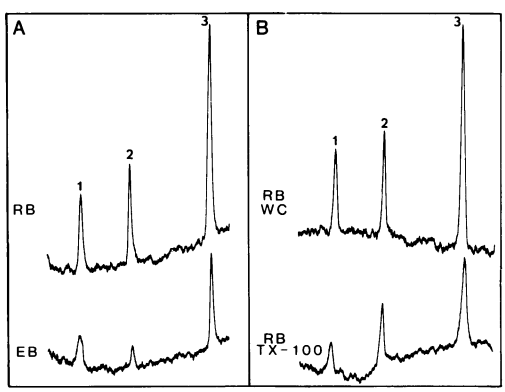

FIG. 5. (A) Microdensitometric tracings of fluorograms of PBPs 1, 2, and 3 in *C. trachomatis* RB and EB preparations. The concentration of [^{3}H]penicillin was 50 pmol/ml. PBP 1 is on the left. (B) Microdensitometric tracings of fluorograms of PBPs in intact RB whole cells (RB WC) and in RBs first lysed in Triton X-100 (TX-100) before the addition of [^{3}H]penicillin (50 pmol/ml).

TABLE 2. Muramic acid and DAP contents in whole cells and SDS-insoluble fractions of *C. trachomatis*, *E. coli*, and *N. gonorrhoeae*

Preparation	Content (% [dry wt])[a]		
	Total amino acids	Muramic acid	DAP
Whole cells			
C. trachomatis EB	55.3	<0.02	<0.02
C. trachomatis RB	48.1	<0.02	<0.02
E. coli K802	50.5	0.47	0.35
N. gonorrhoeae JS1	42.3	0.49	0.41
SDS-insoluble fraction[b]			
C. trachomatis EB	1.3	<0.02	<0.02
E. coli K802	56.1	1.16	0.92
N. gonorrhoeae JS1	32.6	4.13	2.82

[a] Hydrolyzed preparations were applied to a Beckman 118CL analyzer.

[b] Yields (percent, dry weight, of whole cells) were 5, 17, and 3% for *C. trachomatis*, *E. coli*, and *N. gonorrhoeae*, respectively.

toplasmic membrane (6, 15). Yet, a strain of this species had at least 50 times as much muramic acid in its SDS-insoluble fraction as *C. trachomatis*. We would predict that an organism such as *C. trachomatis* that has a smaller cell volume than *E. coli* would require even more muramic acid, on a weight basis, to cover its surface than *E. coli* would.

Another aminosugar may take the place of muramic acid in a chlamydial peptidoglycan. However, the spherical envelopes that comprise the Sarkosyl-insoluble fraction of *C. trachomatis* EB are completely disrupted when immersed in SDS (7). This is not the case with *E. coli*; a rod-shaped sacculus remains after SDS treatment (34, 42). Garrett et al. (13) and Caldwell et al. (7) have suggested that the shape and rigidity of the EB envelope may be determined by a material, possibly protein, that is neither peptidoglycan nor an analogous polymer of aminosugars. This suggestion is similar to that of Henning (18) who has proposed that the outer membrane of *E. coli* is the shape-determining structure of this species (16, 36). An extension of these proposals is that a tetrapeptide, analogous to that found in peptidoglycan and predictably containing D-alanine (3), cross-links a protein or lipoprotein layer in *C. trachomatis*. There is some basis for suggesting this; in *E. coli*, the tetrapeptide is covalently bound, not only to muramic acid and other tetrapeptides, but also to Braun's lipoprotein (5). However, if a chlamydial protein layer were extensively and covalently cross-linked, we would expect it not to be readily disrupted by SDS, and as a consequence, the SDS-insoluble residue should have a higher amino acid content than that found in *C. trachomatis* in the present study. The lack of evidence of cross-linking of either a muramic acid-containing layer or a protein layer suggests to us that penicillin, when it inhibits the division of chlamydiae, is acting on a target not necessarily involved in transpeptidation reactions. Although the role of this target (or these targets) in chlamydial division and cellular reorganization remains to be determined, *C. trachomatis* would appear to be a useful model for studying the effect of penicillin on bacterial cell division.

ACKNOWLEDGMENTS

We thank Barbara Nichols (Francis I. Proctor Foundation, University of California, San Francisco) for performing electron microscopy and Susan Smaus for assisting in preparing the manuscript.

LITERATURE CITED

1. **Alexander, J. J.** 1968. Separation of protein synthesis in meningopneumonitis agent from that in L cells by differential susceptibility to cyclohexamide. J. Bacteriol. **95**:327–332.
2. **Barbour, A. G.** 1981. Properties of penicillin-binding proteins in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **19**:316–322.
3. **Blumberg, P. M., and J. L. Strominger.** 1974. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol. Rev. **38**:291–335.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Braun, V., and V. Bosch.** 1972. Sequence of the murein-lipoprotein and the attachment site of the lipid. Eur. J. Biochem. **28**:51–69.
6. **Braun, V., H. Gnirke, U. Henning, and K. Rehn.** 1973. Model for the structure of the shape-maintaining layer of the *Escherichia coli* cell envelope. J. Bacteriol. **114**:1264–1270.
7. **Caldwell, H. D., J. Kromhout, and J. Schachter.** 1981. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. **31**:1161–1176.
8. **Eagle, H.** 1954. The binding of penicillin in relation to its cytotoxic action. III. The binding of penicillin by mammalian cells in tissue culture (HeLa and L strains). J. Exp. Med. **100**:117–124.
9. **Eagle, H., and A. D. Musselman.** 1949. The slow recovery of bacteria from the toxic effects of penicillin. J. Bacteriol. **58**:475–490.
10. **Eaton, M. D., T. F. Dozois, A. Van Allen, V. L. Parish, and S. Schwalm.** 1947. Studies of inhibition of the agents of murine and feline pneumonitis by penicillin. J. Immunol. **58**:251–267.
11. **Fox, G. E., E. Stackebrandt, R. B. Hespell, J. Gibson, J. Maniloff, T. A. Dyer, R. S. Wolfe, W. E. Balch, R. S. Tanner, L. J. Magrum, L. B. Zablen, R. Blakemore, R. Gupta, L. Bonen, B. J. Lewis, D. A. Stahl, K. R. Luehrsen, K. N. Chen, and C. R. Woese.** 1980. The phylogeny of prokaryotes. Science **209**:457–463.
12. **Furness, G., D. M. Graham, and P. Reeve.** 1960. The titration of trachoma and inclusion blennorrhoea viruses in cell cultures. J. Gen. Microbiol. **23**:613–619.
13. **Garrett, A. J., M. J. Harrison, and G. P. Manire.** 1974. A search for the bacterial mucopeptide component, muramic acid, in *Chlamydia*. J. Gen. Microbiol. **80**:315–318.
14. **Georgopapadakou, N. H., and F. Y. Liu.** 1980. Penicillin-binding proteins in bacteria. Antimicrob. Agents Che-

mother. **18**:148–157.
15. **Ghuysen, J. M.** 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol. Rev. **32**:425–464.
16. **Haller, I., and U. Henning.** 1974. Cell envelope and shape of *Escherichia coli* K12. Crosslinking with dimethyl imidoesters of the whole cell wall. Proc. Natl. Acad. Sci. U.S.A. **71**:2018–2021.
17. **Hamre, D., and G. Rake.** 1947. Studies on lymphogranuloma venereum. V. The action of some antibiotic substances and sulfonamides in vitro and in vivo upon the agents of feline pneumonitis and lymphogranuloma venereum. J. Infect. Dis. **81**:175–190.
18. **Henning, U.** 1975. Determination of cell shape in bacteria. Annu. Rev. Microbiol. **29**:45–60.
19. **Hobson, D., E. Rees, F. W. A. Johnson, and I. A. Tait.** 1977. Effect of penicillin on *Chlamydia trachomatis* in vitro and in vivo, p. 227–229. *In* D. Hobson and K. K. Holmes (ed.), Nongonococcal urethritis and related infections. American Society for Microbiology, Washington, D.C.
20. **Jenkin, H. M.** 1960. Preparation and properties of cell walls of the agent of meningopneumonitis. J. Bacteriol. **80**:639–647.
21. **Jones, J. B., B. Bowers, and T. C. Stadtman.** 1977. *Methanococcus vannielii*: ultrastructure and sensitivity to detergents and antibiotics. J. Bacteriol. **130**:1357–1363.
22. **Kingsbury, D. T.** 1969. Estimate of the genome size of various microorganisms. J. Bacteriol. **98**:1400–1401.
23. **Kingsbury, D. T., and E. Weiss.** 1968. Lack of deoxyribonucleic acid homology between species of the genus *Chlamydia*. J. Bacteriol. **96**:1421–1423.
24. **Kramer, M. J., and F. B. Gordon.** 1971. Ultrastructural analysis of the effects of penicillin and chlortetracycline on the development of a genital tract *Chlamydia*. Infect. Immun. **3**:333–341.
25. **Manire, G. P.** 1966. Structure of purified cell walls of dense forms of meningopneumonitis organisms. J. Bacteriol. **91**:409–413.
26. **Manire, G. P., and A. Tamura.** 1967. Preparation and chemical composition of the cell walls of mature infectious dense forms of meningopneumonitis organisms. J. Bacteriol. **94**:1178–1183.
27. **Matsumoto, A.** 1973. Fine structures of cell envelopes of *Chlamydia* organisms as revealed by freeze-etching and negative staining techniques. J. Bacteriol. **116**:1355–1363.
28. **Matsumoto, A., and G. P. Manire.** 1970. Electron microscopic observations on the effects of penicillin on the morphology of *Chlamydia psittaci*. J. Bacteriol. **101**:278–285.
29. **Matsumoto, A., and G. P. Manire.** 1970. Electron microscopic observations on the fine structure of cell walls of *Chlamydia psittaci*. J. Bacteriol. **104**:1332–1337.
30. **Mohr, V., and H. Larsen.** 1963. On the structural transformation and lysis of *Halobacterium salinarium* in hypotonic and isotonic solutions. J. Gen. Microbiol. **31**:267–280.
31. **Moulder, J. W., D. L. Novosel, and J. E. Officer.** 1963. Inhibition of the growth of agents of the psittacosis group by D-cycloserine and its specific reversal by D-alanine. J. Bacteriol. **85**:707–711.
32. **Perkins, H. R., and A. C. Allison.** 1963. Cell-wall constituents of rickettsiae and psittacosis-lymphogranuloma organisms. J. Gen. Microbiol. **30**:469–480.
33. **Schachter, J., and H. D. Caldwell.** 1980. Chlamydiae. Annu. Rev. Microbiol. **34**:285–309.
34. **Schwarz, U., and W. Leutgeb.** 1971. Morphogenetic aspects of murein structure and biosynthesis. J. Bacteriol. **106**:588–595.
35. **Shiao, L. C., S. P. Wang, and J. T. Grayston.** 1967. Sensitivity and resistance of TRIC agents to penicillin, tetracycline, and sulpha drugs. Am. J. Ophthalmol. **63**:1558–1568.
36. **Sonntag, I., H. Schwarz, Y. Hirota, and U. Henning.** 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. **136**:280–285.
37. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur. J. Biochem. **72**:341–352.
38. **Stock, J. B., B. Rauch, and S. Roseman.** 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **252**:7850–7861.
39. **Tamura, A., and G. P. Manire.** 1967. Preparation and chemical composition of the cell membranes of developmental reticulate forms of meningopneumonitis organisms. J. Bacteriol. **94**:1184–1188.
40. **Tamura, A., and G. P. Manire.** 1968. Effect of penicillin on the multiplication of meningopneumonitis organisms (*Chlamydia psittaci*). J. Bacteriol. **96**:875–880.
41. **Treharne, J. D., J. Day, C. K. Yeo, B. R. Jones, and S. Squires.** 1977. Susceptibility of chlamydiae to chemotherapeutic agents, p. 214–222. *In* D. Hobson and K. K. Holmes (ed.), Nongonococcal urethritis and related infections. American Society for Microbiology, Washington, D.C.
42. **Weidel, W., and H. Pelzer.** 1964. Bagshaped macromolecules—a new outlook on bacterial cell walls. Adv. Enzymol. **26**:193–232.
43. **Weiss, E.** 1950. The effect of antibiotics on agents of the psittacosis-lymphogranuloma group. I. The effect of penicillin. J. Infect. Dis. **87**:249–263.
44. **Weiss, E., S. Schramek, N. N. Wilson, and L. W. Newman.** 1970. Deoxyribonucleic acid heterogeneity between human and murine strains of *Chlamydia trachomatis*. Infect. Immun. **2**:24–28.
45. **Woese, C. R., L. J. Magrum, and G. E. Fox.** 1978. Archaebacteria. J. Mol. Evol. **11**:245–252.