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# Synthesis of Disulfide-Bonded Outer Membrane Proteins during the Developmental Cycle of *Chlamydia psittaci* and *Chlamydia trachomatis*

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**The disulfide bond cross-linked major outer membrane protein (MOMP) of the extracellular elementary bodies (EBs) of *Chlamydia psittaci* was reduced to its monomeric form within 1 h of entry of EBs into host cells by a process which was inhibited by chloramphenicol, while monomeric forms of three cross-linked cysteine-rich proteins could not be detected in Sarkosyl outer membrane complexes at any time in either extracellular or intracellular forms of *C. psittaci*. Synthesis and incorporation of the MOMP into outer membrane complexes were detected early in the infection cycle (12 h postinfection), while synthesis and incorporation of the cysteine-rich proteins were not observed until reticulate bodies had begun to reorganize into EBs at 20 to 22 h postinfection. By 46 h postinfection, the intracellular population of *C. psittaci* consisted mainly of EBs, the outer membrane complexes of which were replete with monomeric MOMP and cross-linked cysteine-rich proteins. Upon lysis of infected cells at 46 h, the MOMP was rapidly cross-linked, and infectious EBs were released. The status of the MOMP of intracellular *Chlamydia trachomatis* was similar to the status of the MOMP of *C. psittaci* in that the MOMP was largely uncross-linked at 24 and 48 h postinfection, but formed interpeptide disulfide bonds when it was exposed to an extracellular environment late in the developmental cycle. In contrast to *C. psittaci*, only a fraction of the cross-linked MOMP of infecting EBs of *C. trachomatis* was reduced by 4 h postinfection, and reduction of the MOMP was not inhibited by chloramphenicol. Exposure of extracellular EBs of *C. trachomatis* and *C. psittaci* to dithiothreitol reduced the MOMP but failed to stimulate metabolic activities normally associated with reticulate bodies.**

*Chlamydia psittaci* and *Chlamydia trachomatis* are obligate intracellular parasitic bacteria which have a developmental cycle (17). This cycle is characterized at one extreme by the elementary body (EB) form, which is adapted for extracellular survival and infection of new hosts, and at the other extreme by the reticulate body (RB) form, which is adapted for intracellular survival and multiplication. Both life cycle forms possess a cell envelope which consists of an outer membrane and an inner membrane (5, 19) but lacks or is deficient in peptidoglycan (1, 6, 13, 18). Despite this deficiency, EBs are osmotically stable, although RBs are readily lysed by ultrasonic treatment (18). The EB outer membrane includes a 40-kilodalton (kDa) major outer membrane protein (MOMP) (4, 12, 15) and at least three cysteine-rich proteins (a 60-kDa doublet and a 12-kDa peptide), all of which are extensively disulfide bond cross-linked to form a supramacromolecular lattice (2, 7, 10, 14). In contrast, the outer membrane of the RB form is deficient in the cysteine-rich proteins, and the MOMP of RBs is not extensively cross-linked (7, 10). Recently, Hackstadt et al. (7) demonstrated that treatment of EBs of *C. trachomatis* serovar L2 with dithiothreitol (DTT) converted cross-linked MOMP to monomers with a concomitant loss of osmotic stability and infectivity and a gain in the ability to transport and oxidatively decarboxylate glutamic acid. Sarov and Becker (16) similarly demonstrated that mercaptoethanol-treated EBs of *C. trachomatis* can be induced to incorporate UTP into RNA. A possible role for the MOMP in the reduced state to function as a transport channel was further suggested by the observations of Bavoil et al. (2). These investigators demonstrated that liposomes containing reduced MOMP treated with iodoacetamide to block the spontaneous formation of disulfide cross-links possess 10-fold-higher porin activity than liposomes constructed with untreated MOMP.

The present study was undertaken to define the temporal sequence of synthesis and disulfide cross-linkage of outer membrane proteins during the chlamydial developmental cycle and to further investigate the role of cross-linked outer membrane complexes in maintaining the permeability barrier and osmotic stability of EBs of chlamydiae.

## MATERIALS AND METHODS

**Growth of organisms.** *C. psittaci* 6BC was grown in cell line 929 L cells, and *C. trachomatis* serovar L2 was grown in cell line 229 HeLa cells (12). RBs and EBs were harvested at 20 and 48 h, respectively, and were purified by using Renografin (E. R. Squibb & Sons, Princeton, N.J.) density centrifugation (9). Purified EBs were treated for 5 min with 0.5% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) to lyse any remaining RBs which copurified with the EBs. In some experiments EBs were released from infected cells by treating the cells with 0.5% Nonidet P-40 for 5 min at 4°C and were partially purified by differential centrifugation before outer membranes were prepared (8). For experiments in which EBs were intrinsically labeled, *C. psittaci* and *C. trachomatis* were grown in $5 \times 10^7$ host cells in 100 ml of growth medium containing 250 μCi of either [$^{35}$S]cysteine (1,055 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) or [$^{35}$S]methionine (1,345 Ci/mmol; Amersham).

**Outer membrane complex preparation and electrophoresis.** Sarkosyl outer membrane complexes (SOMCs) were pre-

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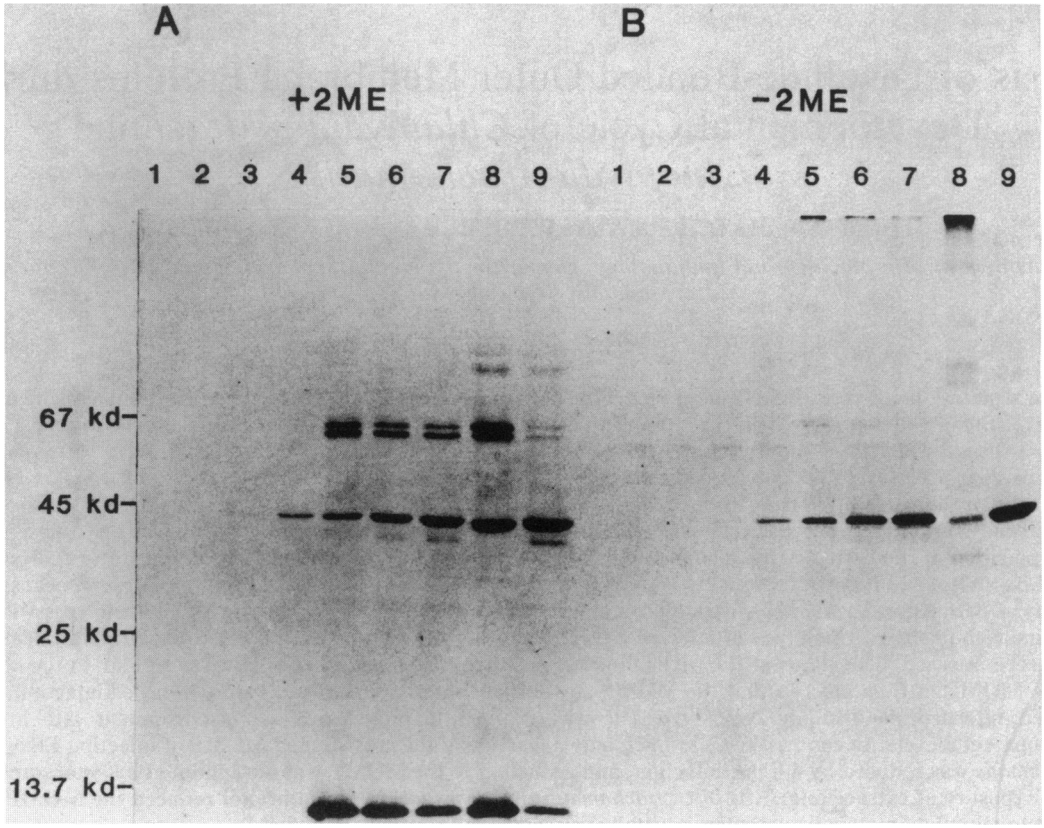


FIG. 1. Synthesis of outer membrane proteins by *C. psittaci*. *C. psittaci*-infected L cells were pulsed with [$^{35}$S]cysteine at the times indicated below, and outer membrane complexes were prepared 2 h later as described in Materials and Methods. Proteins were separated by SDS-PAGE in the presence (A) and absence (B) of 2ME, and radioactivity was detected by autoradiography. Lane 1, 4 h; lane 2, 8 h; lane 3, 12 h; lane 4, 16 h; lane 5, 20 h; lane 6, 24 h; lane 7, 30 h; lane 8, 44 h, harvested EBs; lane 9, 44 h, intracellular chlamydiae. kd, Kilodaltons.

pared by extracting EBs and RBs with 2% sodium lauryl sarkosinate (Sarkosyl; Sigma) containing 50 mM iodoacetamide (10). Whole organisms and outer membranes were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in the presence or absence of 10% (vol/vol) 2-mercaptoethanol (2ME) as previously described (10). Gels were stained with Coomassie brilliant blue R-250 and dried, and radioactive bands were visualized by autoradiography, using Kodak X-Omat AR film.

**Isotopic labeling studies.** For the experiment in which *C. psittaci*-infected cells were pulsed with [$^{35}$S]cysteine, $10^8$ L cells were infected with ten 50% infective doses of *C. psittaci* (8), and monolayer cultures containing $5 \times 10^6$ L cells in 25-cm$^2$ tissue culture flasks were prepared. At various times postinfection, the growth medium was replaced with 1 ml of medium 199 (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal calf serum and 50 μCi of [$^{35}$S]cysteine (1,055 Ci/mmol). After 2 h of incubation at 37°C, the radioactive medium was decanted, and the cells were washed three times with Dulbecco phosphate-buffered saline. Outer membrane complexes were prepared either by direct extraction of the infected cells with 4.5 ml of 2% Sarkosyl containing 50 mM iodoacetamide or by extraction of Nonidet P-40-harvested EBs with Sarkosyl-iodoacetamide.

For the iodoacetamide labeling study, outer membrane complexes were prepared by extracting extracellular EBs of *C. psittaci* or L cells infected for 1.0 h with *C. psittaci* (intracellular EBs) with 2% Sarkosyl containing 50 mM unlabeled iodoacetamide and were suspended in 400 μl of 2% SDS in 0.1 M Tris hydrochloride (pH 8.0)–10% (vol/vol) glycerol. The preparations were split equally, and either 100 μl of water or 100 μl of 100 mM DTT was added to each. Both preparations were heated at 100°C for 2 min, cooled, and incubated for 1 h at 4°C following the addition of 50 μl of 20 mM [$^{14}$C]iodoacetamide (2.5 μCi; Amersham). The reaction was terminated by adding 10 μl of 2ME, and 20 μl portions of the preparations were subjected to electrophoresis.

**Metabolic studies.** EBs and RBs (50 μg of protein) were suspended in 100 μl of buffer containing 100 mM Tris hydrochloride (pH 7.4), 0.5 M KCl, 5 mM $MgCl_2$, and 5 mM $KPO_4$ (pH 7.0) with and without 10 mM DTT. For protein synthesis, 100 nmol of ATP, 1.12 nmol of creatine phosphate (Sigma), 3 μg of phosphocreatine kinase (Sigma), 10 nmol each of 19 unlabeled amino acids, 5 μCi of [$^{35}$S]methionine ($8.4 \times 10^6$ cpm), and 20 pmol of unlabeled methionine were added, and the incorporation of radioactivity into trichloroacetic acid-precipitable material was determined after 1 h of incubation at 37°C. For transport studies, 1 μCi of either [$^3$H]ATP or [$^3$H]GTP (850,000 cpm; ICN Pharmaceuticals Inc., Irvine, Calif.) was added with unlabeled nucleotide to a final concentration of 5 μM, and the uptake after 5 min of incubation at 37°C was measured by filtering the mixture onto 0.2-μm microporous filters and washing three times with 5 ml of Dulbecco phosphate-buffered saline. A back-

ground value, which was obtained when incubation was carried out with chlamydiae that had been heated at 60°C for 3 min, was subtracted from all sample values.

**Turbidity and infectivity studies.** EBs and RBs (approximately 300 μg of protein) were suspended in 1.5 ml of distilled water and incubated at 37°C with and without 10 mM DTT. After 1.5 h of incubation, the optical densities of the suspensions were measured at 600 nm through a 1-cm light path. Samples then were removed for titration, and the optical densities were determined after Nonidet P-40 was added to a final concentration of 0.5%. EBs were titrated by determining the 50% infective dose for $5 \times 10^6$ L or HeLa cells and calculating the number of infectious units, using the Poisson distribution as previously described (8), except that 5 mM DTT was present for all samples during the 1-h attachment phase of *C. psittaci* to L cells.

## RESULTS

**Synthesis and incorporation of disulfide bond cross-linked proteins into outer membranes during the developmental cycle of *C. psittaci*.** L cells infected with *C. psittaci* were pulsed for 2 h with [$^{35}$S]cysteine at various times postinfection, and the incorporation of labeled proteins into SOMCs was analyzed by SDS-PAGE and autoradiography. The complexes were prepared by direct extraction of the infected cells with 2% Sarkosyl containing iodoacetamide to alkylate free sulfhydryl groups and to prevent the spontaneous formation of disulfide bonds. The synthesis and incorporation of the MOMP into SOMCs were detected as early as 12 to 14 h postinfection and at all times thereafter (Fig. 1A). The newly synthesized MOMP migrated as a 43-kDa monomer in the absence of reducing agent, indicating that the MOMP was maintained in its reduced form by intracellular *C. psittaci* (Fig. 1B). In contrast, synthesis and incorporation of the cysteine-rich 60-kDa doublet and the 12-kDa protein were first detected at 20 to 22 h postinfection (Fig. 1A, lane 5), the time in the developmental cycle when some of the intracellular RBs are in the process of reorganizing to EBs. The cysteine-rich proteins continued to be synthesized and incorporated into SOMCs until the infection was terminated at 46 h; however, in contrast to the MOMP, these proteins failed to migrate as monomers in the absence of reducing agent (Fig. 1B). When EBs were harvested by treating 46-h-infected cells with Nonidet P-40 before SOMCs were prepared, most of the MOMP failed to migrate as a monomer suggesting that the MOMP was rapidly cross-linked when it was exposed to an extracellular environment (Fig. 1B, lane 8).

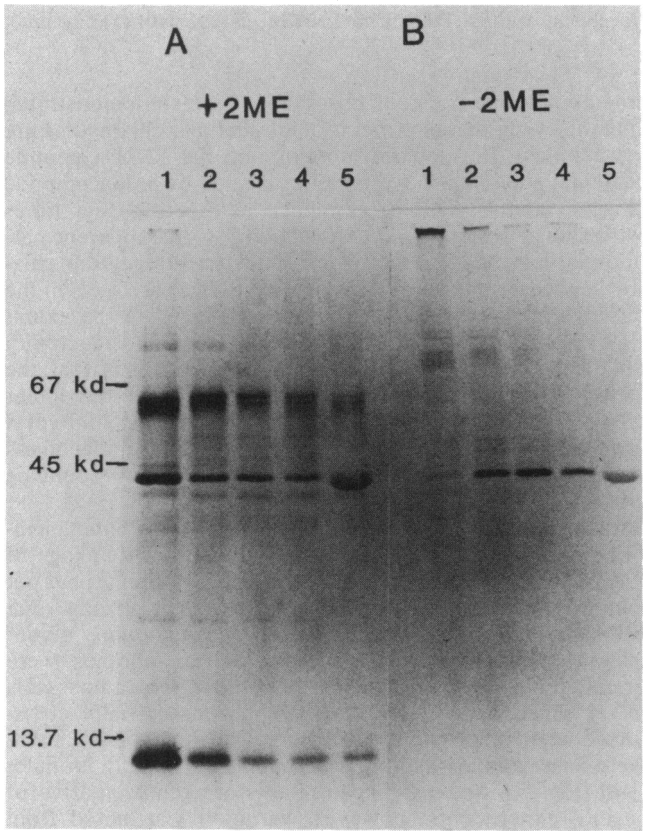


FIG. 2. Fate of outer membrane proteins following infection of L cells with *C. psittaci*. L cells were infected with [$^{35}$S]cysteine-labeled EBs, outer membrane complexes were prepared from the same number of infected cells at the times postinfection indicated below, and proteins were separated by SDS-PAGE in the presence (A) and absence (B) of 2ME. An autoradiograph of the gel is shown. Lane 1, Zero time; lane 2, 1 h; lane 3, 3 h; lane 4, 6 h; lane 5, 22 h. kd, Kilodaltons.

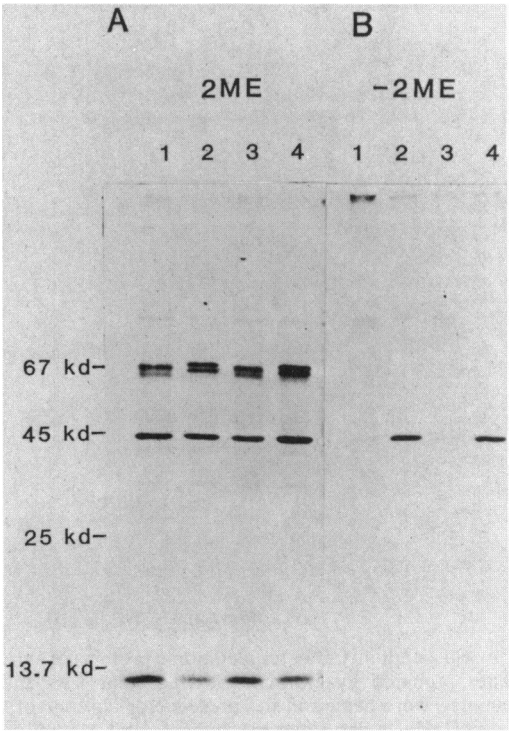


FIG. 3. Effects of inhibitors of protein synthesis on the reduction of *C. psittaci* outer membrane proteins. The experiment was conducted as described in the legend to Fig. 2, except that chloramphenicol (100 μg/ml) and cycloheximide (100 μg/ml) were present as indicated below. Lane 1, Zero time; lane 2, 1.5 h; lane 3, 1.5 h, chloramphenicol present; lane 4, 1.5 h, cycloheximide present. kd, Kilodaltons.

**Fate of disulfide bond cross-linked outer membrane proteins of EBs following infection.** To determine the fate of EB outer membrane proteins following infection, L cells were infected with [$^{35}$S]cysteine-labeled EBs of *C. psittaci*, and SOMCs were prepared between 1 and 22 h postinfection and analyzed by SDS-PAGE (Fig. 2). By 1 h postinfection most of

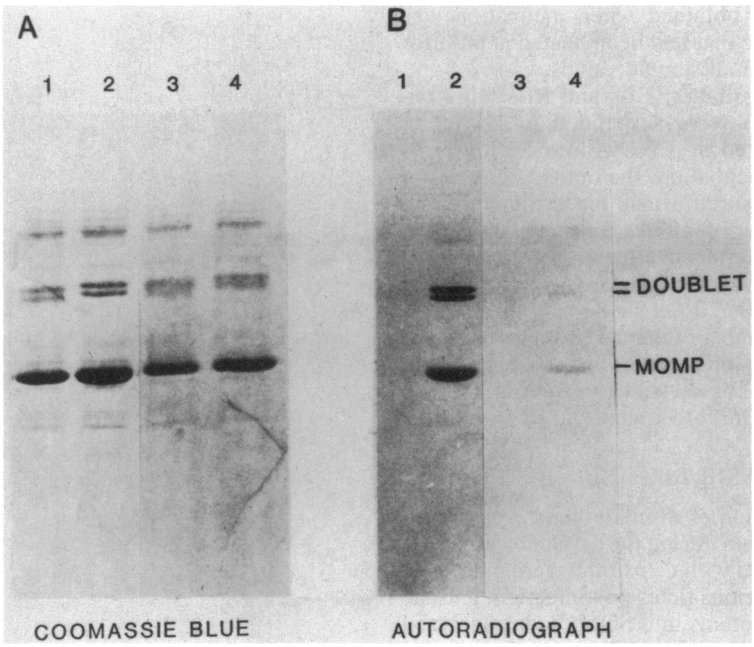


FIG. 4. Susceptibility of outer membrane proteins of extracellular and intracellular *C. psittaci* EBs to alkylation with iodoacetamide. Outer membranes prepared by extracting extracellular EBs and 1-h-infected L cells (intracellular EBs) with Sarkosyl containing unlabeled iodoacetamide were heated in the presence or absence of DTT, reacted with [$^{14}$C]iodoacetamide, and subjected to electrophoresis in the presence of 2ME as described in Materials and Methods. Lane 1, Extracellular, without DTT; lane 2, extracellular, with DTT; lane 3, intracellular, without DTT; lane 4, intracellular, with DTT.

the cross-linked MOMP of infectious EBs was reduced to the monomeric form, while the cysteine-rich proteins failed to migrate as monomers in the absence of reducing agent. Label could be detected in both the MOMP and the cysteine-rich proteins as late as 22 h postinfection. Although this experiment was not highly quantitative, it appears that the MOMP and the cysteine-rich proteins in the infecting EBs were not broken down to a significant extent as EBs reorganized to RBs, but rather were diluted out as RBs multiplied. Reduction of the MOMP apparently required chlamydial protein synthesis but not host protein synthesis since reduction was inhibited when the infection was carried out in the presence of chloramphenicol but not when the infection was carried out in the presence of cycloheximide (Fig. 3).

The reduction of cross-linked outer membrane proteins upon entry of EBs into host cells was further investigated by determining the susceptibility of the proteins to alkylation with iodoacetamide. Outer membrane complexes from extracellular (zero-time) and intracellular (1-h-postinfection) EBs were again prepared by extraction with Sarkosyl containing unlabeled iodoacetamide to block free sulfhydryl groups. The preparations then were incubated with and without DTT, labeled for 1 h at 4°C with [$^{14}$C]iodoacetamide, and electrophoresed in the presence of a reducing agent. With this method, cysteine residues of outer membrane proteins that were engaged in disulfide bonds were labeled after their reduction with DTT (but not in the absence of prior treatment with DTT), while cysteine residues that existed as free sulfhydryls were blocked by the unlabeled iodoacetamide and consequently were not labeled (with or without prior treatment with DTT). Consistent with the previous results that indicated that the MOMP of extracellular EBs is cross-linked, the MOMP of DTT-treated extracellular EBs was well labeled (Fig. 4B, lane 2), while the MOMP of DTT-treated intracellular EBs was poorly labeled with [$^{14}$C]iodoacetamide (Fig. 4B, lane 4). In apparent contradiction to the previous observations which demonstrated that the cysteine-rich proteins of intracellular chlamydiae are cross-linked, the doublet proteins and the 12-kDa peptide (data not shown) also were poorly labeled by iodoacetamide (Fig. 4, lane 4). This experiment was repeated four times with similar results. One explanation for this apparent paradox is that the cysteine-rich proteins of intracellular chlamydiae were sufficiently cross-linked to not enter gels in the absence of reducing agent, yet were reduced to the extent that some of their cysteine residues were free to react with unlabeled iodoacetamide. A second possibility is that the cysteine-rich proteins existed as monomers in intracellular chlamydiae, but that a proportion of the free sulfhydryl groups spontaneously formed interpeptide disulfide bonds during outer membrane preparation before they could be alkylated with unlabeled iodoacetamide.

**Disulfide bond cross-linkage of *C. trachomatis* outer membrane proteins.** The status of the disulfide cross-linkage of the MOMP of *C. trachomatis* serovar L2 was similar, but not identical, to the status of the MOMP of *C. psittaci*. The MOMP of 24- and 48-h intracellular *C. trachomatis* organisms was largely reduced when outer membranes were prepared by direct Sarkosyl extraction of infected host cells in the presence of iodoacetamide but was partially cross-linked when outer membranes were prepared from EBs that were harvested at 48 h by lysing infected cells with Nonidet P-40 (Fig. 5A and B). These results are representative of several experiments; however, variation was noted from experiment to experiment. For example, in some experiments a significant porportion (40 to 50%) of the MOMP was found to be cross-linked in intracellular chlamydiae late in the developmental cycle, while in other experiments the MOMP of 48-h extracellular chlamydiae was completely cross-linked. This variation may have been due to differences from experiment to experiment in the ratio RBs to EBs in 48-h infected cells. Unlike the MOMP of *C. psittaci*, the

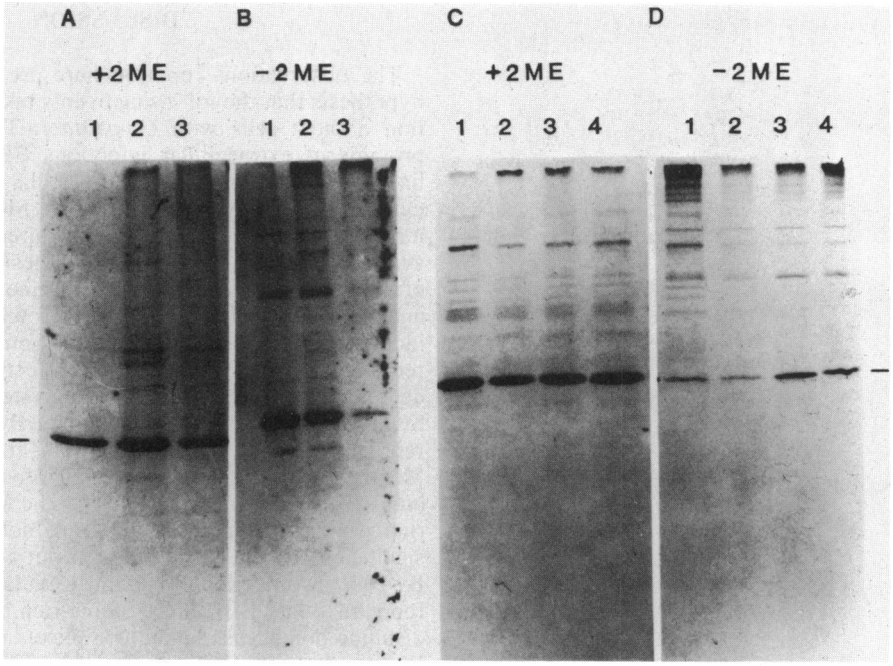


FIG. 5. SDS-PAGE profile of *C. trachomatis* outer membrane proteins prepared at various times during the developmental cycle. (A and B) HeLa cells were infected with *C. trachomatis* in the presence of [$^{35}$S]methionine, and outer membrane complexes were prepared by direct extraction of infected cells at 24 h (lane 1) and 48 h (lane 2) postinfection or by extraction of EBs harvested at 48 h (lane 3). (C and D) HeLa cells were infected with [$^{35}$S]cysteine-labeled EBs in the presence or absence of chloramphenicol (100 μg/ml), and outer membrane complexes were prepared by extracting infected cells at the following times: lane 1, zero time; lane 2, 2 h; lane 3, 4 h; lane 4, 4 h, chloramphenicol present. Autoradiographs of the gels are shown.

MOMP of *C. trachomatis* EBs was not reduced by 2 h postinfection and was only partially reduced by 4 h postinfection; also, this partial reduction was not inhibited by chloramphenicol (Fig. 5C and D). Longer incubation (8 to 12 h) did not increase the proportion of MOMP that was reduced (data not shown).

**Effect of DTT on the osmotic stability, infectivity, and metabolism of EBs.** Workers in our laboratory previously have demonstrated that RBs of *C. psittaci* are capable of transport and synthetic activities which are not detected in EBs (9, 11). It has been suggested that the disulfide cross-linked MOMP of EBs may preclude diffusion of substances through pores formed by the MOMP in the outer membranes of uncross-linked RBs (2, 7). It also has been suggested that the disulfide cross-linked lattice in the outer membranes of EBs renders extracellular chlamydiae osmotically stable (2, 7, 10, 14). We found that DTT-treated EBs of *C. trachomatis* lost infectivity and were lysed when they were suspended in distilled water (Table 1), confirming the observations of Hackstadt et al. (7). EBs of *C. psittaci* were found to be relatively stable in DTT; however, DTT-treated EBs of *C. psittaci* were lysed by the addition of Nonidet P-40. Treated EBs also lost infectivity, but only when 5 mM DTT was present during the attachment phase (1 h) of the titration

TABLE 1. Effect of DTT on EBs and RBs[a]

| Organism | Prepn | Presence of DTT | Optical density at 600 nm[b] | | | Infectivity (infectious units per ml, $\times 10^{-9}$) | ATP transport (cpm) | GTP transport (cpm) | Protein synthesis (cpm) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Zero time | 1.5 h | With Nonidet P-40[c] | | | | |
| *C. psittaci* 6BC | EBs | − | 1.07 | 1.07 | 1.05 | 4.71 | 402 | 209 | 1,083 |
| | | + | 1.08 | 0.86 | 0.44 | 0.21 | 390 | 277 | 1,229 |
| | RBs | − | 0.44 | 0.46 | 0.22 | ND[d] | 74,527 | 9,279 | 939,906 |
| | | + | 0.41 | 0.40 | 0.19 | ND | 67,483 | 8,648 | 524,089 |
| *C. trachomatis* serovar L2 | EBs | − | 1.25 | 1.08 | 0.90 | 1.42 | 326 | 165 | 873 |
| | | + | 1.23 | 0.43 | 0.29 | 0.17 | 423 | 294 | 1,345 |
| | RBs | − | 0.62 | 0.57 | 0.39 | ND | 37,258 | 3,940 | 191,396 |
| | | + | 0.56 | 0.51 | 0.28 | ND | 32,318 | 3,445 | 76,489 |

[a] EBs and RBs were incubated at 37°C with or without 10 mM DTT, and infectivity, turbidity, transport, and protein synthesis were measured after 1.5 h as described in Materials and Methods.

[b] The optical densities at 600 nm of *C. psittaci* and *C. trachomatis* RBs in phosphate-buffered saline before they were suspended in distilled water were 1.58 and 1.63, respectively.

[c] Nonidet P-40 was added after 1.5 h, and the optical density at 600 nm was determined.

[d] ND, Not determined.

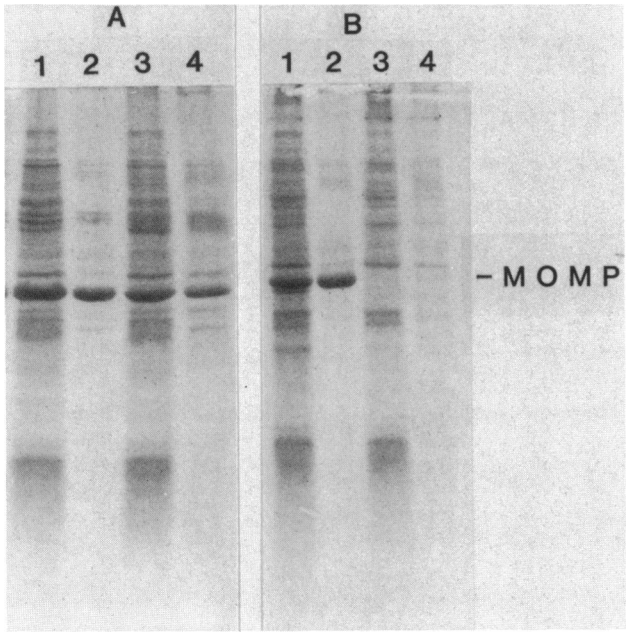


FIG. 6. SDS-PAGE profile of *C. psittaci* EBs treated with DTT. EBs were treated with 10 mM DTT for 1 h at 37°C and washed once with phosphate-buffered saline containing 50 mM iodoacetamide before whole EBs and SOMCs were subjected to electrophoresis in the presence (A) and absence (B) of 2ME. Lane 1, DTT-treated EBs; lane 2, SOMCs from DTT-treated EBs; lane 3, untreated EBS; lane 4, SOMCs from untreated EBs.

process. As expected, RBs were lysed in distilled water whether DTT was present or not (Table 1).

Significant transport of ATP and GTP and incorporation of [$^{35}$S]methionine into protein were not detected in DTT-treated EBs even though these metabolic activities were readily measured in both treated and untreated RBs (Table 1). We also were unable to demonstrate incorporation of GTP into RNA by DTT-treated EBs of chlamydiae (data not shown). Our failure to detect metabolic activities in host-free EBs may have been due in part to the harvest procedure, which included lysis of host cells (and osmotically unstable forms of chlamydiae) with a nonionic detergent. Purified EBs of *C. trachomatis* obtained by sonic lysis of infected cells with glass beads in a sonic bath transported 83.8 pmol of ATP per mg of protein in the absence of DTT and 107 pmol/mg of protein in the presence of DTT. These values are considerably lower than the 438 pmol of ATP transported by RBs of *C. trachomatis* (calculated from the data in Table 1) and may reflect contamination of the EB preparation with RBs or intermediate forms.

**Attempts to reduce disulfide bonds in host-free chlamydiae.** Treatment of intact host-free EBs of *C. psittaci* with DTT reduced the MOMP to monomers, but had no effect on the cysteine-rich proteins (Fig. 6). Similar treatment of EBs of *C. trachomatis* also reduced the MOMP (data not shown). However, incubation of EBs with 10 mM reduced glutathione, 100 U of glutathione reductase, 1 mM NADH, 1 mM NADPH, 50 μM UTP, 50 μM GTP, 50 μM CTP, 1 mM ATP and an ATP-regenerating system, and a mixture of 20 amino acids, each present at 100 μM both in the presence and the absence of sonic lysates of uninfected cells, failed to reduce the cross-linked outer membrane complexes.

## DISCUSSION

The observations reported here are consistent with the hypothesis that the following events take place during infection of host cells with *C. psittaci*. The outer membrane proteins of extracellular infectious EBs are highly cross-linked, forming a supramacromolecular lattice. Within 1 h of entry of EBs into host cells, the MOMP is reduced to monomers by a process which requires chlamydial protein synthesis but not host protein synthesis. The susceptibility of the cysteine residues of the cysteine-rich 60-kDa doublet and the 12-kDa outer membrane protein to alkylation with iodoacetamide suggests that these proteins may be partially reduced; however, at no time postinfection was it possible to detect monomeric forms of the cysteine-rich proteins in SOMCs of intracellular chlamydiae. By 12 h postinfection, reorganization of EBs to RBs is complete, monomeric MOMP is synthesized and inserted into the outer membrane, and RBs divide by binary fission. The MOMP and cysteine-rich proteins of infecting EBs are not broken down to a significant extent, but are diluted out by RB multiplication. By 20 h postinfection, when intracellular RBs have begun to reorganize into EBs, the cysteine-rich proteins are found as disulfide bond cross-linked complexes in outer membranes. Because only SOMCs were examined, we did not determine at what time in the cycle the cysteine-rich proteins are first synthesized. It is possible that they are synthesized early but are stably incorporated into SOMCs only after 20 h postinfection or that they are not synthesized until 20 h postinfection. Late in the developmental cycle (44 to 48 h), the multiplication of RBs and the reorganization of RBs to EBs is nearly complete, and the outer membranes of intracellular chlamydiae are replete with monomeric MOMP and at least partially cross-linked cysteine-rich proteins. Upon lysis of the host cells and exposure of EBs to an extracellular environment, the MOMP becomes disulfide bond cross-linked (it is also likely that cross-linkage of the cysteine-rich proteins is further consolidated at this time). The mechanism by which the MOMP is cross-linked is not known; however, it is likely that exposure to an oxidizing environment triggers or at least facilitates the process.

It must be emphasized that the topographical relationship of chlamydial outer membrane proteins is not known. The MOMP and the cysteine-rich proteins may each form homopolymers, or they may be cross-linked with each other and other outer membrane proteins to form heteropolymeric complexes.

The requirement of *C. psittaci* protein synthesis for reduction of the MOMP was unexpected in that the reduction process was promptly initiated upon entry of EBs into host cells, yet host-free EBs possess little or no capacity for protein synthesis. The nature of the proteins synthesized is unknown; however, the synthesis of these proteins may represent one of the first metabolic events that occur within intracellular *C. psittaci*. The nature of the natural reductant also is not known; neither reduced glutathione nor NADH plus NADPH promoted reduction of the MOMP complex of host-free EBs.

The status of the MOMP of intracellular *C. trachomatis* serovar L2 was similar to the status of the MOMP of *C. psittaci* in that the MOMP of *C. trachomatis* was present in outer membrane complexes in monomeric form by 24 h postinfection and remained largely monomeric until 48 h postinfection when the cells were lysed, a process that triggered the formation of additional interpeptide disulfide bonds. In contrast to *C. psittaci*, only a fraction of the

cross-linked MOMP of *C. trachomatis* EBs was reduced by 4 h postinfection. This observation may reflect a longer reorganization period for *C. trachomatis* or may indicate that a significant proportion of the *C. trachomatis* EBs which gained entry into host cells were not viable and thus were incapable of reorganization. Also, chloramphenicol failed to inhibit the reduction of the MOMP of *C. trachomatis*. The simplest explanation for this observation is that parasite protein synthesis is not required for the reduction process in *C. trachomatis*; however, it also is possible that protein synthesis by *C. trachomatis* is resistant to chloramphenicol at early stages of the developmental cycle.

The lysis of *C. trachomatis* EBs in a nonisosmotic environment in the presence of DTT reported here confirms the earlier observation of Hackstadt et al. (7) and supports the supposition that one function of the complex is to provide osmotic stability to the extracellular form of the microorganism. It is interesting that the addition of a nonionic detergent following DTT treatment was required for lysis of *C. psittaci* 6BC, an organism which is transmitted from host to host by an aerosol route (as opposed to direct contact for *C. trachomatis*) and which must endure lengthy exposure to extracellular environments. It is conceivable that cross-linked outer membrane complexes are required for the attachment and entry of EBs into host cells; however, the low infectivity of DIT-treated EBs reported here and elsewhere (7) may also be explained by irreversible damage suffered by EBs during their incubation in the presence of DTT.

The demonstration that liposomes prepared with reduced MOMP have porin activity (2) and the observations that treatment of EBs with reducing agents stimulates glutamate oxidation (7) and RNA synthesis (16) suggest that complexes of MOMP, when reduced, may provide channels for the passage of nutrients to specific transport sites in the inner membrane. While we have not investigated glutamate oxidation by chlamydiae, our inability to detect ATP and GTP transport as well as protein and RNA synthesis in DTT-treated EBs obtained by lysis of host cells with nonionic detergent suggests that simple reduction of cross-linked MOMP to monomeric form is not sufficient to induce EBs to a metabolically active state. It is possible that additional intracellular events, such as the chloramphenicol-inhibited events which take place in EBs of *C. psittaci* upon entry into host cells or reduction of the cysteine-rich proteins, are necessary for activation of transport activities. The reason for our failure to reproduce the observations of other investigators is not known but may be related to the different techniques used to obtain EBs. In support of this explanation, we noted that gradient-purified EBs of *C. trachomatis* obtained by mild sonic disruption of infected cells transported ATP about 20% as efficiently as RBs. This activity, which was stimulated about 30% by DTT, may have been due to contaminating RBs or intermediate forms that were not present in EB preparations from detergent-lysed cells.

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## LITERATURE CITED

1. **Barbour, A. G., K.-K. Amano, T. Hackstadt, L. Perry, and H. D. Caldwell.** 1982. *Chlamydia trachomatis* has penicillin-binding proteins but not detectable muramic acid. J. Bacteriol. **151:** 420–428.
2. **Bavoil, P., A. Ohlin, and J. Schachter.** 1984. Role of disulfide binding in outer membrane structure and permability in *Chlamydia trachomatis*. Infect. Immun. **44:**479–485.
3. **Bose, S. K., and R. G. Paul.** 1982. Purification of *Chlamydia trachomatis* lymphogranuloma venereum elementary bodies and their interaction with HeLa cells. J. Gen. Microbiol. **128:**1371–1379.
4. **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.
5. **Friis, R. R.** 1971. Interaction of L cells and *Chlamydia psittaci*: entry of the parasite and host responses to its development. J. Bacteriol. **110:**706–716.
6. **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.
7. **Hackstadt, T., W. J. Todd, and H. D. Caldwell.** 1985. Disulfide-mediated interactions of the chlamydial major outer membrane protein: role in the differentiation of chlamydiae? J. Bacteriol. **161:**25–31.
8. **Hatch, T. P.** 1975. Competition between *Chlamydia psittaci* and L cells for host isoleucine pools: a limiting factor in chlamydial multiplication. Infect. Immun. **12:**211–220.
9. **Hatch, T. P., E. Al-Hossainy, and J. A. Silverman.** 1982. Adenine nucleotide and lysine transport in *Chlamydia psittaci*. J. Bacteriol. **150:**662–670.
10. **Hatch, T. P., I. Allan, and J. H. Pearce.** 1984. Structural and polypeptide difference between envelopes of infective and reproductive life cycle forms of *Chlamydia* spp. J. Bacteriol. **157:** 13–20.
11. **Hatch, T. P., M. Miceli, and J. A. Silverman.** 1985. Synthesis of protein in host-free reticulate bodies of *Chlamydia psittaci* and *Chlamydia trachomatis*. J. Bacteriol. **162:**938–942.
12. **Hatch, T. P., D. W. Vance, and E. Al-Hossainy.** 1981. Identification of a major envelope protein in *Chlamydia* spp. J. Bacteriol. **146:**426–429.
13. **Manire, G. P., and A. Tamura.** 1967. Preparation and chemical composition of the cell walls of mature infectious forms of meningopneumonitis organisms. J. Bacteriol. **94:**1178–1183.
14. **Newall, W. J., V, and R. B. Jones.** 1983. Disulfide-linked oligomers of the major outer membrane protein of chlamydiae. J. Bacteriol. **154:**998–1001.
15. **Salari, S. H., and M. E. Ward.** 1981. Polypeptide composition of *Chlamydia trachomatis*. J. Gen. Microbiol. **123:**1083–1089.
16. **Sarov, I., and Y. Becker.** 1971. Deoxyribonucleic acid-dependent ribonucleic acid polymerase activity in purified trachoma elementary bodies: effect of sodium chloride on ribonucleic acid transcription. J. Bacteriol. **107:**593–598.
17. **Schachter, J., and H. D. Caldwell.** 1980. Chlamydiae. Annu. Rev. Microbiol. **34:**285–309.
18. **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.
19. **Tamura, A., A. Matsumoto, G. P. Manire, and N. Higashi.** 1971. Electron microscopic observation on the structure of envelopes of mature elementary bodies and developmental reticulate forms of *Chlamydia psittaci*. J. Bacteriol. **105:**355–360.