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Interaction of Chlamydiae and Host Cells In Vitro

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INTRODUCTION

One of the most uncomplicated ways to study the behavior of an intracellular parasite is to mix populations of parasites and host cells in vitro and see what happens. Although the variety of interactions is more limited in vitro than in vivo, there are still many possible outcomes of contact between parasite and host cell. These outcomes are determined by a number of variables, many of which can be experimentally manipulated. Since much of the effect of an intracellular parasite on its multicellular host is the sum of its effects on all the infected cells in that host, populations of host cells maintained in vitro and infected with intracellular parasites are valid models of infectious disease. Because intact hosts are infinitely more complicated than populations of a single cell type, the usefulness of such models is limited. The occurrence of a particular host cell-intracellular parasite reaction in vitro raises the possibility that it may also occur in vivo, but other experimental approaches are required to show whether or not it actually does occur.

The genus *Chlamydia* was originally constituted with two species, *Chlamydia trachomatis* and *Chlamydia psittaci* (338). *C. trachomatis* was later subdivided into three biovars—trachoma, lymphogranuloma venereum (LGV), and mouse (314). A third species, *Chlamydia pneumoniae*, has now been proposed (151). In phenotype, it most closely resembles the trachoma biovar (239). *C. pneumoniae* is an important agent of pneumonia in humans, and it has no known reservoir in other mammals or in birds. The degree of DNA relatedness between *C. trachomatis* and *C. psittaci* has been reported to range from less than 10% to almost 30% (94, 132, 226), and *C. pneumoniae* DNA exhibits less than 10% relatedness to the DNA of the other two species (94). However, the monophyletic origin of the genus is unquestionable. The 16S rRNA sequences in *C. trachomatis* biovar LGV and *C. psittaci* 6BC differ from each other by only 5% while at the same time being deeply separated from all other known eubacterial 16S rRNA sequences (503). The 16S rRNA of *C. pneumoniae* closely resembles that of the other two chlamydial species (502a). The 16S rRNA genes of the trachoma and LGV biovars are closely related, and the corresponding genes in *C. trachomatis* biovar mouse and *C. psittaci* strain guinea pig inclusion conjunctivitis (GPIC) share partial but not complete nucleotide sequence homology with the *C. trachomatis* biovar LGV gene (340). All three species have proteins with extensively shared sequences (69, 70, 185, 500, 531, 532). Of the three biovars of *C. trachomatis*, LGV and trachoma exhibit almost 100% DNA homology, but the DNA relatedness of the mouse biovar to the other two biovars is 30 to 60% (132, 510). There is a very high level of sequence homology between proteins of the trachoma and LGV biovars (166, 354, 500, 532). It is a matter of conjecture just when the three chlamydial species diverged, but it must have been a long time ago, perhaps as early as the great radiation of the invertebrate phyla (503).

Practical considerations limit the intensive study of any microbial species to observation of a relatively few strains, so there always remains the unanswered question of how well these often arbitrarily chosen strains represent the species as a whole. This question is more of a problem with *C. psittaci* than with *C. trachomatis*. Of the three biovars of *C. trachomatis*, only biovars trachoma and LGV have been well characterized regarding their interactions with host cells in vitro. The two biovars have been further separated by indirect microimmunofluorescence into 15 often closely related serovars (152, 494, 495). LGV, which infects lymphatic cells to produce the disease for which it is named, exists in three serovars, L1, L2, and L3. The trachoma biovar is a parasite of the squamocolumnar cells of mucous membranes, which it infects by two different modes of transmission (412). Serovars A, B, Ba, and C are associated with trachoma, a follicular conjunctivitis spread by close person-to-person contact, whereas serovars D through K are commonly associated with sexually transmitted infections. There are no nonhuman reservoirs for any of these chlamydial infections. Compared with the trachoma biovar, LGV has relatively few well-characterized strains. By far the most frequently used are strains 434L and 440L, isolated from patients with classical LGV (413). More than a score of trachoma isolates belonging to both the A through C and the D through K groups of serovars have been employed in cell culture investigations. A group of *C. trachomatis* strains once thought to belong to the trachoma biovar and labeled "fast" because they quickly kill chicken embryos after yolk

sac inoculation has been shown to belong to the LGV biovar (serovar L2) (493). They will be referred to as *C. trachomatis* biovar LGV. Biovars trachoma and LGV often differ sharply in their behavior in cell culture, but within each biovar, little variation among the different strains examined has been recorded. Therefore, unless there is some special reason for designating the precise strain used, any strain of serovar A through K will be called biovar trachoma and any strain of serovar L1, L2, and L3 will be called biovar LGV.

C. psittaci is genetically (132) and phenotypically (351, 435, 436) more diverse than *C. trachomatis*. Comparison of 29 strains of *C. psittaci* growing in mouse fibroblasts (L cells) revealed numerous differences in growth rate and other characteristics (435). Strains designated as *C. psittaci* have been isolated from humans and a very large number of avian and mammalian species, in which they produce a broad spectrum of disease (413, 451). However, despite this well-documented heterogeneity, most investigations of the interaction of *C. psittaci* with cells in culture have been conducted with only two strains. They are the 6BC strain, which was isolated from a parrot in 1941 by K. F. Meyer and B. Eddie (details of the isolation were never published), and the meningopneumonitis/Cal 10 (MN/Cal 10) strain, which was isolated in 1936 from ferrets inoculated with throat washings from a suspected human case of influenza (130). The two strains exhibit almost complete DNA homology (94, 132, 226), and with only a few exceptions, their behavior in vitro is virtually identical. How well these strains represent the species as a whole is uncertain. Strain 6BC is atypical in that it is one of the few *C. psittaci* isolates that is sensitive to sulfadiazine (259, 338), and the natural host for MN/Cal 10 is unknown. MN/Cal 10 has all the attributes of an avian strain of *C. psittaci*.

The interaction of chlamydiae with host cells in culture has been studied with only a limited number of established cell lines and primary cell cultures. For *C. psittaci*, the L-929 line of mouse fibroblasts (403) has been by far the most frequently used. In light of the well-known ability of most strains of *C. psittaci* to grow in many different cell types, both in vitro and in vivo, the present picture of *C. psittaci*-host cell interaction would probably have not been changed by using a wider variety of host cells. For *C. trachomatis*, cell lines HeLa 229 (a line derived from a human cervical carcinoma [415]), McCoy (a mouse fibroblast line of uncertain origin [147]), and BHK-1 (a line of diploid hamster fibroblasts [447]) have been widely employed. Most *C. psittaci* strains and the LGV biovar of *C. trachomatis* readily multiply in a variety of cell cultures, but biovar trachoma infects many cell lines only with difficulty (97, 393). In efforts to find host cell populations that more closely resemble the squamocolumnar cells of mucous membranes naturally infected by the trachoma biovar, primary cultures of human amnion (169, 231, 323), human genital epithelium (71, 295, 520), and conjunctival and corneal epithelium (341) have been tried as in vitro hosts for the trachoma biovar. Mouse trophoblasts have also been used (20). These host cell populations are all susceptible to infection, and they may prove to offer advantages over the usual cell lines. Investigations with professional phagocytes have generally employed unelicited mouse peritoneal macrophages. *C. psittaci* enters mouse peritoneal macrophages in the absence of antibody and complement at the same rate as L cells and, with low infecting doses, multiplies well (520). *C. trachomatis* is also ingested by mouse peritoneal macrophages, but once ingested, it grows poorly (234, 535) or not at all (245, 470). The effect of the growth medium on chlamydia-host cell interactions has not been investigated extensively. Eagle's minimal essential medium (116) and medium 199 (296) have been the most frequently used.

Most strains of *C. psittaci* and the LGV and mouse biovars of *C. trachomatis* may be titrated for infectivity by the formation of plaques in L-cell monolayers, but the trachoma biovar cannot (19), probably because it does not initiate secondary cycles of infection (133). However, the infectivity of chlamydiae, the plaque formers included, is most often titrated in terms of inclusion-forming units, i.e., the number of inclusions (number of infected cells) produced under standard conditions (134, 238). For *C. psittaci* MN/Cal 10, the ratio of the number of elementary bodies (EBs) counted by an electron microscopic method of enumeration (419) to plaques produced in L cells is close to unity (131). It is probable, but not proven, that one inclusion-forming unit equals one infectious EB. Therefore, in the interests of simplicity, multiplicities of infection, i.e., the number of infectious units per host cell, will be expressed as EBs per host cell, regardless of how infectivity was measured.

ENTRY

Chlamydiae reproduce by means of a developmental cycle (496) that consists of the alternation of two cell types, EBs and reticulate bodies (RBs). EBs never divide. They are released from infected host cells and enter uninfected ones, where they reorganize into RBs. RBs never infect new host cells (466). Their role is to multiply and eventually transform themselves into a new generation of EBs.

What happens during entry is critical to the fate of both chlamydiae and host cells. Extant entry mechanisms probably appeared relatively late in chlamydial phylogeny, when ancestors of the different kinds of present-day chlamydiae diverged from each other and evolved different relationships with new hosts and new host cells (310, 311). Consequently, concepts of chlamydial entry must allow for the possibility that different species and biovars enter host cells by different mechanisms. All differences aside, there are two indisputable common characteristics of chlamydial entry. First, chlamydiae enter host cells that usually are not actively phagocytic (nonprofessional phagocytes [369]), and second, entry terminates in the appearance of the chlamydial cell in a membrane-bound vacuole (inclusion) in the host cell cytoplasm. Even the incompletely characterized chlamydialike organisms that parasitize the cells of invertebrates have these hallmarks of chlamydial entry (for examples, see references 171, 331, and 421).

Nature of the Chlamydial Cell Surface

It will be helpful to examine some of the properties of the chlamydial cell surface before considering the initial interaction of the chlamydial cell with host cells. Only EBs enter nonprofessional phagocytes at measurable rates (466). RBs of *C. psittaci* MN/Cal 10 are ingested by mouse peritoneal macrophages but are quickly destroyed (50). The entry-promoting property of the EB lies within structures in its cell wall. Cell walls of *C. psittaci* 6BC and MN/Cal 10 prepared by either of two methods (204, 274) attach to and are interiorized by L cells as rapidly as whole EBs (119, 255). EBs of *C. psittaci* MN/Cal 10 enter L cells in the presence of chloramphenicol and rifampin (131, 481) and after the L cells have been inactivated with UV light (313), indicating that the entry-promoting structure is an intrinsic property of the EB

wall and is not dependent on chlamydial macromolecular synthesis at the time of entry.

The surfaces of chlamydial EBs are hydrophobic and negatively charged at neutral pH, with isoelectric points of about pH 5 (25, 416, 431, 486). The major outer membrane proteins (MOMPs) of both *C. trachomatis* biovars have identical isoelectric points (pI = 5.3 to 5.5) (25). Host cells are also negatively charged at pH 7. The negative charges on the surfaces of both host and parasite represent mainly free carboxyl groups. With both interacting surfaces carrying a net negative charge, the question arises as to how chlamydiae ever attach to host cells. It has been suggested that negative chlamydial ligands are neutralized by electrostatic interaction with host ligands, thus leading to the binding of chlamydiae to host cells by powerful van der Waals forces (183). Cations, Ca^{2+} in particular, are required for the attachment of *C. psittaci* 6BC to L cells, and this requirement is abolished when the free carboxyl groups on the L-cell surface are blocked (183). Divalent cations facilitate the binding of LGV to HeLa cells, but they are not absolutely required (429).

Chlamydial cell walls resemble the walls of gram-negative host-independent bacteria with the important exception of having no peptidoglycan (see "Effect of antibacterial agents on conversion of RBs to EBs" below). Both chlamydial cell types contain large and approximately equal amounts of a MOMP with an apparent molecular mass of about 40 kDa. The MOMP of *C. psittaci* is structurally different from the various *C. trachomatis* MOMPs, which in turn differ to a lesser degree from each other, according to serovar and biovar (67). EBs also have lesser amounts of 12-, 59-, and 62-kDa outer membrane proteins exceptionally rich in cysteine residues and occurring in much smaller amounts in RBs (177, 325, 534). Instead of a more conventional network of peptide cross-linkages in the peptidoglycan layer, a network of disulfide bonds among the outer membrane proteins imparts rigidity to the chlamydial envelope (177, 324). The extent of disulfide bond cross-linking in the *C. psittaci* 6BC RB is less than in the EB (177). There is also some evidence that the MOMP of *C. psittaci* 6BC is more highly cross-linked than that of *C. trachomatis* (177, 324). EB surfaces have patches of regularly spaced hemispheric projections that are specializations of the plasma membrane (154, 282, 327). There is some evidence that RBs also have comparable surface projections (279).

Kinetics of Attachment and Ingestion

Association of EBs with host cells has been monitored by measuring changes in free and cell-associated infectious units by counting inclusions (51, 241, 246, 468) or plaques (131, 188, 189) and by measuring the binding of chlamydiae intrinsically labeled with radioactive amino acids to host cells (29, 131, 192, 238, 246, 497). Entry of chlamydiae into host cells has been divided into attachment and ingestion stages (368) by treating host cells that have been inoculated with chlamydiae with trypsin or other proteinases (51, 52, 193, 433, 497). Attached but not ingested EBs are dissociated from host cells, and only the internalized ones remain. With *C. psittaci* 6BC, rates of attachment are about the same in suspension or monolayer culture (62). With the trachoma biovar of *C. trachomatis*, attachment is more efficient in suspension than in monolayer (246), but almost all experiments with both trachoma and LGV biovars have been done in monolayer cultures.

The rate and extent of association (sum of attached and ingested chlamydiae) of *C. psittaci* and the trachoma and LGV biovars of *C. trachomatis* with host cells are proportional to the multiplicity of infection over a very wide range (45, 52, 62, 131, 189, 238, 246, 433, 468, 497). There is disagreement concerning the extent to which *C. psittaci* MN/Cal 10 attaches to L cells at 4°C. The ratio between binding at 4°C to that at 37°C has been reported to be only 1:20 (131) or almost 1:1 (192). The explanation may lie in different experimental conditions. *C. trachomatis* significantly binds to host cells at a low temperature. At 0°C, the attachment of biovar LGV to HeLa cells is 12% as much as at 37°C (238); with McCoy cells, binding at the two temperatures is almost identical (433). For the trachoma biovar, attachment to either of these host cells at 0 to 4°C is 30 to 50% of that at 35 to 37°C (192, 238, 246). The fraction of attached *C. trachomatis* EBs that is subsequently ingested varies from 10% to almost 100% (238, 246, 433). There is little ingestion of attached *C. psittaci* MN/Cal 10 or *C. trachomatis* biovars trachoma and LGV at 0 to 4°C, but when the chlamydia-host cell complex is warmed to 37°C, ingestion rapidly occurs (192, 193, 497).

At low multiplicities of infection (10 to 50 EBs per host cell), attachment of *C. psittaci* 6BC to L cells is quickly followed by ingestion, so that after 60 min at 37°C, almost all the attached EBs have been internalized (52). With inocula of 500 to 5,000 EBs per L cell, the initial rates of attachment and ingestion increase with multiplicity of infection, but attachment stops before all the free EBs are bound, and ingestion ceases before all the bound chlamydiae have been internalized. This cessation in attachment and entry is most likely due to injury to the L cells incurred when they ingest large numbers of EBs (see "Destruction of Host Cells" below) rather than to saturation of the available binding sites on the L-cell surface. Attachment of the trachoma biovar of *C. trachomatis* to McCoy cells also stops while fully infectious EBs remain unassociated with host cells (246, 434). However, this occurs at such low multiplicities of infection that damage to the McCoy cells by previously attached biovar trachoma EBs is not a plausible explanation.

Precise comparisons cannot be made of the extent and rate of association between host cells, *C. psittaci* 6BC and MN/Cal 10, and *C. trachomatis* biovars trachoma and LGV because of the various conditions for measurement employed in different laboratories. However, even though it is much easier to produce visible inclusions in host cells with either biovar LGV or *C. psittaci* than with biovar trachoma, the available data reveal no differences between species and biovars that are distinctly greater than differences reported for the same strain by different investigators. The fraction of a purified, radioactively labeled chlamydial population that associates with host cells under optimal conditions is a rough, imperfect measure of extent of association. This fraction is about 80% for *C. psittaci* 6BC (51, 193), 10 to 50% for biovar LGV (29, 45, 238, 433), and 10 to 30% for biovar trachoma (192, 238, 433, 434). Rates of association are also not markedly different. Depending on the multiplicity of infection and other variables, EBs of *C. psittaci* MN/Cal 10 and 6BC and *C. trachomatis* biovars trachoma and LGV bind to host cells in a linear fashion for 1 to 3 h, after which the rate of association declines (29, 51, 52, 62, 131, 238, 433, 434, 497). Although L cells are not generally regarded as active phagocytes, they take up *C. psittaci* 6BC at a rate and to an extent that is 10 to 100 times those observed for *Escherichia coli* or polystyrene latex spheres (62). Comparable data are not available for other chlamydia-host cell systems.

Modification of Entry

Of all the stages in the developmental cycle, entry is by far the easiest to modify. Investigators have sought to alter the course of chlamydial entry in hopes of learning more about the event itself and improving the sensitivity of cell culture methods for diagnosing infections with the trachoma biovar.

Centrifugation. Most cell culture procedures for identification of the trachoma biovar in clinical specimens (101) involve centrifugation of the inoculum onto the host cell monolayer with a force of approximately 1,000 × *g* (508). Increases in the percentage of infected cells as measured by inclusion counts, sometimes as much as 100- to 200-fold, have been consistently reported (100, 147, 148, 208, 374, 395, 515). Although the value of centrifugation in clinical diagnosis is undisputed, how it exerts its effect is poorly understood. Centrifugation-enhanced infection of monolayers is not unique to chlamydiae. The infection of cell cultures with *Rickettsia prowazekii* (508), *Rickettsia conorii* (352), *Coxiella burnetii* (508), *Yersinia enterocolitica* (488), *E. coli* (488), and *Shigella flexneri* (404) is also increased by centrifugation. The most frequently offered explanation is that centrifugation enhances parasite-host cell contact. Within the genus *Chlamydia*, the effect of centrifugation is minimal with the LGV biovar of *C. trachomatis* and with the 6BC strain of *C. psittaci* (246). It is most pronounced with the trachoma biovar and with other *C. psittaci* strains such as those causing GPIC (365), sheep abortion (435, 436), and calf polyarthritis (435, 436). Quantitative comparison of centrifugation effects is not possible because of varied experimental conditions and because it has not been generally realized that the 12-mm flat-bottomed vials widely used as cell culture vessels in centrifugation-aided infection provide a very unfavorable environment for unaided infection, even with a centrifugation-indifferent strain such as *C. psittaci* 6BC (246).

For at least one centrifugation-sensitive organism, *C. psittaci* GPIC, explanation on the basis of increased contact is not enough (365). When McCoy cells were infected with GPIC and biovar LGV (centrifugation indifferent), approximately equal numbers of the two kinds of EBs became host cell associated and equal fractions were ingested whether or not the inocula were centrifuged onto the McCoy cell monolayers, but the monolayers inoculated with GPIC had some 15-fold-fewer inclusion-bearing cells when the centrifugation step was omitted. This suggests that centrifugation overcomes some impediment to infection with GPIC, not at entry, but at some point later in the developmental cycle. As will be discussed below in "Interaction of *C. trachomatis* and other strains of *C. psittaci* with lysosomes", GPIC EBs that have entered host cells with the aid of centrifugation are less likely to fuse with lysosomes than are those that have been ingested in its absence (366). Whether or not other centrifugation-sensitive chlamydiae respond similarly is not known. For further discussion of the effect of centrifugation on the relation between chlamydiae and host cells, see reference 344.

Alteration of chlamydial and host cell surfaces with physical and chemical agents. The effect of treatment with a variety of physical and chemical agents on the binding of chlamydiae to host cells has been studied with the aim of identifying the chemical structures responsible for this association. As has been emphasized elsewhere (183), the effect of such treatments must be treated with caution. Reduction in attachment after exposure to a reagent specific for a structure suspected of playing a role in the attachment process does not necessarily mean that the target structure has been destroyed or blocked. Conversely, a negative result does not necessarily mean that the target structure is not involved.

Attachment of EBs of *C. psittaci* 6BC to L cells is not altered by treating the EBs with proteases and detergents, but it is drastically reduced by gentle heating (as little as 3 min at 60°C) (51, 183). Oxidation of *C. psittaci* 6BC with periodate and acetylation with acetic anhydride also reduce attachment (183), a fact that points to a role for carbohydrates and amino groups on the chlamydial surface. Destruction of attachment capacity by mild heating suggests protein denaturation, but resistance to proteases does not support the suggestion.

Mild heating of *C. trachomatis* biovar LGV reduces its attachment to L and HeLa cells (45, 62, 238). Heating inhibits the attachment of biovar trachoma serovar B to HeLa cells (238), but it does not affect the association of a serovar A strain to McCoy cells (246). Trypsin has no effect on EBs of biovar LGV (45, 160, 454), but it drastically curtails attachment and infectivity of a biovar trachoma serovar B strain for HeLa cells (454). This differential effect of trypsin probably reflects the way in which it cleaves the MOMPs of the two biovars (454). The MOMP genes for *C. trachomatis* serovars B and L2 code for amino acid sequences that are more than 90% homologous (18, 442). The nonhomologous amino acid residues are contained in four variable domains. The biovar LGV MOMP is cleaved at a single site in variable domain IV, whereas the biovar trachoma MOMP is split at one site in variable domain II and one site in IV that is different from the biovar LGV site in that domain. These results suggest that, although a totally intact MOMP may not be required for attachment, tryptic cleavage at some sites is deleterious to the entry process. They also provide a good example of why modification experiments must be interpreted carefully. It would be interesting to know if the *C. psittaci* MOMP, like that of biovar LGV, is split by trypsin at an attachment-indifferent site.

The effects of alterations in the host cell surface on chlamydial attachment have also been studied. Incubation of L cells with as little as 10 μg of trypsin per ml reduces attachment of *C. psittaci* 6BC by as much as 80% (51, 62, 183). Other proteases act similarly. Once trypsin is removed, the trypsin-cleaved structures are regenerated in a few hours, and the regeneration is blocked by cycloheximide (51). These results implicate trypsin-sensitive host proteins in the attachment of *C. psittaci* to L cells, but the relation is not necessarily specific. Trypsin also inhibits the binding of polystyrene latex spheres by L cells (62) and the recognition of migration-inhibiting factor (102) and complement (256) by macrophages. Pretreatment of HeLa or L cells with trypsin and other proteases also interferes with the attachment of *C. trachomatis* biovar LGV (45, 62). Oxidation and acetylation of L cells inhibits the binding of *C. psittaci* 6BC by L cells (183), again implicating proteins (possibly glycoproteins) in the *C. psittaci*-host cell interaction.

Although pretreatment of host cells with the polyanions heparin and dextran sulfate uniformly inhibits chlamydial attachment and inclusion formation (29, 45, 239, 241), the effect of pretreatment with polycations such as DEAE-dextran (DEAE-D) depends on the particular chlamydial strain used for infection. Cultures of HeLa or McCoy cells treated with DEAE-D before infection with the trachoma biovar produce 10 to 100 times more inclusions than untreated cultures (239, 241, 242, 246, 393, 394). Attachment of labeled *C. trachomatis* biovar trachoma EBs to DEAE-D-

treated host cells is also increased (47, 239, 431). In contrast, when DEAE-D-treated host cells are inoculated with *C. trachomatis* biovar LGV, inclusion formation and attachment are either unaffected or slightly decreased (45, 239, 242, 431). This difference holds for a number of different trachoma and LGV strains. The DEAE-D effect was first observed in a strain of *C. psittaci* isolated from sheep (170). Later work separated strains from sheep and cattle into serotypes 1 and 2 (409, 410). Treatment of L cells with DEAE-D increases the number of infected cells when they are infected with *C. psittaci* serotype 2 but not when they are infected with serotype 1 (435, 436). As with centrifugation, the infection-promoting action of DEAE-D is not limited to chlamydiae. It also facilitates infection of cell cultures with several viruses (for a review, see reference 170). The DEAE residues on DEAE-D probably modify the surface charge of host cells so that they offer more attractive targets for the binding ligands of some chlamydiae but not of others, although the possibility that the uncharged dextran moiety plays a role cannot be ignored (170, 431). What this alteration might be is not known. Equally a mystery is why DEAE-D promotes infection with some chlamydiae and not others. There is little or no difference in isoelectric points among the chlamydial species and biovars, but there are variations in masses and isoelectric points of some of the minor outer membrane proteins of biovars trachoma and LGV (25). Other evidence also suggests that the charge distribution may be different in the two biovars (431). Whether these differences are enough to account for the sharp difference in DEAE-D effect remains to be seen. The basis for the differential response of *C. psittaci* strains has not been investigated. It may be that the evolutionary distance between *C. trachomatis* and *C. psittaci* is so great that there is no single explanation for the infection-promoting effect of DEAE-D. A final unanswered question is why the inhibitory effect of dextran sulfate and the stimulatory effect of DEAE-D are both abolished when the chlamydial inoculum is centrifuged on the host cell monolayer (246, 431).

Modification of carbohydrate side chains on the host cell surface. The carbohydrate side chains of the plasma membrane glycolipids and glycoproteins have been implicated as possible attachment sites of *C. trachomatis*. *N*-Acetylneuraminic acid (sialic acid) usually occurs at the end of carbohydrate side chains in membrane gangliosides (for a review, see reference 390). Its free carboxyl group is largely responsible for the net negative charge of the host cell surface. Bacterial neuraminidase (sialidase), which splits off *N*-acetylneuraminic acid from the oligosaccharide side chain, has been reported to reduce the ability of HeLa 229 cells to attach labeled *C. trachomatis* biovar trachoma EBs and to suppress inclusion formation (47, 241). However, other investigators have not observed inhibition of attachment of the trachoma biovar to either HeLa or McCoy cells treated with the enzyme (6, 218). There is agreement that pretreatment of host cells with neuraminidase does not alter their interaction with the LGV biovar (6, 47, 218, 241). *N*-Acetylneuraminic acid has been reported to inhibit (6) or not inhibit (47, 433) the attachment of *C. trachomatis* biovar LGV to HeLa and McCoy cells. These irregular results, together with the observation that cultures of mosquito cells that have no detectable *N*-acetylneuraminic acid residues bind biovar trachoma EBs just as well as McCoy cells do (6), suggest that *N*-acetylneuraminic acid residues on the host cell surface are not required for attachment of chlamydiae.

Like *N*-acetylneuraminic acid, *N*-acetylglucosamine has been implicated as a possible binding site for *C. trachomatis*, and once again the evidence is not strong. *N*-Acetylglucosamine occurs terminally on complex oligosaccharides bound covalently to membrane glycolipids. The lectin wheat germ agglutinin binds specifically to these *N*-acetylglucosamine residues (146). Pretreatment of L cells with wheat germ agglutinin has been reported to inhibit (254) or not to inhibit (183) the attachment of *C. psittaci* 6BC to L cells. Short polymers of *N*-acetylglucosamine (chitobiose, chitotriose, etc.) are said either to inhibit (433) or not to inhibit (6) the association of labeled *C. trachomatis* biovar LGV EBs with McCoy cells. The effect of wheat germ agglutinin is strongly influenced by the conditions under which it reacts with host cells. This lectin reduces the association of *C. trachomatis* biovar LGV with McCoy cells at 0 but not at 37°C (433). Adherence of the LGV biovar to HeLa and McCoy cells is enhanced by pretreatment with wheat germ agglutinin, but only when the chlamydiae have just been passaged in HeLa cells (42). Finally, a mutant line of BHK-21 cells without terminal *N*-acetylglucosamine residues binds labeled biovar LGV EBs just as well as wild-type BHK-21 cells (6). As with *N*-acetylneuraminic acid, terminal *N*-acetylglucosamine residues on host cell surfaces do not seem to be required for attachment of chlamydiae.

Modification with antibody and complement. It has been known since the early days of chlamydial research that antibody raised in chickens and rabbits with crude preparations of infectious chlamydiae neutralized the infectivity of these organisms for mice and chicken embryo yolk sac in a strain-specific manner. With the advent of modern methods of chlamydial investigation, it became possible to ask which antigens give rise to neutralizing antibodies and at what point in the developmental cycle these antibodies act. The ability of *C. trachomatis* biovars trachoma and LGV to form inclusions in cultured cells is neutralized in the presence of complement by polyclonal antibodies against intact chlamydiae and by monoclonal antibodies against MOMP (68, 198, 263, 345, 356, 454). Polyclonal antibody raised against EBs blocks attachment and ingestion of *C. trachomatis* biovar LGV in HeLa cells (497, 514) and *C. psittaci* 6BC in L cells (62). Monoclonal antibodies to epitopes in variable regions II and IV of the MOMP inhibit attachment of *C. trachomatis* biovars LGV and trachoma to HeLa cells (454). Other antibodies to MOMP, both polyclonal (68) and monoclonal (345), neutralize the infectivity of the two *C. trachomatis* biovars without preventing attachment. It appears that different antibodies may neutralize chlamydial infectivity at different times in the developmental cycle.

Identification of Receptors on Chlamydial and Host Cell Surfaces

Chlamydial proteins that bind to host cells have been identified by separating chlamydial polypeptides by electrophoresis and reacting them with surface-iodinated host cell extracts or isolated host cell membranes. In one investigation, a number of biovar LGV and trachoma strains of *C. trachomatis* displayed two proteins with apparent molecular masses of 18 and 32 kDa that bound to extracts of HeLa cells (514), whereas two *C. psittaci* strains had only a single binding component of 17- to 19-kDa (159). A second study revealed that single biovar LGV and trachoma strains have 18- and 31-kDa proteins that bind to membranes from either HeLa or McCoy cells (514) and that one *C. psittaci* strain (MN/Cal 10) has 16- and 30-kDa proteins that are bound by HeLa cells (513). The gene encoding the 31-kDa protein has

been cloned and expressed in *E. coli* (219). These putative chlamydial adhesins have many of the properties to be expected of ligands that attach chlamydiae to their host cells. They are found in isolated cell wall fractions (514). They appear only on infectious EBs and not on noninfectious RBs (159, 514), and the 18-kDa protein has been shown to be surface exposed (150). Heparin, already identified as an inhibitor of attachment, is also bound by the 18- and 32-kDa proteins (159). Polyclonal antibody prepared against the binding proteins from *C. trachomatis* biovar LGV inhibits both attachment and inclusion formation with biovar LGV and HeLa cells (514). Antiserum to the recombinant 31-kDa protein also neutralizes chlamydial infectivity (219). It is possible that the differences in entry behavior among the chlamydial species and biovars reflects, at least in part, slight differences in the chlamydial binding proteins. The differences between *C. psittaci* and *C. trachomatis* have already been pointed out. In addition, the several *C. trachomatis* serovars tested differed slightly in the apparent molecular weights of their 32-kDa binding proteins (159).

The MOMP must be added to the list of putative adhesins. By sheer abundance alone, it demands consideration. More persuasive reasons are the presence in variable domains II and IV of attachment-sensitive trypsin cleavage sites (455) and epitopes for attachment-inhibiting monoclonal antibodies (454). It has been suggested that the MOMP functions as a chlamydial adhesin by promoting electrostatic and hydrophobic bonding with host cells (454). Since only EBs bind to host cells, it must be assumed that the binding sites in variable domains II and IV are functional only when the MOMP is cross-linked with disulfide bonds.

Surface-iodinated cells from a number of cell lines of mouse, monkey, and human origin all bind the 18- and 31-kDa proteins from both *C. trachomatis* biovars and the 16- and 29-kDa proteins from *C. psittaci* MN/Cal 10 (218). The association of the two biovar LGV binding proteins to HeLa cells was abolished by pretreatment of the host cells with trypsin (218), just as it is with whole EBs. When the binding of two serovars of biovar trachoma and one serovar of biovar LGV to HeLa and McCoy cells was measured at 4°C by use of serovar-specific biotinylated monoclonal antibodies, the binding of any one serovar was competitively inhibited by the others (490). Chlamydiae heated at 56°C for 5 min or treated with trypsin failed to compete. These results suggest that a common receptor for heat-labile, trypsin-sensitive moieties on the chlamydial surface is widely distributed on host cells.

Ingestion

In the ingestion of a chlamydial EB, the host cell expends oxidative and glycolytic energy, but the EB neither expends energy nor synthesizes protein. Ingestion is blocked by fluoride, cyanide, and dinitrophenol (131, 238), which must act by inhibiting energy metabolism of the host cell because the EB has none to inhibit (see "Energy metabolism" below), but chloramphenicol, which shuts down protein synthesis in *C. psittaci* MN/Cal 10 (481), has no effect on ingestion (131, 481). Thin-section electron micrographs of host cells prepared immediately after exposure to chlamydiae show EBs with their cell envelopes in close contact with the plasma membrane of the host cell, which is often invaginated at the point of contact (12, 13, 29, 131, 187, 189, 245). In electron micrographs made 2 to 4 h later, many of the *C. psittaci* 6BC and MN/Cal 10 EBs have been ingested and appear in the host cell cytoplasm as single unchanged EBs within compact membrane-bound vacuoles (inclusions) (131, 187, 193, 251, 261). Soon after ingestion, this picture also holds for *C. trachomatis*, but later in the developmental cycle, some of the *C. trachomatis* inclusions fuse with each other (36, 40, 72, 193, 233, 384), an event that does not regularly happen with *C. psittaci*. *C. pneumoniae* multiplies in host cells by means of a developmental cycle like that of other chlamydiae, but its mode of attachment and ingestion is unusual (82, 237). EBs of *C. pneumoniae* are typically pear shaped, not spherical like those of other chlamydiae. They preferentially first attach to host cells by the pointed end and then bind to other parts of the host cell surface by means of cell wall protrusions. The host cell is invaginated, and the *C. pneumoniae* EBs are internalized in vacuolated inclusions.

EBs of *C. psittaci* are preferentially bound to the microvilli of host cells. This binding was first observed in the intestinal epithelial cells of calves (115) and later in L cells, in which the attached EBs of *C. psittaci* MN/Cal 10 are then transported to the bases of the microvilli, where they enter the host cells by way of invaginated pits in the plasma membrane (193). Microfilaments are not visible at the site of ingestion, and the EBs are not sequentially engulfed by pseudopodia. The entry of *C. psittaci* MN/Cal 10 into L cells via coated pits resembles the internalization of colloidal gold-labeled α_2-macroglobulin, a marker for endocytosis in clathrin-coated vesicles (113). When L cells are simultaneously exposed to gold-labeled α_2-macroglobulin and EBs, they often appear together in coated pits and cytoplasmic vacuoles. Although clathrin has yet to be unequivocally identified in the pits containing attached chlamydiae, ingestion of *C. psittaci* MN/Cal 10 by L cells most likely occurs by receptor-mediated endocytosis (the clathrin-coated vesicle pathway; reviewed in references 402 and 428) rather than by conventional phagocytosis (reviewed in reference 428).

It is not clear whether *C. trachomatis* enters host cells by means of microfilament-dependent phagocytosis or receptor-mediated endocytosis or indeed whether both pathways operate. In a study of the ingestion of the LGV biovar by HeLa cells (497), EBs entered host cells in vacuoles that were not clathrin-coated pits, although coated pits and vesicles were seen in adjacent portions of host cells. Cytochalasin D, a powerful inhibitor of microfilament function (75), markedly interfered with the uptake of EBs of biovar LGV. Vinblastin, an inhibitor of microtubule function, also interfered with ingestion, but inhibitors of receptor-mediated endocytosis such as monodansylcadaverine did not. However, a moderate reduction in ingestion of biovar LGV and trachoma EBs by monodansylcadaverine has been reported by others (432). Hyperimmune rabbit serum prevented ingestion of already attached antibody (497). These results point to microfilament-dependent phagocytosis as the mode of ingestion. However, a comparable investigation with the trachoma biovar and McCoy cells is best interpreted in terms of receptor-mediated endocytosis (192). *C. trachomatis* biovar trachoma EBs bound to microvilli and to coated pits at the bases of the microvilli and on the smooth surfaces of the host cells, after which they were ingested in coated vesicles. Immunogold labeling with antibody to clathrin showed that the gold marker was localized in chlamydia-containing pits and vesicles.

These different conclusions were reached with different experimental techniques, different host cells, and different biovars of *C. trachomatis*. Any or all of these variables could account for the conflicting pictures of *C. trachomatis* ingestion. One variable that does make a difference is the orientation of the host cell on its substrate. The natural host cells

for the trachoma biovar are the epithelial cells of the genital tract and conjunctiva. Like all epithelial cells that line body cavities, these cells are polarized; that is, one specialized portion of the plasma membrane, the apical domain, faces the lumen and engages in transport activities, and another specialized area anchors the cell to the collagen-rich basement membrane (117, 387). Hormonally maintained (estrogen or estrogen plus progesterone) primary cultures of human endometrial glands grow in polarized orientation on collagen-coated plates and in unpolarized arrays on plastic (522). When *C. trachomatis* biovar trachoma was inoculated onto the apical surfaces of polarized cells, many more EBs were observed attached in coated pits and vesicles than when it was inoculated onto the unpolarized cell sheets. Polarized sheets of HeLa cells could also be used if the cultures were obtained while they were still monolayers. Again, more EBs were seen in coated pits and vesicles after infection of polarized HeLa cell cultures than after infection of unpolarized ones. This demonstration that the mode of chlamydial entry may be influenced by the physical orientation of the host cell should encourage continued efforts to reproduce more closely in vivo conditions in cell culture models.

Role for Cyclic Nucleotides and Ca^{2+} in Entry?

Because the intracellular concentrations of cyclic AMP (cAMP) and cGMP, which respond to a variety of external stimuli, are known to regulate many cell functions, including endocytosis, a role in the control of chlamydial entry has been sought for the cyclic nucleotides. When added to HeLa cells before infection with biovar trachoma or LGV, dibutyryl cGMP increases and dibutyryl cAMP decreases inclusion formation and chlamydial amino acid uptake (498, 499). cGMP stimulates inclusion formation only when it is added before or at the time of infection. Prostaglandins, which increase intracellular cGMP levels, increase the inclusion-promoting effect of cGMP and prostaglandin inhibitors abolish it, suggesting a role for endogenous prostaglandin synthesis (499). The intercellular level of free calcium ion (Ca^{2+}) also regulates a number of cell functions, either by itself or in conjunction with cAMP. Ca^{2+} acts by changing the configuration of Ca^{2+}-binding proteins such as calmodulin (reviewed in reference 186). Ca^{2+} facilitates the attachment and ingestion of chlamydiae, but it is not an absolute requirement (183, 429). However, the calcium antagonist verapamil almost completely suppresses the production of a new crop of infectious chlamydiae in African green monkey kidney cells infected with *C. trachomatis* biovar LGV by blocking the developmental cycle at the RB stage (418). This suggests additional functions of Ca^{2+} beyond its role in entry. The role of calmodulin in entry and early development of chlamydiae was studied by using inhibitors of calmodulin, notably trifluperazine (TFP). The results were confusing. In one study with HeLa cells and the trachoma and LGV biovars of *C. trachomatis*, TFP inhibited inclusion formation and chlamydial amino acid incorporation when added up to 9 h after infection, but attachment and ingestion were not affected (224). In a second investigation with TFP, the calmodulin inhibitor not only reduced inclusion formation and amino acid incorporation but also inhibited attachment and ingestion (497), and in a third investigation, TFP did not affect binding or internalization (429).

Some General Considerations

All intracellular parasites that live in nonprofessional phagocytes appear to have evolved some adaptation that facilitates their entry into host cells (309). In animals naturally infected with chlamydiae, the principal host cells are nonprofessional phagocytes, and different sets of host cells are preferentially infected by different kinds of chlamydiae. These preferences are likely to be determined in large part by variations in entry mechanism, although it is likely that other early events in chlamydial multiplication also play a role. The implicit assumption in many investigations has been that chlamydiae have evolved one or more surface structures that bind to complementary structures on the surfaces of host cells. This assumed adaptation must be one-sided, because host cells have been under no pressure to evolve better ways for binding chlamydiae. The effects of modification of chlamydial and host surfaces on the entry process suggest that charged groups on both reactants participate in binding and that these groups reside on proteins and glycoproteins, but efforts to implicate specific charged groups have been inconclusive. These suggestions have been supported by the identification and isolation of chlamydial surface proteins that have some of the characteristics predicted for the hypothetical chlamydial ligands. So many putative chlamydial ligands have now been identified that it is hard to see how all can function in the binding of one EB to one host cell. However, it should be remembered that when the invasion process in other intracellular parasites (the shigellae, for example [165]) has been subjected to genetic analysis, it has been found to depend on the concerted action of many genes and gene products. Considering the great diversity of hosts, host cells, and manifestations of disease encountered in chlamydial infections (24, 412, 451), remarkably few differences in entry behavior have been demonstrated among the members of the genus *Chlamydia*, and those that have been demonstrated tend not to follow the phylogenetic separation, possibly because these differences developed relatively late in the evolutionary history of the genus. Superficially at least, the LGV biovar of *C. trachomatis* behaves more like the MN/Cal 10 and 6BC strains of *C. psittaci* than like the closely related trachoma biovar, which in turn has more in common with mammalian strains of *C. psittaci* such as GPIC than with biovar LGV. Only *C. pneumoniae* is unique, possibly because of long isolation in human hosts. An attractive hypothesis is that as ancestral chlamydiae diverged into new hosts and encountered new potential host cells, they evolved novel ways of getting inside those cells, thus accounting for differences in entry behavior among extant chlamydiae. Unfortunately, present information is inadequate to evaluate this hypothesis. A detailed discussion of how well the principles of coevolution explain chlamydial diversity is presented elsewhere (311).

EARLY INTRACELLULAR EVENTS, FROM ENTRY TO ONSET OF MULTIPLICATION

Once a chlamydial EB has entered a prospective host cell, it may or may not be destroyed by host defense mechanisms. If it escapes destruction, it may or may not enter into a normal developmental cycle by differentiating into an RB. This section will examine the possibilities. Discussion of host cell defense mechanisms will be restricted to those of nonimmune cells.

Destruction of Chlamydiae

When intracellular parasites enter host cells, they must avoid destruction by whatever antiparasitic activities the host cells are able to mobilize. One such capability shared by both professional and nonprofessional phagocytes is fusion of their lysosomes with parasite-containing phagosomes and release of acid hydrolases into the resulting phagolysosomes. Intracellular parasites have evolved several ways to avoid being killed by lysosomal enzymes (309). Along with a number of other host-dependent microorganisms of diverse phylogenetic origin, chlamydiae may evade lysosomal killing by the stratagem of not provoking the fusion of lysosomes with parasite-laden phagosomes. Not every chlamydia-containing phagosome escapes fusion with lysosomes. Whether or not there is phagosome-lysosome fusion depends on the kind of host cell, the kind of chlamydial cell, what has been done to that particular chlamydial cell, and the conditions under which it has been ingested.

Interaction of *C. psittaci* MN/Cal 10 and 6BC with host cell lysosomes. The interaction of chlamydia-containing phagosomes with lysosomes has been most extensively studied with the MN/Cal 10 and 6BC strains of *C. psittaci*. Application of the results of these investigations to *C. trachomatis* and other strains of *C. psittaci* will be discussed shortly. Twelve to 20 h after L cells have ingested infectious EBs of *C. psittaci* 6BC and MN/Cal 10, the cytoplasm of the L cells contains inclusions in which the EBs have differentiated into RBs and started to divide. There is no morphological evidence of fusion with lysosomes, and the inclusions do not stain for acid phosphatase (131, 232). In contrast, 12 h after heated, noninfectious EBs have been ingested, the L cells contain numerous myelin figures and lysosomes, each associated with a phagocytosed and degenerating EB (131). In isopycnic gradients of the cytoplasmic fraction of L cells inoculated 8 h previously with ^{14}C-labeled infectious *C. psittaci* MN/Cal 10, chlamydial inclusions and lysosomes marked by acid phosphatase activity appear as distinct bands, whereas with inocula of heat-inactivated EBs, the two bands broaden and overlap extensively (131). With *C. psittaci*, failure to provoke fusion with L-cell lysosomes depends not on preservation of infectivity, but on preservation of as-yet-undefined structures in the EB cell wall. UV-inactivated (119) and chloramphenicol-inhibited (131, 481) or rifampin-inhibited (481) *C. psittaci* MN/Cal 10 neither multiplies nor triggers phagosome-lysosome fusion in L cells. However, these postulated structures may be inactivated by heating or by combination with antibody, because heated (119, 131) and antibody-treated (131) EBs enter L cells in phagosomes that quickly fuse with lysosomes. That the structures responsible for inhibition of phagosome-lysosome fusion reside in the chlamydial cell wall is demonstrated by the observation that cell walls purified (274) from *C. psittaci* MN/Cal 10 EBs and labeled with a tritiated Bolton-Hunter reagent release label in trichloroacetic acid-soluble form almost as slowly as similarly labeled intact infectious EBs (119). With the 6BC strain in L cells (232) and the calf polyarthritis strain in bovine fetal spleen cells (476), *C. psittaci* inclusions remain free of lysosomal enzyme activity for about 24 h after infection. By that time, RBs have multiplied extensively, many have differentiated into EBs, and the developmental cycle is drawing to a close. Lysosomes are then released into the cytoplasm of the host cells, which begin to disintegrate and lyse (232, 476).

The developmental cycle of *C. psittaci* in mouse peritoneal macrophages is identical in all important respects to the cycle observed in cell lines (251). *C. psittaci* MN/Cal 10 multiplies well in mouse peritoneal macrophages when the multiplicity of infection is not greater than 1 (520). At higher multiplicities, many of the macrophages are rapidly destroyed (see "Destruction of Host Cells" below). Infectious EBs are ingested in phagosomes that do not fuse with ferritin-labeled lysosomes, whereas heat-inactivated or antibody-coated EBs are promptly destroyed in ferritin-marked phagolysosomes (520). Untreated EB walls do not appear in ferritin-labeled phagolysosomes; heated EB walls do (119). When mouse macrophages ingest infectious *C. psittaci* MN/Cal 10 EBs, acid phosphatase levels do not rise, but when they ingest heat-killed EBs, there is an increase in enzyme activity (300). Thus, at a low multiplicity of infection, the relation between *C. psittaci* and lysosomes is much the same in mouse macrophages and L cells.

The ability of macrophages to ingest a wider range of objects than L cells has been exploited to provide more information about the peculiar relation between *C. psittaci* and lysosomes. RBs are noninfectious, but they are readily ingested by mouse peritoneal macrophages. When radioactively labeled RBs of *C. psittaci* MN/Cal 10 are taken up by macrophages, their label is rapidly released in trichloroacetic acid-soluble form, indicating that they are being destroyed (520). It thus appears that the elements in the EB wall required for evasion of phagosome-lysosome fusion are either absent from the RB wall or present in an inactive state. However, the constant presence of the EB wall is not required. By 8 to 12 h after infection, there are no longer any EBs in the *C. psittaci* inclusions, and yet these inclusions do not provoke lysosomal fusion for many hours thereafter (232, 476). When mouse macrophages simultaneously ingest infectious EBs of *C. psittaci* MN/Cal 10 and whole cells of either *Saccharomyces cerevisiae* or *E. coli*, the yeast- and bacteria-laden phagosomes fuse with lysosomes and their contents are degraded to the same extent as in macrophages that phagocytized *S. cerevisiae* or *E. coli* alone (118). This shows that the chlamydia-induced inhibition of fusion is not generalized but is strictly limited to the phagosomes containing intact EBs.

One explanation for all the results just described is that there are two pathways for entry of chlamydiae into host cells—one for the chlamydial cells (and cell walls) that meet the structural requirements for evasion of fusion and one for those that do not. Only in the former pathway do chlamydiae end up in phagosomes with membranes that do not provoke fusion. This idea was tested by isolating *C. psittaci* 6BC-bearing phagosomes from mouse peritoneal macrophages 1 h after they had been inoculated with either infectious or heat-inactivated EBs (530). As determined by polyacrylamide gel electrophoresis, the phagosome membranes surrounding the two kinds of chlamydial cells shared several proteins, but a small number of proteins were unique to each kind of phagosome. Further work is needed to decide if such small differences in membrane proteins are enough to account for the occurrence or nonoccurrence of lysosomal fusion.

Interaction of *C. trachomatis* and other strains of *C. psittaci* with lysosomes. Information on the interaction of *C. trachomatis* with lysosomes of nonprofessional phagocytes is limited. As judged by electron microscopic observation and acid phosphatase assay, there is no phagosome-lysosome fusion at any time during the growth of *C. trachomatis* biovar LGV in BHK-21 cells (245, 471), and when McCoy cells are inoculated with another strain of LGV, only about 10% of the ingested EBs are associated with thorotrast-

labeled lysosomes at 4 h (366). When the inoculum has been heated, 75% of the EBs appear in thorotrast-laden lysosomes. There are no comparable observations for the trachoma biovar. Since biovar trachoma organisms do indeed reproduce in suitable host cells, it is obvious that some infected biovar trachoma EBs must escape destruction in lysosomes, but it is not at all obvious how many EBs do not escape and how important an obstacle lysosomal attrition is to the reproduction of trachoma organisms in vitro and in vivo. The same uncertainties apply to the relation of *C. trachomatis* to the lysosomes of mouse peritoneal macrophages. The same inoculum of biovar LGV or trachoma produces far fewer inclusions in macrophages than in HeLa cells (234), yet *C. trachomatis* is just as toxic to macrophages as is *C. psittaci* (see "Destruction of Host Cells" below). This makes it difficult to distinguish between destruction of *C. trachomatis* by macrophages and destruction of macrophages by *C. trachomatis*. However, after inoculation of admittedly toxic doses of biovar LGV, damaged EBs appear in macrophage lysosomes within 80 min (245). The low yield of infectious progeny from macrophages infected with either *C. trachomatis* biovar may reflect the destruction of a significant fraction of ingested EBs by lysosomal action, but, as with the comparable possibility with biovar trachoma and nonprofessional phagocytes, direct evidence is lacking.

The behavior of strains of *C. psittaci* other than MN/Cal 10 or 6BC has been studied with the calf polyarthritis (476) and GPIC (366, 470) strains of *C. psittaci*. Once again, the heterogeneity of the species asserts itself. As already stated, there is no association of the calf polyarthritis strain with lysosomes of bovine fetal spleen cells until near the end of the developmental cycle (476). In mouse macrophages, *C. psittaci* GPIC behaves like *C. trachomatis*; it does not grow, but it is toxic even at low multiplicities of infection (470). With GPIC and McCoy cells, about half of the ingested EBs are in thorotrast-labeled lysosomes 4 h after infection without the aid of centrifugation, but when the inoculum is centrifuged onto the host cell monolayer, only about 10% of the GPIC EBs become associated with the McCoy cell lysosomes (366). The latter degree of association is about the same as that achieved in parallel experiments with the LGV biovar either with or without centrifugation. Heating the EBs before either unaided or centrifuge-aided infection greatly increases the proportion of ingested EBs that end up in thorotrast-labeled lysosomes (366). It has been concluded (366) that with GPIC and McCoy cells, evasion of lysosomal fusion is dependent not only on the integrity of heat-sensitive fusion-inhibiting structures in the EB cell wall, but also on the mode of entry. Only centrifuge-aided entry clothes the EB in a phagosome membrane that does not fuse with lysosomal membranes.

The studies on *C. psittaci* GPIC suggest that destruction in lysosomes may account for a large part of the difficulty with which *C. trachomatis* biovar trachoma produces inclusions in most cell lines and that centrifugation enhances infection by bringing biovar trachoma EBs into host cells surrounded by a nonfusing phagosome membrane. *C. psittaci* GPIC resembles the trachoma biovar both in manifestations of disease in the natural hosts (320) and in its behavior in cell culture (365, 366, 435), but because the two chlamydial species diverged a long time ago, the resemblances are probably the results of convergence. However, some observations support the suggestion. With the same inoculum of *C. trachomatis* biovar trachoma, human fetal tonsil cells produce only 15% as many inclusions as HeLa cells, yet the association of ^{3}H-labeled EBs to the two cells lines is almost identical (238), indicating that attachment is not the limiting step. Infection of mouse macrophages with biovars LGV and trachoma is stimulated two- to threefold by centrifugation (234), perhaps because lysosomal fusion is thereby reduced. A direct test of the effect of centrifugation on *C. trachomatis* biovar trachoma is needed. Factors other than destruction in lysosomes must also limit the multiplication of the trachoma biovar in many cell lines. When 10 different cell lines were identically infected with a biovar trachoma strain with the aid of centrifugation, there was more than a 100-fold difference in the resulting inclusion counts (97).

Interaction of chlamydiae with monocytes and monocyte-derived macrophages. Human peripheral blood monocytes (HPBMs) ingest and support the multiplication of *C. psittaci*, but the infectious yield is less than in macrophages or cell lines (275, 396, 398). *C. trachomatis* biovars LGV and trachoma do not multiply in freshly cultured HPBMs (275, 526). The mechanism that restricts chlamydial multiplication in HPBMs is oxygen independent. However, in sharp contrast to their dissimilar behavior in mouse peritoneal macrophages, both *C. psittaci* and biovar LGV multiply equally well in macrophages derived from HPBMs by 7- to 14-day culture in vitro (275). The infectious yield for both *C. psittaci* MN/Cal 10 and the LGV biovar is 1,000-fold greater than for *C. psittaci* in HPBMs but less than in a control cell line. Macrophages derived by culturing HPBMs for 8 days or longer support good growth of biovar LGV, but the multiplication of biovar trachoma is still almost as strongly suppressed as in HPBMs cultured for only 1 day (526). Lysosomes in 1-day-cultured HPBMs fuse with both *C. trachomatis* biovars, but 8-day-cultured lysosomes fuse only with phagosomes containing biovar trachoma EBs (526). Further work is needed to explain the different response to chlamydial infection in HPBMs, HPBM-derived macrophages, and mouse peritoneal macrophages.

Interaction of chlamydiae with PMNs. The initial cellular response to chlamydial infection consists primarily of polymorphonuclear leukocytes (PMNs) (236), which are the only myeloid cells whose lysosomes readily fuse with phagosomes containing intact EBs of either *C. trachomatis* or *C. psittaci*. EBs of both the LGV and trachoma biovars of *C. trachomatis* activate complement in the absence of antibody and exert a strong chemotactic effect on human PMNs (286). Human peripheral blood PMNs rapidly ingest *C. psittaci* (378) and the trachoma (378, 525) and LGV (525, 540) biovars. At multiplicities of infection from 1:1 to 100:1, about 50% of the inoculum is ingested in 15 min, either in the presence or absence of complement. Inclusion counts of homogenates of infected PMNs inoculated back into susceptible cell lines drop rapidly with both *C. psittaci* MN/Cal 10 and *C. trachomatis* biovar LGV (378, 527, 540). Infectivity of EBs ingested by PMNs drops from 10- to 1,000-fold in 1 to 4 h, the decrease being greater in the presence of complement (527) than in its absence (378). At 15 min, half of the ingested biovar trachoma and LGV EBs are in phagosomes and half are in phagolysosomes identified by peroxidase activity (525). At 1 h, most are in phagolysosomes and some are completely disintegrated (525). When [^{3}H]uridine-labeled EBs of *C. psittaci* MN/Cal 10 and the trachoma biovar are ingested by PMNs, 75% of the label becomes trichloroacetic acid-soluble in 4 h, and electron micrographs show the presence of both intact and degraded EBs (378). By 10 h, 80 to 95% of the ingested *C. psittaci* MN/Cal 10 has been killed (378), and, by 24 h, there is a 1,000-fold drop in the infectivity of biovar LGV (540).

Both oxygen-dependent and oxygen-independent mecha-

nisms are active in the killing of chlamydiae by PMNs. EBs of both *C. trachomatis* biovars induce a chemiluminescent response in PMNs that is indicative of the activation of oxygen-dependent mechanisms (430, 540). The chemiluminescent response to biovar trachoma is stronger than that to biovar LGV (430). Additional direct evidence for the operation of an oxygen-dependent antichlamydial mechanism is the observation that cell-free myeloperoxidase-H_2O_2-halide preparations from PMNs almost completely inactivate both *C. trachomatis* biovars in 30 min at 37°C (525, 527). Myeloperoxidase release by PMNs is stimulated by *C. trachomatis* biovars trachoma and LGV (477). Data on release of toxic oxygen-derived molecules are conflicting. It has been reported that *C. trachomatis* biovars trachoma and LGV inhibit the respiratory burst in PMNs at the level of NADPH oxidase (469) and that they do not cause an increase in superoxide levels in PMNs (477), but in another study (539), it was found that the LGV biovar stimulates the production of superoxide anions and hydrogen peroxide by PMNs. PMNs from individuals deficient in myeloperoxidase inactivate chlamydiae normally, indicating that other mechanisms are also important (527). Proteins separated from PMN granules exhibit at least two oxygen-independent antichlamydial activities (377). Crude granule extracts kill both *C. psittaci* MN/Cal 10 and the trachoma biovar. When the extracts are fractionated, only those fractions containing lysozyme inactivate biovar trachoma, whereas only lysozyme-free proteins of mass 13,000 kDa inactivate *C. psittaci* MN/Cal 10. Highly purified PMN lysozyme also kills only the trachoma biovar and not *C. psittaci* (377). These observations have a twofold interest. First, the differential sensitivity of the two species to lysozyme may be important in understanding the fate of these species in macrophages. Second, it is a surprise that even one species of *Chlamydia* is killed by lysozyme. Lysozyme hydrolyzes the 1—4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine residues in the carbohydrate backbone of peptidoglycan, which cannot be found in either chlamydial species (see "Effect of antibacterial agents on conversion of RBs to EBs" below). Lysozyme purified from hen egg white has been reported either to inactivate (227) or not to inactivate (197, 512) *C. trachomatis* biovar trachoma. Further study of the effect of lysozymes from both PMNs and egg white is needed.

Destruction of Host Cells

In the early days of chlamydial research, it was discovered that intravenous injection of heavily infected chicken embryo yolk sac emulsions into mice killed the mice within 4 to 24 h, long before death could be attributed to chlamydial multiplication (370). This rapid lethal action is referred to as the chlamydial "toxin." Toxin has been demonstrated in *C. trachomatis* biovars LGV (370, 472), trachoma (32), and mouse (370) as well as in numerous strains of *C. psittaci* (271, 370). Rapidly lethal toxicity is intimately associated with EBs (370) and cannot be demonstrated in RBs (83). It is heat labile (370), and it cannot be prevented by simultaneous administration of chlortetracycline (272). The toxic dose is very large, i.e., 10^7 to 10^8 EBs per mouse (370, 472). The chlamydial toxin is neutralized by antibody with a specificity nearly identical to that obtained in neutralization of infectivity (32, 273, 370). Chlamydial cell walls absorb neutralizing antibody but are not in themselves rapidly toxic to mice (392).

There is an in vitro correlate to the killing of mice by massive injections of chlamydiae. Mouse peritoneal macrophages and many cell lines are destroyed within a few hours by inoculation of appropriate multiplicities of either *C. trachomatis* or *C. psittaci*. The near identity of requirements for expression of chlamydial toxicity in vivo and in vitro leaves little doubt that the two phenomena are different manifestations of the same chlamydia-host cell interaction.

Demonstration of chlamydial toxicity in vitro. Multiplication-independent toxicity of chlamydiae for host cells in vitro was first demonstrated with mouse peritoneal macrophages, and only later was it realized that, with an adequate multiplicity of infection, chlamydiae were also immediately toxic to the cell lines routinely used for in vitro propagation. An inoculum of the order of 10 to 100 intact EBs of *C. trachomatis* biovars trachoma (235, 470) and LGV (235, 470, 471) and *C. psittaci* 6BC (230), MN/Cal 10 (521), and GPIC (470) per host cell damages macrophages within 2 to 10 h, depending on the exact multiplicity of infection and the particular chlamydial strain inoculated. Visual evidence of damage includes rounding up, vacuolization, pyknosis, and, by 20 to 24 h, detachment from the substrate and lysis. Trypan blue and other vital stains are no longer excluded, lysosomal (acid phosphatase) and cytoplasmic (lactic dehydrogenase) enzymes are released into the medium, and the uptake of labeled amino acids is depressed. In electron micrographs of macrophages 3 h after inoculation with low toxic doses of *C. psittaci*, there are many vacuoles, usually free of EBs, into which acid phosphatase has been released, and in the cytoplasm are myelin whorls, swollen mitochondria, and other degenerating organelles (229). With high toxic doses, mitochondria, organelles, and myelin figures have largely disappeared. Heat-killed (235, 470, 471, 521) and antibody-combined (230, 235, 471, 521) EBs are not toxic for macrophages. RBs of *C. psittaci* are not immediately toxic for mouse macrophages even at an RB-to-macrophage ratio of 1,000:1 (50).

Relation of immediate toxicity to chlamydial entry. An inoculum of 500 to 1,000 intact EBs of *C. psittaci* 6BC per L cell is immediately toxic (313). About 40% of an inoculum of this size has been ingested by 1 h (52). In 30 to 60 min, monolayers begin to round up and detach from their substrates, and suspensions fail to attach and spread when they are plated out. The L cells begin to die as early as 8 h, and all are dead at 20 h. In the range of 5 to 500 EBs of *C. psittaci* per L cell, the time of multiplication-independent death is inversely proportional to the multiplicity of infection. The infected L cells maintain normal oxidative and glycolytic metabolism for at least 5 h, but they incorporate less [^{14}C]isoleucine into protein and have smaller pools of nucleotide triphosphates (313). Free, bound, and medium levels of the lysosomal enzyme acid phosphatase are unchanged (313), but K^+ and other intracellular inorganic ions are leaked into the medium (79).

Entry is a prerequisite for immediate toxicity. Mere contact of *C. psittaci* and L cells is not enough. Prolonged contact of EBs and L cells under conditions that do not permit ingestion—for example, at 4°C or after treatment of the L cells with trypsin and cycloheximide—does not result in immediate toxicity (313). UV-inactivated EBs enter L cells just as rapidly as intact ones, and they are equally toxic, whereas heat-inactivated and antibody-combined EBs, which are ingested much more slowly, are not toxic (313). EB walls, which are also rapidly ingested (119, 255), are immediately toxic to mouse macrophages and L cells at very high multiplicities of infection (523). The ingested *C. psittaci* 6BC EBs need not synthesize new macromolecules in order

to be immediately toxic. The unabated toxicity of UV-inactivated chlamydiae shows that DNA synthesis is not needed, and concentrations of chloramphenicol and rifampin sufficient to completely inhibit chlamydial synthesis of protein and RNA do not block the appearance of immediate toxicity (313).

Immediate toxicity is also produced by other chlamydiae in other cell lines. An inoculum of 1,000 EBs of *C. psittaci* 6BC per host cell is toxic to HeLa cells and monkey kidney cells (313), although HeLa cells are less susceptible to damage than L cells (79). With *C. psittaci* MN/Cal 10 and *C. trachomatis* biovars mouse and LGV, an inoculum of 500 EBs per host cell is immediately toxic to L cells (313). *C. trachomatis* biovar trachoma was not toxic to HeLa cells at an unspecified multiplicity (235), and 450 EBs of biovar LGV per BHK-21 cell did not elicit immediate toxicity (472).

Release of lysosomal enzymes in response to ingestion of large numbers of EBs is a possible but unlikely explanation of multiplication-independent immediate toxicity. There is no rise in the level of free acid phosphatase when L cells ingest toxic doses of *C. psittaci*, and RBs and heated or antibody-combined EBs, which are ingested by macrophages in phagosomes that quickly fuse with lysosomes, are not toxic either to mice or to macrophages. Chlamydial cell walls contain a lipopolysaccharide that in many respects resembles the endotoxin of host-independent gram-negative bacteria (see "Modification of plasma membranes of host cells" below), and it has been suggested that the toxic death of mice and cultured cells might be caused by massive contact with chlamydial endotoxin. However, toxic doses of *C. psittaci* MN/Cal 10 kill endotoxin-responsive (C3H/HeN) and endotoxin-nonresponsive (C3H/HJ) mice at the same time after injection, and macrophages from nonresponsive mice are only slightly more sensitive to EB toxicity in vitro (200). In addition, chlamydial lipopolysaccharide (LPS) engenders genus-specific antibody (66), whereas antibody neutralization of multiplication-independent toxicity is strain specific, both in mice and in cell culture.

Role for chlamydial toxin in natural infections? The chlamydial toxin is probably an artifact. The large doses of chlamydiae needed for immediate damage to mice or cultured cells are unlikely to exist outside the laboratory. Only if the natural cell targets of chlamydiae are exquisitely sensitive to immediate toxic injury is there much chance of this phenomenon playing any role in the pathogenesis of chlamydial infection. It remains, nevertheless, a fascinating example of the interaction of chlamydiae and host cells.

Common Determinant for Entry, Avoidance of Lysosomal Fusion, and Immediate Toxicity

The most plausible explanation of immediate toxicity is that a lesion is produced in the plasma membrane of a host cell when it ingests a chlamydial cell with a cell wall containing a particular structure or set of structures. The lesion permits a massive leakage of inorganic ions and enzymes that quickly leads to cell death. Each act of ingestion produces an independent lesion, and the more lesions produced, the faster the host cell dies. In natural infections—and in most laboratory infections as well—the multiplicity of infection is low and the host cells are not killed before a new generation of chlamydiae is produced.

The structure or set of structures required for expression of immediate toxicity has the same attributes as the structure that enables chlamydiae to enter nonprofessional phagocytes with unusual facility and, once inside, to avoid lysosomal fusion. They are present in EBs but not RBs, they are located in the cell wall, and they are destroyed by heat and blocked by combination with antibody, but they are not inactivated by UV light. These structures may lie wholly or in part in the MOMP. Evidence for participation of the MOMP in chlamydial entry has already been presented (see "Identification of Receptors on Chlamydial and Host Cell Surfaces" above), and monoclonal antibodies to the MOMP passively neutralize the toxicity of *C. trachomatis* biovar trachoma for mice (533).

The linkage between entry, avoidance of lysosomal fusion, and immediate toxicity is clearest in the 6BC and MN/Cal 10 strains of *C. psittaci*. For these chlamydiae, one explanation for the linkage is that an infectious EB has on its surface a host cell-binding ligand with the properties just described. Its structure is probably complex, and it may contain one or more of the host cell-binding proteins described above in Entry. During the long association of chlamydiae with their eucaryotic hosts, the chlamydial ligand has evolved a complementarity to a particular set of structures on the host cell surface. EBs bind to these structures by means of their ligands, and ingestion follows rapidly. Thus, EBs enter the host cell enveloped in a phagosomal membrane derived from a particular secretion of the plasma membrane of the host cell that carries a marker that discourages fusion with lysosomes. A similar explanation has been offered for the failure of intracellular vacuoles containing *Toxoplasma gondii* to fuse with lysosomes (210). When EBs enter at sites containing the fusion-inhibiting marker, the integrity of the plasma membrane is not completely restored. If a large number of EBs enter a single host cell, both small and large molecules are not retained, and the host cell dies. When chlamydiae in which the structure of the chlamydial ligand has been disrupted by heat, blocked by antibody, or obscured in the reorganization of EBs into RBs enter host cells, they are ingested in phagosomes derived from other sections of the plasma membrane that have no signal to stop lysosomal fusion, and the plasma membrane reseals tightly behind them. These chlamydiae do not cause immediate toxicity, and they are destroyed by lysosomal enzymes. This is purely an explanation for the linkage. No attempt has been made to guess the nature of the putative common determinant and how it brings about such diverse consequences of chlamydia-host cell interaction.

Future investigations may reveal a common basis for the association between entry, avoidance of lysosomal fusion, and immediate toxicity in *C. psittaci* 6BC and MN/Cal 10 and the effect of centrifuge-aided entry on lysosomal fusion in *C. psittaci* GPIC and possibly the trachoma biovar of *C. trachomatis* as well. They all may represent different expressions of a single recurring theme—that there is more than one way for a chlamydia to get inside a host cell and that how it gets in determines the fate of the chlamydia and the fate of the host cell.

Initiation of Multiplication

Like many other intracellular parasites (309), chlamydiae have evolved distinct infectious and reproductive forms in order to meet the often conflicting demands of extracellular survival and intracellular multiplication. On entering a host cell, the chlamydial EB promptly begins to reorganize into an RB. This simple statement generates several important questions. What component of the intracellular environment provides the signal for the EB-RB transformation? How is this signal transmitted to the phagosome-enclosed EB? What

are the first steps in reorganization of an EB into an RB? How are these events regulated? Presently, none of these questions can be answered completely, but enough progress has been made to take the phenomenon out of the black-box stage.

Study of the differentiation of EBs into RBs often demands the availability of preparations of EBs virtually devoid of RBs and vice versa. The density of EBs is 1.21 and that of RBs is 1.18, so the two chlamydial cell types can be separated by differential centrifugation (50, 131, 461, 466). For preparation of RBs, infected cells are harvested early in the developmental cycle before many EBs have appeared, and EBs are prepared by harvesting host cells at the end of the cycle.

First detectable events in reorganization of EBs into RBs. RBs differ from EBs in many ways, both biological and chemical (reviewed in references 175, 280, 326, and 496). The RB has an osmotically and mechanically fragile cell wall, probably because the MOMP is no longer cross-linked with disulfide bonds (161); it has a fibrillar nucleoid in contrast to the highly compacted nucleoid of the EB (93); it is noninfectious; and it is metabolically active. Identification of the very first changes in the just-ingested EB that start it along the road to becoming a mature, dividing RB is prerequisite to understanding how the EB-RB reorganization is initiated and controlled. The first biological property lost is infectivity. The number of infectious *C. psittaci* Texas turkey recovered from McCoy cells begins to decline as early as 1 h after inoculation (467), and by 2 h, only 1 to 5% of an inoculum comprising 50 to 1,000 infectious units per L cell can be found in *C. psittaci* 6BC-infected L-cell lysates (313). Ability to avoid lysosomal fusion and to cause immediate toxicity probably declines just as rapidly.

The first biochemical event that can be detected in chlamydiae after they have been ingested by host cells is the synthesis of protein (359). Synthesis of chlamydial protein in L cells infected with *C. psittaci* occurs as early as 15 min after infection. Early protein synthesis has been demonstrated both in infected L cells treated with cycloheximide and in host-free chlamydiae reisolated from L cells soon after infection. The conditions under which host-free chlamydiae synthesize protein and the methods used to demonstrate this synthesis will be described below in "Synthesis of protein". Under comparable conditions, EBs of either *C. psittaci* 6BC or *C. trachomatis* biovar LGV isolated from host cells at the end of the developmental cycle do not synthesize detectable protein (179). Dactinomycin, a general inhibitor of RNA synthesis (135), almost completely inhibits early protein synthesis in host-free chlamydiae, whereas rifampin, which inhibits initiation of transcription (135), only partially inhibits synthesis. This suggests that early protein synthesis by host-free chlamydiae depends both on translation of messages synthesized in vivo and on initiation of transcripts in vitro.

The second early biochemical event is the reduction of the disulfide-bond-cross-linked MOMP to its monomeric form within 1 h after entry of *C. psittaci* EBs into L cells, as demonstrated by the appearance of monomeric MOMP in electrophoretically separated sodium lauryl sarkosinate extracts (180). Reduction of MOMP is inhibited by chloramphenicol but not by cycloheximide, indicating that chlamydial-protein synthesis is required for reduction of MOMP but that host protein synthesis is not. Entry of *C. trachomatis* biovar LGV EBs into HeLa cells is followed by reduction of only part of their MOMPs, even after 4 h, and the reduction is not inhibited by chloramphenicol (180).

The roles played by synthesis of protein and reduction of the MOMP in initiation of the EB-RB reorganization are not known, but there are some hints. Protein synthesis in RBs depends on the transport of ATP and ADP by an ATP-ADP exchange mechanism not possessed by EBs (176, 179), and liposomes prepared from reduced (but not cross-linked) MOMP contain pores with exclusion limits compatible with passage of adenine nucleotides (26). Reduction of the MOMP to provide channels for passage of ATP and other metabolites from the host cell into the newly ingested chlamydial cell could be the event that turns on protein synthesis and starts the reorganization. Unfortunately for this explanation, reduction of the MOMP in itself requires synthesis of chlamydial protein. Postulating protein synthesis as the first event leads to a similar problem. This dilemma is almost certainly only apparent, the result of our incomplete knowledge of early events.

Search for signal for conversion of EBs to RBs. Intracellular parasites must receive the signal for initiation of growth and multiplication when they enter host cells. Nevertheless, there are so many differences between extracellular and intracellular environments—temperature, pH, redox potential, and concentrations of inorganic ions and low-molecular-weight metabolites, to list some of the most obvious—that it is not easy to identify the effective signal(s) for each parasite. There have been a few successes, particularly with the obligate intracellular bacterium *Coxiella burnetii*. It multiplies intracellularly only in phagolysosomes, which have a pH of about 5. Host-free *C. burnetii* utilizes glutamate, glucose, and tricarboxylic acid cycle intermediates very sluggishly at pH 7 and very vigorously at pH 5, with accompanying generation of ATP (162, 163). At pH 5, in the presence of glutamate and appropriate precursors, host-free *C. burnetii* synthesizes DNA, RNA, and protein (81, 164, 538). It is likely that the acidic pH of the phagolysosome is the signal for *C. burnetii* to start intracellular growth and multiplication.

Several investigators have tried to induce host-free EBs to perform enzymatic reactions known or assumed to occur in RBs on the implicit assumption that any activity provoked in EBs in vitro is probably involved in the early stages of chlamydial differentiation. High-resolution ^{31}P nuclear magnetic resonance was used to demonstrate ATPase activity in host-free EBs of the trachoma and LGV biovars of *C. trachomatis* that had been treated with 2-mercaptoethanol (347). This reagent reduces disulfide-bond-cross-linked MOMP to its monomeric form (182, 329). Monoclonal antibody to the MOMP reduced the ATPase activity of 2-mercaptoethanol-treated EBs, a further suggestion that MOMP acts as a porin in ATP transport. EBs treated with 2-mercaptoethanol transport ATP, whereas untreated ones do not. Host-free EBs of *C. trachomatis* biovar LGV (but not of *C. psittaci* 6BC [175]) exhibit a DNA-dependent RNA polymerase activity that is doubled by treatment with 2-mercaptoethanol (407, 408). [^{3}H]UTP is linearly incorporated for 1 h in the presence of all four nucleotide triphosphates, and the incorporation is inhibited by dactinomycin and rifampin. Host-free EBs of *C. trachomatis* biovar LGV and *C. psittaci* MN/Cal 10 lose infectivity and osmotic stability when treated with dithiothreitol (161). Their cross-linked MOMPs are reduced to monomers, but their compact nucleoids do not become fibrillar. Dithiothreitol increases the rate of glutamate oxidation in LGV EBs by about fivefold, but it only doubles the rate in *C. psittaci* MN/Cal 10 EBs (161). There is no induction of ATP transport or protein synthesis in *C. psittaci* EBs treated with dithiothreitol (179, 180). The

promotion of enzymatic activity in host-free EBs of *C. trachomatis* biovar LGV by exposure to reducing agents is consistent with the often-expressed idea that reduction of the disulfide-bond-cross-linked MOMP to a monomeric state is a critical early event in the differentiation of EBs into RBs (26, 161, 180, 325, 347). Why dithiothreitol reduces the disulfide bonds of *C. psittaci* 6BC MOMP without stimulating enzymatic activity remains to be explained. The nature of the reducing agent operating in infected host cells has not been determined. Neither glutathione nor a combination of NADH and NADP brings about reduction of the cross-linkages in EBs of *C. psittaci* 6BC (180). They may not be the intracellular reducing agents, or, if they are, other events such as synthesis of protein and reorganization of the EB nucleoid may also be required. The signal that sets all this activity in motion remains unknown. As discussed above in Entry, cAMP and cGMP, which act as regulatory signals in many procaryotic and eucaryotic cells, have an effect on the chlamydial developmental cycle, but they most likely affect the differentiation of RBs into EBs (220, 221) and not vice versa.

Regulation of EB-RB reorganization. Even in pathogens much more amenable to genetic analysis than the chlamydiae, the mechanisms that regulate response of the pathogen to the host environment are poorly understood (128, 292), and regulatory mechanisms in chlamydiae are poorly understood indeed. Although host-free EBs synthesize protein in an environment far removed from the inside of a host cell, the electrophoretic profiles of chlamydial proteins synthesized inside and outside of host cells are the same (359). Therefore, any plausible regulatory mechanisms must account for the commitment of the EB to a particular set of temporally regulated proteins at the time of its derivation from an RB. It has been suggested that chlamydiae may have a series of promoters that are sequentially recognized by an RNA polymerase modified by activating proteins present only at specific times in the developmental cycle (359). Evidence has been presented that cycle-specific promotor sequences are recognized by a cascade of distinct sigma factors in *C. psittaci* 6BC (178), and *C. trachomatis* biovar mouse contains a protein homologous to the sigma-70 protein of *E. coli* (121). Promoters for two *C. trachomatis* genes have been described, but they appear to be most active in the middle, not in the beginning, of the cycle (120, 443).

MULTIPLICATION

The two preceding sections described only a brief segment of the developmental cycle, with a time scale in minutes. Entry, avoidance of lysosomal fusion, and initiation of EB reorganization all occur almost simultaneously, and all are determined by the structure of the EB cell wall and the changes that occur therein. For multiplication to proceed, the EB wall must disappear and be replaced by the RB wall, which has a different set of equally essential properties. The events described in this section occupy the greater part of the developmental cycle, with a time scale in hours. Chlamydial multiplication is the product of structural and metabolic interactions between chlamydiae and host cells so complex that a brief overview of the entire process may be helpful.

Once started, reorganization of EBs into RBs proceeds rapidly via numerous morphologically intermediate stages so that by 8 to 12 h after infection, almost pure RB populations are seen. Among the many changes associated with reorganization are increase in size (the volume of RBs is about 10 to 100 times that of EBs), alteration in structure of the cell wall and nucleoid, and increase in number of ribosomes. Infectivity is lost, and there is a great increase in the variety and intensity of metabolic activity. Once mature RBs have appeared, multiplication occurs by binary fission, and daughter cells are more or less equal in size. Chlamydiae multiply in membrane-bound cytoplasmic vacuoles traditionally called inclusions. The phagocytic membrane surrounding the ingested EB becomes the inclusion membrane enclosing a microcolony of dividing RBs. As the microcolony grows, the inclusion membrane enlarges to contain it. All the energy sources and synthetic intermediates the multiplying EBs get from their host cell must pass through the inclusion membrane. Individual cells of *C. trachomatis* and *C. psittaci* look very much alike, but their inclusions are distinctively different in appearance. Titratable intracellular infectivity abruptly rises about 20 h after infection and is correlated with the appearance of EBs in thin-section electron micrographs of infected host cells. Extracellular infectivity appears soon thereafter, and the number of infectious chlamydiae in infected cells and in the medium increases logarithmically until about 40 h after infection. Numerous chlamydial cells intermediate between RBs and EBs are seen. Many RBs continue to divide long after the first EB appears, so that there are always RBs and intermediate forms among the many EBs in the terminal chlamydial population. There is no sharply defined end to the developmental cycle. It is generally assumed that the cycles of *C. psittaci* and *C. trachomatis* biovar LGV are 48 h long and that the cycle of the trachoma biovar is somewhat longer. However, *C. psittaci*-bearing inclusions may lyse anywhere from 30 to 60 h after infection, depending on strain and multiplicity of infection, and *C. trachomatis*-infected host cells may remain intact for many hours after chlamydial multiplication has ceased. The host cell cycle is not disrupted at low multiplicities of infection, and some infected cells continue to divide. However, most investigations on the biochemistry of chlamydial multiplication have employed multiplicities of infection high enough to infect nearly every cell in the host population. At these multiplicities, host cells cease to make macromolecules and fail to exclude trypan blue and other vital stains before the developmental cycle has been completed. The yield of new infectious units per host cell ranges from less than 100 to more than 1,000, the equivalent of 8 to 12 doublings of a single invading EB. Chlamydiae are not known to utilize directly any large host molecules. They synthesize few low-molecular-weight metabolites and depend on the host cell for both synthetic intermediates and energy in the form of ATP. Multiplying RBs must compete with their host cells because metabolic pools of the host are the feedstock for both host and chlamydial biosynthesis. In the early stages of multiplication, the mass of the host biosynthetic apparatus is much greater than that of the parasite, and the chlamydiae are sometimes at a distinct disadvantage in the competition.

Biochemistry of Chlamydial Multiplication

Multiplication of chlamydiae in cultured host cells is accompanied by many changes in the metabolism of infected host cells. However, even with homogeneous populations of host cells infected with enough chlamydiae to infect virtually every host cell, interpretation of these changes is not easy. Any perturbation of uninfected cell metabolism may result from the activity of either host or parasite alone or from the combined activity of both, thus confronting the investigator

with a simple equation and two unknowns. Approximate solutions to such equations have been sought by comparing infected host cell populations with uninfected ones, by employing reagents that selectively inhibit either host cell or parasite metabolism, by isolating and analyzing chlamydiae after a period of intracellular multiplication, and by studying the metabolism of host-free organisms. Each approach has yielded valuable information about chlamydial multiplication, and each approach has had its limitations.

Energy metabolism. L cells infected with the 6BC or MN/Cal 10 strain of *C. psittaci* utilize glucose twice as fast as uninfected cells, and most of the extra glucose utilization is accounted for by glycolysis to lactate (142, 305). Respiration of infected L cells is either the same as (305) or 25% higher than (143) that of uninfected cells. The increase in respiration observed in the latter study was ascribed to increased oxidative phosphorylation. Stimulation of both glycolysis and respiration are responses of the L cell to infection. Respiration is increased in infected cells in which chlamydial multiplication has been blocked by nutritional deprivation (143), and the increase in glycolysis accompanying infection is not prevented by chloramphenicol (305) but is completely inhibited by cycloheximide, which does not affect chlamydial growth (223). In the range of 1.5×10^{-3} to 1.0×10^{-2} M, multiplication of *C. psittaci* is proportional to glucose concentration, and at limiting glucose levels, NADP markedly increases the yield of infectious organisms (142). As *C. psittaci* multiplies in L cells, ATP levels fall and ADP levels rise concomitantly (143). Antimycin A, which inhibits oxidative phosphorylation by blocking conversion of cytochrome *b* to cytochrome c_1 (78), reduces ATP levels in infected L cells and prevents the multiplication of *C. psittaci* (144). Pretreatment of FL cells (a human amnion line) with ethidium bromide, an inhibitor of mitochondrial function (541), makes them incapable of supporting the growth of *C. trachomatis* biovar LGV (27). However, *C. trachomatis* biovar LGV grows as well in a mutant Chinese hamster cell line with less than 10% of the normal mitochondial respiration as in the parent Chinese hamster line or in HeLa cells (346). An equilibrium labeling technique has been used to show that *C. psittaci* growing in L cells incorporates added uridine and adenine into its RNA at rates consistent with the chlamydiae drawing exclusively on the ribonucleoside triphosphate pools of the L cell for precursors (172). These investigations on infected host cells are all consistent with the idea that chlamydial multiplication depends on ATP and other high-energy metabolites generated by the dissimilation of glucose by the host (304).

Experiments with host-free chlamydiae give further support to this "energy parasite" hypothesis. The first studies were done with populations of *C. psittaci* MN/Cal 10 or *C. trachomatis* biovar LGV from chicken embryo yolk sac or allantoic fluid in which there were two to three times as many EBs as RBs. Later investigations have shown that the metabolic activity of mixed chlamydial populations is due almost entirely to their RB content. EB-RB mixtures carry glucose through part of the pentose cycle. $^{14}CO_2$ is evolved from [1-^{14}C]glucose (334, 506, 509). CO_2 evolution is absolutely dependent on ATP and divalent cations and in their presence is further increased by NADPH (506). CO_2 evolution from glucose proceeds much more slowly than in most host-independent, glucose-utilizing bacteria. The amount of CO_2 evolved is always less than the amounts of ATP and NADPH added. The ATP requirement is for phosphorylation of glucose to glucose-6-phosphate by hexokinase (506), which may be of host origin (487). Extracts of RB-EB mixtures of *C. psittaci* MN/Cal 10 contain at least two enzymes of the pentose-phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), which are electrophoretically distinct from the dehydrogenases of the chicken embryo host (312). Isolated populations of *C. psittaci* MN/Cal 10 RBs and EBs also degrade glucose (509) and glucose-6-phosphate (506) to pyruvate, which is then decarboxylated (507, 511). They also carry out part of the tricarboxylic acid cycle by deaminating glutamate to α-ketoglutarate and decarboxylating it to succinate, which is not further metabolized (507). Host-free *C. psittaci* MN/Cal 10 does not consume oxygen (7, 509). Flavoprotein and cytochrome respiratory enzymes are not present (7, 8, 508a), although exogenous cytochrome *c* is reduced in the presence of NADH (7, 8, 465). These studies on host-free RB-EB mixtures make it unlikely that such preparations generate ATP by either substrate-level or oxidative phosphorylation. Again, as with the experiments on intact infected host cells, the conclusion that chlamydiae are energy dependent rests on negative findings.

Positive evidence for the energy parasite hypothesis has been furnished by direct demonstration of the use of host ATP for chlamydial biosynthesis in host-free RBs. ATP-ADP translocases, enzymes in adenylate nucleotide transport systems that exchange ATP and ADP, were discovered in mitochondria and chloroplasts (489). In these organelles, translocases couple excretion of ATP produced by oxidative or photosynthetic phosphorylation with uptake of ADP. In intracellular parasites, ATP-ADP translocases work in the opposite direction; i.e., ATP is taken up and ADP is excreted. Translocase activity in intracellular parasites was first demonstrated in *R. prowazekii* (518). In host cells with active phosphorylation, the rickettsiae get their ATP from the host by exchanging ADP for ATP, but when host ATP is limited or when the rickettsiae are in transit from one host cell to another, the rickettsiae make ATP on their own (15). Chlamydiae resemble *R. prowazekii* in that they exchange ADP for host-generated ATP by means of a translocase, but they differ from rickettsiae in that they have not been shown to produce their own ATP, even in the complete absence of an exchangeable exogenous source. One of the EB's adaptations for extracellular survival may have been dispensing with the necessity for an energy source to preserve its structural integrity.

EB-free RBs from L cells infected with *C. psittaci* 6BC transport ATP and ADP by an ATP-ADP exchange mechanism, but EBs free of RBs do not take up ATP (176). Most of the ATP transported into RBs is hydrolyzed to ADP by a Mg^{2+}-dependent, oligomycin-inhibited ATPase, and the ADP is exchanged for more extracellular ATP. In the presence of ATP, RBs transport lysine against the electrochemical gradient. Lysine uptake is inhibited by oligomycin, and RBs lose lysine to the extracellular medium when the ATP concentration is reduced. The dependency of lysine transport on the breakdown of ATP is best explained by assuming that hydrolysis of ATP to ADP generates a proton motive force (293) that produces an energized RB that is active in transporting lysine and other host-generated metabolites into chlamydial cells (176). RBs of *C. trachomatis* biovar LGV have an ATP-ADP translocase similar to that of *C. psittaci* 6BC (175). Both ATPase activity and inclusion formation are inhibited by oligomycin in *C. trachomatis* biovar LGV growing in HeLa cells (346). Examples of the dependency of chlamydial biosynthesis (and thus chlamydial multiplication) on exogenously supplied ATP will be provided in the next section.

Synthesis of protein. In chlamydia-infected host cells, synthesis of parasite protein, unlike that of DNA and RNA, cannot be detected in the face of continued host protein synthesis without the help of radioactively labeled precursors. When L cells are infected with *C. psittaci* MN/Cal 10 at a multiplicity of 100 and when the incorporation of [^{14}C]lysine into trichloracetic acid-insoluble fractions of isolated chlamydiae is determined at the end of successive 5-h labeling periods, protein synthesis is first evident 15 to 20 h after infection (414). During this time, the RBs formed from the infecting EBs have probably undergone two or three divisions. The rate of [^{14}C]lysine incorporation into chlamydial protein increases until the 30- to 35-h labeling period, after which it declines. The rate of labeled lysine incorporation into *C. psittaci* MN/Cal 10 protein parallels the rates of labeled cytidine uptake into chlamydial DNA and RNA (414), as would be predicted for an organism multiplying by binary fission.

The use of specific inhibitors of eucaryote protein synthesis to suppress synthesis of host protein in chlamydia-infected cells simplifies the task of measuring uptake of labeled amino acids into parasite protein by eliminating the necessity of isolating the labeled protein. One such inhibitor, cycloheximide (135), has no effect on the multiplication of *C. psittaci* in L cells in concentrations (2 to 5 μg/ml) that eliminate over 90% of the protein synthesis in uninfected L cells (1). In L cells infected with 100 EBs of *C. psittaci* MN/Cal 10 per host cell and treated with cycloheximide 1 h before the addition of labeled amino acids, incorporation into trichloroacetic acid-insoluble fractions of whole infected L cells begins to rise 12 to 15 h after infection and continues to rise through the 27- to 30-h labeling period, after which it declines (1). At its peak, incorporation of labeled amino acids into the protein of *C. psittaci*-infected, cycloheximide-treated L cells equals the incorporation into uninfected L cells in the absence of cycloheximide. That the cycloheximide-insensitive protein synthesis in infected host cells is a chlamydial activity is demonstrated by the virtually complete inhibition of this synthesis by the inhibitors of procaryotic protein synthesis (135), chloramphenicol and chlortetracycline.

Cycloheximide is also without effect on the multiplication of *C. trachomatis* biovar LGV in FL cells, but it reduces protein synthesis in uninfected FL cells by only 50% (28). Therefore, emetine, another specific inhibitor of eucaryotic protein synthesis (155), has been used to follow the course of protein synthesis in FL cells (28). In the presence of 1 μg of emetine per ml, incorporation of ^{14}C-labeled amino acids into FL-cell protein is inhibited by 98% without reducing the yield of *C. trachomatis*, but incorporation into *C. trachomatis*-infected host cells rises at 12 h after infection and continues to rise until 42 h. Protein synthesis in *C. trachomatis*-infected, emetine-treated FL cells is completely suppressed by rifampin, an inhibitor of the DNA-dependent RNA polymerase of procaryotes (135) to which both *C. trachomatis* biovar LGV (30) and *C. psittaci* MN/Cal 10 (481) are exquisitely sensitive.

Both cycloheximide and emetine do something more than just suppress synthesis of host protein so that synthesis of chlamydial protein becomes apparent. Cycloheximide increases the infectious yield of *C. psittaci* 6BC in L cells more than 10-fold (173), and it has a comparable effect on the yield of the trachoma biovar of *C. trachomatis* in McCoy cells (35, 385). Emetine similarly enhances the infectious yield of *C. trachomatis* biovar LGV in FL cells (28) and of *C. trachomatis* biovar trachoma and *C. psittaci* sheep abortion strain in McCoy cells (342). Under most conditions of in vitro culture, chlamydial multiplication does not inhibit synthesis of host protein (1, 2, 414), and cycloheximide and emetine probably act by preventing the host from competing with the parasite for amino acids and possibly other essential metabolites as well (173). Evidence for this conclusion will be presented below in "Amino acid metabolism." The highly successful use of cycloheximide to increase the sensitivity of methods for isolating *C. trachomatis* biovar trachoma from clinical specimens (101, 385) suggests that one of the reasons the trachoma biovar grows less readily in cell culture than the LGV biovar or most strains of *C. psittaci* might be that chlamydiae of the trachoma biovar experience unusual difficulty in competing with their hosts for amino acids. However, limited data on the enhancement of infectious yield by cycloheximide and emetine do not support such a suggestion (28, 173, 342, 385). There is no real difference in the response of the two *C. trachomatis* biovars and *C. psittaci* to these inhibitors of eucaryotic protein synthesis. The action of cycloheximide is unpredictable. It varies with concentration, host cell, chlamydial strain, and the laboratory in which it is measured. In a single study, the effect of cycloheximide on the infectious yield from 22 strains of *C. psittaci* growing in L cells varied from enhancement to inhibition to no effect at all (435). Exposure of host cells to ionizing radiation (147) or to 5-iododeoxyuridine (515) has also been used to improve the efficiency of isolation of the trachoma biovar from clinical specimens. It is not known if these inhibitors of DNA function exert a differential effect on the growth of *C. trachomatis* biovar trachoma compared with other chlamydiae.

By examining the incorporation of [^{35}S]methionine of high specific activity into the protein of host-free *C. psittaci* obtained from a large number of L cells, synthesis of chlamydial protein may be detected as early as 15 min after infection (359). Several electrophoretically distinct proteins are made immediately after infection and are synthesized either not at all or in greatly reduced amounts in later stages of the developmental cycle. One protein of molecular weight 35,000 is particularly prominent.

The development of methods for isolating a number of outer membrane proteins has made it possible to determine their times of synthesis in the chlamydial developmental cycle. In L cells infected with *C. psittaci* 6BC and labeled with [^{35}S]cysteine, synthesis and incorporation of monomeric MOMP into outer membrane complexes is first detected 12 h after infection and continues throughout the developmental cycle (180). By 48 h, the intracellular chlamydial population consists mainly of EBs with outer membrane complexes containing MOMP still in the monomeric form. When the L cells lyse at 46 h, the MOMP is rapidly cross-linked and infectious EBs are released. In HeLa cells infected with *C. trachomatis* biovar LGV and labeled with [^{35}S]cysteine, synthesis of MOMP is first seen 12 to 18 h after infection (325). The MOMP of *C. trachomatis* becomes progressively cross-linked during the latter half of the developmental cycle. The outer membrane of the chlamydial EB contains proteins of very high cysteine content that are described by various authors as 12-, 59-, and 62-kDa proteins or as a 12-kDa protein and a 60-kDa doublet (177, 180, 325, 405, 534). The molecular weight polymorphism of the 59- to 62-kDa protein is the result of processing at alternate signal peptide cleavage sites (9). In *C. psittaci* (180) and in all three biovars of *C. trachomatis* (325, 405), synthesis of these cysteine-rich outer membrane proteins is not observed until about 20 h after infection, the time when RBs begin to

reorganize into EBs. Synthesis then continues throughout the developmental cycle. In *C. trachomatis*, the 60-kDa doublet is extensively cross-linked throughout the growth cycle, but the 12.5-kDa protein becomes so only during the last 24 h (325). Since, as already described above in Entry, *C. trachomatis* EBs contain 18- and 32-kDa host-binding proteins not found on RBs (159, 514), these proteins must be synthesized late in the developmental cycle. Proteins specified by *C. trachomatis* biovar LGV plasmids are also synthesized late, between 24 and 48 h after infection (339).

Like all organisms so far examined, chlamydiae synthesize heat shock proteins (stress proteins), a small number of highly conserved proteins produced in response to heat and other forms of stress (260, 299). Their exact function is unknown, but it is thought that they protect against stress and perhaps play roles in normal growth and development. Heat shock proteins are the dominant antigens recognized in the immune response to many pathogens (528). When the temperature of a HeLa-cell culture is raised from 37 to 45°C, 10 to 20 new chlamydial proteins appear (361). However, the only heat shock proteins that have been studied in any detail are those sharing extensive amino acid sequences with the *E. coli* heat shock proteins hsp 70, DnaK, and GroE. *C. trachomatis* biovars LGV and trachoma and *C. psittaci* GPIC synthesize throughout their developmental cycles a 57- to 60-kDa polypeptide homologous with the GroE protein of *E. coli* (302, 303). Because it produces eye inflammation in *C. trachomatis*-sensitized animals, it has been postulated that this protein is responsible for some of the host damage seen in infection with *C. trachomatis* (301, 302). It is not clear whether chlamydiae synthesize one or two 75-kDa heat shock proteins. It has been reported that *C. trachomatis* biovar LGV synthesizes a DnaK-like 75-kDa protein early in the developmental cycle (38, 39), and a 75-kDa protein homologous with *E. coli* hsp 70 protein and located on the surface of *C. trachomatis* biovar trachoma EBs has also been described (99). Heat shock-mediated induction of the mRNA for a DnaK-like protein in the mouse biovar depends on de novo synthesis of chlamydial proteins, but the induction of the mRNA for another heat shock protein (pg82) does not (361), indicating that there are multiple pathways for induction of chlamydial stress proteins. The presence of heat shock proteins in chlamydiae is of particular interest because, as has been suggested by several investigators, these proteins may protect chlamydiae against the stresses likely to be encountered in the hostile intracellular habitat (306, 307).

Taken as a whole, the studies on synthesis of specific proteins during the course of the developmental cycle demonstrate that a temporally regulated synthesis of proteins parallels and is most certainly responsible for the structural and functional changes that constitute the cycle.

Amino acid metabolism. The amino acids that chlamydiae incorporate into protein are either synthesized de novo or obtained from the amino acid pools of the host, which are the sum of contributions from the growth medium, de novo host synthesis, and normal turnover of host protein. As measured by omission of single amino acids from a medium adequate for growth of both host cells and chlamydiae, the amino acid requirements of chlamydiae are not highly dependent on the host cell (86, 335), but they vary significantly from strain to strain, even among closely related strains. *C. psittaci* 6BC does not multiply in L cells when glutamine, phenylalanine, tyrosine, tryptophan, cysteine, threonine, isoleucine, leucine, or valine has been omitted from the growth medium (16). *C. psittaci* 6BC grows in L cells just as well without arginine as with it, but the final infectious titer of the MN/Cal 10 strain of *C. psittaci* is reduced 100-fold in its absence (479). This is one of the few recorded ways in which these two strains of *C. psittaci* behave differently in cell culture. *C. trachomatis* biovar LGV does not multiply in FL cells when arginine, histidine, or lysine is left out of the growth medium, whereas the multiplication of *C. psittaci* 6BC is undiminished by such omission (335). *C. psittaci* GPIC, which in its entry behavior resembles the trachoma biovar more than strains of *C. psittaci* such as 6BC and MN/Cal 10 (see Entry above), reveals its true colors by multiplying in McCoy cells without arginine, histidine, lysine, or serine in the growth medium (86). *C. pneumoniae* requires all the amino acids of Eagle's medium (116) except lysine, and *C. trachomatis* biovar trachoma serovar E needs all the Eagle's medium amino acids except threonine (240).

The differential requirement of *C. psittaci* 6BC and *C. trachomatis* biovar LGV for arginine, histidine, and lysine is of particular interest because these amino acids are all required for growth of host cells in culture (116). The MN/Cal 10 strain of *C. psittaci* contains each of these amino acids (274), and it is likely that other strains do as well. One, but not the only (see reference 16), explanation for the difference is that only *C. psittaci* 6BC synthesizes the three amino acids itself. *C. psittaci* MN/Cal 10 decarboxylates diaminopimelic acid (the precursor of lysine in lysine-independent, free-living bacteria) to lysine and may satisfy part of its lysine needs in this way (318), although lysine from the growth medium is also incorporated into chlamydial protein (173). In cycloheximide-treated L cells, the label of [^{14}C] glutamate appears in the arginine of both the 6BC and MN/Cal 10 strains of *C. psittaci* (479). It is not known whether chlamydiae synthesize histidine or any of the several amino acids that are not required by either parasite or host.

Host-free RBs of *C. psittaci* transport lysine (176) and methionine (179) by mechanisms entirely dependent on exogenous ATP, which is hydrolyzed to ADP in order to establish an energized membrane. Other amino acids are probably taken into RBs by similar mechanisms. How amino acids pass through the inclusion membrane has never been studied.

In suspensions of L cells that have grown for 24 h in medium 199 (296), the level of isoleucine is too low to support further division of the L cells, and when such cultures are infected with *C. psittaci* 6BC, the chlamydiae do not multiply but persist in a latent state (173). The minimum concentration of isoleucine that must be added to the infected cultures to initiate chlamydial multiplication is the same as that required to restore L-cell division. No level of isoleucine will permit one to multiply without the other. The minimum isoleucine level is determined solely by the total L-cell density, not by the density of infected L cells. The addition of cycloheximide to 24-h-infected L-cell suspensions initiates *C. psittaci* 6BC multiplication in the absence of added isoleucine or L-cell division (173), probably because cycloheximide, by inhibiting synthesis of host protein, makes all of the limited amount of isoleucine arising from normal turnover of host protein available for synthesis of chlamydial protein. *C. psittaci* 6BC growing in 24-h-old L-cell suspensions plus cycloheximide incorporates five times as much host-derived isoleucine into its proteins as does *C. psittaci* 6BC growing in freshly fed host cell suspensions with no cycloheximide (173). These results suggest that chlamydiae and host cells draw on the same amino acid pools for synthesis of protein and that they compete with

each other for the amino acids of the host pools. With respect to *C. psittaci* 6BC, L cells, and isoleucine, it appears that the parasite can get enough amino acid for protein synthesis only when the concentration of isoleucine is sufficient to permit L-cell division but that this restriction can be removed by inhibition of host protein synthesis.

Cycloheximide also reduces the amino acid requirements of other chlamydiae. In its presence, the requirement for growth of *C. psittaci* GPIC in McCoy cells, as measured by single amino acid omission, drops from six amino acids to only three—leucine, valine, and phenylalanine (5, 86)—and the addition of arginine, serine, and lysine is no longer required for multiplication of *C. trachomatis* biovar LGV (5, 335). The amino acid requirements for growth of 15 chlamydial strains in cycloheximide-treated McCoy cells are different at the levels of species, biovar, and serovar (5). Of the 12 amino acids in Eagle's minimum essential medium (116), arginine, cysteine, lysine, and threonine are not needed by any strain. Most strains need added leucine, valine, and phenylalanine, although a few have unique sets of requirements. *C. trachomatis* is distinguished from *C. psittaci* by its histidine demand; the LGV biovar of *C. trachomatis* needs methionine and the trachoma biovar does not; *C. trachomatis* serovars A through C (classical trachoma) require tryptophan, but serovars D through I (nonclassical oculogenital infections) have no need of it. This is a rare metabolic difference between the two groups of serovars. When McCoy cells are starved for 2 days in Earle's balanced salt solution, treated with cycloheximide, and infected with a freshly isolated D/E serovar of *C. trachomatis*, chlamydial multiplication absolutely requires the addition of cysteine, histidine, isoleucine, leucine, phenylalanine, and valine to the growth medium; five other amino acids stimulate multiplication but are not required (216). It may be that starvation reduces host amino acid pools beyond the level achieved with cycloheximide alone, or it may be that the freshly isolated *C. trachomatis* strain (only six passages in McCoy cells) is more dependent on exogenous amino acids than the laboratory-adapted strains used in other studies on amino acid requirements. The possibility that passage through different hosts modifies chlamydial properties has never been seriously examined.

The amino acids that must still be furnished by the growth medium in the presence of cycloheximide are those that are otherwise at too low a concentration in the host pools to support chlamydial multiplication, even in the absence of competition from the host's protein-synthesizing machinery. The extreme strain-to-strain variability in the amino acids that must be supplied in the growth medium for chlamydiae to multiply in cycloheximide-inhibited host cells suggests that different chlamydiae need these amino acids for different reasons. In cycloheximide-treated McCoy cells, turnover of host protein and chlamydial biosynthesis appear to provide all the essential amino acids needed for growth of *C. psittaci* GPIC except leucine, valine, and phenylalanine (4, 5). Why only these three amino acids are required in the growth medium is not immediately evident. Their minimum required concentrations are only 5 to 20% of that found in Eagle's medium. They do not appear in strain GPIC protein in unusually high amounts, they are not degraded in infected cell cultures, and leucine, at least, enters McCoy cells at the same rate as the nonessential amino acid threonine (4). For this particular chlamydia-host cell pair, the requirement is generated by amino acid antagonisms (86). Valine is not needed if isoleucine is also omitted, the phenylalanine requirement is abolished when tryptophan is not added, and leucine can be omitted if both isoleucine and valine are also left out. Amino acid antagonisms are common among bacteria, but the leucine-isoleucine-valine antagonism is rare. It has been suggested that antagonisms occur when the extracellular presence of one amino acid reduces the intracellular level of a second below its stimulatory threshold (86). If the second amino acid is synthesized by chlamydiae, then the first may also antagonize its biosynthesis (86). There are other examples of the role of amino acids in regulating chlamydial multiplication. Both the trachoma and LGV biovars of *C. trachomatis* grow to 100-fold-higher titers in FL cells in the presence of the nonessential amino acid serine than in its absence, although *C. psittaci* 6BC is indifferent to the presence of this amino acid (335), and the growth of *C. pneumoniae* in HeLa cells is increased by the complete or near complete depletion of lysine and methionine (240). Since chlamydial EBs (but not RBs) contain three outer membrane proteins (12, 59, and 62 kDa) exceptionally rich in cysteine that are not synthesized until RBs begin to change into EBs (see "Synthesis of protein" above for references), it is not surprising that cysteine deprivation specifically blocks the RB-to-EB conversion. As judged by electron microscopy (444) or staining with acridine orange (3), omission of cysteine from cultures of cycloheximide-treated McCoy cells infected with *C. trachomatis* biovar trachoma blocks differentiation of RBs into EBs. The effect is cysteine specific; omission of other amino acids does not inhibit the conversion. Addition of cysteine to cysteine-deprived cultures partially restores infectivity, and the restoration is blocked by chloramphenicol. Impairment of the conversion of RBs to EBs by cysteine deprivation occurs in 3 strains of *C. psittaci* and 10 other strains of *C. trachomatis*, although the block is not as severe as in the intensively studied strain. The three cysteine-rich outer membrane proteins are probably a part of all chlamydial EBs.

Synthesis of nucleic acids. The genomes of *C. trachomatis* biovar LGV (406) and *C. psittaci* MN/Cal 10 (281) are double-stranded DNA molecules with molecular weights of 660×10^6. The chlamydial genome is among the smallest of all procaryotic genomes; only the *Mycoplasma* genome is smaller (372). The trachoma and LGV biovars of *C. trachomatis* also carry a common, closely related 7.5-kb plasmid (91, 184, 212, 215, 339, 439). Its consistent presence in a large number of *C. trachomatis* isolates has suggested an essential role in reproduction of this species. However, one *C. trachomatis* biovar LGV isolate has no 7.5-kb plasmid (355). Genes of the 7.5-kb plasmid are expressed in HeLa cells (339), and one gene product shares amino acid sequences with the DnaK protein of *E. coli* (91, 438). Some, but by no means all, strains of *C. psittaci* have a 7.5-kb plasmid that is not related to the *C. trachomatis* plasmid of the same size (212, 264, 284, 339, 473). It is unlikely that this plasmid is required for *C. psittaci* reproduction. Rare chlamydial isolates are also infected with viruses (171, 383). One chlamydial virus has been isolated from a duck strain of *C. psittaci* (450). It is a 22-nm icosohedral virus with a single-stranded, 4.8-kb DNA that is related to but distinct from the bacterial viruses ϕX174 and S13. So far, the virus has infected only avian strains of *C. psittaci*. Does this mean that chlamydiae have restriction endonucleases? The RNAs of *C. psittaci* MN/Cal 10 RBs are predominantly 21S, 16S, and 4S rRNAs (466), whereas EBs contain mainly 4S rRNAs (462). RBs of *C. psittaci* MN/Cal 10 have about three times as much total RNA as EBs (466). The 16S rRNA of *C. psittaci*, *C. trachomatis*, and *C. pneumoniae* is of an apparently unique eubacterial variety (340, 502a, 504).

In L cells heavily infected with *C. psittaci* MN/Cal 10, the absolute amounts of both RNA and DNA begins to increase 10 to 15 h after infection (189, 462). At peak concentration, there is about half again as much DNA and RNA in infected host cells as in uninfected ones. In FL cells inoculated with the LGV biovar of *C. trachomatis* (36) and in HeLa cells infected with the trachoma biovar (87), there is a comparable absolute increase in RNA and DNA, but it occurs more slowly. Autoradiography shows that the newly made nucleic acids appear mainly in chlamydial inclusions (36, 87, 96, 294, 462).

The newly synthesized chlamydial RNA is almost all rRNA. Centrifugation of phenol extracts of ^{32}P-labeled L cells infected with *C. psittaci* MN/Cal 10 yields three peaks corresponding to the 21S, 16S, and 4S RNAs in ribosomes from purified chlamydiae (459, 462). In *C. trachomatis* biovar LGV-infected FL cells, the newly synthesized RNA appears as 23S, 17.2S, and 16S species (158). The 23S and 16S species are rRNAs, and the 17.2S RNA is a precursor of the 16S rRNA. As measured by incorporation of tritiated cytidine, synthesis of chlamydial RNA is first detected 10 to 15 h after infection, reaches a maximum rate at 25 to 30 h, and then declines (158, 414). At its peak, the rate of RNA synthesis in *C. psittaci* MN/Cal 10 equals that of uninfected host cells (414, 462). Synthesis of chlamydial RNA is inhibited by rifampin (158, 481) and dactinomycin (462), but only if the latter drug is added not later than 5 h after infection.

Thymidine, the customary label for DNA, is incorporated into chlamydial DNA with extraordinary inefficiency (see "Nucleoside metabolism" below). Consequently, either cytidine or uridine is used as a label for chlamydial DNA, and the much larger quantity of simultaneously labeled RNA must be removed by digestion with RNase (96) or hydrolysis with alkali (2, 414). The rate of DNA synthesis in [^{3}H]cytidine-labeled *C. psittaci* MN/Cal 10-infected L cells rises and falls in a temporal pattern identical to that just described for RNA (414), and the highest rate achieved is equal to that of uninfected L cells. As estimated by quantitative autoradiography, the DNA of an ornithosis strain of *C. psittaci* is made 2.5 times as fast as that of its HeLa cell host (96).

Although cycloheximide and emetine inhibit nucleic acid as well as protein synthesis in eucaryotic cells (122, 123, 155), cycloheximide only incompletely blocks synthesis of RNA (2). Emetine is a more effective inhibitor of RNA synthesis and was used to establish the precursor nature of the 17.2S RNA of *C. trachomatis* biovar LGV (158). The same concentration of cycloheximide that blocks protein synthesis in L cells also reduces incorporation of [^{14}C]uridine into L-cell DNA by more than 90%, although it takes 3 h to achieve this level of inhibition (2). The uptake of labeled uridine into the DNA of *C. psittaci* MN/Cal 10 growing in cycloheximide-treated L cells follows the same time curve established by isolating chlamydial DNA from uninhibited L cells (2, 414). During the time that multiplication of *C. psittaci* is most rapid, the amount of uridine incorporated into chlamydial DNA doubles every 2 h, a rate that is consistent with the 2- to 3-h generation time established for an ornithosis strain of *C. psittaci* by microcinematography (360).

Host-free RBs of *C. psittaci* MN/Cal 10 incorporate labeled UTP and GTP into RNA in the presence of P_i (to inhibit chlamydial polynucleotide phosphorylase) and all four ribonucleoside triphosphates by means of a DNA-dependent RNA polymerase (460). The incorporation is inhibited by dactinomycin (460) and rifampin (175). As described above in "Initiation of Multiplication", mercaptoethanol-treated EBs of *C. trachomatis* biovar LGV incorporate [^{3}H]UTP into RNA when supplied with the four ribonucleoside triphosphates by a mechanism inhibited by dactinomycin and rifampin (407, 408). Whether such a reaction also occurs in *C. trachomatis* RBs is not known. Host-free RBs of *C. psittaci* 6BC and *C. trachomatis* biovar LGV incorporate the four deoxyribonucleoside triphosphates into DNA that hybridizes to chlamydial DNA but not to host cell DNA (181). The requirement for TTP as one of the four deoxyribonucleoside triphosphates does not fit with the many observations that thymidine is very poorly incorporated into the DNA of chlamydiae multiplying inside host cells. This discrepancy will be considered below in "Nucleoside metabolism." Although host-free synthesis of protein and RNA is totally dependent on the presence of ATP, host-free synthesis of DNA not only does not require ATP but is actually inhibited by it (175). How ATP inhibits DNA synthesis is not yet known.

Nucleoside metabolism. When the RNA and DNA of HeLa cells and L cells are tagged with a variety of radiolabels and then infected with *C. psittaci* (an ornithosis strain or MN/Cal 10), the prelabeled nucleic acids are not broken down faster than in uninfected host cells and their radiolabels do not preferentially appear in newly synthesized chlamydial RNA and DNA (96, 257). The precursors for chlamydial biosynthesis of nucleic acid must come from the cytoplasmic nucleoside and nucleotide pools of the host or from de novo synthesis by the chlamydiae themselves. Both L cells and *C. psittaci* incorporate exogenous cytidine, uridine, adenosine, and guanosine into their RNA and DNA (482). Adenine and guanine, but not cytosine and uracil, are also good precursors of host and chlamydial RNA. However, in a line of HeLa cells deficient in the enzyme that catalyzes the conversion of guanine into GMP, *C. psittaci* does not incorporate growth medium guanine into its RNA (76). These observations suggest that chlamydiae depend on their hosts for RNA precursors at the nucleotide level, a suggestion consistent with the already described findings that ribonucleoside triphosphates are the immediate precursors of *C. psittaci* RNA both in infected host cells (172) and in host-free RBs (460).

Investigations on the precursors of chlamydial DNA have been dominated by the early discovery that thymidine is incorporated very poorly into the DNAs of *C. trachomatis* and *C. psittaci* (36, 96, 294, 348, 414, 440). There were also early hints that the impediment to chlamydial utilization of thymidine is not absolute (36, 96). Exogenous deoxyadenosine and deoxyguanosine are equally efficient precursors of DNA in *C. psittaci* and L cells, but deoxycytidine, the other pyrimidine deoxyribonucleoside, is as poor a precursor of chlamydial DNA as thymidine, although it, too, is readily incorporated into host DNA (482). When *C. psittaci* 6BC is grown in either L cells (174) or mouse macrophages (278) in the presence of much higher levels of [^{3}H]thymidine than used in previous studies, autoradiographs show significant incorporation into chlamydial inclusions, although it is still much less than in the host cell nuclei. Comparison of the incorporation of high concentrations of labeled thymidine into L-cell nuclei and purified *C. psittaci* 6BC shows that the chlamydiae sequester thymidine nucleotides from L-cell pools 265 times less efficiently than their hosts (174). The most likely explanation of this disparity is that *C. psittaci* comes in a poor second in competing with the L cells for thymidylate pools, a situation that may be aggravated by localization of these pools in the nucleus, away from the cytoplasmic chlamydial inclusion (174). However, cyclohex-

imide, which might be expected to abolish host competition, does not increase the efficiency of thymidine utilization by *C. psittaci* 6BC (174). Absence of host competition would explain why *C. psittaci* 6BC has no difficulty in using TTP and dCTP for synthesis of DNA in host-free systems (175, 180). Another possibility that cannot be ignored is that the deoxypyrimidine nucleoside triphosphates are not readily transported across the inclusion membrane.

Although *C. psittaci* can, albeit inefficiently, utilize exogenous thymidine, it does not depend on host thymidylate pools for synthesis of DNA. *C. psittaci* MN/Cal 10 and 6BC multiply just as well in L cells deficient in thymidine kinase, which converts thymidine to TMP, as they do in L cells containing the enzyme, and thymidine kinase is not induced in *C. psittaci*-infected, thymidine kinase-deficient L cells (174, 258). The best-characterized thymidine kinase-less cells are mammalian cell lines and bacteria that have been deliberately selected for this deficiency. Lack of thymidine kinase in a naturally occurring organism such as *C. psittaci* is rare, but perhaps not as rare as once thought. In a recent study of thymidine utilization by marine bacteria, 4 of 41 isolates showed no thymidine kinase activity (203). In thymidine kinase-deficient host cells, there is no incorporation of even high concentrations of [^{3}H]thymidine into chlamydial DNA, which shows that a functioning host thymidine kinase is necessary for utilization of thymidine for synthesis of chlamydial DNA (174). It has been suggested that the main source of thymidylate residues in *C. psittaci* DNA is the conversion of dUMP to TMP by a chlamydial thymidylate synthetase (96, 258, 482). It is not known how well this picture of thymidylate metabolism, drawn almost exclusively from studies on the 6BC and MN/Cal 10 strains of *C. psittaci*, fits other chlamydiae.

Synthesis of glycogen. The accumulation of glycogen in *C. trachomatis* inclusions in epithelial cells from conjunctival scrapings from trachoma patients was reported long before the trachoma agent was grown in chicken embryos or cell cultures and its relationship to *C. psittaci* was established (379). Glycogen is found only in inclusions of *C. trachomatis* (all three biovars, although the mouse biovar has been but little studied) and never in those of *C. psittaci* (149) or *C. pneumoniae* (151). The presence or absence of glycogen, usually established by staining with iodine, is one of the characteristics that differentiates *C. trachomatis* from the other two chlamydial species (314).

As characterized by physical, chemical, and enzymatic methods, the great bulk of polysaccharide isolated from *C. trachomatis* biovar LGV grown in BHK cells is a glycogen with an average chain length of 14 to 16 glucose residues (136). About 5% of the polysaccharide is either not glucose or is glucose groups linked differently than in glycogen. Glycogen appears in inclusions of *C. trachomatis* 20 to 30 h after infection, reaches a maximum at 30 to 60 h, and then slowly declines (36, 124, 137, 149, 280). Multiplication of *C. trachomatis* and accumulation of glycogen follow parallel curves. Glycogen is deposited in the RBs in the form of discrete particles visible by electron microscopy that are released into the intrainclusion space when the RB envelopes rupture (280). As the infection progresses, inclusions and host cells disintegrate to release glycogen into the growth medium, where it is slowly hydrolyzed (124, 137).

The biosynthesis of glycogen is brought about by the enzyme glycogen synthetase, which adds glucose units to a preexisting glycogen primer. In mammalian cells, the glucose donor is UDP-glucose, formed from UTP and glucose-1-phosphate (249), whereas in bacteria, the donor is ADP glucose, made from ATP and glucose-1-phosphate (153). The glycogen synthetase active in *C. trachomatis* biovar LGV-infected HeLa cells is of the bacterial type (206, 502). Lysates of HeLa cells infected with *C. trachomatis* incorporate ADP-glucose into glycogen at rates that increase as the developmental cycle proceeds. Uninfected HeLa cell lysates incorporate only UDP-glucose into glycogen and at a much slower rate. Additional evidence that the glycogen in *C. trachomatis* inclusions is made by chlamydial enzymes comes from inhibitor studies. Glycogen synthesis is inhibited by penicillin (36, 149, 280) and chloramphenicol (137, 502) but not by cycloheximide (502). However, it appears that there is an as-yet-undefined host influence. The yield of glycogen is independent of the multiplicity of infection, it does not always correlate with the extent of chlamydial multiplication (124, 137), and, in different cells infected with the same strain of *C. trachomatis*, the rates and magnitudes of glycogen synthesis and breakdown may differ widely (502).

Synthesis of glycogen from glucose-6-phosphate, which is transported into both *C. trachomatis* and *C. psittaci* (487, 506), requires at least three enzymes: phosphoglucose isomerase to convert glucose-6-phosphate into glucose-1-phosphate, ADP-glucose pyrophosphorylase, and glycogen synthetase. Addition of each glucose unit to the glycogen backbone requires the expenditure of two host-generated ATPs, although this has not yet been demonstrated in host-free RBs. It may be assumed that *C. trachomatis* has all three enzymes and that *C. psittaci* lacks one or more. Granular cytoplasmic deposits of glycogen are common among many species of *Bacillus* and *Clostridium* and among many genera of the family *Enterobacteriaceae* (103). In these bacteria, glycogen storage occurs when nitrogen is limiting but carbon is still available and is regarded as a means of storing carbon in an osmotically inert form that can be used for macromolecule synthesis when nitrogen is no longer limiting. It is hard to see how glycogen accumulation could serve such a function in obligately intracellular *C. trachomatis*, but it is also hard to believe that the genes for at least three enzymes would have been conserved in a genome as small as that of *C. trachomatis* unless they contributed to survival. Perhaps synthesis and accumulation of glycogen, originally as a carbon store in a remote free-living ancestor, have acquired new and as yet unknown functions in the intracellular multiplication of *C. trachomatis* that have no counterpart in the other two chlamydial species.

Synthesis of lipids. The composition of chlamydial lipids has been reviewed elsewhere (326). Of particular interest to a discussion of the biosynthesis of chlamydial lipids is the presence in chlamydiae of lipids not found in their host cells, notably the C 15:0 and C 17:0 anteiso branched-chain fatty acids and phosphatidyl glycerol (126, 127, 140, 205, 269, 326). It is hard to follow the biosynthesis of chlamydial lipids inside of host cells because the chlamydiae do not make nearly as much lipid as their hosts and because they progressively inhibit host lipid synthesis as the infection proceeds. However, in monkey kidney (MK-2) cells infected with *C. psittaci*, the chlamydial branched-chain fatty acids accumulate, and there is a severalfold increase in the rate at which labeled serine is incorporated into phosphatidyl ethanolamine at 25 to 30 h after infection (126, 127). Cycloheximide (2 μg/ml for 24 h) inhibits 90% of the lipid biosynthesis of L cells without affecting the multiplication of *C. trachomatis* biovar LGV (373). When [^{3}H]isoleucine is used to label newly synthesized branched-chain fatty acids and phospho-

lipids, the synthesis of lipids in infected L cells increases from 12 h after infection to a maximum at 25 to 30 h that is 100 times the rate in cycloheximide-treated, uninfected L cells (373). Host-free mixtures of RBs and EBs prepared from allantoic fluid from *C. psittaci*-infected chicken embryos incorporate the carbon of labeled aspartate, isoleucine, and glucose-6-phosphate into trichloroacetic acid-insoluble fractions, and almost all the label appears in the branched-chain fatty acids of phosphatidyl ethanolamine and phosphatidyl choline (140, 511). The incorporation of aspartate and isoleucine depends on the presence of exogenous ATP, and the uptake of glucose-6-phosphate, the best label of the three, is stimulated by it (511). Why the demonstrated chlamydial biosynthesis of lipid is limited to their unique branched-chain fatty acids is not immediately evident.

Metabolism of B vitamins. *C. psittaci* 6BC does not grow in L cells that have been kept for 2 days in balanced salt solution (17). When synthetic media deficient in a single B vitamin are added to the depleted L cells, only the medium lacking thiamine fails to support chlamydial growth. It is not known why a thiamine requirement is so easily demonstrated. Prolonged depletion of the L cells in vitamin-deficient media shows that when thiamine is present, pantothenate, niacin, pyridoxin, and choline are not absolutely required but are needed for maximal growth. No requirement for biotin, inositol, riboflavin, or folate is evident, even after long depletion. Although negative results may only mean incomplete depletion, riboflavin is probably not required because there are no riboflavin respiratory enzymes in chlamydiae, and some chlamydiae may make their own folates.

The growth of *C. psittaci* 6BC in chicken embryos and in cell culture is inhibited by sulfadiazine, and the growth suppression is reversed competitively by *p*-aminobenzoic acid and noncompetitively by folic acid and folinic acid (297, 298). Folate analogs such as aminopterin inhibit the growth of *C. psittaci* 6BC as well as that of the MN/Cal 10 strain, which is not subject to growth inhibition by sulfadiazine, and this inhibition is also reversed by folic and folinic acids (297, 298). Trimethoprim, a 2,4-diaminopyrimidine that inhibits dihydrofolate reductase (135), suppresses the growth of *C. trachomatis* biovars trachoma and LGV in HeLa cells and in chicken embryos, and the suppression is reversed by folinic acid (376). From a taxonomic point of view, the relation of the 6BC strain to sulfonamides is anomalous. It is the only well-characterized strain of *C. psittaci* (259) or *C. pneumoniae* (151) that is not sulfonamide resistant, whereas all well-characterized strains of *C. trachomatis* are sulfonamide sensitive (259), and growth inhibition by sulfadiazine is one of the differentiating characteristics of the three chlamydial species (314). This selective action of sulfonamides on the different chlamydial species has important implications when interpreted in terms of their mode of action, which is inhibition of the synthesis of folates from simpler components (135). The chlamydial host does not make folate. It requires the preformed vitamin and is, therefore, sulfonamide insensitive. Therefore, if sulfadiazine inhibits growth of a chlamydia, it may be supposed that the chlamydia synthesizes folate and that this synthesis is vital to its multiplication. Conversely, it may be supposed that a chlamydia that is resistant to growth inhibition by sulfadiazine but still sensitive to folate analogs has lost the ability to synthesize folates and acquired the ability to utilize host-supplied folates.

Two sulfonamide-susceptible chlamydiae (*C. psittaci* 6BC and *C. trachomatis* mouse pneumonitis) and two insusceptible strains (*C. psittaci* MN/Cal 10 and feline pneumonitis) were grown in chicken embryos, and mixtures of EBs and RBs were separated from infected allantoic fluid (90). Bioassay and bioautography with the folate requirers *Lactobacillus casei* and *Pediococcus cerevisiae* revealed that each strain has a set of compounds active for the assay organisms that is different from the sets of the other three chlamydiae and of the chicken embryo host. L cells infected with *C. psittaci* 6BC contain over twice as much folate as do uninfected L cells (194).

When EB-RB mixtures of *C. trachomatis* mouse pneumonitis are incubated at 37°C for 6 h, there is a severalfold increase in their growth-promoting activity for *P. cerevisiae* as measured against a folinic acid standard (88, 89). This increase appears to come from two kinds of enzymatic activity, synthesis of folinic acid from small-molecule precursors and hydrolysis of folate conjugates inactive for *P. cerevisiae* to folinic acid. Less-extensive data indicate that the same kind of reactions also take place in *C. psittaci* MN/Cal 10, which is sulfonamide insensitive and should, according to the hypothesis just proposed, not be making its own folate. To further confuse the issue, folinic acid synthesis from low-molecular-weight precursors in EB-RB mixtures is inhibited by sulfadiazine in *C. trachomatis* mouse pneumonitis but not in *C. psittaci* MN/Cal 10. Although the experiments with host-free chlamydiae offer reasonable evidence that chlamydiae synthesize folates, they fall short of explaining just why *C. trachomatis* is susceptible to growth inhibition by sulfadiazine and *C. psittaci* usually is not. It would be worthwhile to reinvestigate folate synthesis and sulfonamide susceptibility with modern methods of preparing pure RB populations and identifying and quantitating folates.

In L cells, NADP stimulates the multiplication of *C. psittaci* 6BC (142), and in host-free *C. psittaci* MN/Cal 10, it increases the release of CO_2 from labeled glucose (506) and brings about the reduction of added cytochrome *c* (7, 8, 464). These effects of NADP deserve further investigation in light of the failure of many host-independent bacteria to transport NAD intact (for references, see reference 14). Many activities of host-free *R. prowazekii* are also enhanced by NAD (15). This obligately intracellular bacterium does not transport NAD intact, and the source of its intracellular NAD has yet to be established (14).

Some dark areas. Although the metabolic interactions between parasite and host that result in chlamydial multiplication have been defined in broad outline, there remain many dark regions of the chlamydial cell-host cell interaction that must still be illuminated. It is generally agreed that chlamydiae exploit the cytoplasmic pools of their hosts for energy-rich compounds and synthetic intermediates. The ATP-ADP translocase and the ATP-dependent transport system for lysine are models for how such exploitation might occur, but there must be many other specific, energy-requiring transport systems to bring across the chlamydial cytoplasmic membrane the many constituents of the host cytoplasmic pools required for chlamydial multiplication. It has been suggested that the ATP-ADP translocase is part of a complicated network of nucleotide transport mechanisms that allow chlamydiae to use a number of different host-generated energy-rich compounds both as substrates and as energy sources (175). A GTP-GDP translocation system has already been demonstrated in *C. psittaci* 6BC and *C. trachomatis* biovar LGV (175). There must be transport systems for amino acids other than those already described for lysine and methionine. In view of the importance of cysteine to the

RB-to-EB transformation, study of cysteine transport should be particularly interesting. In addition to the substrates for macromolecule synthesis that must be imported in stoichiometric amounts, cofactors and vitamins that are required intact may also have specific mechanisms for their transport. For example, utilization of folate by free-living, sulfonamide-resistant bacteria is dependent on an active energy-requiring process (519), and the same is probably true for *C. trachomatis*.

Localization of the synthesis of specific constituents of chlamydial cells to particular segments of the growth cycle and identification of their roles in chlamydial multiplication are just beginning. A few good starts in this direction have been described in previous sections, but far too little is known about the time of synthesis or function of most chlamydial constituents. Substances found in chlamydiae but not in host cells should make especially good objects of investigation. For example, among the chlamydial lipids and glycolipids there are the C 15:0 and C 17:0 anteiso branched-chain fatty acids, phosphatidyl glycerol, and the 3-hydroxy long-chain fatty acids and 3-deoxy-D-*manno*-2-octulosonic acid of the chlamydial lipopolysaccharide. Of these, only the synthesis of the branched-chain fatty acids has been studied so far.

Perhaps the greatest darkness of all enshrouds the failure of host-free chlamydiae to support the synthesis of macromolecules for more than a very few hours and even then at rates far less than those achieved in host cells. The many reasons for this failure and the substantial benefits that would accrue if it were ever overcome have been discussed in detail elsewhere (175).

Role of the Inclusion Membrane in Chlamydial Multiplication

Like many other intracellular parasites, both eubacterial and eucaryotic, chlamydiae multiply in inclusions separated from the main intracellular space of the host cell by inclusion membranes that are the outgrowths in time and space of the phagocytic vacuoles in which they entered their hosts (309). All the raw materials of parasite multiplication supplied in the growth medium or synthesized by the host cell must be transported across these membranes. It is widely assumed that intracellular parasites modify surrounding host membranes in ways that contribute to successful multiplication, but just what these modifications are and how they are brought about have not been satisfactorily determined for any intracellular parasite.

During the course of the chlamydial developmental cycle, the membrane enclosing a phagocytic vacuole with its single entering EB expands to surround a mature inclusion containing as many as several hundred RBs, EBs, and intermediate bodies while remaining intact until late in the developmental cycle (11, 131, 187, 251, 283, 364). Inclusion membranes are so remarkably stable that intact inclusions may be released from host cells by ultrasonic energy or by homogenization and purified by centrifugation or filtration through fine-mesh sieves (41, 279, 529). Intact inclusions of *C. trachomatis* biovar LGV have been separated from BHK-21 cells 23 h after infection (41), and inclusions of *C. psittaci* MN/Cal 10 and 6BC have been prepared from L cells at 1 h (529) and 20 h (279). At 1 h, the membranes surrounding single *C. psittaci* 6BC EBs exhibit 10 major electrophoretic bands, 7 of which correspond in mobility to proteins of the L-cell surface (529). Comparable data for 20-h inclusion membranes are not available. Comparison of membranes from 20-h *C. psittaci* inclusions with plasma membranes of L cells by the freeze-replica technique suggests that the morphologies of the two structures are different (279). RBs in inclusions separated from L cells 20 h after infection have surface projections that are probably homologous with the previously described surface projections of EBs (279). In thin-section electron micrographs, it appears that the RBs are directly connected with the host cytoplasm through the canals of these projections, and it has been suggested that the connections may provide an avenue of interchange between RBs and the main cytoplasmic compartment of the host (279).

Since cycloheximide prevents neither multiplication of chlamydiae (1) nor expansion of the membrane enclosing the growing inclusion (84, 437), synthesis of inclusion membranes does not seem to depend on continued synthesis of host protein. This possibility has been explored by studying the incorporation of newly synthesized proteins into the internal membranes of L cells infected with *C. psittaci* MN/Cal 10 and labeled with ^{14}C-amino acids in the presence and absence of cycloheximide (448). Membranous organelles from whole L-cell homogenates were separated by centrifugation on discontinuous sucrose gradients. In the absence of cycloheximide, incorporation of labeled amino acids into the membrane proteins of *C. psittaci*-infected L cells is not significantly depressed for 20 h after infection, but after that time, it stops almost completely. When 5 μg of cycloheximide per ml is added 2 h before the labeled amino acids, incorporation into the membranes of both uninfected and infected (0 to 30 h) L cells is almost completely inhibited. If large amounts of proteins synthesized by *C. psittaci* had been incorporated into the inclusion membrane, then a cycloheximide-resistant incorporation of the amino acid label should have been observed. It appears that most of the membrane proteins used to expand the inclusion membrane are not newly synthesized by either host or parasite, although minor contributions from either source cannot be ruled out. When [^{14}C]glucosamine, which is incorporated mainly into membrane glycoproteins, was used to label L-cell membranes instead of ^{14}C-amino acids, a different set of results was obtained (449). Infection with *C. psittaci* MN/Cal 10 has no effect on incorporation of glucosamine into membrane proteins for as long as 30 h after infection. In uninfected L cells, cycloheximide almost completely inhibits glycosylation, but in infected host cells, there is a low but significant cycloheximide-resistant uptake of labeled glucosamine into membrane fractions that increases as chlamydial multiplication proceeds. This cycloheximide-insensitive glycosylation is probably a chlamydial activity, and it is tempting to conclude that the inclusion membrane is modified by chlamydia-catalyzed glycosylation. However, more work is needed to show that it is the inclusion membrane that is glycosylated and that its properties are thereby altered in ways favoring chlamydial multiplication.

Biogenesis of inclusion membranes from preexisting membranes of the host cell is not hard to visualize in light of the large and continuous exchange of membranes among the organelles of eucaryotic cells (156, 428). What is hard to visualize is how the host cell alone can possibly assemble a whole repertoire of inclusion membranes, each with a set of properties tailored to meet the particular needs of each of the many intracellular parasites that multiply in intracytoplasmic inclusions. For example, *C. psittaci* uses host ATP and *C. burnetii* does not. It is more plausible to assume that *C. psittaci* somehow inserts an ATP-ADP translocase into its inclusion membranes, whereas *C. burnetii* does not, than to

assume that the host cell "knows" when the translocase is needed. When the malarial parasites *Plasmodium lophurae* (478) and *Plasmodium knowlesi* (328) are separated from their erythrocyte hosts still enclosed in vacuolar membranes, macromolecule precursors are transported across the vacuolar membranes, and the plasmodia complete at least one cycle of development. Comparable experiments with chlamydiae, although very difficult, would not be impossible.

The relation of mitochondria to the chlamydial inclusion has interested many investigators because mitochondria generate the ATP that chlamydiae absolutely require. Close juxtaposition of mitochondria and inclusions has been noted in *C. psittaci* infections (131, 275, 280, 452), but not in *C. trachomatis* infections (73). Since both chlamydial species use host ATP, close association between mitochondria and inclusions does not seem to be a necessity. It has already been mentioned that *C. trachomatis* biovar LGV grows normally in a mitochondrion-deficient mutant Chinese hamster kidney cell line (346). In addition, other intracellular parasites, such as legionellas (196) and toxoplasmas (211), also grow in intracytoplasmic inclusions that often lie close to mitochondria, even though these parasites are not known to use host ATP.

Effect of Chlamydial Multiplication on Host Cells

Nowhere is the role of multiplicity of infection more dominant than in determining the effect of chlamydial multiplication on host cells. With infecting doses of the order of a single EB per host cell, the effect is minimal and infected cells may continue to divide. However, most studies on the biochemistry of chlamydial multiplication have employed populations in which more than 90% of the host cells have been infected. At the multiplicity of infection required to achieve this level of infection, chlamydial growth brings about changes in host cells that lead ultimately to death.

Effect on progression through the cell cycle and cell division. When HeLa cells are infected with an ornithosis strain of *C. psittaci* sufficient to infect 18% of the host cells (about one EB for every five HeLa cells), nearly all inclusion-bearing cells complete the cell cycle without delay (96). However, with multiplicities of infection in excess of about one EB per HeLa cell (more than 40% infected cells), G_1 (the period from the end of M [mitosis] to the start of S [DNA synthesis]), G_2 (the time from the end of S to the onset of M), and possibly M are prolonged. The duration of S is doubled, with a proportional decrease in rate of DNA synthesis. However, only at extremely high multiplicities of infection (90% or more of host cells are infected) is DNA synthesis prevented or indefinitely prolonged. In L cells infected with one or two EBs of *C. psittaci* 6BC (about 70% infected cells) and plated out at a very low density so that the clones produced by each cell can be observed individually, the mean generation time of infected L cells is twice as long as that of uninfected cells (195). Half of the infected L cells divide once, and half divide more than once. When the input multiplicity is increased 10-fold (>90% infection), half of the infected cells divide once before most of the cells detach from the substrate at 3 days. With a further fivefold increase in multiplicity, less than 10% of the infected cells manage to divide. In HeLa cell populations inoculated with not more than about five EBs per host cell (up to 90% infected cells), there is no inhibition of host DNA synthesis or progression through the cell cycle in the infected cells that successfully divide (43). However, at 2 to 3 days after infection, cell division is blocked in McCoy cells containing large inclusions of *C. trachomatis* biovar trachoma (72). It has been noted in infections with both an ornithosis strain of *C. psittaci* (96) and *C. trachomatis* biovar trachoma (72) that the larger the inclusion, the more drastic the effect on cell division.

In host cells infected with *C. psittaci* 6BC (195, 332), *C. trachomatis* biovar LGV (43), and *C. trachomatis* biovar trachoma (72), cell division may produce either two infected daughter cells or one infected daughter cell and one uninfected daughter cell. With *C. psittaci* 6BC, two infected cells probably arise from a parent with multiple inclusions, but with *C. trachomatis* biovar trachoma, division and sharing of a single inclusion also occurs (72). It may be that the creation of uninfected host cells from infected ones plays a role in limiting the spread of infection at naturally infected sites and in establishing persistent infections, both in vitro and in vivo.

Although events in the host cell nucleus may be disrupted by chlamydial multiplication, the nucleus itself is not required for that multiplication. Enucleate fragments of HeLa cells obtained by microdissection support normal growth of an ornithosis strain of *C. psittaci* (95), and L cells enucleated with cytochalasin B are still good hosts for *C. psittaci* 6BC (172) and *C. trachomatis* biovar mouse (349).

Effect on internal structures of host cells. Intracytoplasmic bodies as large as chlamydial inclusions must have some effect on the internal structures of the cells in which they reside. The wonder is that the effects are not greater than they actually are. In McCoy cells infected with *C. trachomatis* biovar trachoma, a single mature inclusion occupies an area of 500×10^{-6} m^2 (72), an area greater than that of an entire uninfected cell and more than 1,000 times greater than the calculated area of a single freshly ingested EB and its enclosing phagosomal membrane. In McCoy cells with single large oval-shaped inclusions of the trachoma biovar of *C. trachomatis*, damage to cytoskeletal elements (microfilaments, intermediate filaments, and microtubules) is minimal, probably because the cytoskeleton is distorted to accommodate the expanding inclusion instead of being destroyed (73). This finding almost certainly applies to the LGV biovar and, with less certainty, to *C. psittaci* as well. The two *C. trachomatis* biovars multiply in similar inclusions, but in host cells infected with *C. psittaci*, inclusions are multiple, small, and irregularly shaped or single, large, and lobular. There is also much variation among strains (435).

When L and bovine fetal spleen cells are inoculated with *C. psittaci* 6BC (232) or lamb polyarthritis (475, 476), the fine structure of the host cell remains intact and lysosomal enzymes are localized for 20 to 25 h after infection, although RBs have multiplied extensively to produce large inclusions. Concomitant with the appearance of the first of the new crop of EBs, lysosomal enzymes are released (232, 475, 476). Focal cytoplasmic degeneration is followed by dislocation and destruction of cytoplasmic organelles (mitochondria last of all), degeneration of nuclei, lysis, and death. Inclusions, both membranes and contents, remain intact (476). The stimulus for lysosomal release is not known. Because the release of lysosomal enzymes is closely associated with destruction of host cell structures, it is usually assumed that the relation between the two events is causal, although direct evidence is lacking and other causes cannot be excluded. The role of lysosomal enzymes in the cytopathology of *C. trachomatis* infections has not been studied.

Specialized activities of host cells are intuitively suspect as being particularly susceptible to inhibition by multiplying intracellular parasites. For chlamydia-infected host cells,

ciliary beating comes immediately to mind. The epithelial cells of the genital tract are ciliated, and both chlamydiae and cilia depend on host ATP for their activity. *C. trachomatis* biovar LGV decreased ciliary beating frequency in human nasal polyp cultures as early as 24 h after infection, and by 48 h, half of the cilia were paralyzed (265). However, in other studies, chlamydial infection did not measurably decrease ciliary activity in human, bovine, and murine oviducts (199, 358) and in human fallopian tube organ cultures (92).

Modification of plasma membranes of host cells. Like a number of other intracellular parasites (214, 225, 309, 401), chlamydiae modify the outer membranes of their host cells, presumably in ways that are beneficial to the parasites. Chlamydial LPS appears in the plasma membranes of L, McCoy, and HeLa cells in which *C. trachomatis* biovars LGV and trachoma (217, 380, 382) and *C. psittaci* sheep abortion and ornithosis (380, 382) are multiplying.

The outer membranes of all chlamydiae have an LPS that resembles the LPSs of other gram-negative bacteria, the Re LPS of *Salmonella typhimurium* in particular (49, 66, 111, 112, 330). It contains the epitope for the genus-specific (group) antigen of chlamydiae (49, 66), traditionally demonstrated by complement fixation. The epitope consists of a trisaccharide of 3-deoxy-D-*manno*-octulosonic acid in 2.8 and 2.4 linkage, the former linkage being unique to chlamydiae (48). Chlamydial LPSs also contain D-glucosamine and 3-hydroxy fatty acids typical of LPSs in general, as well as a 22-carbon 3-hydroxy fatty acid found only in chlamydiae (49, 330). Other epitopes, consisting probably of different combinations of 3-deoxy-D-*manno*-octulosonic acid, are shared with the LPSs of other bacteria (48, 66). The function of LPS in the chlamydial economy is not known. However, the conservation of identical LPS epitopes in the two chlamydial species through long separation from other bacteria and from each other (503) indicates that there is a function and that it is vital. Cell cultures infected with *C. trachomatis* biovars trachoma and LGV and the ARK-2 strain of *C. psittaci* secrete a second antigen with genus specificity, a glycolipid structurally unrelated to LPS (453).

As measured with genus-specific complement-fixing antibody, LPS is present at all times in the developmental cycle of *C. trachomatis* biovar trachoma in HeLa cells (375). The amount of LPS increases concomitantly with increase in chlamydial mass. Some of the LPS is host cell associated, and some is in the growth medium. Of the cell-associated LPS, only about half sediments with the EBs. When L, McCoy, or HeLa cells are infected with either *C. psittaci* or *C. trachomatis* and appropriately fixed with formaldehyde or glutaraldehyde, indirect immunofluorescence staining with either polyclonal (380, 382) or monoclonal (217) antibody to LPS reveals the presence of LPS not only in inclusions but also on the plasma membranes and surface blebs of infected cells and on the membranous structures of adjacent uninfected cells. Electron microscopy with an immunoperoxidase reagent confirms the presence of LPS on the surfaces of chlamydia-infected McCoy cells (382). That the chlamydial LPS visualized at the surfaces of infected cells is indeed on their plasma membranes has been shown by doubly infecting L cells with *C. trachomatis* biovar LGV and vesicular stomatitis virus, whose virions are enveloped with portions of the host cell plasma membrane (217). As predicted, vesicular stomatitis virions made in *C. trachomatis*-infected L cells are enriched for chlamydial LPS.

LPS is released from chlamydial cells as 30- to 80-nm single-membrane vesicles, or blebs, formed by evagination of the RB outer membrane (446). Immune electron microscopy shows that they contain LPS. Blebs appear in inclusions at about 24 h and increase in number as the developmental cycle proceeds. Bleb formation may occur during reorganization of RBs into the much smaller EBs (217). Other gram-negative bacteria also produce similar outer membrane blebs (80, 110, 441).

Shedding of LPS, and possibly other outer membrane components as well, may be only a by-product of chlamydial multiplication, of no use to the parasite but of value to the host's immune system as an external marker of chlamydial presence, be it patent or latent. On the other hand, if chlamydiae have evolved mechanisms for modifying host cell membranes in ways that increase the reproductive potential of the chlamydiae, then what better way than to insert chlamydial LPS into those membranes? Accumulation of LPS in cell membranes decreases their fluidity (217), and it has been suggested that LPS-induced changes in membrane fluidity may affect entry and interaction with lysosomes (217). Since LPS passes through the inclusion membrane on its way to the periphery of the host cell, it may also change the properties of that membrane. Immune electron microscopy fails to visualize LPS on the inclusion membrane and other internal membranes, but this may be a failure of technique (382).

Effect on host cell biosynthesis. As they studied the biosynthesis of chlamydial protein, nucleic acid, and lipid in infected cell cultures (see "Biochemistry of Chlamydial Multiplication" above), investigators often noted the effect of chlamydial multiplication on the biosynthesis of corresponding constituents of host cells. For reasons already described, these experiments were done with host populations inoculated at a high multiplicity of infection to ensure the infection of virtually every host cell. Under these conditions, host cell synthesis of RNA (189, 414, 462) and DNA (1, 2, 189, 414, 483) is inhibited. Inhibition is first apparent 15 to 25 h after infection, and it increases as the infection proceeds. Under identical conditions, protein synthesis in infected host cells does not decrease (1, 414), but the increase expected of logarithmically multiplying cells does not occur (1). All these experiments employed L cells infected with *C. psittaci* MN/Cal 10. How the results apply to other host cell-chlamydia interactions is not known. Host lipid synthesis in MK-2 and L cells inoculated with high multiplicities of *C. trachomatis* biovar LGV is also inhibited (126, 373).

Similar inhibition of host biosynthesis obviously does not occur in host cells minimally infected with chlamydiae because these cells progress through the cell cycle and divide normally. Even in heavily infected host cells, inhibition of host biosynthesis is not apparent until well into the developmental cycle, when extensive multiplication of RBs has generated large multiple inclusions. It is most likely that at this time host macromolecule and lipid biosynthesis is disrupted by a combination of structural displacement and metabolite competition on the part of the large chlamydial biomass.

LATE EVENTS, FROM REORGANIZATION OF RBs TO RELEASE FROM HOST CELLS

Reorganization of RBs into EBs

In the very dawn of chlamydial research, examination of Giemsa-stained impressions of spleens from mice infected with a parrot-derived strain of *C. psittaci* led to the proposal

that infectious chlamydial cells (EBs) arise from large forms now called RBs (31). Subsequent work has abundantly confirmed this hypothesis.

Structural changes during reorganization of RBs into EBs. The conversion of RBs into EBs has been followed in host cells infected with *C. psittaci* 6BC, MN/Cal 10, Texas turkey, another ornithosis strain, and feline pneumonitis (131, 187, 251, 261, 262, 353); *C. trachomatis* biovars trachoma, LGV, and mouse (13, 157, 187, 353, 358, 445); and *C. pneumoniae* (82). A variety of cell lines, including L, McCoy, and HeLa, as well as mouse macrophages and oviducts have been used. Although the interval between entry and beginning of reorganization is dependent on the length of the developmental cycle of the particular chlamydia being observed, once started, the sequence of events leading from RB to EB appears to be much the same for all chlamydia-host cell combinations. Through several divisions of intermediate transitional forms between RBs and EBs, the RB progresses toward the EB by reduction in size, internal condensation, differentiation of electron-dense nucleoids, and formation of rigid cell walls. RBs changing into EBs tend to lie close to the inclusion membrane, where they are close to host cell mitochondria and endoplasmic reticulum (353).

In going from RB to EB, the MOMP becomes cross-linked by disulfide bonds, and three cross-linked proteins (12, 59, and 62 kDa) exceptionally rich in cysteine are added to the outer membrane (see "Amino acid metabolism" above). Although synthesis of a new MOMP starts as early as 12 h into the developmental cycle in *C. trachomatis* biovar LGV (325) and strain mouse pneumonitis (405) and in *C. psittaci* 6BC (180), labeled cysteine is not incorporated into the cysteine-rich proteins until 18 to 21 h. These proteins are extensively cross-linked at the time of their incorporation into the outer membrane (180, 325). In contrast, the MOMP of *C. psittaci* 6BC growing in L cells is not cross-linked 46 h after infection but is immediately cross-linked at the moment of host cell lysis and release of infectious EBs (180). In *C. trachomatis* biovar LGV growing in HeLa cells, the MOMP has been reported either to remain largely in monomeric form until host cell lysis (180) or to become progressively cross-linked (to a maximum of 60%) during the last 24 h of the developmental cycle (325). Intracellular formation of disulfide bonds could be brought about by specific chlamydial enzymes (325), or it could occur nonenzymatically when slowing chlamydial metabolism allows the levels of ATP and reducing agents such as NADPH and reduced glutathione to fall so low that sulfhydryl groups are oxidized to disulfide (26). In a similar way, cross-linking at the time of lysis could occur when the EBs are released into the more highly oxidizing extracellular environment.

Regulation of RB-to-EB conversion. The external signal that starts the reorganization of EBs into RBs must be something about the inside of the host cell that is not like the outside (see "Search for the signal for conversion of EBs to RBs" above). Although initiation of EB reorganization is not truly synchronous, most of the EBs that have infected a population of host cells begin to reorganize into RBs within a few hours of entry, and by the time dividing RBs are seen, mature undifferentiated EBs are hard to find (13, 131, 187). The dynamics of the RB-to-EB shift are quite different. As judged by either electron microscopic observation or the appearance of titratable infectivity, some RBs begin their differentiation into EBs from 15 to 25 h after infection, whereas others continue to divide into undifferentiated daughter RBs until the end of the developmental cycle and lysis of the host cell 24 to 48 h later (16, 131, 187, 261, 445). At all times after intermediate forms first appear, the chlamydial population within a single inclusion consists of a mixture of dividing RBs, intermediate forms, and mature EBs. This utter lack of synchronicity makes it unlikely that the signal for reorganization of RBs into EBs is generated by some change in the intrainclusion milieu brought about by continued multiplication of the RB population. It seems that the signal must originate at different times in individual RBs.

Like the change of EBs into RBs (see "Initiation of Multiplication" above), the transformation of RBs into EBs also involves the synthesis of proteins not made at other times in the developmental cycle. In addition to the three cysteine-rich outer membrane proteins, proteins that bind to eucaryotic cell membranes (159, 514) or to chlamydial DNA (491) are also found in EBs but not in RBs. One of the DNA-binding proteins appears to be identical with the largest of the cysteine-rich proteins of the outer membrane. The expression of sets of temporally regulated proteins in the developmental cycle is now well-established. It has been suggested that this regulation is achieved by a series of promoters specifically recognized in sequence by RNA polymerase either modified by cycle-dependent sigma factors or mediated by DNA-binding proteins (121, 178, 359). What remains to be determined is why different RNA polymerase modifiers are synthesized at different times in the developmental cycle.

When HeLa cells are infected with the trachoma biovar, cAMP reversibly inhibits the conversion of RBs to EBs (221). RBs have a cAMP-binding protein absent in EBs. In the presence of cAMP, small, immature inclusions containing noninfectious chlamydiae without the binding proteins characteristic of EBs are formed. When synthesis of the MOMP is used as a marker for general protein synthesis, cAMP abolishes the increased rate of MOMP synthesis normally seen 18 to 36 h after infection (220). The action of cAMP is at the level of transcription. It decreases the concentration of MOMP mRNA in *C. trachomatis* biovar LGV-infected HeLa cells, and it blocks transcription of a recombinant MOMP gene in vitro. These results suggest that the RB-to-EB conversion is under bidirectional cyclic nucleotide control, with cGMP acting as a stimulator and cAMP acting as an inhibitor.

Effect of antibacterial agents on conversion of RBs to EBs. Antibiotics that inhibit chlamydial synthesis of protein and RNA (chloramphenicol, chlortetracycline, erythromycin, and rifampin) block both reorganization steps in the developmental cycle, depending on when they are added to host cells (85, 125, 131, 157, 191, 233, 481). Given early, they prevent EBs from changing into RBs; given late, they keep RBs from reorganizing into EBs. However, there are other antibiotics and antibacterial agents that disrupt the chlamydial cycle at a single point, the reorganization of RBs into EBs. These agents fall into two groups on the basis of their known sites of action in host-independent eubacteria. Penicillin and D-cycloserine inhibit the synthesis of peptidoglycan, whereas 5-fluorouracil, aminopterin, trimethoprim, sulfamethoxazole, and hydroxyurea inhibit the synthesis of DNA.

At a concentration of 0.1 U/ml, penicillin G (benzyl penicillin) inhibits by 50% the production of infectious EBs by *C. psittaci* MN/Cal 10 growing in L cells; at 1 U/ml, inhibition is almost complete (465). This is the same order of susceptibility exhibited by many host-independent bacteria. Although there is some strain variation, other chlamydiae are comparably sensitive to penicillin (21, 209). Other β-lactam antibiotics are generally less active than benzyl penicil-

lin (190). As first observed by light (492, 505) and later by electron (13, 151, 233, 283, 458) microscopy, penicillin blocks the conversion of RBs into EBs in several strains of *C. psittaci* and *C. trachomatis* growing in cultured cells and in chicken embryo yolk sac. In L cells infected with *C. psittaci* MN/Cal 10 and treated with penicillin, reorganization of the invading EBs into RBs is not prevented, but by 12 h, normal division of RBs is disrupted, and many very large, abnormal RBs are produced (283). Several investigators have noted the resemblance of the large abnormal RBs produced in the presence of penicillin to the spheroplasts formed by host-independent bacteria exposed to penicillin in osmotically protective media analogous. Unorganized masses of RB cytoplasmic membranes accumulate inside and outside the RBs, and empty or near-empty vesicles pinch off from the RBs. Abnormal "penicillin forms" continue to accumulate for at least 40 h. Synthesis of protein and nucleic acid by *C. psittaci* MN/Cal 10 and 6BC in the presence of penicillin is either uninhibited (465) or only slightly inhibited (480). RBs made in penicillin-treated host cells have intact cell walls and cytoplasmic membranes, and the amino acid content of RB walls isolated after 40 h in penicillin is the same as that of 18-h RB walls without the antibiotic (465). However, normal mature EBs do not appear. In *C. trachomatis* biovar LGV, penicillin is without effect on synthesis of the MOMP, but synthesis of the 60-kDa cysteine-rich outer membrane protein is inhibited (77). This may explain why the RB-to-EB change is so sensitive to penicillin.

The action of D-cycloserine on the chlamydial developmental cycle is similar to that of penicillin. In concentrations comparable to those inhibiting the growth of susceptible free-living bacteria, D-cycloserine suppresses the production of infectious EBs by several strains of *C. psittaci* and all three biovars of *C. trachomatis* in chicken embryos (259, 317) and in cell culture (197, 480). Most strains of *C. trachomatis* are distinctly more susceptible to this antibiotic than are most strains of *C. psittaci* (259). As revealed by light (197, 317, 480) and electron (131a) microscopy, host cells infected with chlamydiae and treated with D-cycloserine contain very large and abnormal RBs closely resembling those produced in the presence of penicillin. Mature EBs are not visible.

The disruption of normal cell division in RBs and blockage of their conversion into EBs by penicillin and D-cycloserine would be perfectly understandable as inhibition of cell wall synthesis in terms of their well-known effects on peptidoglycan synthesis (135, 388, 501) were it not that muramic acid, the sine qua non of eubacterial peptidoglycan structure, occurs in chlamydiae either in insignificant amounts or not at all. Amino acid analysis failed to detect muramic acid in either EBs or RBs of *C. psittaci* MN/Cal 10 (274, 463) and *C. trachomatis* biovar LGV (21), a method involving conversion to [^{3}H]muramicitol found only traces of muramic acid in either *C. trachomatis* biovar LGV or *C. psittaci* MN/Cal 10 (138), and capillary gas chromatographic separation and mass spectrographic detection have set the limits for muramic acid in *C. psittaci* MN/Cal 10 as <0.006% and in *C. trachomatis* biovar trachoma as <0.02% (129). For comparison, *Legionella* spp. contain 0.1 to 0.3% muramic acid. The chlamydial levels of muramic acid, if they are really above background, are far too low to permit the synthesis of a peptidoglycan that would cover the entire chlamydial cell. The absence of muramic acid in eubacteria is not as unusual as once thought. In addition to chlamydiae, budding bacteria such as *Planctomyces* spp. (228) and *Isosphaera pallida* (145), the scrub typhus agent *Rickettsia tsutsugamushi* (10), and mycoplasmas (372) also lack muramic acid. However, of all these peptidoglycan-deficient bacteria, only the chlamydiae are susceptible to penicillin and D-cycloserine, and only the chlamydiae have penicillin-binding proteins. In peptidoglycan-containing bacteria, such proteins are considered equivalent to penicillin-sensitive enzymes (501). *C. trachomatis* biovar LGV has three penicillin-binding proteins (81, 61, and 36 kDa) whose 50% binding concentrations for benzyl penicillin are of the same order of magnitude as the growth-inhibitory concentrations (21).

The response of peptidoglycan-containing bacteria to penicillin is complex, and the precise molecular mechanism(s) by which penicillin exerts its action is still not completely understood (135, 388, 501). The antibiotic reacts with penicillin-binding proteins in the bacterial membrane to disrupt elongation, septum formation, and cell separation, with lysis often being the final result. These events appear to be the result of interference with the final steps in peptidoglycan synthesis, in which tetrapeptide bridges are formed between two adjacent peptidoglycan strands and a terminal D-alanine residue is eliminated by the action of penicillin-sensitive transpeptidases and D-D-carboxypeptidases. It has been proposed that there is a structural analogy between β-lactam molecules and the carboxy terminal end (D-alanyl-D-alanine) of the peptidoglycan side chain. There are other penicillin-sensitive enzymes, but their roles in peptidoglycan synthesis are not clear. D-Cycloserine inhibits peptidoglycan synthesis and interferes with bacterial growth and division because, as a structural analog of D-alanine, it inhibits incorporation of that amino acid into the peptidoglycan side chain (135, 388). D-Cycloserine inhibits both the conversion of L-alanine to its D-stereoisomer and the synthesis from D-alanine of D-alanyl-D-alanine, in which form D-alanine is added to the peptidoglycan side chain in a reaction not sensitive to D-cycloserine. This antibiotic also inhibits the transport of D-alanine and glycine.

There are really only two ways to explain the effect of penicillin on the peptidoglycanless chlamydiae, and both have a definite air of implausibility. These explanations are without precedent, but since the relation of chlamydiae to penicillin is unique, novel explanations are required. First, as has already been suggested (21, 138), the penicillin-binding proteins (= penicillin-sensitive enzymes) may catalyze much the same set of reactions as do the penicillin-binding proteins of peptidoglycan-containing bacteria. They could participate in the formation of an essential part of the chlamydial cell wall architecture that resembles a peptidoglycan side chain but is linked to structures other than the *N*-acetylmuramyl–*N*-acetylglucosamine backbone of the peptidoglycan molecule. Second, during the long phylogenetic isolation of chlamydiae, the penicillin-binding proteins may have assumed new catalytic functions completely unrelated to peptidoglycan synthesis but nevertheless essential to assembling normal chlamydial cell walls. The action of D-cycloserine on chlamydiae can be explained in the same either/or fashion. The link between penicillin and D-cycloserine is D-alanine—in different ways, each antibiotic keeps D-alanine from taking its customary position in cross-linked peptidoglycan. It might be rewarding to investigate the role of this amino acid in chlamydial wall structure. The most compelling reason for leaning toward the first explanation is that chlamydial multiplication is inhibited by two antibiotics that inhibit peptidoglycan synthesis at different steps and, at least under some conditions, by an enzyme, lysozyme, that hydrolyzes the glycosidic bonds of the peptidoglycan carbo-

hydrate backbone (see "Interaction of chlamydiae with PMNs" above). It is hard to believe that this is just a coincidence. Neither explanation offers a mechanism for inhibition of synthesis of the 60-kDa outer membrane protein by penicillin.

Several antibacterial agents that directly or indirectly inhibit DNA synthesis also block the developmental cycle at the RB-to-EB step. 5-Fluorouracil prevents synthesis of DNA by specifically inhibiting thymidylate synthetase, the enzyme that probably supplies chlamydiae with their thymidine nucleotides (see "Nucleoside metabolism" above). 5-Fluorouracil has no effect on *C. trachomatis* biovar LGV in HeLa cells for 24 h, after which the RBs become abnormal in appearance and do not develop into EBs (13). Trimethoprim also prevents the synthesis of thymidine nucleotides by inhibiting reduction of dihydrofolate to the tetrahydrofolate required by thymidylate synthetase (135). It inhibits multiplication of *C. trachomatis* biovar trachoma in HeLa and McCoy cells (167, 376). By 48 h, some RBs have started to change into EBs, but most are defective. Aminopterin, another inhibitor of dihydrofolate reductase, probably also inhibits multiplication of *C. psittaci* Texas turkey at the RB-EB conversion step (362). Sulfamethoxazole has an effect on the trachoma biovar similar to that of trimethoprim (376). It, too, prevents synthesis of thymidine nucleotides by inhibiting formation of tetrahydropteroic acid, a precursor of tetrahydrofolate (135). Hydroxyurea strongly inhibits DNA synthesis by an unknown mechanism. At 5×10^{-2} M, it completely inhibits reproduction of *C. trachomatis* biovar LGV in FL cells, but at 5×10^{-4} M, hydroxyurea permits development of RBs but prevents their conversion into EBs (391). Nalidixic acid inhibits DNA gyrase (135). It is a poor inhibitor of *C. psittaci* multiplication in L cells, but at high concentrations it also blocks reorganization of RBs into EBs (481). It appears that there is an unusual stress on DNA synthesis at the time of RB reorganization, possibly related to inadequate concentrations of thymidine nucleotides being synthesized via the thymidylate synthetase pathway. Why this might be so is not known.

Release from Host Cells

The developmental cycle ends with release of chlamydiae from host cells. However, release is always incomplete; as much infectivity—or more—remains associated with host cells as is released into the growth medium. This is true of *C. trachomatis* biovars trachoma and LGV (126, 137, 445) and *C. psittaci* MN/Cal 10 and Texas turkey (189, 414, 467). There is no preferential release of EBs. RBs and intermediate forms left at the close of the developmental cycle are set free along with the EBs. They do not contribute to infectivity, but in intact hosts they undoubtedly serve as antigenic stimuli.

One mode of release shared by the *C. trachomatis* biovars trachoma (34, 72, 73) and LGV (474) and *C. psittaci* 6BC, calf polyarthritis, and MN/Cal 10 (115, 232, 474, 476) is lysis of host cells followed by exit of chlamydiae. In a time-lapse microcinematographic study of *C. trachomatis* biovar trachoma in McCoy cells, inclusions were seen to burst while still inside host cells (72). Cellular organelles were disrupted, the host cells were lysed, and the chlamydiae diffused away from the disrupted cells. Because lysosomal enzymes are released just prior to lysis of infected cells (see "Interaction of *C. psittaci* MN/Cal 10 and 6BC with host cell lysosomes" above), it has been suggested that these host enzymes are the lytic agents (232, 476).

Intact inclusions are also released from host cells. This mode of expulsion has been most effectively studied by scanning electron microscopy. *C. trachomatis* biovar trachoma distends and then ruptures the cell membranes of McCoy cells with subsequent extrusion of both chlamydial inclusions and McCoy cell nuclei (105). However, in some host cells, the inclusion is extruded through a focal distention of the cytoplasmic membrane of the host cell without apparently affecting the rest of the cell surface. When *C. trachomatis* biovar trachoma has grown in HeLa cells for 72 h, deep, cavernous openings appear in the infected cells, and in the depths of these openings are seen chlamydial cells attached to smooth membranes (474). By 120 h, these openings have "healed over" and are covered by membranes without the microvilli and blebs characteristic of the unaltered HeLa cell surface. When HeLa cells are inoculated with either *C. trachomatis* biovar LGV or *C. psittaci* MN/Cal 10, only host cell lysis is observed (474). It has been suggested that in contrast to those of other chlamydiae, inclusions of *C. trachomatis* trachoma biovar are transported to the host cell surface and extruded by a process resembling exocytosis that allows release of infectious chlamydiae without concomitant death of host cells (474). Such a mechanism might explain why *C. trachomatis* biovar LGV and *C. psittaci* form plaques in cell culture monolayers, whereas the trachoma biovar does not (19). The pertinence of this mechanism to the establishment of persistent chlamydial infections in vitro will be considered in the next section.

Mechanisms of release from specialized cells and tissues of intact host cells may, without doubt, be more complicated than in cell culture monolayers and suspensions. In a study of the release of a calf polyarthritis strain of *C. psittaci* from the intestinal epithelial cells of calves, at least three distinct modes of release were noted: rupture of infected cells, expulsion of whole infected cells into the intestinal lumen, and extrusion and pinching off of large chlamydia-containing pseudopods (115).

PERSISTENT INFECTION OF CELL CULTURES

Persistent chlamydial infections in birds and nonhuman mammals were recognized soon after *C. psittaci* was first isolated (288, 427, 451). Two well-known strains, the mouse biovar of *C. trachomatis* (for references, see reference 288) and the GPIC strain of *C. psittaci* (320), were isolated from laboratory animals showing minimal signs of infection. An early report that humans are also persistently infected with *C. psittaci* (141) has been extended to *C. trachomatis* infections (202, 333). There are case reports of human infections with *C. psittaci* (291) and *C. trachomatis* biovar LGV (98) that persisted for 10 to 20 years. Although persistent infections have been studied in intact animals (289, 290, 337, 371), most laboratory investigations on the nature of persistent infection with chlamydiae have been done in cell culture.

In the discussion that follows, any cell culture from which both infectious chlamydiae and viable host cells may be repeatedly recovered for many days will be said to be persistently infected, and no attempt will be made to categorize the infection as chronic or latent. Emphasis will be placed on defining the mechanisms whereby chlamydiae and susceptible host cells manage to coexist. The several ways of achieving persistent chlamydial infection in cell culture presage the existence of different routes to the persistently infected state.

Persistent Infections in Which Chlamydial Multiplication Is Inhibited

Persistent infection of McCoy cells with the Texas turkey strain of *C. psittaci* is obtained by addition of 25 μg of aminopterin per ml not more than 20 h after infection (362). When thymidine is also added to reverse aminopterin toxicity for the McCoy cells, there are no visible inclusions and no infectivity for fresh McCoy cells for as long as 4 weeks, although chlamydial multiplication may be restored at any time by the addition of folinic acid. Here, for once, the mechanism is self-evident. The explanation of aminopterin-induced persistence is that this inhibitor of dihydrofolate reductase prevents reorganization of RBs into EBs (see "Effect of antibacterial agents on the conversion of RBs to EBs" above) and that a rare RB survives passage from host cell to host cell to await removal of the aminopterin block by the addition of folinic acid. Under appropriate conditions, other inhibitors of chlamydial multiplication are probably also capable of producing states of persistent infection. The effect of penicillin is reversible after contact with infected cells for as long as 48 h (209, 465), and growth inhibition by rifampin and chloramphenicol is reversible for as long as 5 h (481).

Persistent Infections in Which Both Host Cell and Chlamydial Multiplication Are Inhibited

Cell cultures may also be persistently infected with chlamydiae when nutrients essential to parasite multiplication are omitted from the growth medium. Because chlamydiae have no known nutritional requirement not shared by their hosts (see "Amino acid metabolism" and "Metabolism of B vitamins" above), the duration of deficiency-induced persistent infections is limited by how long the host cells can survive in the nutritionally inadequate medium. When L cells depleted by a 2-day stay in inorganic salts and glucose are infected with *C. psittaci* 6BC, recoverable infectivity quickly declines and no signs of infection are visible by either light or electron microscopy (285). However, for as long as 4 days after infection, a normal developmental cycle is promptly initiated by addition of all the amino acids (16) and B vitamins (17) required for multiplication of *C. psittaci*. When L cells have been depleted of isoleucine by growing in suspension culture in medium 199 (296) for 1 day, ingestion of *C. psittaci* 6BC proceeds normally, but chlamydial multiplication does not begin until isoleucine is added (173). The most likely explanation of deficiency-induced states is also simple. Omission of an essential nutrient blocks multiplication not only of chlamydiae but of host cells as well. For a limited time, both host and parasite survive in nonmultiplying states from which they may be revived in the presence of the missing nutrient. The severe retardation of the differentiation of *C. trachomatis* biovar trachoma RBs into EBs in cysteine-deficient media (3, 444; see also "Amino acid metabolism" above) suggests that persistent infections of cell cultures with *C. trachomatis* may also be obtained by nutritional deprivation.

Persistent Infections under Conditions Capable of Supporting Unrestricted Multiplication of Both Chlamydiae and Host Cells

Persistent infections in the absence of inhibitors or deficiencies have been established in a number of ways, and no single mechanism can account for them all. There are probably several different mechanisms operating singly or in concert and modified by numerous factors such as the density and nutritional state of the host cell population. Persistently infected cell cultures in the absence of any impediment to unlimited growth of either host or parasite require the establishment of an equilibrium between multiplication of host cells and chlamydiae that allows indefinite coexistence. When one chlamydial strain destroys the host cell population under customary conditions of culture, its destructive potential must be mitigated. When the EBs generated by another strain in the first turn of the developmental cycle do not readily infect a new set of host cells (this occurs mainly with the trachoma biovar of *C. trachomatis*; see "Kinetics of Attachment and Ingestion" above), something may have to be done to increase the efficiency of chlamydial transit between host cells.

Ways of establishing persistent infections under nonlimiting growth conditions. After a lag of days to weeks of host cell multiplication and no sign of chlamydiae, inoculation with multiplicities of infection near the limiting dilution gives persistent infection of rabbit cornea cells with *C. psittaci* sheep polyarthritis and 6BC (417), of McCoy cells with *C. psittaci* GPIC (336), and of HeLa cells with *C. trachomatis* biovar trachoma (484). Cell cultures that eventually become persistently infected after inoculation with low and nondestructive multiplicities of chlamydiae have not been studied in detail, and definite mechanisms cannot be offered. However, this method of initiation is probably successful when the efficiency of chlamydial transit from infected cell to new host cell is low, so that at each turn of the developmental cycle, enough new cells are infected to maintain a chlamydial presence but there are not enough to pose any threat to the continuity of the host cell population.

Another way to reach a state of persistent infection is to inoculate high multiplicities of infection of chicken embryo-grown seed into host cell populations relatively resistant to egg-derived chlamydiae. After an interval of several weeks during which chlamydiae are present in only a very small fraction of the host cell population, abundant chlamydial growth occurs without destruction of all the host cells, and host and parasite coexist indefinitely. Persistent infection of HeLa cells with *C. psittaci* MN/Cal 10 (270) and of Chang's human liver (CHL) cells (later shown to be contaminated with HeLa cells [139, 244]) with *C. psittaci* 6BC (332) have been established in this manner. In *C. psittaci* MN/Cal 10-HeLa cell cultures (270), inclusions were seen after 2 weeks and large numbers of chlamydiae were produced after 12 weeks. The fraction of the HeLa cell population containing inclusions varied, but precise figures were not reported. Cultures that continuously yielded chlamydiae with only slight depression of the normal HeLa cell growth rate were kept for almost a year by twice-weekly feeding. *C. psittaci* MN/Cal 10 from persistently infected cultures established new persistent infections faster and was more infectious for chicken embryo yolk sac than the original egg-grown seed. All evidence points to the emergence of a *C. psittaci* genotype capable of multiplying extensively in HeLa cell populations without materially reducing their growth rates.

A large inoculum of yolk sac-grown *C. psittaci* 6BC immediately infected only 2 to 5% of the cells in CHL cell monolayers (332). When the infected cultures were transferred every 10 to 14 days, the fraction of CHL cells bearing inclusions rose from less than 10% at the 4th transfer to 95% at the 10th, declined to 23% by the 23rd transfer, and rose again to 70% by the 30th passage. The CHL cells multiplied 3- to 10-fold between transfers, depending on the number of

cells destroyed by infection, and chlamydial infectivity released into the medium was proportional to the number of infected CHL cells. Because the fluctuations in percentage of infected host cells suggested the emergence of both chlamydial and host cell variants, *C. psittaci* was reisolated from passage 10, and CHL cells (cured of chlamydiae with tetracycline) were reisolated from passage 23. Passage 10 *C. psittaci* infected parent CHL cells much more efficiently than the original yolk sac inoculum, and passage 23 CHL cells were more resistant to infection with passage 10 *C. psittaci*, particularly at lower multiplicities of infection. It thus appears that the fluctuations in percentage of infected CHL cells during successive transfers are due to selection: first, of a *C. psittaci* that more efficiently infects CHL cells, and second, of a CHL cell that is more resistant to the newly emergent chlamydial population. The parent CHL cell population contained a clonal type very similar to the host cell population isolated at passage 23.

Persistent infection of McCoy cells with the trachoma biovar of *C. trachomatis* may result from a population shift toward a *C. trachomatis* genotype that passes from host cell to host cell more efficiently than the wild stock does (247, 248). Half of the cells in a McCoy cell population were infected with *C. trachomatis* biovar trachoma with the aid of centrifugation and cycloheximide (see "Entry" above), but because of the low rate at which cells were infected without this help, the proportion of inclusion-bearing McCoy cells fell to less than 1% and remained at that level during weekly feeding or transfer. Then, 100 to 150 days after infection, large numbers of inclusion-bearing cells suddenly appeared and the cell sheets were destroyed. However, some cells always survived to repopulate the flasks after the cells had been washed and fed. Thereafter, the persistently infected cultures went through many 2- to 4-week cycles of chlamydial multiplication in which the fraction of inclusion-positive cells rose from <1 to 50 to 100% of the monolayers followed by destruction of most host cells and repopulation of the McCoy cell sheet by a few survivors. Most of the surviving host cells were initially uninfected. None of the 11 McCoy cell clones isolated from cultures with 1 to 15% inclusion-positive cells ever produced inclusion-bearing cells. The McCoy cell clones from persistently infected cultures were indistinguishable from wild-type host cells in their susceptibility to infection with wild-type or persistent-infection *C. trachomatis* with or without centrifugation. The behavior of the chlamydiae while they were in persistently infected cultures was so different from that of wild-type *C. trachomatis* biovar trachoma that their identity was confirmed by the microimmunofluorescence test (494). The *C. trachomatis* biovar trachoma isolated from persistently infected cultures containing nearly 100% infected host cells could not be distinguished from wild-type *C. trachomatis* in rates of attachment and ingestion by wild-type and persistent-infection host cells, in yield of infectious chlamydiae per McCoy cell, or in resistance to extracellular inactivation. The only measurable difference was that when the *C. trachomatis* from persistently infected cultures was used to start new persistent infections, the first massive chlamydial multiplication and host cell destruction occurred after 2 weeks instead of the 7 to 10 weeks required when the initial inoculum was wild-type *C. trachomatis*. These findings suggest the selection of a genotype of the trachoma biovar more adept at establishing a persistently infected state than the wild type, presumably by facilitating cell-to-cell chlamydial transit. The basis for this assumed increase in efficiency is not immediately evident.

Persistent infections with several strains of *C. psittaci* have been set up in McCoy and L-cell cultures. These host cell-chlamydia systems differ from the ones just discussed in that virtually all the cells in McCoy and L-cell populations are infected and destroyed by the inoculum used to initiate the persistent infection. No period of "adaptation" is needed. Monolayers are infected with enough *C. psittaci* to completely destroy the cell sheet. The dead cells are washed off, fresh medium is added, and the apparently empty flasks are reincubated for 2 to 3 weeks, when scattered colonies of host cells become visible. Some contain cells with inclusions (as revealed by Giemsa staining), and some do not. These colonies are then allowed to grow up into cell sheets that are persistently infected with *C. psittaci*. This general method has successfully established persistent *C. psittaci* infection with the 6BC (315) and sheep abortion strains (350) in L cells and with several sheep and goat strains in McCoy cells (386). Such persistently infected cultures do not always behave the same, and despite a common method of initiation, several different mechanisms seem to be in operation.

When L-cell monolayers were infected with enough of a sheep abortion strain of *C. psittaci* to destroy them in 10 to 15 days, rare host cells survived to repopulate the cultures (350). Then, at intervals of about 30 days, the proportion of inclusion-bearing cells increased from a few percent to almost 100%, the monolayers disintegrated, and a few survivors began to regenerate the monolayers. Within a single cycle, extracellular and intracellular infectivity rose concomitantly with the number of inclusion-bearing cells. *C. psittaci* sheep abortion from an infection that had persisted for more than 7 months destroyed L-cell monolayers faster and with smaller inocula than the wild-type chlamydiae did. Although L cells spontaneously cured of persistent infection showed unchanged susceptibility to *C. psittaci*, persistently infected L cells had a reduced capacity to ingest EBs attached to their surfaces. Immunofluorescence often visualized many more L cells with surface-attached particulate chlamydial antigen (EBs?) than cytoplasmic inclusions. When a population of persistently infected L cells with 15% inclusions and 80% surface-attached antigen was centrifuged in the presence of cycloheximide, 80% of the host cells developed inclusions. Persistently infected L cells were more resistant to superinfection with either wild-type or persistent-infection *C. psittaci*, and this resistance disappeared in the presence of cycloheximide. Persistent infection of L cells with *C. psittaci* sheep abortion appears to depend in part on selection of a chlamydial genotype that spreads more rapidly among the cells of persistently infected monolayers and in part on reversible depression of the capacity of persistently infected L cells to ingest attached chlamydiae.

When L cells were infected with 0.01 to 10 EBs of the 6BC strain of *C. psittaci* and plated out in medium 199 at a density of 4,000 cells per cm^2, the resultant monolayers were destroyed 4 to 14 days later, depending on the multiplicity of infection (308, 315, 316, 319). Two to 3 weeks later, a few L-cell colonies appeared. Some contained cells with inclusions, some did not, and, as was learned later, all were persistently infected. Approximately 1 in every 10^5 L cells gave rise to a persistently infected colony. Freshly cloned L-cell and *C. psittaci* populations, as well as host and parasite populations isolated from persistently infected cultures, generated persistently infected colonies at about the same frequency. It thus appears that the capacity of L cells and *C. psittaci* to interact in such a way as to establish a persistent relationship is an inherent property of most of the cells in wild populations. Persistently infected cultures could

be kept in a single flask for several months with only washing and feeding after each episode of host cell destruction. However, most experiments were done with cultures dispersed when the fraction of inclusion-positive cells reached 1 to 5% and replated in medium 199 at a density of 4,000 L cells per cm^2. Such monolayers were consistently destroyed 12 to 14 days later. Regularly cycling cultures always contained infectious chlamydiae. Shortly before destruction of the monolayers, there were more than 100 EBs per L cell, half in the growth medium and half associated with L cells. The dividing L cells that grew out after each episode of massive host cell destruction grew more slowly than wild-type L cells and in the early stages of regrowth were largely without visible inclusions. However, nine clones obtained from cultures with 25% inclusion-positive cells all gave rise to persistently infected populations, although at first all were inclusion free. This suggests that all L cells in persistently infected populations, even the ones that do not have visible inclusions, are infected with *C. psittaci*. This suggestion is borne out by other properties of persistently infected L cells. First, they were almost completely resistant to superinfection with inocula of either wild-type or persistent-infection *C. psittaci* several hundred times larger than required to infect >99% of wild-type L-cell populations because of a defect in the first step of entry: they failed to attach added ^{14}C-labeled *C. psittaci* 6BC. Resistance to superinfection could be circumvented by centrifuging the inoculum onto the host cells or by pretreating the persistently infected L cells with DEAE-D. Why *C. psittaci* 6BC mimics the trachoma biovar of *C. trachomatis* in its interaction with persistently infected L cells (see Entry above) is not known, but this mimicry must be accounted for in any complete explanation of the effects of centrifugation and DEAE-D on chlamydial entry. As in persistent infection of L cells with a sheep abortion strain of *C. psittaci* (350), there was an L-cell-associated but not ingested chlamydial fraction that entered the persistently infected L cells and multiplied normally when they were centrifuged or treated with DEAE-D. Second, both inclusion-positive and inclusion-negative L cells from persistently infected cultures had a pattern of surface-exposed plasma membrane proteins different from the patterns of uninfected and acutely infected wild-type L cells. By polyacrylamide gel electrophoresis and autoradiography of surface proteins iodinated with iodine 135 by lactoperoxidase-catalyzed iodination, there was a new 35-kDa band and a generalized reduction in intensity of labeling of bands in the 60- to 100-kDa range. It would be interesting if these changes could be related to disappearance of chlamydia-binding surface proteins. Superinfection refractoriness and abnormal surface protein patterns disappeared simultaneously when L cells were cured of persistent infection, either spontaneously or with tetracycline or rifampin. Third, the persistently infected state, characterized by resistance to superinfection, abnormal surface protein patterns, and ability to shift into overt production of infectious chlamydiae and destruction of host cells, could be maintained for at least 3 months in the absence of visible chlamydial inclusions. The L cells that exhibit such altered behavior without visible signs of chlamydial infection must harbor a chlamydial presence in some previously unrecognized state. The term "cryptic body" has been used to designate this postulated chlamydial presence (315). In situ hybridization or immune electron microscopy should reveal its morphological identity. The isolation of 11 uninfected clones from McCoy cells persistently infected with *C. trachomatis* biovar trachoma (248) makes it most unlikely that cryptic bodies play a role in this infection. This is also true of L-cell *C. psittaci* sheep abortion infections (350) in which the L cells were cured of persistent infection by several passages at low density.

L cells have been persistently infected with a *C. trachomatis* strain, serovar unspecified, isolated from the human genital tract (420). From the ease with which it infects L cells, this isolate may be biovar LGV. In the early stages, the infections resemble the persistent infections established in McCoy cells with biovar trachoma of *C. trachomatis* (247, 248). However, after six to seven cycles of chlamydial multiplication and L-cell destruction and regrowth, the L-cell monolayers were not destroyed, and inclusions typical of a normal developmental cycle were not seen by light microscopy. This behavior is reminiscent of the *C. psittaci* 6BC L-cell infections in which the existence of a cryptic chlamydial body has been suggested (315). Electron microscopy revealed the presence of three kinds of inclusion-bound structures in 10 to 50% of the L cells: (i) RBs in inclusions of a size normally seen 8 to 12 h after infection, (ii) 200-nm bodies that might be RB protoplasts, and (iii) dense oval bodies, 100 nm in diameter and bounded by single membranes. The ability of these structures to give rise to productive developmental cycles is not known. It is possible that the 100-nm bodies are related to the cryptic bodies postulated for persistent infection of L cells with *C. psittaci* 6BC (315).

When McCoy cell monolayers were infected with inocula of a dozen different strains of *C. psittaci* isolated from sheep and goats, all the monolayers were destroyed in 1 to 2 weeks, and all the repopulated cultures were persistently infected (386). However, at least two kinds of persistent infections were generated. Type 1 infections were produced mainly by strains of intestinal origin that were noninvasive for mice (no splenic infection after footpad inoculation) and formed small plaques on McCoy cells. In type 1 infections, the monolayers were regenerated within 3 days of destruction. Thereafter, they looked and behaved like wild, uninfected McCoy cells, except that they continuously produced infectious chlamydiae. The type 1 infections appear to be different from all other persistent *C. psittaci* infections thus far described.

Type 2 persistent infections were mostly produced from mouse-invasive sheep abortion and conjunctivitis strains that formed large plaques on McCoy cells, although some also originated from noninvasive intestinal strains (386). In these infections, scattered colonies of McCoy cells did not appear until 2 to 4 weeks after initial destruction. When the monolayers were repopulated, they underwent repeated cycles of chlamydial multiplication, host cell destruction, and host cell regrowth similar to those already described for L cells and the 6BC strain of *C. psittaci* (315). On a number of occasions, cultures that eventually behaved as type 2 populations gave no sign of chlamydial infection for 2 to 3 months after destruction of the monolayers by the initiating infection. Both type 1 and 2 persistent infections were maintained by passage at low McCoy cell density, and persistently infected clones were isolated from persistently infected host cell populations with few or no inclusion-bearing cells (385a). These observations suggest that persistent infections of McCoy cells with mammalian strains of *C. psittaci* and of L cells with the avian 6BC strain of *C. psittaci* are maintained by similar mechanisms. Some of the chlamydiae from persistently infected cultures were more virulent for mice and sheep than the corresponding wild-type strain. For example, a strain that did not invade the spleens of mice and did not cause pregnant ewes to abort gave rise to

a type 2 infection that yielded chlamydiae that invaded mouse spleens and produced abortion in pregnant ewes.

Avenues to coexistence of host and parasite in persistent infections. For coexistence to be achieved in the absence of external barriers to multiplication of chlamydiae and host cells, unlimited increase in the population size of one component of the host-parasite system at the expense of the other must be prevented by other means. In persistent infections in which the chlamydiae are capable of rapid cell-to-cell spread, either initially (315, 350, 386, 420) or after a state of persistent infection has been produced (247, 248, 270, 332), equilibrium between chlamydial and host cell proliferation is frequently maintained by means of biphasic cycles of (i) multiplication of largely inclusion-free host cells and (ii) chlamydial multiplication and host cell destruction. The forces that initiate and maintain these cycles are largely unknown.

Numerous attempts have been made to change the rate at which cycling persistent infections proceed from a stage at which only a small fraction of the host cell population is inclusion positive to one in which nearly all the cells have inclusions. In rabbit cornea cells persistently infected with *C. psittaci* 6BC and sheep abortion, raising the incubation temperature to 39°C or lowering the medium pH to 6.6 increases the rate of appearance of inclusions (417), but these changes have no effect on persistent infections with *C. psittaci* 6BC in L cells (308). An analog of cAMP, 5'-guanylimidophosphate, hastens the increase in inclusion-positive cells in a cycling persistent infection of BHK cells with *C. trachomatis* biovar trachoma (266), possibly by antagonizing the inhibitory action of cAMP on synthesis of chlamydial protein (220, 221). There are several ways to retard the appearance of inclusions. In some systems, the time from replating to destruction of the resulting monolayer is inversely proportional to the initial host cell density (247, 248, 308). In McCoy cells persistently infected with *C. trachomatis* biovar trachoma, this is because the cell sheets are destroyed by cell-to-cell transfer of infection from a few centers of chlamydial multiplication (248). Cell-to-cell transfer of wild-type *C. trachomatis* biovar trachoma also occurs in McCoy cell cultures in which there is an unusually high degree of contact among host cells (34). In L cells persistently infected with *C. psittaci* 6BC, shifting from medium 199 to Eagle's medium indefinitely prolongs the appearance of large numbers of inclusion-bearing cells, but the cultures remain persistently infected, as shown by return to medium 199 (308). In contrast, an identical change in medium has no effect on persistent infections with a sheep abortion strain of *C. psittaci* in L cells (350). Penicillin, added when the fraction of inclusion-bearing cells is low, sharply reduces the rate at which the persistently infected host cells become inclusion positive (248, 308, 350). It probably slows cell-to-cell transfer by blocking the RB-to-EB step (see "Effect of antibacterial agents on conversion of RBs to EBs" above). This explains the effect of penicillin on the *C. trachomatis* biovar trachoma McCoy cell (248) and *C. psittaci* sheep abortion L cell (350) systems. The effect of penicillin on infections in which it is postulated that every host cell is always infected must be something else (308). It is not clear how a few cells survive destruction of virtually the entire host population at the end of each cycle of persistent infection and then proliferate to start the next cycle. The division of infected host cells (see "Effect on progression through the cell cycle and cell division" above) to produce one inclusion-bearing and one inclusion-free cell has been suggested as a mechanism for generating uninfected host cells in chlamydia-infected populations (332). The same mechanism has been proposed to account for the appearance of uninfected host cells in L-cell populations 100% infected with *C. burnetii* (389). Inclusions may also divide simultaneously with host cells so that they are shared between daughters (381). This may be a way to ensure persistence of an infection with minimal damage to the host cell population. Host cells may survive infection by releasing (332, 474) or dissolving (332) inclusions without being destroyed in the process. They may also become more resistant to the entry of both wild-type and persistent-infection chlamydiae after establishment of persistent infection. This refractoriness may represent the selection of a more resistant genotype (332) or the occurrence of reversible changes that disappear when the host cells are cured of persistent infection (315, 316, 319, 350). Resistance to superinfection may be partial (332, 350) or almost complete (315, 316, 319). All these phenomena may contribute to the survival of rare host cells through periods of extensive chlamydial multiplication and to their continued resistance to infection during regrowth of the host cell population. Just which, if any, of them is most important in a particular persistent infection remains to be seen.

Pertinence of Persistent Infections In Vitro to Persistent Infections In Vivo

Somewhere in the back of the mind of everyone who studies chlamydiae in cell culture always lurks the unanswered question, "Will my experiments contribute to the understanding of natural infections?" This seems to be especially true of persistent infections. No matter how interesting or revealing an in vitro persistent infection is in its own right, the question always arises as to its pertinence to persistent infections in vivo. Production of persistently infected cell cultures by adding an inhibitor of chlamydial multiplication or by withholding a nutrient essential to growth has sometimes been regarded as a laboratory curiosity, but the demonstration that gamma interferon (IFN-γ) reversibly blocks growth of *C. psittaci* 6BC by inducing an enzyme that breaks down tryptophan, an essential amino acid for chlamydiae (61; see also "Action of IFN-γ" below), suggests that this in vitro mechanism may well have its in vivo counterpart. Long-time persistent chlamydial infections, whether in cell culture or in natural hosts, must be based on establishment of a mutually acceptable balance between multiplication of chlamydiae and their host cells. Stasis continued long enough ends in death, and the only way that host cell and chlamydial populations can persist for weeks, months, and even years is by multiplying. Considering the relatively few investigations on the subject, it is remarkable how many different ways to produce persistent chlamydial infections in cell culture have been described and how differently these persistent infections behave. It seems unlikely that chlamydiae, with one of the smallest of all eubacterial genomes, would have evolved such a variety of ways of interacting with host cells without at least some of them being of adaptive value. The "normal" developmental cycle by which chlamydiae reproduce themselves in acutely infected cell cultures may not be their only mode of multiplication in intact hosts.

Also remarkable is the frequency with which population shifts favoring new chlamydial genotypes have occurred during persistent infection of cell cultures, although propagation of the same chlamydial strains by acute infection of the same cell cultures very rarely results in population

changes. The selection of genotypes better adapted to conditions of persistent culture (247, 270, 332, 350) is not surprising, but the selection of strains of *C. psittaci* more virulent for sheep by persistent infection of mouse (McCoy) cells (386) is not easily explained. Could it be that persistent infections of natural hosts are fertile breeding grounds for new strains of chlamydiae?

INTERACTION OF CHLAMYDIAE WITH IMMUNE CELLS AND IMMUNE MOLECULES IN VITRO

Natural and experimental chlamydial infections provide a protection against reinfection that is usually incomplete, transient, and strain specific (for reviews, see references 24, 54, 252, and 301). Although the full complexity of the cell-mediated and antibody-mediated responses to chlamydial infection can be explored only in the intact host, some aspects of acquired chlamydial immunity can be studied in vitro. The fate of chlamydiae in cells from nonimmunized and immunized animals can be compared, the interaction between chlamydiae and immunocompetent cells can be investigated, and the effects of products of immunocompetent cells on the survival and growth of chlamydiae in other cells can be measured.

Fate of Chlamydiae in Cells from Immune Animals

Although it has long been known that cell-mediated immunity to intracellular parasites can be demonstrated by in vitro challenge of macrophages from immunized animals (267, 268), little work of this nature has been done with chlamydiae. *C. psittaci* MN/Cal 10 multiplied in peritoneal macrophages from nonimmunized guinea pigs but failed to grow in comparable macrophage populations from immunized animals (33). The limited growth of *C. trachomatis* biovars trachoma and LGV in macrophages from nonimmunized mice was not further decreased in macrophages from immunized mice (234). These observations have not been followed up.

Interaction of Chlamydiae with Lymphocytes In Vitro

In intact hosts, lymphocytes respond to the presence of chlamydiae by exhibiting cytotoxic activity and by secreting antibodies and cytokines. In culture, purified human and mouse B lymphocytes are stimulated to proliferate and produce polyclonal antibody by *C. trachomatis* biovars trachoma and LGV and by *C. psittaci* MN/Cal 10 (22, 253). Stimulated human B cells needed T-cell help for antibody synthesis, but mouse cells did not. The proliferation-inducing factor was not chlamydial LPS. Chlamydiae have been reported to stimulate (367) or not to stimulate (22, 253) T lymphocytes to proliferate. A survey of 10 lymphoblastoid-myeloid cell lines showed that *C. trachomatis* biovar LGV multiplied in some lines, entered others but did not multiply, and attached to still other lines but did not enter (23). The interaction of these lines with chlamydiae may be indicative of the complexity of lymphocyte-chlamydia interaction in intact hosts.

Interaction of Chlamydiae with Cytotoxic Cells In Vitro

The possibility that chlamydial infection elicits the appearance of cytotoxic T lymphocytes specifically reactive with chlamydia-infected host cells has been tested in vitro. The demonstration of chlamydial antigen on infected cell surfaces (see "Modification of plasma membranes of host cells" above) satisfies one of the requirements for cytotoxic T-cell activity, the other being the presence of appropriate class I major histocompatibility antigens. Spleen cells from mice immunized with *C. psittaci* MN/Cal 10 5 days previously primarily killed only *C. psittaci*-infected L cells and mouse macrophages (243). However, cytotoxic T-cell activity was not found in cells from spleens, lymph nodes, and peritoneal exudate taken from mice immunized with *C. trachomatis* biovar LGV and assayed against infected L cells (343). The different results may reflect the way different strains of mice react to different kinds of chlamydiae.

Interaction of Chlamydiae with Antibody In Vitro

The infectivity of chlamydiae is neutralized by combination with antibody and complement, usually by inhibition of attachment to host cells (see "Modification with antibody and complement" above), but later stages may also be affected. Polyclonal (68) or monoclonal (345) antibodies to the MOMP inhibit inclusion formation by *C. trachomatis* biovars trachoma and LGV without affecting the attachment of labeled EBs to host cells, indicating that the developmental cycle is stopped at an as-yet-undefined point beyond attachment. Transport of ATP may be inhibited (345). Antibody against intact *C. psittaci* 6BC slows the association of labeled EBs to L cells (62), but the antibody-coated EBs that are ingested fuse with lysosomes, whereas uncoated EBs do not (131), again suggestive of a postentry point of action for antibody.

Action of Cytokines on Chlamydial Multiplication In Vitro

Cytokines are immunoregulatory polypeptides secreted by cells of the mononuclear phagocyte lineage and by some other cells as well. Interferons, tumor necrosis factor alpha (TNF-α), and interleukin-1 (IL-1) are synthesized in chlamydia-infected cells, and interferons—IFN-γ, in particular—and TNF-α induce host cells to suppress chlamydial multiplication.

Action of IFN-α and IFN-β. It was established that chlamydiae in cell culture both induce and are inhibited by interferon (168, 207, 222, 287, 456) when interferon was still thought only to inhibit viral reproduction and when the multiplicity of interferon molecules had not been realized. Judging from the cells and viral inducers used to produce it, the interferon of these early studies probably was a mixture of IFN-α and IFN-β (250). More recently, mitogen-induced mouse IFN-α and IFN-β (337) and recombinant mouse IFN-α AD (104) have been used to restrict the growth of *C. trachomatis* biovar LGV in L and McCoy cells, respectively. Inhibition of the entry of *C. trachomatis* biovar LGV into host cells was never observed. Inhibition later in the developmental cycle has been reported to occur only at the RB-to-EB conversion step (397) or at both the EB-to-RB and RB-to-EB stages (104, 222). Under the same conditions used for *C. trachomatis* biovar LGV, IFN-α and IFN-β had no effect on growth of *C. psittaci* 6BC and MN/Cal 10 (63). However, despite this interesting suggestion of a species-based dichotomy of action for IFN-α and IFN-β, most of the work on interferon and chlamydiae has been done with IFN-γ.

Action of IFN-γ. Upon stimulation by antigen, a set of helper T cells secretes the cytokine IFN-γ. Originally called macrophage-activating factor (322), IFN-γ activates both professional and nonprofessional phagocytes to destroy in-

gested microorganisms. In macrophages, IFN-γ stimulates the production of microbicidal reactive metabolites of molecular oxygen as well as that of other antimicrobial factors whose action does not depend on molecular oxygen (322). IFN-γ inhibits the multiplication of many eucaryotic and procaryotic intracellular parasites (65, 309), the chlamydiae included (53, 424). It is thought to be especially important in defense against pathogens that are not effectively destroyed by host cells.

IFN-γ secreted by antigen-sensitized (56, 57) or mitogen-stimulated mouse spleen cells (321) inhibited the multiplication of *C. psittaci* 6BC in mouse macrophages by oxygen-independent mechanisms. IFN-γ prevented *C. psittaci* 6BC growth in oxygen-deficient host cells (L cells and monocyte-derived macrophages from patients with chronic granulomatous disease) (398). The factor in extracts of sensitized lymphocytes that activates L cells and human macrophages to contain the growth of *C. psittaci* 6BC was positively identified as IFN-γ by blocking its action with polyclonal (59) and monoclonal (399) antibodies against IFN-γ but not with antibodies against IFN-α and IFN-β. Final proof of identity was provided by showing that human recombinant IFN-γ activates human macrophages to prevent the growth of *C. psittaci* 6BC (398). IFN-γ also activates host cells to restrict the growth of *C. trachomatis*. Mouse fibroblasts (L cells) and mouse peritoneal macrophages were activated by mouse IFN-γ to inhibit the growth of the LGV biovar (399, 535), and mouse IFN-γ reduced the number of inclusion-bearing cells in McCoy cells treated with the mouse biovar (516). Human IFN-γ inhibited the growth of the trachoma biovar in primary cultures of human conjunctival epithelium (457). It also activated HeLa, HEp-2 (a human epidermoid lung carcinoma line), and T24 cells (an epithelial cell line derived from a human bladder carcinoma) to restrict the growth of *C. trachomatis* biovar LGV (107, 423). IFN-γ plays a role in resistance of mice to infection with the mouse (516) and LGV (536, 537) biovars, but its relative importance in host defense against chlamydial infection has not been determined.

The effect of IFN-γ on chlamydial growth has been analyzed with the LGV biovar of *C. trachomatis* and human IFN-γ in HEp-2 cells (423) or mouse IFN-γ in L (399) or McCoy (107) cells. Compared with untreated controls, infected cell cultures exposed to IFN-γ have fewer infected cells, fewer chlamydiae per inclusion, and smaller infectious yields. In host cells to which IFN-γ was added 18 to 24 h before infection, 25 IU/ml reduced the inclusion count by 50% (399), and 15 IU/ml gave a 50% reduction in infectious yield (423). When IFN-γ was added to cell cultures 24 h before infection and never removed, as little as 25 IU/ml completely and indefinitely suppressed growth of *C. trachomatis* biovar LGV. When IFN-γ was added at the time of infection, the delay in initiation of chlamydial multiplication was proportional to the IFN-γ concentration. An initial concentration of 25 IU/ml delayed growth for about 48 h; 400 IU/ml delayed it indefinitely. IFN-γ added to cell cultures as late as 18 to 24 h after infection still exerted a measurable slowing effect on chlamydial multiplication, indicating that IFN-γ activation of host cells occurs relatively rapidly. IFN-γ does not act directly on chlamydial cells. It neither kills them extracellularly nor prevents their ingestion. Protein and RNA synthesis by host cells is required, as evidenced by blocking of IFN-γ-mediated activation by pretreatment with cycloheximide or dactinomycin (399).

Thin-section electron microscopy of untreated and IFN-γ-treated infected cells made at different times in the developmental cycle of *C. trachomatis* biovar LGV has been used to establish the point or points in the cycle at which IFN-γ-activated host cells inhibit chlamydial multiplication (107, 109, 399, 423). Somewhat different conclusions have been drawn, possibly because different host cells and different times of addition and concentrations of IFN-γ were used. Under appropriate conditions, it appears likely that almost every intracellular stage in the developmental cycle may be interfered with—the differentiation of EBs into RBs, the multiplication of RBs, and the conversion of RBs back into EBs. This would be indicative of a generalized slowing down of chlamydial activity rather than an absolute block at some particular point in the developmental cycle.

The crucial discovery that led to understanding how IFN-γ-activated host cells prevent chlamydial multiplication was the discovery that IFN-γ blocks the growth of the eucaryotic intracellular parasite *T. gondii* in cultured fibroblasts by inducing them to degrade tryptophan (357). IFN-γ induces the synthesis of the enzyme indoleamine-2,3-deoxygenase, which degrades tryptophan to kynurenine. Tryptophan also reversed the IFN-γ-induced inhibition of *C. psittaci* 6BC multiplication in T24 cells (61) and of *C. trachomatis* biovar LGV in HEp-2 cells (422), but it failed to block the action of IFN-γ on McCoy cells infected with *C. trachomatis* biovar LGV (108). When 10 ng of human recombinant IFN-γ was added to T24 cells 24 h before infection with *C. psittaci* 6BC, the appearance of inclusion-bearing cells was strongly suppressed (61). Tryptophan added 1 h after infection reversed the IFN-γ-mediated inhibition of chlamydial growth in proportion to its concentration. A concentration of 10 μg/ml gave measurable reversal, and at 100 μg/ml, reversal was almost complete. Tryptophan had no effect on chlamydial growth when the T24 cells had not been incubated with IFN-γ, and neither isoleucine nor lysine reversed the IFN-γ-mediated inhibition of chlamydial growth. Comparable results were obtained with *C. trachomatis* biovar LGV and HEp-2 cells (422). When 100 IU of recombinant human IFN-γ per ml was added to HEp-2 cultures 24 h before infection with the LGV biovar, chlamydial reproduction was completely blocked. As little as 40 μg of tryptophan per ml also added 24 h before infection completely restored infectious yield to the uninhibited level. The concentration of IFN-γ required for 50% reduction in infectious yield depended on the concentration of tryptophan added to the growth medium. With 10 μg of tryptophan per ml, the 50% inhibitory concentration of IFN-γ was 5 IU/ml, but with 40 μg/ml, the 50% value rose to 33 IU/ml. Inhibition of *C. trachomatis* biovar LGV growth in IFN-γ-induced HEp-2 cells was completely reversed by tryptophan addition 1 day after infection, partially reversed by addition at 2 days, and reversed hardly at all at 3 days. In both *C. psittaci* (55) and *C. trachomatis* (422) infections, addition of tryptophan later than 3 days after inoculation of host cells activated with IFN-γ failed to restore chlamydial multiplication, probably because prolonged tryptophan deprivation was irreversible.

In IFN-γ-treated T24 cells, tryptophan was degraded at rates proportional to the IFN-γ concentration, but there was no tryptophan degradation in untreated cells (61). The primary degradation products were kynurenine and *N*-formylkynurenine formed from kynurenine by a constitutive enzyme. Tryptophan was also degraded to kynurenine and *N*-formylkynurenine by monocyte-derived human macrophages (74) and human conjunctival epithelial cells (457) activated by human IFN-γ to inhibit growth of *C. psittaci* 6BC and *C. trachomatis* biovar trachoma, respectively. In

both systems, IFN-γ-mediated inhibition was reversed by tryptophan. Thus, tryptophan destruction is a widely distributed mechanism for IFN-γ-activated restriction of chlamydial growth. However, it is not the universal mechanism for all intracellular parasites; IFN-γ-induced inhibition of the growth of *R. prowazekii* in mouse and human fibroblasts was not reversed by tryptophan (485).

IFN-γ-mediated activation of host cells to restrict multiplication of chlamydiae has been proposed as a mechanism for establishing persistent chlamydial infections in vivo (53, 54, 424). For the reasons advanced above in Persistent Infection of Cell Cultures, a choking down of chlamydial multiplication rather than a total stoppage is most likely to establish the state of mutually tolerable rates of chlamydial and host cell multiplication required for indefinitely persisting infections. IFN-γ-mediated inhibition of chlamydial growth in cell culture and its reversal by tryptophan may well turn out to be another example of the induction of persistent infection by nutritional deprivation, discussed above in Persistent Infection of Cell Cultures, but more information is needed before it can be concluded that the intracellular concentration of tryptophan is the only thing that matters. The single omission of tryptophan from an otherwise adequate medium inhibits the growth of *C. psittaci* 6BC, and growth may be restored by the not-too-long-delayed addition of tryptophan (16). However, the tryptophan requirements of the *C. trachomatis* strains used in the IFN-γ studies are not known. The only strain of *C. trachomatis* for which the effect of a single omission of tryptophan has been determined is a serovar E trachoma biovar (240). It, too, requires tryptophan for growth. The tryptophan requirements of all chlamydial strains used to explore the IFN-γ–tryptophan relationship should be determined. In view of the widely differing amino acid requirements of different chlamydial strains (see "Amino acid metabolism" above), their dependence on tryptophan cannot be taken for granted. Second, if there is any doubt about the tryptophan requirements of chlamydiae, there is none at all about the requirements of mammalian cells: they need tryptophan (116). The in vitro antiproliferative activity of IFN-γ parallels its antichlamydial activity (107) and is also reversed by the addition of tryptophan to the culture medium (106). This suggests that the concentration of tryptophan in IFN-γ-activated cells is too low to support normal levels of tryptophan incorporation into cell proteins. Could the effect of tryptophan scarcity on chlamydiae be, at least in part, the indirect effect of interference with host cell activities? The experience of those who have studied amino acid deprivation of chlamydia-infected cells has been that amino acid levels low enough to stop chlamydial multiplication also stop host cell multiplication (16, 173).

When inhibition of chlamydial multiplication is desired, IFN-γ is usually added to cell cultures 24 h before infection. However, if IFN-γ is added at the time of infection, it acts as a cytotoxic cytokine for L cells infected with *C. psittaci* 6BC (58, 60, 64). Cytotoxicity, as measured by release of labeled thymidine and staining with trypan blue, was dependent on the concentration of recombinant mouse IFN-γ, with maximum host cell killing obtained with 5 to 10 IU/ml. IFN-γ was not toxic for uninfected L cells, and in infected L-cell populations, the percent cytotoxicity approximated the percentage of infected cells in the range of 17 to 70%. Cytoxicity developed slowly and reached a peak 30 h after infection and addition of IFN-γ. Chloramphenicol added at the time of infection blocked the cytotoxic effect, but the antibiotic given 18 h later did not. This suggests that IFN-γ-mediated cytotoxicity requires chlamydial differentiation and early multiplication. Cycloheximide has a similar effect. Addition at zero hour prevents cytotoxicity; addition delayed for 12 h does not. This implies that synthesis of host protein early after infection is essential for manifestation of cytotoxicity. It is not clear why cell death does not occur until late in infection when required host and parasite activities appear to occur much earlier. How IFN-γ induces a cytotoxic response in *C. psittaci* 6BC-infected L cells is not known. The mechanism does not seem to involve degradation of tryptophan. It remains to be seen if IFN-γ-mediated cytotoxicity occurs in other chlamydia-host cell systems and if this in vitro phenomenon has an in vivo correlate. IFN-γ-mediated cytotoxicity may play a role in the response of mice to infection with the mouse biovar of *C. trachomatis* (58).

Action of TNF-α. TNF-α, a major mediator of the inflammatory response, is elicited in mononuclear phagocytes by a variety of pathogens (37). It inhibits the growth of viruses and both eucaryotic and procaryotic intracellular parasites. TNF-α is produced in the lungs of mice infected with the mouse biovar of *C. trachomatis*, where it plays an as-yet-undefined role in host defense against this infection (517). In cell culture, the effect of TNF-α on chlamydiae resembles that of IFN-γ, and its action is assayed in a similar manner (425, 426). When recombinant human TNF-α is added to HEp-2 cells 24 h before infection with *C. trachomatis* biovar LGV, it reduces the infectious yield in proportion to its concentration. The 50% effective concentration is 0.13 ng/ml, and 99% reduction is achieved with a concentration of 200 ng/ml, which is not toxic for uninfected HEp-2 cells. TNF-α appears to interfere with the developmental cycle chiefly by blocking conversion of RBs to EBs. The TNF-α effect is abolished by cycloheximide, indicating that synthesis of host protein is required. Like IFN-γ, TNF-α increases the degradation of tryptophan, and TNF-α-induced suppression of chlamydial multiplication is reversed by high concentrations of tryptophan.

Not only does the antichlamydial action of TNF-α resemble that of IFN-γ, but the two cytokines also act synergistically to suppress chlamydial multiplication (425, 426). IFN-β also modulates TNF-α action; antibodies against this interferon block the stimulation of tryptophan dissimilation induced by TNF-α. It has been suggested that TNF-α induces the synthesis of both IFN-β and indoleamine-2,3-deoxygenase.

Action of IL-1. IL-1 has multiple functions, including activation of lymphocytes, induction of the synthesis of other cytokines, and mediation of inflammation and tissue destruction (114). IL-1 is produced by HPBMs stimulated by high multiplicities of *C. trachomatis* biovar LGV EBs inactivated by heat or UV light (400), but it is not induced in human monocyte-derived macrophages infected with low multiplicities of the LGV biovar (277). A precise role for IL-1 in resistance to chlamydiae and in host damage caused by chlamydial infection has not been established.

Relation of action of cytokines on cultured cells to role of cytokines in infection of intact hosts. Most of the work with cytokines and chlamydia-infected cell cultures has been done with individual recombinant human and mouse polypeptides free of other cytokine activity. This has allowed unambiguous definition of how single cytokines influence chlamydial growth, but one host cell may produce several different cytokines, and one cytokine frequently affects the release and action of the others. The demonstration that coculture of HEp-2 cells infected with *C. trachomatis* biovar LGV with human monocyte-derived macrophages reduces

infectious yield and chlamydial DNA synthesis (276) may offer a way to approach the problem of how the complex mixture of cytokines encountered in intact hosts affects chlamydial multiplication.

COMPETITION AMONG CHLAMYDIAE AND BETWEEN CHLAMYDIAE AND OTHER INFECTIOUS AGENTS

How chlamydiae compete with host cells has already been described. A single chlamydia may also compete with other chlamydiae like itself and with other infectious agents that may be present. Competition with other infectious agents, particularly on the genital and conjunctival mucosae, could be important in chlamydial infections of intact hosts. Simultaneous genital tract infection of males with *Neisseria gonorrhoeae* and the trachoma biovar of *C. trachomatis* is common in western Europe and the United Kingdom but less common in the United States (201, 213, 411). These double infections may be the consequence of synergism between neisseriae and chlamydiae or, just as likely, of the life styles of their hosts.

When HeLa cells are held at 0°C so that EBs adhere but do not attach, different serovars of *C. trachomatis* compete with each other for attachment sites on host cells (490). However, at 37°C, when attachment is followed by ingestion, competition is not observed. Even multiple infection of BHK cells with *C. trachomatis* biovar LGV does not decrease their susceptibility to fresh chlamydiae added 6 h later (40). At low multiplicities of infection, the rate of attachment of freshly prepared EBs of *C. trachomatis* biovar trachoma actually increases as the multiplicity of infection is raised (46). Chlamydiae multiplying in a single host cell also compete with each other, probably for host-supplied nutrients. When BHK cells are infected with increasing multiplicities of *C. trachomatis* LGV, the infectious yield per inclusion rises from 278 for cells receiving 1 EB to 848 for cells receiving 16 EBs, an undoubted consequence of inclusion fusion (124). However, at the same time, the ratio of infectious units inoculated to infectious units produced drops from 278 (for 1 EB) to 60 (for 16 EBs).

The interaction of chlamydiae with host cells in vitro is also modified by the presence of other infectious agents. The association of *E. coli* and *Staphylococcus aureus* with HeLa cells was increased by simultaneous infection with *C. trachomatis* biovar LGV (44), and pretreatment of cultured human cervical epithelial cells with gonococci increased the number of inclusions obtained by centrifuge-assisted infection with *C. trachomatis* biovar trachoma (524). When African green monkey kidney cells were superinfected with herpes simplex virus type 2 48 h after primary infection with *C. trachomatis* biovar LGV, electron micrographs made 24 h later showed inclusions largely devoid of EBs and RBs (363).

These few investigations show that intra- and interspecific competition can modify the reaction of chlamydiae with host cells in vitro without providing much insight into the mechanisms involved. In light of the many opportunities for comparable competition in natural infections, more intensive study of competitive situations in vitro should be rewarding.

CONCLUSION

The investigations just reviewed lead to the conclusion that, as a first approximation, all chlamydiae behave the same in cell culture. There are differences, but they are insignificant compared with the similarities. This means that all members of the genus share a highly conserved core of characteristics essential to their intracellular way of life that were acquired before the three species diverged from their common ancestor, perhaps even before invasion of the intracellular habitat. Loss of peptidoglycan and appearance of reversible disulfide bond-based cross-linking of the outer membrane proteins may well have been indispensable preadaptations to life inside host cells. The ever-increasing list of chlamydial proteins with amino acid sequences extensively shared among the species and biovars is indicative of this common core. The few differences in the way chlamydiae behave in cell culture and the many differences in the way they behave in their natural hosts stem from genomic changes occurring after divergence. Many of these changes must code for adaptations to life in different hosts and host cells, just the kinds of chlamydial behavior that should be analyzed in cell culture.

That presently employed cell culture models fail to detect subtle differences among chlamydiae should not have been unexpected. A good model of anything is one that faithfully reproduces the behavior of the more complex system being modeled, and faithfulness depends on what is being modeled. The more general the behavior to be reproduced, the less stringent the demands on the model. For example, the chlamydial developmental cycle as worked out in the familiar cell lines of fibroblastic origin (HeLa, L, and McCoy) probably accurately reproduces the main features of the cycle in natural host cells, which are seldom fibroblasts. However, these host cell models cannot explain why some serovars of *C. trachomatis* cause classical trachoma while others cause sexually transmitted oculogenital disease. Perhaps some of the cell culture models using primary cultures of genital tract and conjunctival epithelial cells (see Introduction) will be able to do so. Explanations of why *C. pneumoniae* gets along without a nonhuman host and why the different strains of *C. psittaci* produce such varied manifestations of disease will also require more faithful cell culture models than are presently available.

In cell culture, the chlamydial developmental cycle is particularly vulnerable to interruption at two points. The first critical period comes immediately after entry when the just-ingested EB either reorganizes into an RB or is destroyed. The influences of kind of host cell, kind of chlamydiae, and conditions under which entry has taken place on the fate of the EB have been discussed above in "Destruction of Chlamydiae". The second critical period is late in the developmental cycle, when RBs reorganize into EBs. Reorganization is especially sensitive to the deficiency of amino acids (see "Amino acid metabolism" above), to the presence of antibacterial agents that in host-independent bacteria inhibit the synthesis of peptidoglycan and DNA (see "Effect of antibacterial agents on conversion of RBs to EBs" above), and to the presence of cytokines (see "Action of Cytokines on Chlamydial Multiplication In Vitro" above). If infected cell cultures are good predictors of what goes on during natural chlamydial infections, then these two steps in the developmental cycle should be especially vulnerable to chemotherapeutic and immunological intervention.

Wholly apart from their only partially fulfilled promise of serving as models for natural infections, cell cultures infected with members of the genus *Chlamydia* have yielded results of general biological significance. The interaction of chlamydiae with host cells in vitro provides an extreme example of a general biological phenomenon, the adaptation of an organism to a new habitat. It is extreme because the new habitat is the inside of another cell and because chla-

mydiae have made adaptations that allow them to exploit the resources of the intracellular environment to a greater extent than any other known nonviral parasite. Continued investigation of cell cultures infected with chlamydiae is sure to uncover interesting and unexpected phenomena.

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