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Cytochemical Localization of Glycogen in *Chlamydia trachomatis* Inclusions

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The origin and distribution of glycogen in inclusions of *Chlamydia trachomatis* were demonstrated with silver proteinate stain for electron microscopy. Glycogen particles were detected in all developmental stages of *C. trachomatis*, as well as free in the inclusions. Intrachlamydial glycogen was most common in elementary bodies but was also detected in intermediate forms and reticulate bodies (RBs). Abnormal divisions and breakdown of cytoplasmic membranes were common in RBs. Cytoplasmic contents, including glycogen particles, were released into the inclusions after rupture of the outer membranes of abnormal RBs and intermediate forms. From these observations, we conclude that glycogen in inclusions of *C. trachomatis* originates in the organisms themselves.

Chlamydia trachomatis is an obligate intracellular bacterium, closely related to gram-negative bacteria, that causes serious ocular and genital diseases in humans and lower mammals. Chlamydiae have a unique life cycle that has two principal forms: (i) elementary bodies (EBs), which are extracellular and dormant but infectious forms, and (ii) reticulate bodies (RBs), which are intracellular, metabolically active, reproductive forms. Chlamydiae reproduce intracellularly in vacuoles called inclusions, which are unusual in that they do not fuse with host cell lysosomes and thus form a protected site for the organisms throughout their reproductive cycle. RBs divide by binary fission and, at the end of the growth stage, transform through intermediate forms (IFs) into EBs, which are released to the exterior after rupture of inclusions and host cells. The infectious EBs are then incorporated into other cells, where they transform into RBs and a new inclusion develops.

As the inclusions of *C. trachomatis* mature, they characteristically contain large accumulations of glycogen. When stained with iodine for light microscopy, this glycogen is an important diagnostic feature that distinguishes *C. trachomatis* infections from those of other *Chlamydia* species. As yet, the origin of glycogen in *C. trachomatis* inclusions has not been definitively identified. Metabolic studies have suggested that the chlamydiae, rather than the host cell, are the likely source of the glycogen (2, 4, 6, 13). Matsumoto, using morphological preparations for transmission electron microscopy (TEM), observed particles resembling glycogen in degenerated bodies that he identified as RBs and proposed that these bodies eventually rupture and release the particles into the inclusion (9).

Since the preservation and morphological appearance of glycogen depend upon specimen preparation for electron microscopy (5, 9, 12), it is important to confirm cytochemically that the particles seen in *C. trachomatis* are glycogen. Thiéry's silver proteinate stain for TEM (12), with appropriate controls, is considered one of the most reliable and specific methods for staining glycogen in thin sections (5). With silver proteinate staining, Lépinay and coworkers (8) detected glycogen in *Chlamydia psittaci* organisms although the inclusions are generally considered free of glycogen. In our study, we used a silver proteinate stain to demonstrate the origins of intrainclusion glycogen in *C. trachomatis*-infected cells and to monitor its distribution throughout the development of the inclusion.

Suspension cultures of mouse L929 fibroblasts in minimum essential medium with Hanks' salts and 5% fetal calf serum were inoculated with the LGV strain (L2/434/Bu) of *C. trachomatis* and incubated in 5% CO_2 at 37°C (3). Samples were taken at 15, 22, 30, and 40 h of incubation. No samples were taken after 40 h of incubation because after that time the host cells began to deteriorate and lyse.

For TEM preparations, we used a glutaraldehyde-acrolein fixation protocol previously shown to produce optimal results in the preservation of chlamydial structure (10). This protocol also preserved glycogen well and provided ideal specimens for morphology as well as for cytochemical localization of glycogen. Specimens were fixed in 2.25% glutaraldehyde–2% acrolein in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) on ice for 2 h, pelleted in a microfuge (Beckman Instruments, Palo Alto, Calif.), postfixed for 2 h at 4°C in 2% osmium tetroxide in the same buffer, stained in block with Kellenberger's uranyl acetate (7) plus 4% sucrose for 2 h at room temperature, dehydrated in ethanols and propylene oxide, and embedded in Epox 812 (10).

Silver proteinate staining. The following technique, based on the method of Thiéry (12), was used to localize glycogen. Thin sections were mounted on carbon-stabilized Parlodion-coated or naked gold grids and stained by inversion on droplets of the following solutions: 1% periodic acid for 20 min (followed by washing in distilled water), 0.2% thiocarbohydrazide in 20% acetic acid for 40 min (followed by washing in descending concentrations of acetic acid and several changes of distilled water), and 1% silver proteinate (Protargol; Fluka Chemical Corp., Hauppauge, N.Y.) for 35 min in the dark (followed by thorough rinsing in distilled water). Stained sections were viewed and photographed the same day, because silver grains coalesce over time (11). Silver-stained sections were viewed without additional staining; sections for morphology were stained with 2% aqueous uranyl acetate for 15 min and Reynolds' lead citrate for 10 min. All specimens were

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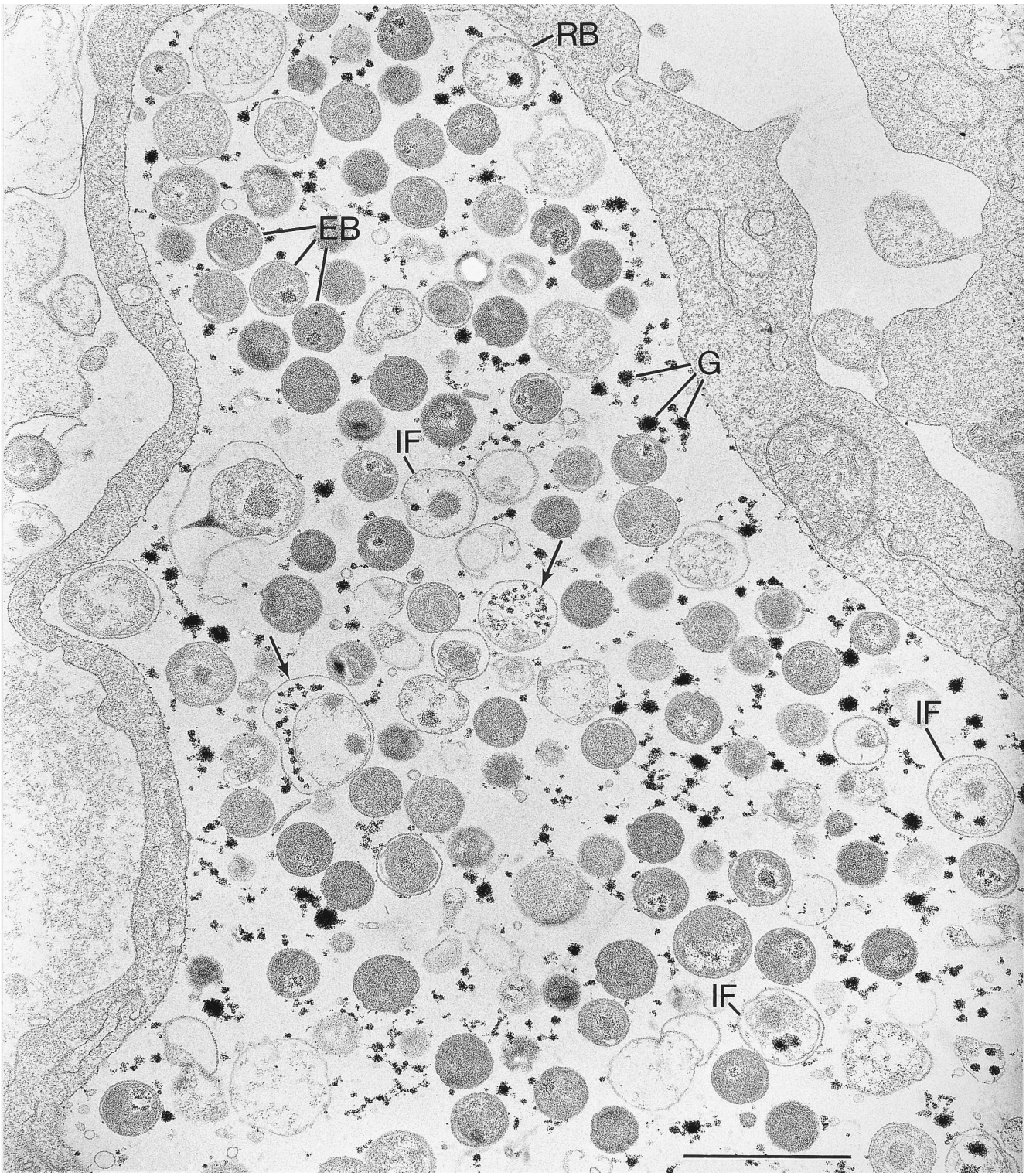
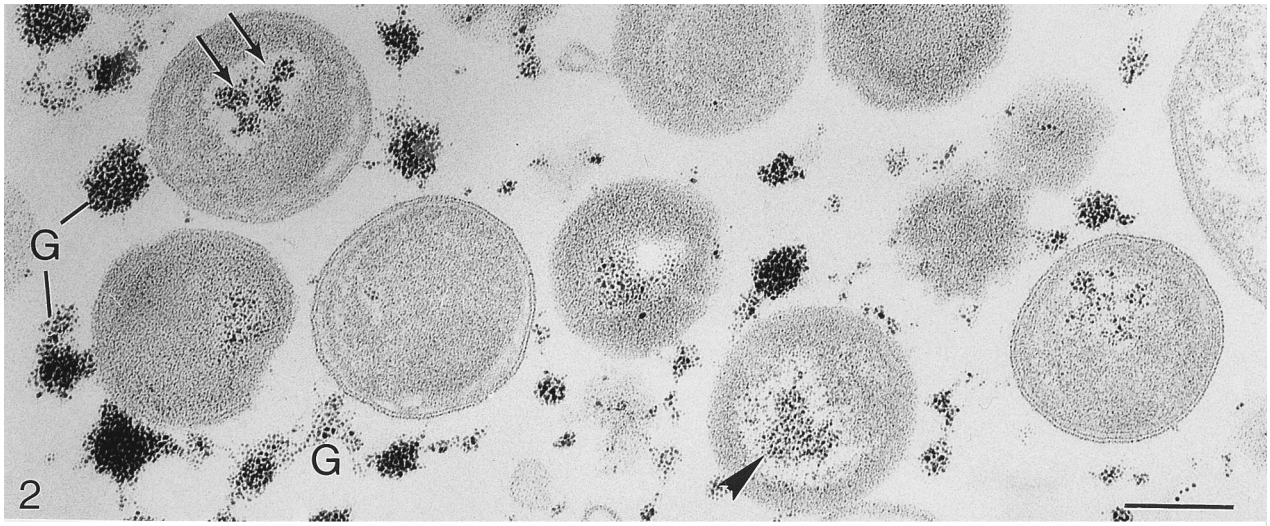


FIG. 1. *C. trachomatis* inclusion after 40 h of incubation. Silver proteinate stain was used. Dense silver deposits mark extracellular glycogen particles (G) and intracellular glycogen particles in EBs, IFs, RBs, and abnormal chlamydial bodies (arrows) in the inclusion. Bar, 1 μm.

examined in either a Siemens Elmiskop 1A or a Zeiss EM 10 CR electron microscope.

As controls, some specimens were digested with amylase before or after embedding in plastic. Pre-embedded specimens were fixed in glutaraldehyde-acrolein as described above to stabilize the ultrastructure before digestion, washed in 0.4 M phosphate buffer (pH 7.4), and digested in 0.5% α-amylase in 0.2 M phosphate buffer (pH 7.4) at 37°C for up to 26 h.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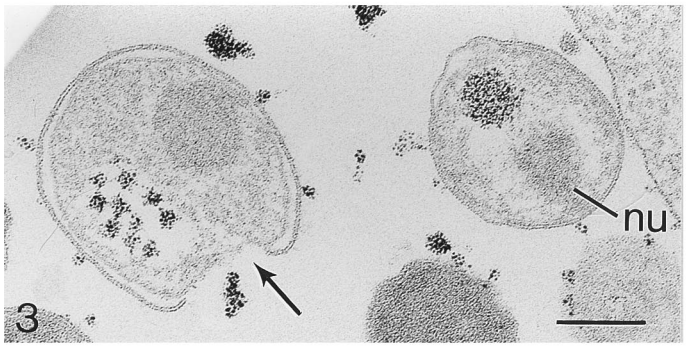
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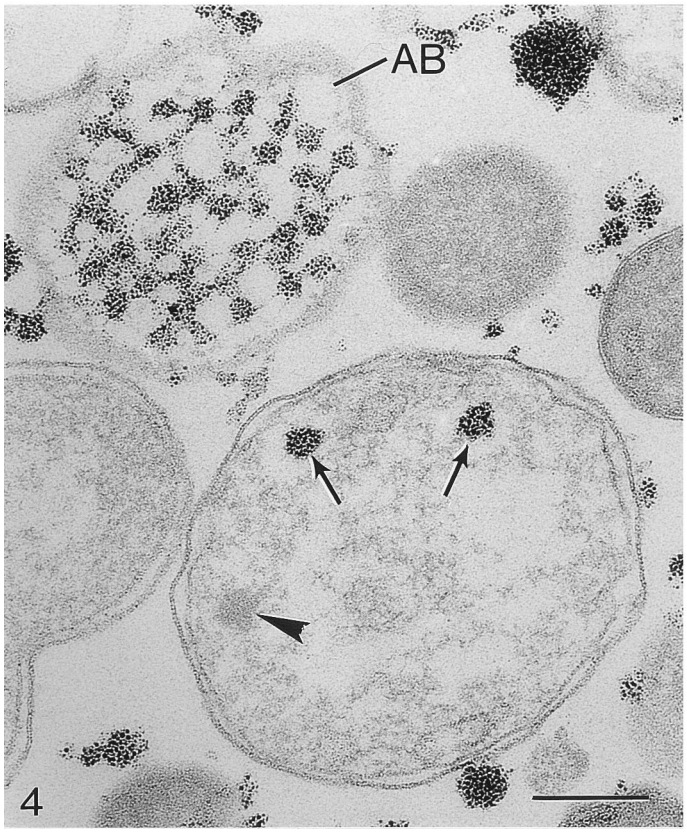
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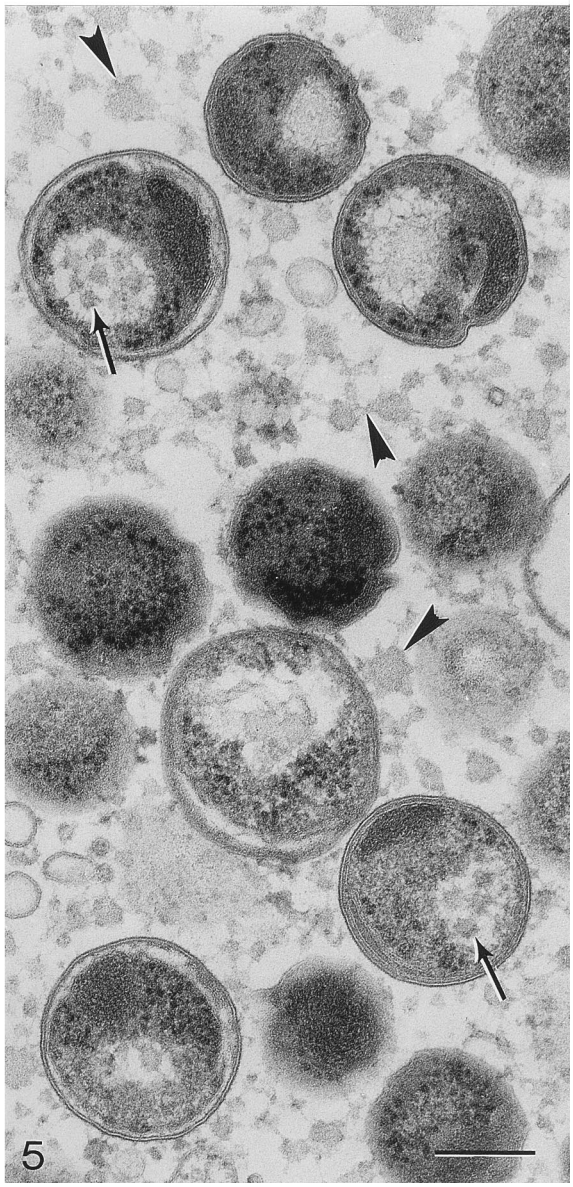
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FIG. 2–5. *C. trachomatis* after 40 h of incubation.

FIG. 2. Silver proteinate stain marks large, single glycogen particles (arrowhead) and small, interconnected glycogen particles (arrows) in EBs. Extracellular glycogen particles (G) in the inclusion are similar in size, configuration, and staining properties. Bar, 200 nm.

FIG. 3. IFs, identified by their condensed nuclear material, or nucleoid (nu), contain single or interconnected glycogen particles stained with silver proteinate. Note that the outer membrane of one IF is incomplete (arrow). Bar, 200 nm.

FIG. 4. An RB contains two glycogen particles (arrows) stained with silver proteinate. A small area of nuclear condensation (arrowhead) indicates that this RB is beginning to transform into an IF. An abnormal body (AB) contains many interconnected glycogen particles. Bar, 200 nm.

FIG. 5. Conventional uranyl acetate and lead citrate stains for TEM show particles in EBs (arrows) as well as extracellular particles (arrowheads) in mature *C. trachomatis* inclusions. These particles cannot be confirmed as glycogen without silver proteinate staining. Note the electron-lucent area surrounding intracellular glycogen particles in both staining preparations (compare Fig. 2 and 5). Bar, 200 nm.

Specimens were then washed three times in phosphate buffer, postfixed in osmium tetroxide, and embedded as described above. For postembedded samples, thin sections were treated with 1% α-amylase for 1 h at 37°C. In control specimens for silver proteinate stain, there was no silver reaction when the periodic acid and/or thiocarbohydrazide conversion step was omitted.

Glycogen in mature inclusions. Silver proteinate stain demonstrated the distribution of glycogen particles in mature inclusions (40 h of incubation) of *C. trachomatis* (Fig. 1). More importantly, it also identified glycogen particles in all developmental stages of *C. trachomatis* organisms in these inclusions (Fig. 1 through 4).

Intrachlamydial glycogen particles were most common in EBs (Fig. 1 and 2). Glycogen particles were also detected in IFs (Fig. 1 and 3) and occasionally in RBs (Fig. 1 and 4). With silver proteinate staining, intrachlamydial glycogen particles ranged in diameter from 20 to 150 nm, were sometimes joined by thin strands giving them a stellate appearance, and closely resembled the free glycogen particles in the inclusions (Fig. 2). Conventional staining for electron microscopy showed comparable extra- and intrachlamydial particles in *C. trachomatis*

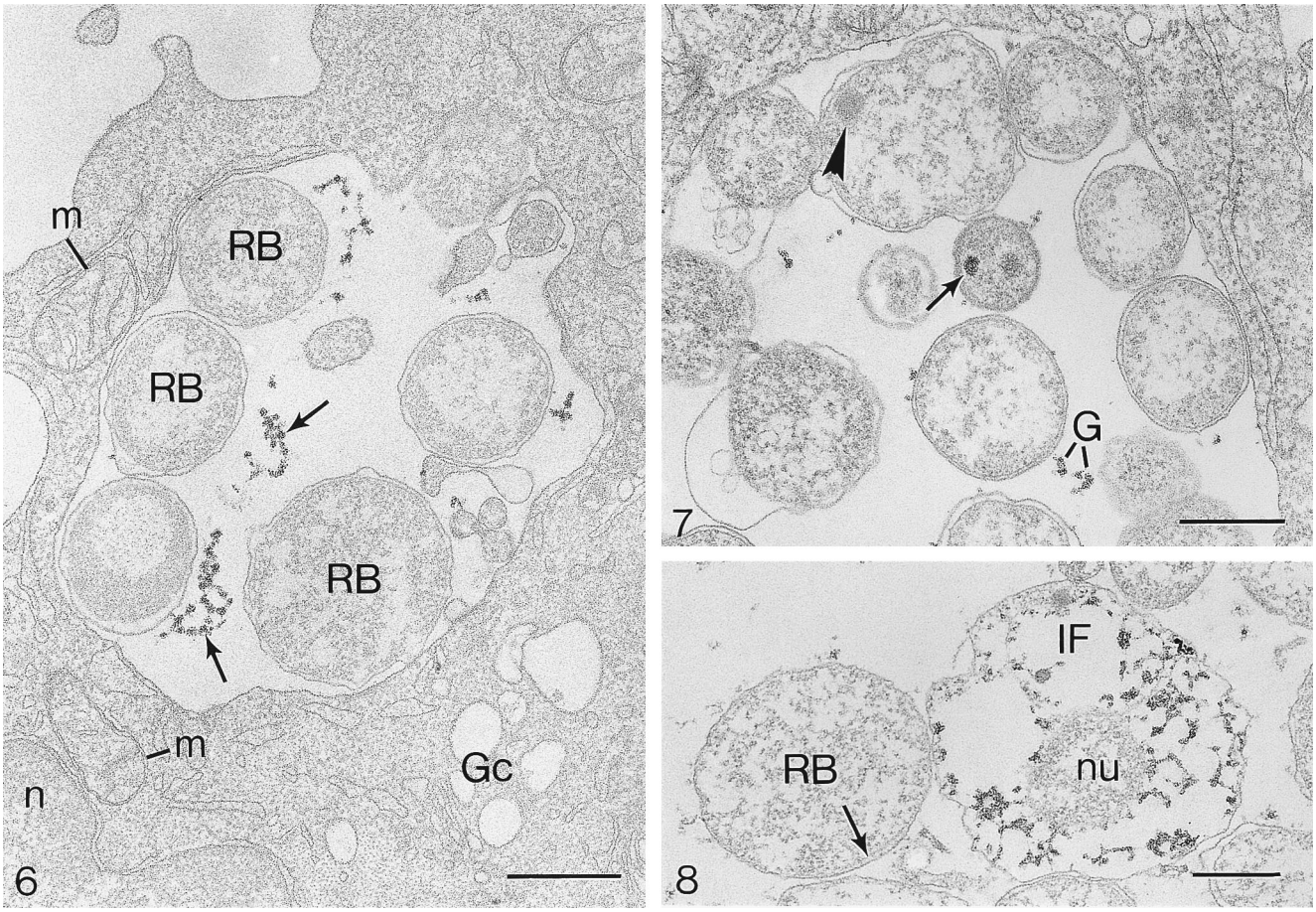

FIG. 6–8. *C. trachomatis* inclusions after 22 h of incubation. Silver proteinate stain was used.

FIG. 6. Silver deposits mark free glycogen particles (arrows) in a small inclusion. Neither RBs nor the host cell cytoplasm is stained. n, nucleus; m, mitochondrion; Gc, Golgi complex. Bar, 500 nm.

FIG. 7. A larger inclusion contains RBs and an IF. One RB (arrowhead) shows beginning nuclear condensation, even as it approaches division; the IF contains a stained glycogen particle (arrow). Free glycogen particles (G) are also in the inclusion. Bar, 500 nm.

FIG. 8. A large, degenerating IF, identified by its nucleoid (nu), contains accumulated silver-stained glycogen particles. Note that the organism has a single membrane. The adjacent RB is also abnormal, with only a small fragment of the cytoplasmic membrane present (arrow). Bar, 500 nm.

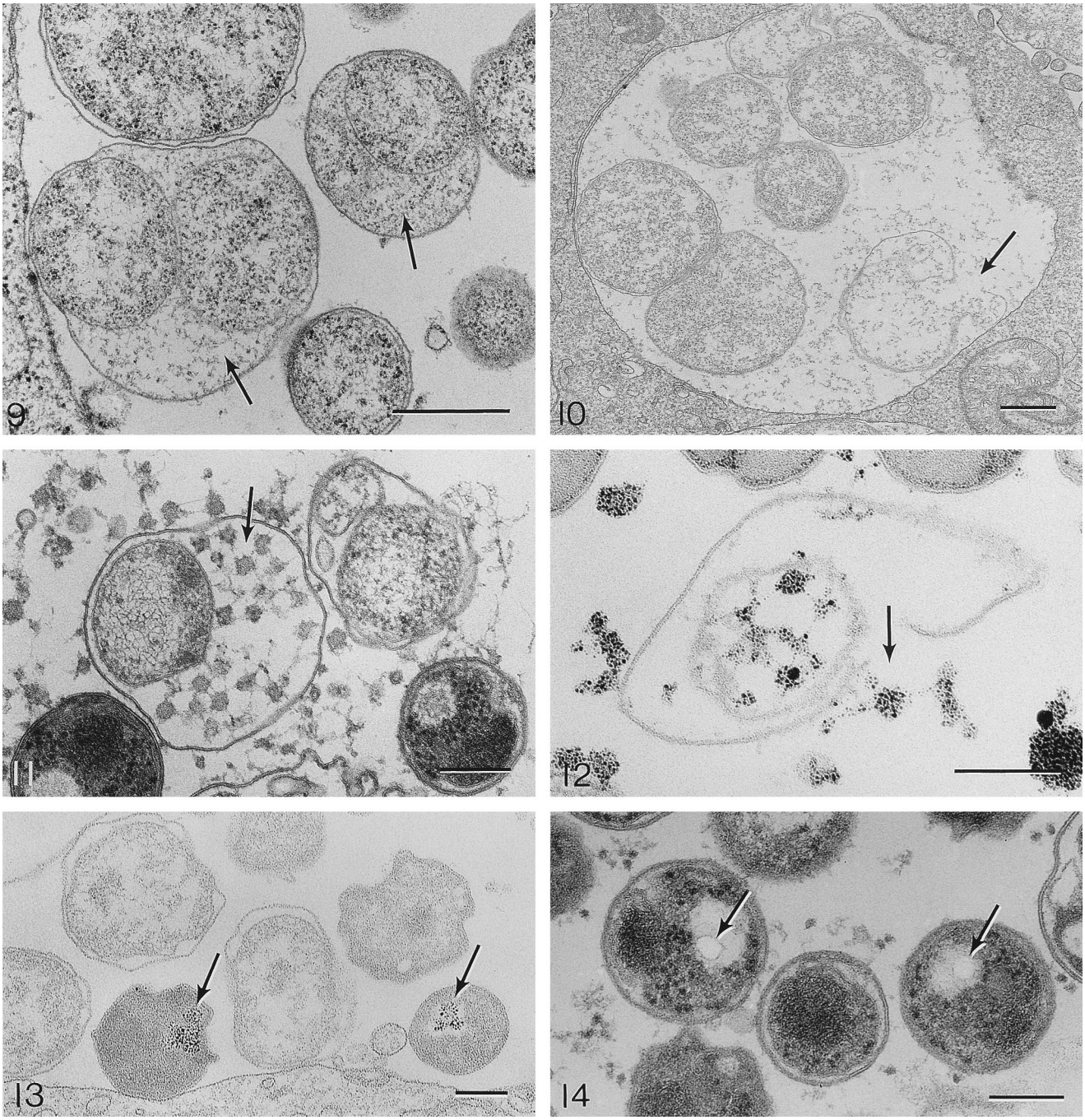

FIG. 9. RBs undergoing abnormal division after 18 h of incubation. Uranyl acetate and lead citrate stains were used. In each organism, the inner membrane of one daughter cell has ruptured, allowing the escape of cytoplasm into the periplasmic space (arrows). Bar, 500 nm.

FIG. 10. An inclusion after 15 h of incubation. Silver proteinate stain was used. The inclusion contains granular material and a ruptured RB with similar material (arrow). However, at this interval of incubation, none of the contents are stained with silver proteinate. Bar, 500 nm.

FIG. 11. An abnormal body after 40 h of incubation, stained with uranyl acetate and lead citrate, contains a number of interconnected, glycogen-like particles (arrow). Bar, 200 nm.

FIG. 12. Silver proteinate stains glycogen accumulations in the remnants of a degenerating cell at 40 h of incubation. A break in the outer membrane (arrow) releases glycogen particles into the inclusion. Bar, 200 nm.

FIG. 13. Specimen (30 h of incubation) fixed and digested with 0.5% amylase for 26 h before embedding. Silver proteinate stain shows that free glycogen particles in the inclusion and those in RBs and IFs are absent, but some glycogen remains in EBs (arrows). Bar, 200 nm.

FIG. 14. EBs (40 h of incubation) treated with 1% amylase for 1 h in thin section and stained with uranyl acetate and lead citrate. Holes in the embedding medium (arrows) indicate where glycogen has been removed. Bar, 200 nm.

inclusions (Fig. 5). With silver proteinate stain, these particles are shown to contain glycogen. At this and all other stages of inclusion development, no glycogen was seen in the cytoplasm of the host cells either morphologically or with silver proteinate staining (Fig. 1 and 6).

Glycogen in developing inclusions. Glycogen was first detected in *C. trachomatis* inclusions with the appearance of IFs (in this study, at 22 h of incubation) (Fig. 6 through 8). Silver stain showed free glycogen particles sparsely scattered in these inclusions (Fig. 6 and 7). Intrachlamydial glycogen particles were rare, but they were seen in some normal (Fig. 7) and degenerating (Fig. 8) IFs. The amount of glycogen gradually increased after 22 h until 40 h of incubation. These findings correspond with those in previous metabolic studies on glycogenesis in *C. trachomatis*-infected cells (4, 6).

Origin of free glycogen in the inclusion. Abnormalities in the division of RBs were common. Often the cytoplasmic or inner membrane of one or both daughter cells was ruptured or degenerated, releasing the cytoplasmic contents into the periplasmic space (Fig. 9). If the outer membrane also ruptured, this material was released into the inclusion (Fig. 10). The high percentage of abnormal and ruptured RBs in developing inclusions of *C. trachomatis* may be due to the fragility of RB membranes. The outer membranes of RBs are not extensively cross-linked like those of EBs and are thus more susceptible to damage by environmental factors (1).

The material released from ruptured RBs after short periods of incubation (before 22 h) was always negative for glycogen with silver proteinate stain (Fig. 10). However, at later stages of incubation, abnormal cells contained accumulations of particles resembling glycogen (Fig. 11) that were positive with silver stain (Fig. 1, 4, and 8) and were released to the exterior after outer membrane rupture (Fig. 3 and 12).

Amylase digestion. Digestion with amylase for 2 h before embedding in plastic completely removed glycogen in the inclusions, RBs, and IFs. However, digestion for up to 26 h only partially removed glycogen from some EBs (Fig. 13). This residual glycogen in EBs may result in part from the fixation period before amylase digestion necessary to preserve the ultrastructure and in part from reduced penetration of the enzyme through the rigid outer membranes of EBs. In samples digested with amylase after embedding, all traces of glycogen, intrachlamydial and free particles, were removed (Fig. 14).

In summary, silver proteinate stain has shown for the first time the distribution of glycogen particles in normal EBs, IFs, and RBs of *C. trachomatis*. Glycogen originates in the organisms themselves and appears to be stored in EBs. Although one cannot rule out the possibility that precursors, unstainable by these cytochemical methods, can be released from RBs and subsequently assembled in the inclusions, we have shown that fully formed glycogen particles are released into the inclusions of *C. trachomatis* from ruptured RBs and IFs.

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REFERENCES

1. **Bavoil, P., A. Ohlin, and J. Schachter.** 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. **44:**479–485.
2. **Becker, Y., P. Mashiah, and H. Bernkopf.** 1962. Biochemical changes in FL cell cultures infected with a trachoma agent. Nature (London) **193:**271–272.
3. **Caldwell, H. D., C. C. Kuo, and G. E. Kenny.** 1975. Antigenic analysis of chlamydiae by two-dimensional immunoelectrophoresis. I. Antigenic heterogeneity between *C. trachomatis* and *C. psittaci*. J. Immunol. **115:**963–968.
4. **Gordon, F. B., and A. L. Quan.** 1965. Occurrence of glycogen in inclusions of the psittacosis-lymphogranuloma venereum-trachoma agents. J. Infect. Dis. **115:**186–196.
5. **Hayat, M. A.** 1989. Principles and techniques of electron microscopy: biological applications, 3rd ed., p. 295. CRC Press, Inc., Boca Raton, Fla.
6. **Jenkin, H. M., and V. S. C. Fan.** 1971. Contrast of glycogenesis of *Chlamydia trachomatis* and *Chlamydia psittaci* strains in HeLa cells, p. 52–59. *In* R. L. Nichols (ed.), Trachoma and related disorders caused by chlamydial agents. Excerpta Medica, Amsterdam.
7. **Kellenberger, E., A. Ryter, and J. Séchaud.** 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4:**671–678.
8. **Lépinay, A., R. Robineaux, J. Orfila, L. Orme-Rosselli, and J. M. Boutry.** 1971. Ultrastructure et cytochimie ultrastructurale des membranes de *Chlamydia psittaci*. Arch. Gesamte Virusforsch. **33:**271–280.
9. **Matsumoto, A.** 1988. Structural characteristics of chlamydial bodies, p. 20–45. *In* A. L. Barron (ed.), Microbiology of chlamydia. CRC Press, Inc., Boca Raton, Fla.
10. **Nichols, B. A., P. Y. Setzer, F. Pang, and C. R. Dawson.** 1985. New view of the surface projections of *Chlamydia trachomatis*. J. Bacteriol. **164:**344–349.
11. **Silva, M. T., and P. M. Macedo.** 1987. Improved Thiéry staining for the ultrastructural detection of polysaccharides. J. Submicrosc. Cytol. **19:**677–681.
12. **Thiéry, J.-P.** 1967. Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J. Microsc. **6:**987–1018.
13. **Weigent, D. A., and H. M. Jenkin.** 1978. Contrast of glycogenesis and protein synthesis in monkey kidney cells and HeLa cells infected with *Chlamydia trachomatis* lymphogranuloma venereum. Infect. Immun. **20:**632–639.