Nucleotide Metabolism In *Chlamydia trachomatis*

By

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NUCLEOTIDE METABOLISM IN CHLAMYDIA TRACHOMATIS

BY

GRAHAM TIPPLES

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Studying the strategies *Chlamydia trachomatis* uses to acquire and metabolize nucleobases, (deoxy)nucleosides, and (d)NTPs, can help in the general understanding of the dynamic process of intracellular parasitism, and can possibly provide insights into new therapies for the treatment of diseases caused by *C. trachomatis*. An *in situ* method using specific inhibitors of prokaryotic or eukaryotic nucleic acid synthesis, and/or mutant host cell lines deficient in specific pathways of nucleotide metabolism enables the differentiation between host and parasite activities and the determination of specific pathways present in *C. trachomatis*. Results indicate that *C. trachomatis* can draw on the host cell's NTP pools with subsequent reduction of the NTPs to dNTPs by a hydroxyurea sensitive chlamydial ribonucleotide reductase (RR). The chlamydial RR is sensitive to the class I RR inhibitor hydroxyurea. Isolation and characterization of hydroxyurea resistant chlamydiae show overexpression of RR. Even though *C. trachomatis* draws significantly on the host NTP pools, the chlamydial infection is not detrimental to the host in terms of the host cell's energy charge (constant at ≈0.98). Chlamydiae are energy parasites requiring ATP from the host; however, the growth of *C. trachomatis* in a mitochondrial deficient host cell line indicates that host oxidative ATP generation is not necessary for chlamydial growth. The discovery of a chlamydial CTP synthetase indicates that *C. trachomatis* is auxotrophic for three (UTP, GTP, ATP) of the four NTPs. The chlamydial CTP synthetase, cloned by functional complementation in *E. coli*, is encoded in an operon. The open reading frames present in the operon encode CMP-KDO synthetase, CTP synthetase, and a 131 amino acid polypeptide of unknown function. Sequencing of DNA isolated from mutant *C. trachomatis* resistant to the CTP synthetase inhibitor cyclopentenyl-cytosine (CPEC), indicates a single point mutation which results in the substitution of a highly conserved aspartic acid residue to a glutamic acid residue.

INTRODUCTION

Chlamydiae

Chlamydiae are obligate intracellular gram negative bacteria that are capable of infecting a wide range of eukaryotic host cells. It is not surprising then that chlamydiae cause a variety of diseases, not only in humans, but also in non-human mammalian and avian species (Fraiz and Jones, 1988). Because of their uniqueness among prokaryotes, chlamydiae are classified into their own order Chlamydiales, family Chlamydiaceae, and genus *Chlamydia*. The genus *Chlamydia* is currently classified into four species: *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, and *C. pecorum* (Moulder *et al*, 1984; Grayston *et al*, 1989; Fukushi and Hirai, 1992). *C. trachomatis* and *C. pneumoniae* are human pathogens, while *C. pecorum* is isolated from ruminants. *C. psittaci* is normally a cause of avian and animal disease, but can cause pneumonia-like disease in humans (psittacosis).

C. trachomatis is divided into trachoma, lymphogranuloma, and mouse biovars based on the particular disease each causes (Moulder, 1988). The mouse biovar consists of a single serotype, MoPn, which causes mouse pneumonitis. Lymphogranuloma serovars L1, L2 and L3 cause the sexually transmitted disease lymphogranuloma venereum. The trachoma biovar encompasses twelve serotypes. Trachoma serovars A, B, Ba, and C cause the disease trachoma - a leading cause of preventable blindness in developing countries (Fraiz and Jones, 1988). In industrialized nations, trachoma serovars D, E, F, G, H, I, J and K are the cause of prevalent sexually transmitted genital infections - these diseases include urethritis, cervicitis, epididymitis, ectopic pregnancy, and pelvic inflammatory disease.

Chlamydiae have evolved to include a unique biphasic life cycle to accommodate survival both intracellularly and extracellularly. The metabolically inert, but infectious elementary body (EB) is the extracellular form. The function of this spore-like form (the EB is sometimes referred to as the *chlamydiospore*) is host to host transmission through the hostile

extracellular environment. The major outer membrane protein (MOMP) as well as three other outer membrane proteins (12, 59, and 62 kDa) are highly cross-linked via disulfide bonds, thereby giving the EB a characteristically condensed and rigid cell wall structure. The EB is capable of attaching to and being phagocytosed by the host cell. The organism is enclosed in a membrane-bound vacuole called the inclusion inside the eukaryotic host cell. It is known that chlamydiae-containing phagosomes do not fuse with lysosomes, although the mechanism for this is unclear. Soon after entry, the condensed EB (≈0.3 μm diameter) differentiates into the larger (≈0.9 μm diameter) reticulate body (RB) (Matsumoto, 1988). Although not much is known about the signals for EB to RB differentiation, reduction of the highly disulfide-cross-linked outer membrane proteins is believed to be a key event in this differentiation process. The reduction of MOMP to its monomeric form occurs within an hour after entry of the EB into the host cell (Hatch *et al*, 1986). The RB is the metabolically active vegetative form which replicates by binary fission (doubling time is ≈2 h). The chlamydial life cycle is asynchronous - a particular inclusion contains both RBs and EBs. At approximately 22 h after entry, metabolic activity (RNA, DNA, and protein synthesis) is at a maximum since the majority of organisms are in the RB form. The process of RB growth and RB to EB differentiation continues until approximately 48 to 72 h at which time the majority of organisms have differentiated back into the EB form. Release of EBs by host cell lysis results in the start of another infection cycle.

The cell wall structure of typical gram-negative organisms consists of an inner phospholipid bilayer, a layer of peptidoglycan surrounding the inner membrane, and an outer lipid bilayer membrane containing lipopolysaccharide (LPS). Both the inner and outer membranes have associated proteins. Chlamydiae are different from most other bacteria because they lack peptidoglycan (Garrett *et al*, 1974). However, they still do exhibit characteristics usually attributed to peptidoglycan. For example, chlamydiae are susceptible to penicillin (Matsumoto and Manire, 1970; Kramer and Gordon, 1971), and also contain penicillin-binding proteins which are usually associated with peptidoglycan biosynthesis in

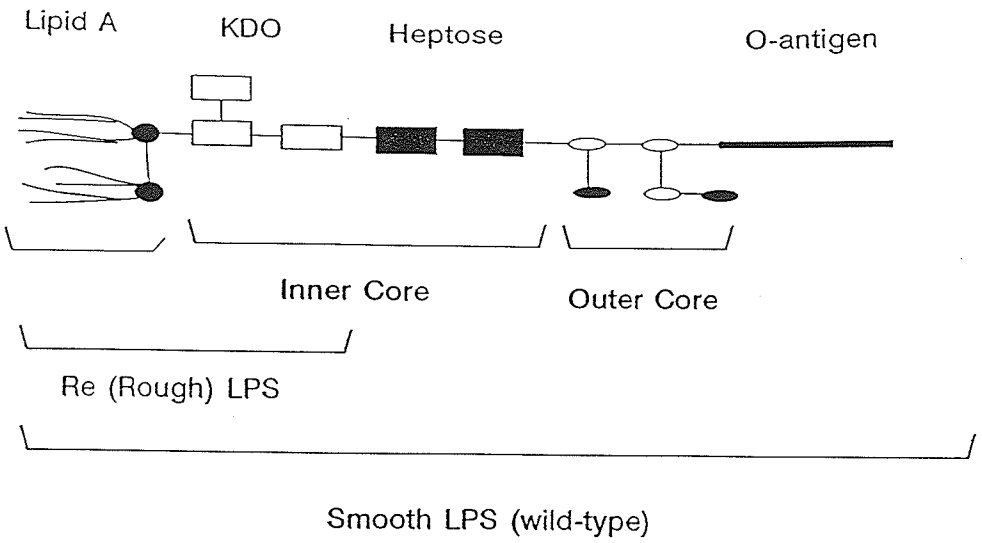
other bacteria (Barbour *et al*, 1982). Penicillin is thought to act by interfering with cross-linking during peptidoglycan biosynthesis. The function of peptidoglycan in most bacteria is to form a rigid barrier between the environment and the cell, thereby ensuring bacterial integrity. The rigidity afforded by the cross-linked outer membrane proteins of the EB are thought to compensate for the absence of peptidoglycan.

It is not surprising that MOMP, which accounts for approximately 60% of the dry weight of the chlamydial cell, is a dominant immunogen on the EB (Caldwell *et al*, 1981). MOMP exhibits antigenic variability among different *C. trachomatis* serovars (Perez-Martinez and Storz, 1985; Brunham *et al*, 1993).

LPS is another major antigenic determinant of chlamydiae. LPS (endotoxin) is a unique molecule found in the outer membrane of most gram-negative bacteria and is responsible for a number of pathophysiological phenomena (Rietschel and Hinshaw, 1984). In typical gram-negative bacteria LPS consists of the following structure: lipid A - inner core - outer core - O-antigen (Raetz, 1990) (Figure 1A). Lipid A is the phospholipid-like hydrophobic membrane-anchoring region. The inner core consists of unique 3-keto-D-*manno*-octulosonic acid (KDO) sugar residues while the outer core consists of heptose plus a number of more common sugar residues. The highly variable O-antigen region consists of repeating oligosaccharide units. Two types of LPS have been found in chlamydiae. The first type of LPS resembles the smooth form LPS (S-LPS) found in wild-type strains of enterobacteria. The S-LPS consists of the lipid A moiety, the inner and outer cores, and the O-antigen region. Chlamydial S-LPS is preferentially seen in chlamydiae propagated in yolk sacs of embryonated eggs (Lukacova *et al*, 1994). The second type, normally seen in tissue-culture grown chlamydiae, resembles the truncated LPS chains of rough *Salmonella* Re mutants (Nurminen *et al*, 1985) (Figure 1B). This chlamydial LPS consists of lipid A with a linear KDO trisaccharide attached via unique linkages:

lipid IV_A (6'→2) αKDO (4→2) αKDO (8→2) αKDO

A.



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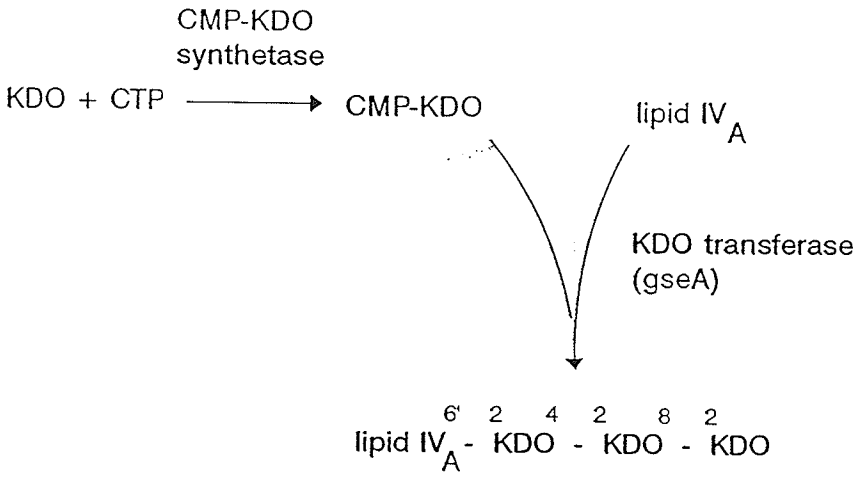


Figure 1: A. Schematic structure of LPS in a typical gram negative bacterium such as *E. coli* and *S. typhimurium*. Closed circles represent polar head groups of lipid A, open rectangles represent KDO sugar residues, solid rectangles represent heptose residues, and open and closed ovals represent outer core sugar residues (Glc, Gal, GlcNAc).

B. Biosynthesis and structure of the genus-specific epitope (LPS) of chlamydiae. Superscript numbers indicate sugar carbon residues involved in the disaccharide linkages.

LPS of different strains are conserved (genus-specific) (Dhir *et al*, 1971; Nurminen *et al*, 1983). The genus-specific LPS epitope is thought to result from the action of a novel KDO transferase encoded by *gseA*; this enzyme is capable of transferring at least three KDO residues to lipid A precursors (Belunis *et al*, 1992). *gseA* has been cloned and sequenced in both *C. trachomatis* (Belunis *et al*, 1992) and *C. psittaci* (Mamat *et al*, 1993). Surprisingly, the deduced amino acid sequences share only 67% homology even though the action of the two gene products is identical. In *E. coli*, CTP:CMP-3-deoxy-D-manno-octulosonate cytidyltransferase (CMP-KDO synthetase), encoded by *kdsB* (Goldman *et al*, 1986) and *KpsU* (Pazzani *et al*, 1993) supplies the activated-sugar precursor, CMP-KDO, which is the substrate for the KDO transferase. Almost all enzymes of lipid A and KDO biosynthesis, including CMP-KDO synthetase and KDO transferase, have been found in the cytosol of *E. coli* (Raetz, 1990). There is evidence presented in this thesis for the existence of a CMP-KDO synthetase in *C. trachomatis*.

Intracellular Parasitism

There are common themes for the survival of intracellular parasites (Finlay and Falkow, 1989; Moulder, 1985): (i) The organism must have some mechanism for attachment to and entry into the host cell, (ii) the invading organism must avoid the host cell defense mechanisms, (iii) the organism must replicate intracellularly, yet at the same time maintain host cell functions which are essential to the parasite, and (iv) progeny parasites must continue the life cycle and invade new host cells. The interaction between the host and the parasite is a dynamic process requiring the parasite to adapt to diverse environmental conditions. There are both benefits and difficulties for a parasite living in an intracellular environment. Benefits include the potential access to many nutrients and metabolites required for parasite growth. Difficulties of surviving intracellularly include dealing with the host cell's intracellular killing mechanisms. Intracellular organisms can multiply free in the cytoplasm (must first escape the

phagosome), in a membrane-bound phagosome (avoids fusion with the lysosome), or in a phagolysosome (must have a mechanism to survive in the presence of the acidic lysosomal contents).

Chlamydiae inhabit phagosomes (membrane-bound vacuole termed the inclusion) and by some unknown mechanism prevent fusion with lysosomes. Chlamydiae potentially have access to all the essential building blocks for the synthesis of DNA, RNA, protein, and lipids (for a review see McClarty, 1994). Exposure to such a nutrient-rich environment would favor the elimination of redundant biosynthetic pathways required for the synthesis of these available metabolites. It has been argued that the unusually small chlamydial genome (1.0×10^6 bp) (Birkelund and Stephens, 1992) may reflect this phenomenon. Whether this is actually true remains to be seen. Access to nutrients in the host cell cytoplasm is influenced by two factors. Firstly, the intracellular parasite must compete with the host cell for these nutrients. There must be a careful balance between exploiting the nutrient rich environment and harming the host cell; an unhealthy host cell does not provide an ideal environment for the parasite to grow. Some of the work in this thesis deals with this question of host-parasite interactions with respect to nucleotide metabolism. Secondly, there are three physical barriers for the nutrients to cross before they can be utilized - the inclusion membrane, the outer membrane, and the cytoplasmic membrane. Many metabolites are in forms which cannot cross membranes in the absence of specific transport systems. For example, highly charged nucleotides require specific transport systems for crossing membranes. No transport systems have been identified on the inclusion membrane but inclusion membrane specific chlamydial antigens have been demonstrated (Rockey and Rosquist, 1994). The outer membrane contains MOMP which may function as a porin in RBs (Bavoil *et al*, 1984). This would allow passive diffusion of hydrophilic molecules across a concentration gradient into the periplasm. As for the cytoplasmic membrane, a proton motive force and specific carrier-mediated transport systems have been shown to be present (Hatch *et al*, 1982).

There are a number of problems encountered when studying chlamydiae, not the least of which is that it is a significant biohazard. Chlamydiae can only be grown in eukaryotic host cells thereby making the generation of purified organism both expensive and labor intensive. Sufficient quantities of metabolically active and pure organisms for protein/enzyme purification is very difficult to achieve. Therefore, it is often more efficient to try a molecular biological approach to first isolate the chlamydial gene of interest and then express it in high levels in *E. coli*. There is no gene transfer system for genetic studies of chlamydiae. For this reason metabolic activities of chlamydiae are often studied *in situ*. For these kinds of experiments, there must be some way to differentiate between host- and parasite-specific activities. The use of specific inhibitors of eukaryotic and prokaryotic biosynthetic activities can be used. For example, cycloheximide specifically inhibits eukaryotic protein synthesis (and indirectly DNA synthesis) while chloramphenicol specifically inhibits prokaryotic protein synthesis. Similarly, aphidicolin inhibits eukaryotic DNA synthesis while norfloxacin inhibits prokaryotic DNA synthesis. In addition to specific metabolic inhibitors, mutant host cell lines with well defined mutations in specific metabolic pathways can be used to study chlamydial metabolism *in situ*.

Nucleotide Metabolism

Nucleotides are involved in many cellular functions (Table 1). A cell must have a continuous and balanced supply of ribonucleotides (ATP, GTP, CTP, UTP) and deoxy-ribonucleotides (dATP, dGTP, dCTP, dTTP) to maintain the fidelity of RNA synthesis and DNA replication, respectively. Nucleoside triphosphates, in particular ATP, are the high energy end products of energy releasing reactions. Many cellular fueling reactions are at least in part regulated by intracellular levels of ATP, ADP, and AMP - the balance between these is termed the energy charge. Other intracellular messengers include cAMP, cGMP, ppGpp, and AppppA. Adenine nucleotides are constituents of a number of coenzymes (NAD^+, $NADP^+$, FMN, FAD, and coenzyme A). Nucleotides are also involved in a number of biosynthetic

Table 1: Cellular functions of nucleotides

Cellular process	Nucleotide
nucleic acid precursors	ATP, GTP, CTP, UTP dATP, dGTP, dCTP, dTTP
high-energy end product of fueling reactions	ATP
intracellular signalling	cAMP, cGMP, ppGpp, 2,5-A, ATP/ADP/AMP
adenine nucleotides as constituents of coenzymes	NAD^+, $NADP^+$, FMN, FAD, coenzyme A
activated intermediates	
(a) oligosaccharide and glycoprotein biosynthesis	CMP-sialic acid GDP-fucose, GDP-mannose UDP- (N-acetylgalactosamine, N- acetylglucosamine, N-acetylmuramic acid, galactose, glucose, glucuronic acid, xylose)
(b) lipid biosynthesis	
(i) phospholipids	CDP-ethanolamine, CDP-choline
(ii) lipopolysaccharides	CMP-KDO

pathways becoming covalently linked to precursor molecules thereby "charging" or "activating" them (for example GDP-mannose, UDP-glucose, CDP-choline, and CMP-KDO). Obviously, nucleotides are of critical importance in a wide range of cellular activities and therefore, the study of nucleotide metabolism is an important aspect of fully understanding the biology of all organisms.

Intracellular parasites such as chlamydiae potentially have access to most of the nucleotides they may require. However, except in a few cases, prokaryotic and eukaryotic cells are impermeable to highly-charged nucleotides because they lack suitable transport systems (Plagemann *et al*, 1988). Studies indicate that chlamydiae can utilize available nucleotides from the host cell cytoplasm (Hatch, 1975; Hatch, 1988; Moulder, 1991).

Presently, it is widely accepted that chlamydiae are energy parasites, being totally dependent on their host cell for high-energy intermediates like ATP (Moulder, 1991). In support of this hypothesis chlamydiae were found to be incapable of ATP generation via oxidative phosphorylation or substrate level phosphorylation (reviewed by Moulder, 1991). Furthermore, Hatch *et al* (1982) have shown that *C. psittaci* 6BC possesses an ATP/ADP translocase whereby ATP can be obtained from the host-cell cytoplasm in exchange for chlamydial ADP. This ATP/ADP translocase is similar to the translocase demonstrated in mitochondria (opposite orientation) and rickettsiae (Winkler, 1976). The ADP-ATP exchange mechanism provides for a net gain of high energy phosphate, but no net gain of nucleotides. Previously, it has been shown that inhibition of host-cell mitochondrial function with ethidium bromide (Becker and Asher, 1971) and mitochondrial respiration with antimycin A (Gill and Stewart, 1970) results in decreased chlamydial growth. In addition, ultrastructural studies indicate that mitochondria are closely associated with chlamydial inclusions (Matsumoto, 1981; Matsumoto *et al*, 1991). Taken together, these results have been interpreted as indicating that mitochondrial generated ATP is essential for chlamydial survival. Evidence in this thesis indicate that this is not entirely true.

Although the above data indicate that chlamydiae are energy parasites with respect to acquisition of ATP, generation of reducing power is another necessity for the fueling of biosynthetic reactions. There appear to be fragments of the Embden-Myerhof-Parnas and/or Entner-Doudoroff pathways present in chlamydiae and also the possibility of the presence of the pentose-phosphate pathway (Moulder, 1991; Hatch, 1988) which may, at least in part, generate some of the necessary reducing power. The pentose-phosphate pathway could supply reduced pyridine nucleotides and precursor metabolites required for biosynthetic reactions.

(Deoxy)nucleotides ((d)NTP) can be generated by *de novo* pathways (from small precursor molecules) or by salvage pathways (re-utilization of preformed bases and nucleosides from the degradation of nucleic acid or salvage from the surroundings). In most prokaryotic and eukaryotic cells, (d)NTPs are generated by *de novo* pathways with varying contributions from the salvage pathways. However, the situation is different in prokaryotic and eukaryotic intracellular parasites. Protozoan parasites (Leishmania, Trypanosoma, Plasmodium, and Toxoplasma) lack the capability to synthesize purine nucleotides *de novo*, but instead rely on the salvage pathways for generation of adenine and guanine nucleotides (Hassan and Coombs, 1988). In contrast, most of the protozoan parasites have retained the ability to *de novo* synthesize pyrimidines as well as salvage pyrimidines (Hassan and Coombs, 1988). The study of nucleotide metabolism in prokaryotic intracellular parasites is minimal compared to the eukaryotes. One prokaryotic parasite which has been studied with respect to its nucleotide metabolism is rickettsiae. Rickettsiae are unable to transport pyrimidine bases, pyrimidine nucleosides, adenine, or adenosine, and also lack the enzymes required for salvage of purines and pyrimidines (i.e. phosphoribosyl-transferases and nucleoside kinases) (Winkler, 1990). The presence of a ribonucleotide reductase (Cai *et al*, 1991), thymidylate synthase (Speed and Winkler, 1991), and deoxycytidine triphosphate deaminase (Speed and Winkler, 1991) in *Rickettsia prowazekkii* have recently been shown.

The mechanism by which chlamydiae obtain a net gain of (d)NTPs is not clear; this is a main question the work in this thesis addresses. Work by Hatch (1975) conclusively shows

that *C. psittaci* draw on the total acid-soluble NTP pools of the host cell for chlamydial RNA synthesis. Host cell DNA synthesis is not required for chlamydial growth since (1) cycloheximide inhibition of host cell DNA synthesis does not impair chlamydial DNA synthesis (Moulder, 1969), and (2) chlamydiae are capable of growing in enucleated host cells (Perara *et al*, 1990). Supporting this is the work by Alexander (1968) which shows that chlamydial DNA synthesis occurs at the same rate in both multiplying and non-multiplying host cells. Chlamydiae are also capable of efficient infection and replication in stationary-phase cells (Moulder, 1991; Schachter, 1988; Schachter and Caldwell, 1980), when the intracellular dNTP concentration of the host cell is extremely low (Spyrou and Reichard, 1988). Tribby and Moulder (1966) have shown that *C. psittaci* growing in mouse L cells can incorporate most exogenously supplied nucleosides and nucleobases into host cell and *C. psittaci* RNA and DNA. They report that purine deoxyribonucleosides are incorporated into chlamydial DNA but that pyrimidine deoxynucleosides are incorporated poorly or not at all into chlamydial DNA. A number of investigators report that exogenously supplied thymidine is not utilized by chlamydiae (Bose and Leibhaber, 1979; Hatch, 1976; Lin, 1968). This minimal thymidine incorporation into chlamydial DNA occurs even when the host cell has a fully active thymidine kinase and incorporation of thymidine into host DNA is occurring (Bose and Leibhaber, 1979; and Hatch, 1976). *C. psittaci* lacks detectable thymidine kinase and grows normally in thymidine kinase-deficient cells (Hatch, 1976). These experiments have led to the hypothesis that *C. psittaci* is unable to utilize thymidine nucleotides from the host cell and that the organism must synthesize its own dTTP. Fan *et al* (1991) have recently presented biochemical evidence for the existence of thymidylate synthase in *C. trachomatis*.

Ribonucleotide reductase (RR) and CTP synthetase are two enzymes involved in regulating intracellular (d)NTP pools. Both of these enzymes are dealt with in detail in this thesis.

RR is the only enzyme found in all prokaryotic and eukaryotic cells studied so far that catalyzes the reduction of the 2' carbon atom of the ribose moiety of ribonucleotides to

generate the corresponding deoxyribonucleotides (reviewed by Reichard, 1988; Stubbe, 1990; Thelander and Reichard, 1979):

$$\text{ribonucleotide} + \text{NADPH} + \text{H}^+ \longrightarrow \text{deoxyribonucleotide} + \text{NADP}^+ + \text{H}_2\text{O}$$

The activity of RR is the rate limiting step of the *de novo* synthesis of dNTPs and is therefore a key enzyme in DNA synthesis. RR reduces all four common (A, C, G and U) ribonucleoside diphosphates/triphosphates to the deoxynucleoside diphosphate/triphosphate while a nucleoside diphosphate kinase raises the dNDP to the triphosphate level (dATP, dCTP, dGTP, and dUTP). Thymidylate synthase generates dTMP from dUMP which is phosphorylated to dTTP by kinases. RR is under complex allosteric regulation and in combination with key salvage enzymes (phosphorylases, kinases, and dCMP deaminase), maintains a balanced supply of dNTPs required for the fidelity of DNA synthesis (Reichard, 1988). There are three classes of RR known (reviewed in *Science* by Reichard, 1993). The prototype of class I RR is the *E. coli* reductase. It is an iron-tyrosyl radical-containing enzyme which has been found in mammalian cells, plant cells, yeast, and some prokaryotes. Class I reductases require oxygen for tyrosyl radical generation and do not function under anaerobic conditions. The class II adenosyl cobalamin-dependent enzymes are found in some prokaryotes (RR of *Lactobacillus leichmannii* is the prototype). Recently, a third class (class III) of RR which only functions under anaerobic conditions has been characterized (Reichard, 1993). The class I reductase has been intensively studied and is known to be functional as a heterodimer ($\alpha_2\beta_2$). The larger (Mr $\approx$ 85,000) R1 subunit (α_2) contains effector binding sites which confer activity and substrate specificity to the reductase (Brown and Reichard, 1969). The smaller (Mr $\approx$ 45,000) R2 subunit (β_2) contains an iron-tyrosyl radical center (Brown *et al*, 1969; Ehrenberg and Reichard, 1972). The actual active site involves both subunits - redox active dithiols of R1, and the tyrosyl-radical and binuclear ferric iron center of R2. The x-ray crystallographic three-dimensional structure has been determined for both the *E. coli* R1 (Eklund, unpublished) and R2 (Nordlund *et al*, 1990) subunits. The reaction requires binding of nucleotides to the specificity sites (ATP specifies CDP or UDP reduction, dTTP specifies GDP reduction, and dGTP specifies ADP reduction)

and activity sites (ATP activates, dATP inhibits) as well as the presence of the reduced form of the redox-active dithiol group. The reduction of a particular nucleotide results in the oxidation of the dithiol group of the R1 reductase subunit. Regeneration of the active enzyme requires reduction of the dithiol group by thioredoxin or glutathionine reductase in the presence of NADPH + H^+. Regulation of mammalian RR is cell cycle dependent with maximal activity during S-phase (Eriksson *et al*, 1984; Engstrom *et al*, 1985). The R2 subunit is produced mainly during S-phase while the R1 subunit is constitutively produced throughout the cell cycle. Hydroxyurea is a specific and relatively potent inhibitor of DNA synthesis in both prokaryotic and eukaryotic cells (Reichard, 1988; Wright *et al*, 1989). The major site of action for hydroxyurea is RR. More specifically, hydroxyurea reduces the tyrosine free radical of class I reductases, thereby inactivating the enzyme (Ehrenberg and Reichard, 1972). Hydroxyurea has been used extensively for the isolation of drug-resistant mutants in mammalian cells (McClarty *et al*, 1987; Reichard, 1988; Wright *et al*, 1990; Wright *et al*, 1989) and viruses (Slabaugh and Matthews, 1986), and in all cases, alterations were detected in ribonucleotide expression. In addition, mammalian cells (Thelander and Thelander, 1988) and bacteria (Platz and Sjoberg, 1980) genetically engineered to overexpress ribonucleotide reductase exhibit increased resistance to hydroxyurea. Furthermore, *E. coli* with mutationally altered ribonucleotide reductase have been identified by a more sensitive phenotype than their wild-type counterpart (Platz *et al*, 1985). The characterization of all these mutants has enormously advanced the understanding of the important function this enzyme plays in the process of proliferation.

In both prokaryotes and eukaryotes, CTP synthetase catalyzes the formation of CTP from UTP in the presence of magnesium, ATP and an amido-group donor:

$$\text{UTP} + \text{ATP} + \text{glutamine} \longrightarrow \text{CTP} + \text{ADP} + P_i + \text{glutamate}$$

This is the terminal reaction of the *de novo* pyrimidine nucleotide biosynthetic pathway. Like RR, CTP synthetase is under complex allosteric control (reviewed in Long and Koshland, 1978; Weinfeld *et al*, 1978), and feedback regulation (McPartland and Weinfeld, 1979) as it is

involved in the stringent regulation of the intracellular CTP pools. Both CTP synthetase from *E. coli* (Long and Koshland, 1978) and mammalian cells (Weinfeld *et al*, 1978) have been thoroughly characterized in terms of their enzymology. GTP is required as a positive effector when glutamine is used as the amido-group donor, however, the reaction can also occur in the absence of GTP using ammonia as the amido-group donor. CTP synthetase exists as a homodimer ($M_r \approx 105,000$) in the absence of substrates, but aggregates to a tetramer ($M_r \approx 210,000$) in the presence of UTP and ATP and Mg^{2+}. UTP and ATP show positive cooperativity while CTP acts as a feedback inhibitor of CTP synthetase. The genes coding for CTP synthetase have been cloned from *E. coli* (Weng *et al*, 1986), *Bacillus subtilis* (Trach *et al*, 1988), *Saccharomyces cerevisiae* (Ozier-Kalogeropoulos *et al*, 1991; Ozier-Kalogeropoulos *et al*, 1994), human (Yamauchi *et al*, 1990), *Azospirillum brasilense* (Zimmer and Hundeshagen, 1992), and *Spiroplasma citri* (Citti *et al*, 1993). The deduced amino acid sequences of these cloned genes show approximately 21% identity (Ozier-Kalogeropoulos *et al*, 1991) and have molecular weights ranging from ≈ 59 to 67 kDa. Most striking are the three highly conserved regions toward the carboxy-terminus which correspond to the glutamine amide transfer domain present in a large number of enzymes with amidotransferase function (Ozier-Kalogeropoulos *et al*, 1991). Being a key enzyme in pyrimidine nucleotide biosynthesis, CTP synthetase has been studied as a potential chemotherapeutic target. A potent inhibitor of CTP synthetase is the cytidine analog cyclopentenylcytosine (CPEC) triphosphate (Kang *et al*, 1989) (see Appendix for structure). CPEC is taken up by cells and is subsequently phosphorylated by kinases to the triphosphate level which then exerts cytotoxic effects mainly, although not entirely, by inhibition of CTP synthetase (Kang *et al*, 1989). The result of the CPEC inhibition of CTP synthetase is a greatly reduced intracellular CTP pool (Glazer *et al*, 1986). CPEC has been studied both as a treatment for cancers (Glazer *et al*, 1985; Moyer *et al*, 1986), and as an anti viral agent (reviewed in DeClercq, 1993). Lymphoblasts resistant to the cytotoxic effects of CPEC have been shown to have decreased uridine/cytidine kinase activity thereby preventing the phosphorylation of CPEC to the cytotoxic triphosphate form

(Blaney *et al*, 1993). Although this decreased kinase activity appears to be the primary mechanism of CPEC resistance, other mechanisms such as an altered CTP synthetase activity (Blaney *et al*, 1993), or loss of CTP feedback inhibition (Whelan *et al*, 1993) also seem to be present.

Work in this thesis was undertaken to not only clarify inconsistencies in the literature, but also to elucidate the pathway by which *C. trachomatis* obtains its dNTPs required for DNA synthesis. This included determining exactly which nucleic acid precursors (deoxyribo- and ribonucleobases, nucleosides, and nucleotides) *C. trachomatis* was capable of transporting from the host cell cytoplasm, determining the presence or absence of *de novo* and salvage pathways for these nucleotides in *C. trachomatis*, and studying particular key nucleotide metabolizing enzymes found to be present in *C. trachomatis*. In addition, other aspects of *C. trachomatis* nucleotide metabolism and how it relates to the complex interaction of host and parasite are also discussed in this thesis.

MATERIALS AND METHODS

1. Materials

[5-^{3}H] cytidine diphosphate (22 Ci/mmol), and [5,6-^{3}H] uridine triphosphate (38 Ci/mmol) were obtained from New England Nuclear Corp. [2-^{14}C] glycine (57.5 mCi/mmol), [U-^{14}C] L-aspartic acid (220.4 mCi/mmol), [γ-^{32}P] ATP (7500 Ci/mmol), [α-^{32}P] dATP (3500 Ci/mmol), and [5,6 ^{3}H] UTP (38 Ci/mmol) were obtained from ICN. [2-^{14}C] cytidine (55 mCi/mmol), [5-^{3}H] cytidine (22 Ci/mmol), [2-^{3}H] adenine (28 mCi/mmol), [8-^{3}H] guanine (56 mCi/mmol), [2-^{3}H] hypoxanthine (25 Ci/mmol), [6-^{3}H] uridine (42 Ci/mmol), [5-^{3}H] uridine (20 Ci/mmol), [2,8-^{3}H]deoxyadenosine (36 Ci/mmol), [8-^{3}H] guanosine (7 Ci/mmol), [8-^{3}H] deoxyguanosine (7 Ci/mmol), [6-^{3}H] deoxyuridine (20 Ci/mmol), [methyl-^{3}H] thymidine (37 Ci/mmol) and [6-^{3}H] uracil (30 Ci/mmol) were obtained from Moravek Biochemicals Inc. Guanazole, hydroxyurea, aphidicolin, norfloxacin, cycloheximide and 8-aminoguanosine (8-AG) were purchased from Sigma. The random primer labelling kit and the DNA cycle sequencing kit were bought from Gibco BRL Life Sciences. Deoxycoformycin (dCF) was a gift from J. Johnston, Manitoba Institute of Cell Biology, Winnipeg. Cyclopentenyl cytosine (CPEC) was kindly provided by V. Marquez, Pharmacology and Experimental Therapeutics, National Institutes of Health, Bethesda, Maryland.

Oligonucleotides were synthesized on a Beckman DNA synthesizer.

2. *Chlamydia trachomatis* strains and propagation

C. trachomatis L2/434/Bu, obtained from R. Brunham, Department of Medical Microbiology, University of Manitoba, Winnipeg, was used throughout these studies. Confluent monolayers of mouse L cells or Chinese-hamster-ovary cells (CHO K1) were infected at a multiplicity of infection of 3 to 5 inclusion-forming units per cell, which resulted

in 90 to 100% infection with little host cell toxicity. *C. trachomatis* was grown as previously described (Peeling *et al*, 1984), and unless otherwise indicated, 1 μg/ml cycloheximide was present in the post-infection (p.i.) medium. Mock-infected (MI) cultures were treated in the same fashion as infected cultures except that *C. trachomatis* was not added. Cycloheximide (1 μg/ml) was always present in MI cell culture medium. *C. trachomatis* L2 stocks were routinely checked for free-living bacterial contamination.

3. Cell lines and culture conditions

The cell lines described in these studies were cultured at 37°C on the surface of plastic tissue culture flasks (Corning Glass Works) in α-minimal essential medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). The mouse L-cell line and its hydroxyurea-resistant derivatives LHF (H^R-5.0) (McClarty *et al*, 1986) and LHH (H^R-30.0) (Hurta and Wright, 1990) were kindly provided by J. Wright, Manitoba Institute of Cell Biology, Winnipeg. The wild-type CHO cell line and the hypoxanthine-guanine phosphoribosyltransferase-negative ($HGPRT^-$) CHO cell line were obtained from J. Hamerton, Department of Human Genetics, University of Manitoba, Winnipeg. For logarithmically growing cultures, approximately 10^5 cells were seeded onto 5-cm dishes and grown for 36 to 40 h at 37°C in 5 ml of MEM-10% FBS to a density of approximately 10^6 cells. The hydroxyurea-resistant phenotypes of LHF (H^R-5.0) and LHH (H^R-30.0) are stable in the absence of hydroxyurea (McClarty *et al*, 1986).

The wild-type Chinese-hamster-lung-fibroblast cell line (CCL 16-B1) and the mutant Chinese-hamster-lung-fibroblast cell line (CCL 16-B2) with defective mitochondrial function as a result of a defect in complex I of the electron-transport chain (Ditta *et al*, 1976), were obtained from I. E. Scheffler, Department of Biology, University of California, San Diego, USA. Both of these cell lines were grown in Dulbecco's modified Eagle medium with 4.5 mg of glucose per ml (D-MEM high glucose, Gibco Laboratories), non-essential amino acids and

10% fetal bovine serum (Intergen). The wild-type Chinese-hamster-ovary cell line (CHO K1), the uridine-requiring mutant CHO K1 cell line (Urd^-A) (Patterson and Carnwright, 1977), the hypoxanthine- or adenine-requiring mutant CHO K1 cell line (Ade^-F) (Patterson, 1975), and the adenine-requiring mutant (Ade^-H) (Patterson, 1976) were generously provided by D. Patterson, Eleanor Roosevelt Institute for Cancer Research, Denver, Colorado. The Urd^-A, Ade^-F, and Ade^-H cell lines were grown in D-MEM, 10% fetal bovine serum, 300 μM proline, supplemented with 30 μM uridine, 30 μM hypoxanthine or 30 μM adenine, respectively. The cytidine requiring mutant CHO K1 cell line (CR^-2) (Kelsall and Meuth, 1988) was kindly provided by M. Meuth, Imperial Cancer Research Fund, Clare Hall Laboratories, UK. The CR^-2 cell line was grown in D-MEM, 7.5% fetal bovine serum, 300 μ M proline, 10 μM thymidine, and 20 μM cytidine.

The uridine/cytidine kinase-deficient Novikoff rat hepatoma cells (UK^-) (Plagemann *et al*, 1978) were obtained from P. Plagemann, University of Minnesota, Minneapolis, MN. The UK^- cell line was grown in D-MEM, and 10% fetal bovine serum. UK^- cells cannot salvage cytidine or uridine because they lack a functional uridine/cytidine kinase. The hypoxanthine/guanine phosphoribosyl transferase (HGPRT) and adenine phosphoribosyl transferase (APRT)-deficient mouse cell line (H^-/A^-; catalogue number GM00346B) was purchased from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden New Jersey. The cell line was grown in D-MEM and 10% fetal bovine serum. H^-/A^- cells cannot salvage purine nucleobases because they lack functional HGPRT and APRT. The wild-type mouse L 929 cell line was provided by K. Coombs, University of Manitoba, Winnipeg. The L 929 cells were grown in D-MEM and 10% fetal bovine serum. The wild-type HeLa 229 cells were obtained from R. Brunham, University of Manitoba, Winnipeg. The HeLa cells were grown in D-MEM and 10% fetal bovine serum.

4. Incorporation of radiolabelled precursors into host cell and *C. trachomatis* DNA

Radiolabelling experiments were done as described by Nicander and Reichard (1983). Briefly, isotope experiments were performed with parallel dishes (5-cm dishes, 5 ml of medium) of logarithmically growing (1.0×10^6 cells per plate) and stationary-phase (3×10^6 to 4×10^6 cells per plate in the presence of 1 μg of cycloheximide per ml) cultures MI or infected with *C. trachomatis*. Three hours before the addition of isotope or inhibitors, the medium volume was reduced to 2.0 ml and buffered with 1 M HEPES (pH 7.4; final concentration, 20 mM). For *C. trachomatis*-infected cultures, medium volume was reduced at 19 h post infection and radiolabel was added at 22 h p.i., a time when L2 DNA synthesis is at a maximum. A ^{3}H- or ^{14}C-labeled nucleobase, nucleoside or deoxynucleoside was added to achieve a final concentration of 0.3 μM. Incubation in the presence of isotope was continued for 3 h. In all cases, incorporation of label into DNA remained linear beyond 3 h and at no time was isotope limiting in the experiments. When radiolabelled precursor incorporation studies were conducted on *C. trachomatis*- infected cultures in the absence of cycloheximide, host and *C. trachomatis* DNA synthesis activities were distinguished from each other by their differing sensitivities to aphidicolin (10 μM) and norfloxacin (200 μM). When required, inhibitors (aphidicolin, norfloxacin, dCF, or 8-AG) were added 2 h before the addition of isotope.

To terminate incubations, the dishes were transferred immediately to an ice bath, medium was sucked off, and the cells were washed rapidly (three times) with 5 ml of ice-cold Tris-buffered saline (pH 7.4) and placed for 1 min in a vertical position on ice before the remaining buffer was removed. When necessary, nucleotides were extracted with 1.5 ml of 60% methanol for 2 h at -20°C. The methanolic solution was removed from the plate and saved for analysis of deoxyribonucleotides. The cells remaining on the dish were dissolved in 0.3 M NaOH and then incubated for 16 h at 37°C to degrade RNA. DNA was precipitated by the addition of 10 ml of 10% trichloroacetic acid (TCA)-0.1 M sodium PP_i. The precipitate

TCA-0.1 M sodium PP_i and ethanol, the dried filters were counted in a liquid scintillation counter (Beckman LS 5000). Methanol extracts were evaporated to dryness in Savant Speed-Vac evaporator, and the residue was redissolved in 0.1 ml of 10 mM Tris-HCl buffer (pH 7.4). Isotope incorporation into dNMP, dNDP, and dNTP was measured by on-line radioactive flow detection (Beckman 171 detector) after separation of the deoxynucleotides by high-pressure liquid chromatography (HPLC) on a 12.5-cm Partisphere C_{18} column (Whatman), using buffer a (double distilled H_2O) and buffer b (0.15 M NaCl, 25 mM tetrabutylammonium dihydrogen [pH 6.0], 5% methanol). For separation of pyrimidine deoxynucleotides (dC, dT), the column ran isocratically for 3.0 min at 1.0 ml/min with 50% buffer b. From 3 to 4 min, a linear gradient changed the buffer from 50% to 100% buffer b. Buffer b then ran isocratically from 4 to 20 min. For separation of purine deoxynucleotides (dA, dG), the column ran isocratically from 2.5 to 20 min. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known deoxynucleotide standards. All analyses were made on duplicate dishes, with results varying by less than 10%. Unless otherwise indicated, all results are normalized to 10^6 cells. For *C. trachomatis*-infected cultures, percent infection was monitored by light microscopy and experiments were not performed unless 90 to 100% of the cells were infected. Under these circumstances, 10^6 is taken to mean approximately 10^6 infected cells. For hydroxyurea- and guanazole-treated cultures, incorporation values are expressed as percentages of the amount of radiolabel incorporated into DNA by untreated controls. The ID_{50} value is the drug concentration required to reduce incorporation of label by 50%.

5. Selection of drug-resistant *C. trachomatis*

a) Hydroxyurea-resistant *C. trachomatis*

The mutant isolation procedure utilized took advantage of the fact that we had hydroxyurea-resistant mouse cell lines (LHF and LHH) as hosts to support *C. trachomatis* growth. Starting with a nonmutagenized population of *C. trachomatis* L2/434/Bu, a series of L2 isolates was sequentially selected in a stepwise manner in the presence of the following concentrations (mM) of hydroxyurea: wild type → 1.0 → 2.0 → 5.0 → 7.5 → 10.0 → 12.5 → 15.0 → 17.5 → 20 → 25. At the selection step between 5.0 and 7.5 mM, the host mouse L-cell line was changed from LHF (resistant to 5 mM hydroxyurea) to LHH (resistant to 30.0 mM hydroxyurea). Our early attempts to isolate *C. trachomatis* mutants involved addition of hydroxyurea at 2 h p.i.; however, this procedure proved unsuccessful. Eventually, we discovered that addition of the selective agent at 22 p.i., a time when RBs were rapidly growing, was much more successful. Passage of EBs produced at each hydroxyurea step was continued in the presence of the selective concentration for at least five growth cycles before progressing to the next drug concentration. In some instances, it was necessary to interrupt the selection protocol with a passage in the absence of hydroxyurea to raise the titer of infectious EBs. The EB isolates so produced were immediately returned to passage in a drug concentration equal to that at the time of interruption. In addition, at several steps in the selection protocol it was necessary to extend the normal 48-h incubation to 96 h to obtain enough EBs for passage to a new culture. The entire selection procedure took place over 1.5 to 2 years. At each selection step between 1 and 25 mM hydroxyurea, an aliquot of EBs was retained and stored at -70°C. Although EB isolates at each selection step appeared phenotypically stable, they were normally passaged in growth medium containing hydroxyurea at the selective concentration.

b) CPEC-resistant *C. trachomatis*

Cyclopentenyl cytosine (CPEC) is a carbocyclic analog of cytidine which, in the triphosphate state (CPE-CTP), is an inhibitor of CTP synthetase. See appendix 2 for the structure of CPEC. Selection of CPEC-resistant *C. trachomatis* mutants made use of the cytidine requiring mutant CHO K1 host cell line, $CR^{-}2$ (Kelsall and Meuth, 1988). $CR^{-}2$ are auxotrophic for cytidine due to non-functional CTP synthetase. Cytidine-starved $CR^{-}2$ cells were used as host during the selection procedure. Starting with a nonmutagenized population of *C. trachomatis* L2/434/Bu, a series of L2 isolates was sequentially selected in a stepwise manner in the presence of medium-supplied CPEC in the following concentrations: 1 μM → 2 μM → 5 μM.

6. Infectivity titration assay

Mouse L cells were seeded onto glass coverslips (4×10^5 cells per coverslip) in MEM-10% FBS and incubated at 37°C for 24 h. The resulting monolayers were infected with 200 μl of serial 10-fold dilutions of chlamydiae in SPG buffer (10 mM NaH_2PO_4[pH 7.4], 5 mM glutamic acid, 250 mM sucrose). Each dilution was allowed to absorb for 1 h at 37°C, and the inoculum was removed. The monolayers were washed (three times) with phosphate buffered saline, fixed with absolute methanol, and then stained for chlamydial inclusions with fluorescein-conjugated anti-*C. trachomatis* monoclonal antibody (commercial kit from Syva Co.). Inclusions were counted in 30 fields at x 400 magnification with the aid of a micrometer. For hydroxyurea- and guanazole-treated cultures, titers are expressed as percentages of the titer of untreated controls. The ID_{50} is the drug concentration which reduces the titer by 50%.

To determine the effect of hydroxyurea or guanazole on the production of infectious EBs, we added various amounts of either drug to *C. trachomatis*-infected cells at 2 h p.i. After incubation at 37°C for 48 h, the infected monolayers were harvested and lysed by sonication,

and the resulting lysates were clarified by centrifugation at 500 x *g* for 10 min. EBs were subsequently pelleted by centrifugation at 30,000 x *g* for 30 min and then resuspended in SPG medium and titered as described above.

7. Measurement of inclusion development

Wild-type mouse L cells and hydroxyurea-resistant mouse L cells (LHF or LHH) were grown on glass coverslips as described above. Confluent or subconfluent monolayers of cells were inoculated with *C. trachomatis* L2 EBs, and various concentrations of hydroxyurea or guanazole were added at 2 h p.i. to growth medium containing 1 μg/ml of cycloheximide when indicated. Cultures were incubated at 37°C, and inclusions were counted after staining at 48 h p.i. with fluorescein-conjugated anti-*C. trachomatis* monoclonal antibody (Syva Co.). The relative inclusion-forming ability was defined as the inclusion-forming ability of drug-treated infected cells divided by the inclusion-forming ability of non-drug-treated infected cells. The ID_{50} value is the drug concentration which reduces relative inclusion-forming ability by 50%.

8. Ribonucleotide reductase assay on crude cell extracts

Crude cell extracts were prepared from logarithmically growing wild-type mouse L cells as previously described (McClarty *et al*, 1986). For mock-infected crude extracts, the starting culture was a confluent monolayer of wild-type mouse L cells that had been cultured in growth medium supplied with 1 μg/ml cycloheximide for 22 h. Wild-type *C. trachomatis* L2 RBs and hydroxyurea-resistant *C. trachomatis* $L2H^R$-10.0 RBs were purified from infected mouse L cells at 22 h p.i. by Hypaque density gradient centrifugation as described previously (Caldwell *et al*, 1981). Hypaque-purified RBs were resuspended in a buffer containing 40 mM HEPES (pH 7.6), 2 mM dithiothreitol, and 2 mM Mg acetate. The suspensions were lysed by sonication (three 30-s pulses at a probe intensity of 40 to 45), and the lysates were clarified by

centrifugation at 25,700 x g for 30 min to remove cell debris. Crude extracts prepared in this fashion were stored at -70°C.

Ribonucleotide reductase activity in crude cell extracts was determined by using [5-^{3}H] CDP as the substrate and snake venom to hydrolyze the nucleotides (Lewis *et al*, 1978; Steeper and Steuart, 1970). The reaction mixture contained, in a final volume of 80 μl, 50 μM [5-^{3}H] CDP (150 cpm/pmol), 40 mM HEPES (pH 7.6), 10 mM dithiothreitol, 2 mM Mg acetate, 4 mM ATP, and a given quantity of cellular extract (usually 150 μg). Reactions were performed for 20 min at 37°C and terminated by boiling for 4 min. Nucleotides were converted to nucleosides by incubating the mixture in the presence of 1 mg of snake venom (20 mg/ml in 100 mM HEPES [pH 8.0]-10 mM $MgCl_3$) for 1 h at 37°C, and then the reaction was terminated by boiling 4 min and 0.5 ml of double-distilled H_2O was added to each assay tube. The tubes were then centrifuged to remove the heat-precipitated material, and the supernatant was loaded onto a column (5 by 80 mm) of Dowex-1-borate to separate cytidine from deoxycytidine (Lewis *et al*, 1978; Steeper and Steuart, 1970). Radioactivity was determined by liquid scintillation counting (Beckman LS 5000 scintillation counter).

9. Measurement of ribonucleotide pools

For each particular cell line, experiments were performed with parallel flasks (75 cm^2) of logarithmically growing cells (7 to 8 x 10^6 cells per flask), with the appropriate supplement(s) included in the medium, and starved cells. To starve cells of their required supplement, medium containing supplement was removed from the flask and the cell monolayer was rinsed with warm phosphate-buffered saline and then medium lacking the required supplement was added. The flasks were then returned to the 37°C incubator for 8 h. Acid-soluble nucleotides were extracted by standard techniques (Van Haverbeke and Brown, 1978). Briefly, the incubations were terminated by placing the flasks on ice, sucking off the media, and washing the cells twice with ice-cold phosphate-buffered saline (PBS). 250 μl of 10%

trichloroacetic acid (TCA) was added to each flask and the cells were scraped from the surface and left on ice for 30 min. The precipitated material was pelleted by centrifugation. The nucleic acid-containing pellet was analyzed for DNA content (Burton, 1956). The nucleotide-containing supernatant was neutralized using 78.1:21.9 (V/V) freon-tri-N-Octylamine (Van Haverbeke and Brown, 1978). NTPs were separated by HPLC on a 12.5 cm Whatman Partisil 5 SAX HPLC column using 100% 0.55 M ammonium phosphate buffer (pH 3.4, 2.55 acetonitrile) at 1 ml/min (Garret and Santi, 1979). For calculation of energy charge ribonucleoside mono-, di-, and triphosphates were separated by gradient elution HPLC at a flow rate of 1 ml/min on a 12.5 cm Whatman C18 reverse-phase column (Ryll and Wagner, 1991). Buffer A was 100 mM KH_2PO_4-K_2HPO_4 (pH 6.0) with 8 mM tetrabutyl-ammonium phosphate (pH 5.3). Buffer B was 100% methanol. A linear gradient of 100-60% buffer A was run from 0 to 15 min. From 15 to 16 min buffer B was increased linearly from 40-100%. From 22 to 23 min buffer A was increased from 0-100%. In all cases nucleotides were identified and quantified by monitoring A_{254} (Beckman 166 UV wavelength detector) and by comparing the absorbance and retention times to that of known standards. All data were plotted and processed with IBM PC50 and Beckman System Gold software.

Measurement of ribonucleotide pools at various time points during the *C. trachomatis* L2 life cycle was carried out as follows. Parallel flasks (75 cm^2) of logarithmically growing HeLa 229 cells (7 to 8 x 10^6 cells per flask) were or were not infected with *C. trachomatis* L2 and cultured in D-MEM containing 10% fetal bovine serum; no cycloheximide was used for these experiments. At 0, 12, 24, 36 and 48 h p.i. duplicate non-infected control flasks and *C. trachomatis* L2-infected flasks were taken from the incubator and acid-soluble nucleotides were extracted and analyzed by HPLC as described above.

The following describes the measurement of nucleotide pools in highly purified reticulate and elementary bodies. Highly purified reticulate and elementary bodies were prepared from infected mouse L cell suspension culture by Hypaque density-gradient centrifugation as previously described (Caldwell *et al*, 1981). RBs were harvested at 22 h p.i.

while EBs were harvested at 44 h p.i. Acid-soluble nucleotides were extracted and analyzed as described above.

10. Measurement of *C. trachomatis* DNA synthesis in the various mutant cell lines

C. trachomatis L2 DNA synthesis activity was measured *in situ* by monitoring the incorporation of radiolabelled nucleic acid precursor into DNA, in the presence of cycloheximide, as described above. This DNA-synthesis assay specifically measures chlamydial DNA-synthesis activity and provides a reliable and accurate estimation of chlamydial growth (McClarty and Tipples, 1991). Unless otherwise indicated, all results are expressed in 10^3 d.p.m. incorporated per 10^6 cells. Throughout this thesis the values presented for *C. trachomatis* L2 DNA synthesis activity represent the actual value obtained for a chlamydiae-infected culture minus the value obtained for an identically treated MI control. Host cell lines were MI and *C. trachomatis* L2-infected under both normal (nucleobase(side) supplement present) and starved (nucleobase(side) supplement not present) conditions.

11. Acid hydrolysis of nucleic acid and subsequent nucleobase analysis

Isotope labelling experiments were performed as described above on logarithmically growing (1 x 10^6 cells per 5 cm plate), stationary phase MI (3 to 4 x 10^6 cells per 5 cm plate, cultured in the presence of 1 μg cycloheximide/ml), and *C. trachomatis*-infected cultures (3 to 4 x 10^6 cells per 5 cm plate, in the presence of 1 μg/ml cycloheximide). At 2 h p.i. radiolabelled nucleobase(side) was added without dilution to give a final concentration of 0.6 μM. After 20 h at 37°C, the incubation was terminated by placing the dish on ice, aspirating off the medium and washing with ice-cold PBS. Total nucleic acid was isolated from the cultures as described by Schwartzman and Pfefferkorn (1981). Briefly, protein was digested with proteinase K, samples were phenol extracted three times, then the nucleic acid was

precipitated with ethanol. DNA and RNA were hydrolyzed to free nucleobases by boiling in 11.3 N perchloric acid for 1 h. The acid hydrolyzed samples were neutralized with KOH and the salt precipitate was removed by centrifugation. Isotope incorporation into nucleobases was monitored by on-line radioactive flow detection (Beckman 171 flow detector) after separation of the nucleobases by HPLC. Pyrimidine nucleobases were separated on a Whatman μBondapak C18 HPLC column using 30% (0.55 M ammonium phosphate pH 3.4, 2.5% acetonitrile) and 70% ddH_2O at a flow rate of 1 ml/min. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known nucleobase standards. Data analyses were done with an IBM PC50 using Beckman System Gold software.

12. Enzymatic hydrolysis of DNA and subsequent deoxynucleoside analysis

Mock-infected and *C. trachomatis* L2-infected cultures were radiolabelled with nucleic acid precursors for 20 h, and total nucleic acids were isolated as described above. RNA was degraded with 0.3 N NaOH and the DNA was precipitated with 10% TCA. The DNA was enzymatically hydrolyzed to deoxynucleosides as described by Baum *et al* (1989). Briefly, DNA was digested with DNase I for 3 h at 37°C followed by incubation with alkaline phosphatase and snake-venom phosphodiesterase for 1 h at 37°C. The digested samples were subjected to HPLC. Isotope incorporation into deoxynucleosides was monitored by on-line radioactive flow detection after separation on a 12.5 cm Whatman μBondapak C18 HPLC column using 100% 200 mM ammonium acetate buffer (pH 4.25, 0.5% acetonitrile) isocratically at 1 ml/min. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known deoxynucleoside standards. Data were analyzed as described above.

13. *E. coli* strains used for molecular cloning

E. coli XL1-blue (obtained from Stratagene) has the following genotype:
endA1 hsdR17($rk^{-}mk^{+}$) supE11 thi-1 lambda^{-} recA1 gyrA96 relA1 F'proAB lac1qZΔM15 Tn10(tet^{r})

E. coli JF646 (Friesen *et al*, 1976; Friesen *et al*, 1978) was obtained from H. Zalkin, Purdue University, Indiana. The genotype is:
F^{-} thi^{-}1 pyrE60 argE3 his4 proA2 thr^{-}1 leu^{-}6 cdd pyrG relA1 mtl^{-}1 xyl^{-}5 ara^{-}14 galK2 lacY1 str^{-}31 nalA λ^{-}supE44 his^{+} recA

14. Bacterial culture media

LB broth, LB agar, SOC broth, and SOB broth were prepared according to Sambrook *et al* (1989).

The selective media for the growth of *E. coli* JF646 consisted of: 1x minimal A salts (60.2 mM K_2HPO_4, 33.1 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, and 1.7 mM sodium citrate), 0.2 mg/ml $MgSO_4$, 5 mg/ml glucose, 0.2 mg/ml thiamine, 1 mg/ml casamino acid, 10 μg/ml uracil, and with or without 50 μg/ml cytidine. H_2O and minimal salts were autoclaved while the other solutions were filter sterilized.

E. coli containing plasmids conferring ampicillin-resistance were selectively grown in the presence of 50 μg/ml ampicillin.

15. Plasmid vectors used for molecular cloning

The 2.69 kb pUC19 plasmid (Sambrook *et al*, 1989) was used as the cloning vector for these studies. pUC19 confers ampicillin resistance and also allows white/blue screening for recombinants in XL1-blue *E. coli* due to the polycloning site being situated in the *lacZ* gene.

The plasmid pMW5 (Weng *et al*, 1986) was obtained from H. Zalkin, Purdue University, Indiana. This plasmid contains a 2.6 kb SalI-PstI *pyrG* fragment (which codes for the *E. coli* CTP synthetase) ligated into the pUC8 plasmid. See appendix 2 for map of pMW5.

16. Preparation of *C. trachomatis* genomic DNA

One liter mouse L cell suspension culture was infected with the appropriate EB stock. After 40 h the cells were collected by centrifugation at 3,000 x *g* for 20 min. The pellet was resuspended in 10 ml HBSS and mouse cells were disrupted by sonication. Cell debris was removed by centrifugation at 500 x *g* for 10 min at 4°C. The resulting supernatant was layered onto 8 ml 35% Hypaque, prepared in 10 mM HEPES buffer (pH 7.0), and ultracentrifuged for 1 h at 44,000 x *g*, in a Beckman SW28 rotor, at 4°C. After resuspending in 2 ml HBSS, the EBs were sonicated for 10 seconds at output setting 4 (Vibracell Sonicator, Sonics and Materials, Danbury, Connecticut). DNase was added to a final concentration of 200 μg/ml and the suspension was incubated for 2 h at 37°C before inactivating the DNase by heating to 95°C for 15 min. The suspension was then layered onto a Hypaque density gradient (7 ml 52%, 8 ml 44%, and 10 ml 40% Hypaque in 10 mM HEPES pH 7.0) and ultracentrifuged for 90 min at 51,000 x *g* at 4°C, in a Beckman SW28 rotor. The EBs, which band at the 52/44% interface, were collected and then centrifuged for 15 min., at 4°C, at 31,000 x *g*. The EB pellet was resuspended and washed three times in HBSS. The final pellet was resuspended in lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 0.1% Triton X-100, 0.5% SDS). An overnight incubation at 50°C with 200 μg/ml proteinase K was then done after which the

suspension was extracted twice with phenol/chloroform and then chloroform. The nucleic acid was precipitated with 2.5 x volumes of 95% ethanol and 1/10th x volume 3M sodium acetate (pH 7.0). After microcentrifugation, the pellet was resuspended in 50 μl TE pH 8. 100μg/ml RNase was added and the suspension was incubated for 3 h at 37°C. The nucleic acid was again precipitated with ethanol/sodium acetate. The nucleic acid pellet was then washed with 70% ethanol. The nucleic acid was resuspended in 50 μl TE pH 8 and quantified by standard methods (UV A_{260} and ethidium bromide stained agarose gel).

17. Preparation of chlamydial genomic DNA library

2 μg *C. trachomatis* L2 DNA was partially digested for an appropriate amount of time at 37°C with the appropriate amount of Hind III restriction enzyme. The reaction mixture was phenol/chloroform extracted, precipitated with ethanol/sodium acetate, and resuspended in 20 μl H_2O. The cloning vector pUC19 was also Hind III digested and then dephosphorylated by standard techniques (Sambrook *et al*, 1989). The ligation reaction was set up in a total volume of 20 μl using approximately 300 ng partially digested genomic DNA and 100 ng dephosphorylated pUC19 vector DNA overnight at room temperature. 0.8 μl of the reaction mixture was then used to transform 40 μl competent *E. coli* MC1061 by electroporation as described below. After 1 h recovery in SOC medium the 1 ml culture was used to inoculate 1 L LB medium. After 2 h, ampicillin was added to a final concentration of 50 μg/ml and the culture was grown overnight at 37°C. The plasmid library was recovered using the alkaline lysis plasmid preparation procedure (Sambrook *et al*, 1989).

18. Preparation of competent *E. coli* for electroporation

10 ml LB was inoculated with a single *E. coli* colony and incubated overnight at 37°C with shaking. The overnight culture was then used to inoculate 1 L SOB broth. This culture was incubated at 37°C until the OD_{600} was between 0.6 and 0.8. The culture was then chilled for 10 min at 4°C. The cells were collected by centrifugation at 3,000 x *g* for 15 min at 4°C. The cells were washed twice in sterilized ice-cold H_2O and collected by centrifugation as before. The pellet was resuspended in 10 ml ice-cold 10% glycerol and centrifuged at 3,000 x *g* at 4°C. The pellet was then resuspended in 2 to 4 ml 10% glycerol, aliquoted into smaller fractions, and stored at -70°C.

19. Complementation screening for chlamydial CTP synthetase

Transformation of *E. coli* was done by electroporation using a Bio-Rad Gene Pulser with a mode of 2.5 kV/resistance high voltage, resistance of 200 ohm, charging voltage of 1.8 kV, desired field strength of 12.25 kV/cm and desired pulse length of 4-5 milliseconds.

40 μl competent *E. coli* JF646 (CTP synthetase deficient) was mixed with 0.8 μl ($\approx$20 ng DNA) plasmid preparation (Hind III partial digest chlamydial DNA library) and transferred to a chilled electroporation cuvette. Immediately following the pulse, 1 ml of SOC was added and the suspension was transferred to a sterile tube and incubated at 37°C with shaking for 90 min. The cells were pelleted by centrifugation at 3,000 x *g* for 10 min. The pellet was washed once in HBSS and resuspended in HBSS. An appropriate amount of the cells were plated onto selective media (lacking cytidine) containing ampicillin and incubated at 37°C.

20. DNA sequencing

The BRL Life Sciences double-stranded cycle sequencing kit was used for sequencing and the protocol supplied with the kit was followed. The reaction products were run on a 6% polyacrylamide gel. After the electrophoresis the gel was dried and then exposed to X-Omat AR film overnight.

The sequencing data was analyzed using PC/GENE software purchased from IntelliGenetics, Inc (Mountain View, California), and also by sequence-homology searching of the data in Genbank.

Analysis of nucleotide sequence for open reading frames coding for polypeptides was done assuming that the start codon/methionine codon was ATG or GTG, and the stop codons were TGA, TAA, or TAG.

21. Incorporation of [6-^{3}H] uracil into recombinant *E. coli* JF646 nucleic acid

Duplicate 5 ml overnight cultures of *E. coli* JF646 harboring pH-1 (plasmid containing the chlamydial CTP synthetase gene), pMW5 (positive control plasmid containing the *E. coli* CTP synthetase gene), or pUC19 (negative control plasmid containing no insert) were grown in LB containing 50 μg/ml ampicillin. The cells were centrifuged (3,000 x g for 10 min) and then resuspended in minimal A selective media. A 1 ml aliquot was diluted to 2 ml in minimal A selective media ($OD_{600} \approx 0.6$) and 10 μl [6-^{3}H] uracil was added. The culture was incubated at 37°C for 3 h and the cells were collected by centrifugation as above. The pellet was resuspended in 500 μl sonication buffer (5 mM imidazole, 0.5 M NaCl, 40 mM Tris-HCl pH 7.9) and then sonicated. The nucleic acid was extracted using phenol/chloroform, then chloroform, and precipitated using ethanol/sodium acetate. The nucleic acid was degraded to free bases by adding 100 μl 11.3 N perchloric acid and incubating in a boiling water bath for 1

h. After neutralization with 100 μl 10 N KOH and centrifugation to remove debris, the free nucleobases in the supernatant were analyzed by HPLC as described above.

22. Extract preparation and conditions for *in vitro* CTP synthetase assay

E. coli JF646 were transformed by electroporation with pH-1 (plasmid containing chlamydial CTP synthetase gene), pMW5 (positive control plasmid containing *E. coli pyrG*), or pUC19 (negative control plasmid containing no insert) and then incubated for 90 min at 37°C in SOC. 1 L LB was then inoculated with the SOC culture and incubated a further 2 h at 37°C. 50 μg/ml ampicillin was added and the culture was incubated overnight at 37°C. The following procedure for extract preparation and CTP synthetase assay conditions was adapted from the procedures of Long and Koshland (1978) and Anderson (1983). The cells were collected by centrifugation at 3,000 x g for 20 min at 4°C. The pellets were weighed and then resuspended in 1.2 ml buffer A (20 mM Tris-acetate pH 7.2, 1 mM EDTA, 1 mM glutamine) per gram of recombinant *E. coli*. 350 μg/ml lysozyme was added and the suspension was frozen to -70°C and then immediately thawed to 4°C. The suspension was sonicated and then centrifuged at 13,000 x *g* for 30 min at 4°C. 0.5 ml 10% streptomycin sulfate was added dropwise for every ml of supernatant and the suspension was stirred for 2 h at 4°C. The suspension was centrifuged as above and then 280 mg ammonium sulfate per ml of supernatant was added slowly and the mixture was stirred for 25 min at 4°C. After centrifugation (as above) the pellet was resuspended in 1 ml buffer A and then dialyzed o/n against 4 L buffer A (no glutamine). The dialyzed extract was then adjusted to contain 10 mM $MgCl_2$, 10% glycerol, 70 mM β-mercaptoethanol, 20 mM glutamine, in 20 mM Tris-acetate buffer, pH 7.2. The protein concentration of the extract was determined using the Bio-Rad protein assay. Aliquots of extract were frozen at -70°C.

The CTP synthetase assay was carried out in a total volume of 100 μl as follows: the prereaction mixture (2 mM glutamine, 0.5 mM ATP, 0.1 mM UTP, 0.1 mM GTP, 10 mM

$MgCl_2$, 1 μCi [5,6-^{3}H] UTP, in 20 mM Tris-acetate buffer, pH 7.2) was equilibrated to 37°C on a sand heating block. The reaction was started with the addition of the appropriate amount of extract and terminated after the appropriate amount of time by adding 20 μl 4 N perchloric acid and immediately placing on ice. The mixture was extracted and neutralized using 1.1 x volumes of 78.1:21.9 (v/v) freon-tri-N-octylamine. 50 μl of the top aqueous layer was used for HPLC analysis. The nucleotides were separated using 0.44 M ammonium phosphate buffer, pH 2.4, containing 2.5% acetonitrile on a Whatman Partisil 5 SAX column at a flow rate of 1 ml/min. CTP formation was measured by monitoring the formation of radioactive CTP using a Beckman 171 radioactive flow detector.

23. Other molecular biology methods

Plasmid purifications, restriction endonuclease digestions, Southern blotting, agarose gel electrophoresis, polymerase chain reaction DNA amplifications, random primer labelling, colony lifts, and hybridizations were performed by standard methods either from the manufacturer's instructions or according to Sambrook *et al* (1989).

RESULTS

A. CHLAMYDIAE - HOST INTERACTION

1. *In situ* studies on *C. trachomatis*

a) Differentiating between host- and *C. trachomatis*-DNA synthesis

A difficulty that arises when studying *in situ* metabolic activities of intracellular parasites is the need to differentiate between host- and parasite-specific activity. Two antibiotics, chloramphenicol and cycloheximide, which selectively inhibit prokaryotic and eukaryotic protein synthesis respectively (Alexander, 1968; Bennett *et al*, 1964), have aided studies on chlamydia-specific protein synthesis. Cycloheximide also causes an indirect inhibition of eukaryotic DNA synthesis activity (Bennett *et al*, 1964; Bose and Leibhaber, 1979). Previous studies have shown that DNA synthesis in the presence of cycloheximide is essentially chlamydia-specific (Bennett *et al*, 1964; Tribby and Moulder, 1966). The depression of host metabolic activity caused by cycloheximide is speculated to result in decreased competition between host and parasite for limiting nutrients (Hatch, 1975).

To assist in the studies on *C. trachomatis* DNA synthesis, it was first necessary to identify specific inhibitors of prokaryotic and eukaryotic DNA synthesis. The most selective and effective pair of inhibitors (Cozzarelli, 1977) were aphidicolin, a eukaryotic DNA polymerase inhibitor, and norfloxacin, a prokaryotic DNA gyrase inhibitor. Figure 2 shows the effect of these inhibitors on host mouse L-cell and *C. trachomatis* DNA synthesis activity as measured by [^{3}H] adenine incorporation. The results indicated that aphidicolin is a very potent inhibitor of eukaryotic mouse L-cell DNA synthesis activity, while having little or no effect on *C. trachomatis* DNA replication (Figure 2A). In contrast, norfloxacin inhibited

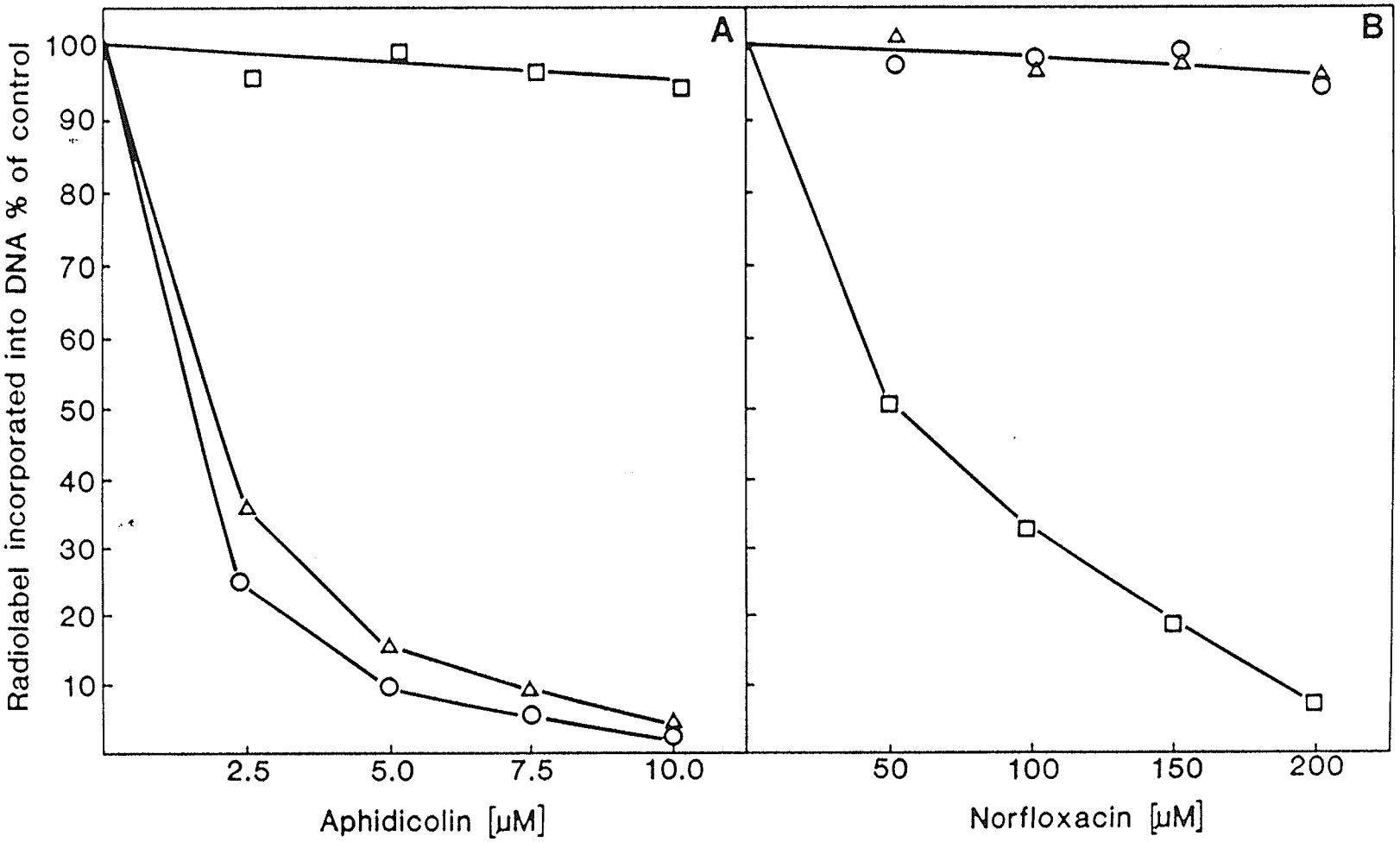

A
B
Radiolabel incorporated into DNA % of control
100
90
80
70
60
50
40
30
20
10
2.5
5.0
7.5
10.0
Aphidicolin [µM]
50
100
150
200
Norfloxacin [µM]

Figure 2: Effect of aphidicolin and norfloxacin on [^{3}H] adenine incorporation into DNA in logarithmically growing mouse L cells (1.0 x 10^6 cells per plate cultured in the presence of cycloheximide) (○), MI mouse L cells (4.0 x 10^6 cells per plate in the presence of 1 μg/ml cycloheximide) (△), and *C. trachomatis*-infected mouse L cells (4.0 x 10^6 cells per plate in the presence of 1 μg/ml cycloheximide) (□). The indicated concentrations of aphidicolin (A) or norfloxacin (B) were added 2 h before the addition of radiolabel. The amount of radiolabel incorporated into DNA in the presence of either inhibitor is expressed as a percentage of the uninhibited control. The following are 100% control values: logarithmically growing mouse L cells, 692,800 dpm/10^6 cells; MI mouse L cells, 9,863 dpm/10^6 cells; and *C. trachomatis*-infected cultures, 152,916 dpm/10^6 cells.

prokaryotic *C. trachomatis* DNA synthesis activity, while having a negligible effect on host mouse L-cell activity (Figure 2B). These inhibitors, as well as cycloheximide, were used throughout these studies to differentiate between host- and parasite-specific DNA synthesis.

In the past there has been conflicting evidence regarding the effect of chlamydial infection on host cell DNA synthesis (Moulder, 1991; Schachter, 1988; Schachter and Caldwell, 1980). It appears that most of the discrepancies result from differences in the initial multiplicities of infection (MOI) used in the various studies (Bose and Leibhaber, 1979). High MOIs, >10 infection-forming units per cell, tend to cause complete cessation of host cell DNA synthesis (and often death), whereas lower MOIs (<5 infection-forming units per cell) may cause little or no inhibition of host cell DNA replication. To obtain close to 100% infection, as judged by light microscopy at 24 h, we routinely used an MOI of 3-5 infection-forming units per cell. The effect of this infection load on host cell DNA synthesis is shown in Table 2. As expected, aphidicolin dramatically inhibited DNA synthesis in uninfected logarithmically growing mouse cells whereas norfloxacin had no effect. Using a similar mouse L cell culture but now infected with *C. trachomatis*, approximately half the incorporation into DNA was inhibited by aphidicolin and half by norfloxacin, indicating that both parasite- and host cell-specific DNAs are being replicated. This indicates that the MOIs used in these experiments did not cause complete cessation of host cell DNA synthesis.

The effects of aphidicolin and norfloxacin on host- and parasite-specific DNA synthesis activity in the presence of cycloheximide are also shown in Table 2. Cycloheximide-treated uninfected mouse L cells (MI cells), incorporated $<2\%$ of the radioactive label compared with logarithmically growing mouse L cells. This small amount of DNA synthesis was sensitive to aphidicolin and resistant to norfloxacin. *C. trachomatis*-infected cycloheximide-treated cultures incorporated substantially more ^{3}H-labelled precursor into DNA than did MI control cultures (Table 2). The DNA synthesis activity in these infected cultures was almost completely unaffected by aphidicolin, indicating that almost all activity was parasite specific.

Table 2: Effect of *C. trachomatis* infection on mouse L-cell DNA synthesis

Culture status[a]	Cycloheximide added[b]	Infection status[c]	Adenine incorporation[d] (10^3 dpm/10^6 cells)	% incorporation remaining[e]		Thymidine incorporation[d] (10^3 dpm/10^6 cells)	% incorporation remaining[e]	
				Aph	Nor		Aph	Nor
Logarithmically growing	—	—	565	5	96	2,266	6	97
	—	+	498	40	44	1,557	7	101
Confluent monolayer	+	—	11	15	98	10	18	103
	+	+	140	98	7	7	13	101

[a] Culture status was either logarithmically growing mouse L cells (1.0 x 10^6 cells/plate) or a confluent monolayer of mouse L cells (4.0 x 10^6 cells/plate).

[b] Experiments were done in the presence or absence of 1 μg/ml cycloheximide as indicated in *Methods*.

[c] Infection status was either noninfected (—) or infected (+) with *C. trachomatis* as described in *Methods*.

[d] Radiolabelled precursor incorporation into DNA was determined after a 3 h labelling period as described in *Methods*. All analyses were made on duplicate dishes, with values varying by less than 10%.

[e] Effect of aphidicolin (Aph, 10 μM) and norfloxacin (Nor, 200 μM) on the incorporation of radiolabelled precursors into DNA. Inhibitors were added 2 h before the ^{3}H labelling period, and incorporation of precursor into DNA was determined as described in *Methods*. The amount of radiolabel incorporated into DNA in the presence of inhibitor is expressed as a percentage of the uninhibited control.

Also shown in Table 2 are the results of an identical series of experiments using [^{3}H] thymidine instead of [^{3}H] adenine. [^{3}H] thymidine was readily incorporated into the DNA of logarithmically growing mouse L cells, and this activity was inhibited by aphidicolin but not norfloxacin. The *C. trachomatis*-infected logarithmically growing culture readily incorporated [^{3}H] thymidine into DNA. However, unlike the [^{3}H] adenine studies, this activity was sensitive to aphidicolin but not norfloxacin. Because of the culture conditions (confluent cells plus cycloheximide), MI mouse L cells incorporate much less [^{3}H] thymidine than do logarithmically growing cells. *C. trachomatis*-infected cultures also incorporated very little [^{3}H] thymidine, and the DNA synthesis activity was inhibited by aphidicolin and resistant to norfloxacin. Taken together, these results indicate that only host mouse L cell DNA synthesis is being detected when thymidine is used as a precursor. In the past it has been observed that exogenously added thymidine either is not incorporated or is poorly incorporated into the DNA of *C. psittaci* (Bennett *et al*, 1964; Hatch, 1976; Lin, 1968).

b) Incorporation of nucleic acid precursors into host and *C. trachomatis* DNA

Work by Tribby and Moulder (1966) established that *C. psittaci* could readily incorporate most exogenously supplied bases and nucleosides into parasite DNA and RNA but that pyrimidine deoxynucleosides were utilized poorly or not at all. However, in these studies, the metabolic activities of the host and parasite were not directly distinguished. The following study reexamines this question of nucleobase and (deoxy)nucleoside precursor utilization in a more direct fashion by taking advantage of the selectivity of aphidicolin and norfloxacin for inhibiting host and parasite DNA synthesis activity, respectively.

The particular radiolabelled precursor was added to the culture medium in tracer amounts (0.3 μM final concentration) such that only minimal perturbation of the host (d)NTP pools should occur. All radiolabelled precursors, with the exception of uracil, were readily incorporated into the DNA of uninfected logarithmically growing mouse L cells (Table 3).

Table 3: Incorporation of various nucleic acid precusors into mouse L-cell- and *C. trachomatis*-specific DNA

Precursor added[a]	Logarithmically growing mouse L cells (10^3 dpm/10^6 cells)[b]	*C. trachomatis*-infected mouse L cells[b]		
			% Incorporation remaining[c]	
		Total incorporation (10^3 dpm/10^6 cells)	Aphidicolin	Norfloxacin
Adenine	604	187	98	14
Adenosine	683	204	97	15
Deoxyadenosine	810	157	96	9
Guanine	104	46	94	17
Guanosine	336	200	93	10
Deoxyguanosine	308	115	101	13
Hypoxanthine	322	198	96	8
Cytidine	519	93	99	15
Deoxycytidine	1,270	12	17	98
Uracil	2.7	0.6	ND[d]	ND
Uridine	430	250	103	16
Deoxyuridine	519	15	19	101
Thymidine	3,663	14	14	102

[a] The various radiolabelled precursors were added to achieve a final concentration of 0.3 μM, and incubation was continued for 3 h. All precursors were ^{3}H labelled with the exception of guanine, which was ^{14}C labelled.

[b] Logarithmically growing mouse L cells (1.0 x 10^6 cells/plate cultured in the absence of cycloheximide) and *C. trachomatis*-infected mouse L cells (4.0 x 10^6 cells/plate cultured in the presence of 1 μg/ml cycloheximide) were seeded and cultured as described in *Methods*. Infected cultures were radiolabelled at 22 h postinfection, and incorporation of precursor into DNA was determined as described in *Methods*. All analyses were made on duplicate dishes, with values varying by less than 10%.

[c] The effect of aphidicolin and norfloxacin on *C. trachomatis*-infected mouse L-cell DNA synthesis activity is expressed as percent incorporation remaining as described in Table 2, footnote *e*.

[d] ND, not determined.

These results indicate that the mouse L cells can transport and convert all the precursors, with the exception of uracil, into at least one of the dNTPs, the required substrate for DNA polymerase.

Results of experiments measuring incorporation of the various precursors into DNA of *C. trachomatis*-infected cycloheximide-treated mouse L cells and the effects of aphidicolin and norfloxacin on this incorporation are also shown in Table 3. Three (adenine, guanine, hypoxanthine) of the four nucleobases tried were efficiently used as DNA precursors: again, the exception was uracil. The detected DNA synthesis activity was inhibited by norfloxacin but unaffected by aphidicolin, a result indicating that the observed activity was parasite-specific. All four ribonucleosides (adenosine, guanosine, cytidine, uridine) tested were good precursors for parasite-specific DNA replication. In addition, the purine deoxynucleosides (dA and dG) were utilized effectively by *C. trachomatis*. In contrast, the results of labelling experiments utilizing the three pyrimidine deoxynucleosides (dC, dU, dT) showed very low levels of incorporation. The small amount of activity demonstrated was sensitive to aphidicolin and resistant to norfloxacin. These results indicate that the observed DNA synthesis activity was host-specific.

c) Incorporation of purine deoxynucleosides into host and *C. trachomatis* DNA

The above results suggested that purine deoxynucleosides (dA and dG) are effective precursors for *C. trachomatis* DNA synthesis but that pyrimidine deoxynucleosides (dC, dU, dT) are utilized by the parasite very poorly or not at all. However, purine deoxynucleosides are much more susceptible than pyrimidine deoxynucleosides to degradative deamination and/or phosphorolysis in most mammalian cells (Martin and Gelfand, 1981). The major pathways involved in deoxyadenosine and deoxyguanosine metabolism in mammalian cells are outlined in Figures 3A and 3B, respectively. When doing the labelling experiments with purine deoxynucleosides, it was consistently noted that extensive incorporation of radiolabel into host

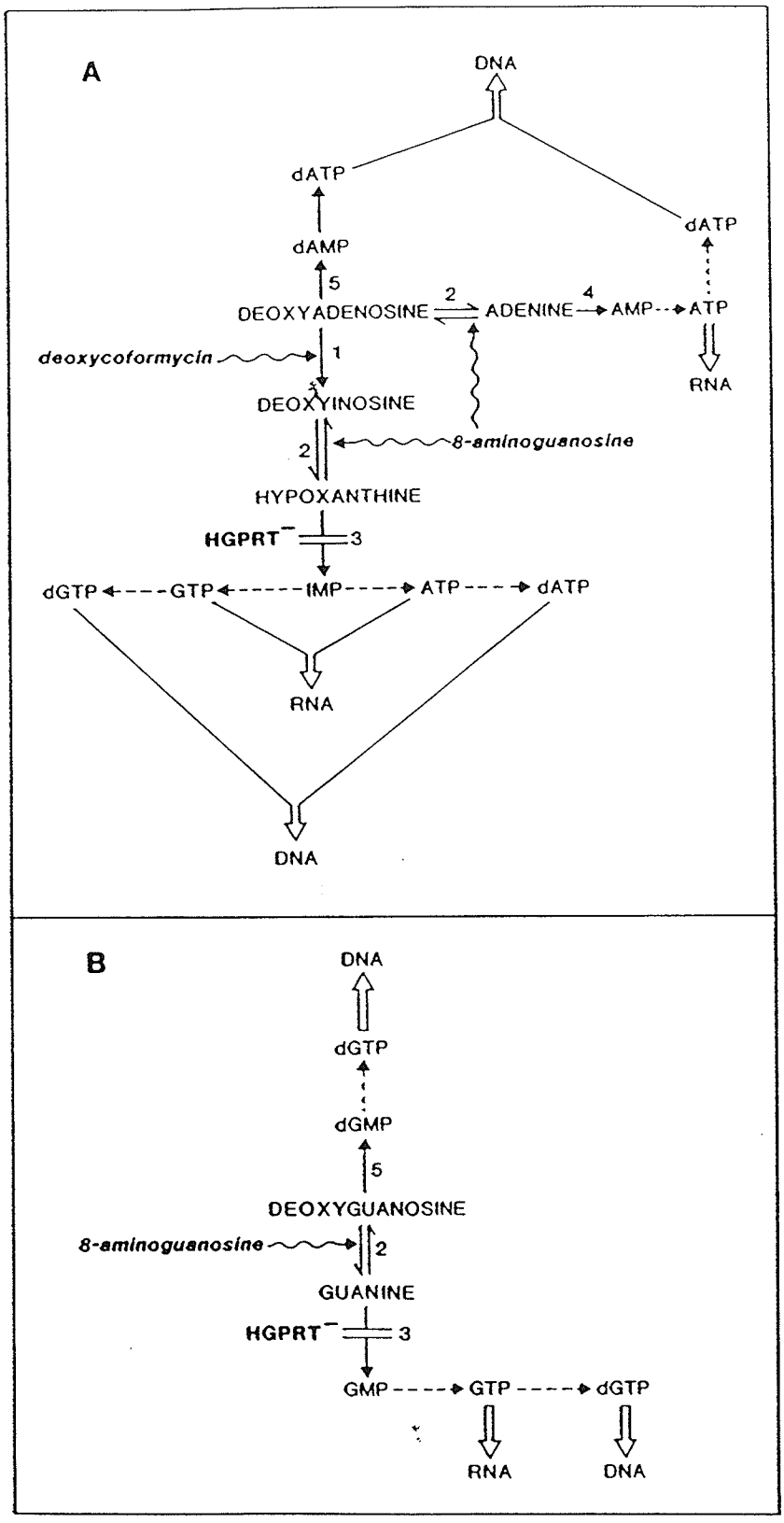

A
DNA
dATP
dATP
dAMP
5
DEOXYADENOSINE
2
ADENINE
4
AMP
ATP
RNA
deoxycoformycin
1
DEOXYINOSINE
8-aminoguanosine
2
HYPOXANTHINE
HGPRT⁻
3
dGTP
GTP
IMP
ATP
dATP
RNA
DNA
B
DNA
dGTP
dGMP
5
DEOXYGUANOSINE
8-aminoguanosine
2
GUANINE
HGPRT⁻
3
GMP
GTP
dGTP
RNA
DNA

Figure 3: Major pathways of deoxyadenosine and deoxyguanosine metabolism in animal cells. Deoxyadenosine (A) and deoxyguanosine (B) metabolism pathways are schematically represented. Not all possible routes of metabolism are included, just major routes relevant to this study. Single arrows do not necessarily imply one-step reactions. Squiggly arrows represent steps inhibited by dCF and 8-AG. $HGPRT^-$ represents the enzyme mutation in $HGPRT^-$ cells. Important enzymes are numbered as follows: 1, adenosine-deoxyadenosine deaminase; 2, purine nucleoside phosphorylase; 3, $HGPRT^-$; 4, adenine phosphoribosyl-transferase; and 5, deoxynucleoside (dC, dG, dA) kinase.

and *C. trachomatis* RNA as well as DNA occurred. This strongly suggested that degradative pathways of purine deoxynucleoside metabolism were being utilized. This complication makes it difficult to distinguish whether *C. trachomatis* is actually taking up dATP and dGTP directly from the host or whether radiolabelled ATP and GTP are being transported with subsequent chlamydia-directed conversion to deoxynucleotides before incorporation into DNA.

To circumvent this problem, a strategy was developed to reduce the amount of purine deoxynucleosides that were degraded and salvaged as ribonucleotides and, as a result, would favor the direct phosphorylation of the added deoxynucleosides. This strategy required the use of a mutant cell line and two specific inhibitors of deoxynucleoside degradation. The mutant was an HGPRT$^-$ Chinese hamster ovary (CHO) cell line. As a result of the HGPRT$^-$ phenotype, these cells can no longer efficiently salvage hypoxanthine or guanine (Figure 3A and B, step 3).

Initially it was necessary to determine whether *C. trachomatis* could grow in wild-type and HGPRT$^-$ CHO cells. Chlamydial replication in these two cell lines was monitored by titrating the number of infectious elementary bodies formed at the end of a growth cycle (48 h p.i.) by the inclusion counting method. It was found that *C. trachomatis* grew as well in the CHO cell lines as it did in mouse L cells. In addition, an identical pattern of nucleotide precursor utilization was found for *C. trachomatis* growing in wild-type CHO cells as was reported in Table 2 for wild-type mouse L cells.

The results of radiolabelling studies in HGPRT$^-$ cells are shown in Table 4. Logarithmically growing HGPRT$^-$ cell DNA was readily labelled when adenine, cytidine, or thymidine was used as a nucleic acid precursor. In keeping with the HGPRT$^-$ phenotype, there was very little labelling of DNA when hypoxanthine, guanosine, or deoxyguanosine was supplied as the precursor and poor incorporation of medium-supplied adenosine and deoxyadenosine. The inefficient utilization of guanosine, deoxyguanosine, adenosine, and deoxyadenosine suggests that the majority of these precursors are channeled through the

Table 4: Incorporation of various nucleic acid precursors into host- and *C. trachomatis*-specific DNA in HGPRT$^-$ cells

Precursor added[a]	Incorporation of precursor into DNA[b] (10^3 dpm/10^6 cells)		
	Logarithmically growing cells	MI cells	*C. trachomatis*-infected cells
Adenine	490	20	202
Cytidine	323	10	86
Thymidine	1,865	29	16
Adenosine	63	8.4	35
Deoxyadenosine	21	2.9	4.3
Guanosine	1.0	0.8	1.2
Deoxyguanosine	5.7	0.7	0.9
Hypoxanthine	6.1	0.9	1.0

[a] The various ^{3}H-labelled precursors were added, and incorporation into DNA was determined as described in Table 2, footnote *e*, and *Methods*. All analyses were made on duplicate dishes, with results varying by less than 10%.

[b] HGPRT$^-$ CHO cells were logarithmically growing (1.0 x 10^6 cells/plate in the presence of 1 μg/ml cycloheximide), MI confluent monolayers (4.0 x 10^6 cells/plate in the presence of 1μg/ml cycloheximide), or *C. trachomatis*-infected confluent monolayers (4.0 x 10^6 cells/plate in the presence of 1 μg/ml cycloheximide). For details, see *Methods*.

catabolic pathway rather than being directly phosphorylated to their corresponding (deoxy)ribonucleotide.

It has been shown in both mouse and human cell lines that one of the consequences of an HGPRT$^-$ phenotype is increased excretion of purines (hypoxanthine, xanthine, guanine) into the culture medium by mutant cells when compared with their wild-type parent strain (Chan *et al*, 1973; Hershfield and Seegmiller, 1977; Ullman *et al*, 1982). This would suggest that even in the absence of HGPRT$^-$, the majority of the intracellular purine (deoxy)ribonucleosides are processed by degradative pathways (Figure 3A and 3B); however, since the mutant cells cannot salvage the free bases, they are excreted by the cells.

In order to reduce the competition between the degradation and kinase pathways for the purine deoxynucleoside precursors, two nucleoside analog inhibitors were used. dCF (Cha *et al*, 1975) and 8-AG (Kayomers *et al*, 1981) are specific inhibitors of the two main purine nucleoside catabolic pathway enzymes, (deoxy)adenosine deaminase and purine nucleoside phosphorylase, respectively (Figure 3). In the presence of dCF and/or 8-AG, less purine deoxynucleoside is degraded, and as a result, more substrate is available for the deoxynucleoside kinase reactions.

Table 5 shows the results of purine deoxynucleoside incorporation studies with logarithmically-growing, MI and *C. trachomatis*-infected wild-type and HGPRT$^-$ cells in the presence and absence of dCF and 8-AG. Again, aphidicolin and norfloxacin were used to distinguish between host and parasite DNA synthesis. An identical series of experiments was also done with radiolabelled cytidine as a precursor as a control to show that dCF and 8-AG do not directly affect host or *C. trachomatis* DNA synthesis. Cytidine was an effective precursor for DNA synthesis in both logarithmically growing wild-type and HGPRT$^-$ cells, and the incorporation was unaffected by the presence of dCF or 8-AG (Table 5). This result indicates that the two inhibitors do not inhibit host cell DNA synthesis. As expected, this host cell activity was sensitive to aphidicolin and unaffected by norfloxacin. Similar results were obtained with MI wild-type and HGPRT$^-$ CHO cells, except that overall incorporation of label

Table 5: Effect of dCF and 8-AG on incorporation of nucleic acid precursors into host- and *C. trachomatis*-specific DNA

CHO cell line	Precursor added	Culture status[a]	Radiolabelled precursor incorporated[b]						Ratio[e]
			Indicated precursor			Precursor + dCF + 8-AG			
			dpm[c]	Aph[d]	Nor[d]	dpm	Aph	Nor	
Wild type	cytidine	LG	658	9	96	671	8	98	0.98
		MI	10	21	98	10	23	105	1.0
		INF	95	96	10	91	99	12	1.04
HGPRT$^-$		LG	498	8	102	503	9	98	0.99
		MI	11	20	101	9.7	26	97	1.13
		INF	78	96	18	75	98	20	1.04
Wild type	deoxyadenosine	LG	1,014	7	99	273	11	102	3.71
		MI	17	20	102	86	14	98	0.20
		INF	167	99	8	68	62	67	2.45
HGPRT$^-$		LG	25	18	98	180	9	99	0.14
		MI	3.1	ND[f]	ND	206	18	92	0.02
		INF	3.0	ND	ND	280	55	78	0.01
Wild type	deoxyguanosine	LG	439	12	101	59	16	104	7.44
		MI	13	23	102	29	19	99	0.45
		INF	105	95	14	28	64	71	3.75
HGPRT$^-$		LG	6.2	16	101	28	14	98	0.22
		MI	2.9	ND	ND	15	29	98	0.19
		INF	1.8	ND	ND	9.2	32	104	0.19

[a] Culture status was defined in Table 4, footnote *b*, and as described in Methods. LG, logarithmically growing; MI, mock-infected; INF, *C. trachomatis*-infected.

[b] Incorporation of the various radiolabelled precursors into DNA was determined in the absence or presence of dCF (7.5 μM) and/or 8-AG (100 μM). Both dCF and 8-AG were added 2 h before ^{3}H label. When required, aphidicolin (Aph) (10 μM) and norfloxacin (Nor) (200 μM) were added simultaneously with dCF and 8-AG. All analyses were performed in duplicate, with results varying by less than 10%.

[c] Values given in this column represent total incorporation of radiolabel in the absence of aphidicolin or norfloxacin. Results are expressed as 10^3 dpm/10^6 cells.

[d] Effects of aphidicolin or norfloxacin are expressed as percent incorporation remaining as described in Table 2, footnote *e*.

[e] Value shown represents the calculated ratio of the total number of counts incorporated into DNA with each precursor in the absence of dCF and/or 8-AG divided by the total incorporation into DNA with each precursor in the presence of dCF and/or 8-AG.

[f] ND, not done.

was decreased. The *C. trachomatis*-infected wild-type and HGPRT$^-$ cells also readily utilized exogenous cytidine as DNA precursor. The total amount of cytidine incorporated was substantially reduced by norfloxacin and unaffected by aphidicolin, results consistent with the measured activity being parasite-specific. Neither dCF nor 8-AG affected the incorporation of cytidine into DNA, a result which indicates that *C. trachomatis* DNA replication is not affected to a significant extent by either inhibitor.

When wild-type CHO cells were infected with *C. trachomatis*, there was efficient incorporation of both dA and dG into DNA. The activity was parasite-specific, as indicated by decreased incorporation in the presence of norfloxacin and essentially no change in the presence of aphidicolin. The addition of 8-AG and/or dCF to infected wild-type cells caused a decrease in the level of incorporation of both labelled dA and dG. Under these conditions, the measured activity was inhibited by both aphidicolin and norfloxacin. These results indicate that both host and parasite DNA synthesis activities were contributing to the total incorporation.

When HGPRT$^-$ cells were infected with *C. trachomatis*, there was no significant incorporation of medium-supplied dA or dG into DNA (Table 5). When *C. trachomatis*-infected HGPRT$^-$ cells were labelled with dA in the presence of dCF and 8-AG, there was significant incorporation of radiolabel into DNA, although not much more than MI control cultures. The measured DNA synthesis activity was inhibited by aphidicolin and by norfloxacin to some extent. When infected HGPRT$^-$ cells were labelled with dG in the presence of 8-AG, the total incorporation of label into DNA was low. The DNA synthesis activity detected was inhibited by aphidicolin and not affected by norfloxacin.

Also shown in Table 5 is the calculated ratio of the total number of counts incorporated into DNA with cytidine, dA, and dG in the absence of dCF and/or 8-AG, divided by the total incorporation into DNA with each precursor in the presence of dCF and/or 8-AG. The significance of these ratios will be addressed in the discussion.

d) Composition of deoxynucleotide precursor pools in mouse L cells

There is a possibility that there is a difference in the proportion of the transported deoxynucleoside precursor that is phosphorylated to the triphosphate level by a *C. trachomatis*-infected cell compared with an uninfected control culture. If this is the case, it may be argued that *C. trachomatis* does not incorporate a significant amount of any deoxynucleoside precursor because the infected host cell does not raise the deoxynucleoside to the deoxynucleoside triphosphate. This question was directly addressed by comparing the deoxynucleotide precursor pools in MI mouse L cells with those in *C. trachomatis*-infected mouse L cells. The results of these experiments are shown in Table 6. In both MI and infected cultures, the presence of dCF and/or 8-AG dramatically reduced the total amount of radiolabel present in the soluble nucleotide pool but did not significantly affect the distribution of the label between mono-, di-, and triphosphate nucleotide derivatives. The reduction in total incorporation likely resulted from blocking of the purine deoxynucleoside degradation pathway. In support of this hypothesis, we found that the majority (>80%) of the soluble nucleotide pool present in MI and infected cells after labelling with dA or dG in the absence of dCF and/or 8-AG was ribonucleotide derivatives and not deoxynucleotides. In contrast, in the presence of dCF and/or 8-AG, the proportion of the total soluble pool that was represented by ribonucleotides was greatly reduced. No trend was observed when the total amount of radioactivity recovered in an individual nucleotide pool from MI cells was compared with the amount recovered from *C. trachomatis*-infected cultures. For example, with deoxyadenosine, less radioactivity was recovered from infected cultures than from MI cultures; however, with thymidine, the reverse result was obtained. More important, there was no significant difference in the proportional distribution of label among deoxynucleoside mono-, di-, and triphosphates when *C. trachomatis*-infected cultures were compared with MI control cultures.

Table 6: Comparison of ^{3}H-labelled nucleotide pools in *C. trachomatis*-infected and MI mouse L cells

[^{3}H] deoxy-nucleoside added[a]	Infection status[b]	^{3}H radiolabel recovered (10^3 dpm/10^6 cells)[c]	Composition of nucleotide pool[d]		
			(d)NMP	(d)NDP	(d)NTP
Deoxyadenosine	MI	3,370	3	16	81
	INF	2,692	<1	32	67
Deoxyadenosine	MI	305	<1	21	79
+ dCF and 8-AG	INF	193	<1	18	82
Deoxyguanosine	MI	206	3	14	83
	INF	73	<1	21	79
Deoxyguanosine	MI	53	4	19	77
+ dCF and 8-AG	INF	32	<1	19	81
Deoxycytidine	MI	158	<1	9	91
	INF	146	<1	<1	99
Thymidine	MI	43	8	18	74
	INF	72	17	15	68

[a] The indicated [^{3}H] deoxynucleoside was added to the culture medium (0.3 μM final concentration) for 3 h, and then the cellular nucleotide pools were extracted with methanol. A portion (10μl) of the extract was analyzed by HPLC, and radioactivity was monitored by on-line radioactive flow detection. dCF (7.4 μM) and/or 8-AG (100 μM) were added as indicated.
[b] Infection status was either MI or *C. trachomatis*-infected (INF) mouse L cells. Culture conditions were as described in Table 4, footnote *b*.
[c] These values represent the total amount of extracted radioactivity which was recovered as nucleotides. All analyses were made on duplicate dishes, with values varying by less than 10%.
[d] These values represent the proportion of the total ^{3}H-labelled nucleotide pool recovered that was present as mono-, di-, and triphosphate nucleotide derivatives. The values are normalized to percentage of the total recovery.

2. NTP pools and energy charge

a) Effect of *C. trachomatis* infection on host-cell NTP pools and energy charge

Since chlamydiae obtain NTPs directly from the host-cell cytoplasm, it was necessary to determine if *C. trachomatis* L2 infection altered the size or composition of the host cells' NTP pools. *C. trachomatis* L2-infected logarithmically growing HeLa cells were harvested at various time points after infection and then acid-soluble nucleotides were extracted and analyzed by HPLC. As a control experiment acid-soluble nucleotide pools were also extracted from uninfected logarithmically growing HeLa cells at the same time points. Figure 4 shows the status of the NTP pools of the *C. trachomatis* L2-infected cells over the course of infection. Despite reaching near confluence, the NTP pools of the uninfected logarithmically growing HeLa cells remained essentially constant over the 48 h time period. In contrast the NTP pools of the *C. trachomatis*-infected cultures decreased to approximately 40-50% of the uninfected HeLa cell control by 24 h p.i. After this time the NTP pools began to rise reaching a value of 60-80% of the uninfected control culture at 48 h p.i. All four NTP pools responded to infection in a similar fashion. These same experiments were repeated with uninfected and *C. trachomatis*-infected confluent monolayers of HeLa cells in the presence of 1 μg/ml cycloheximide, and although the absolute size of the four NTP pools was slightly different they responded to infection in an identical fashion.

Since chlamydiae are energy parasites (Moulder, 1991) the question whether *C. trachomatis* L2 infection had an effect on the host's capacity to maintain its energy charge was examined. The energy charge of a cell reflects the proportion of its adenylate pool which is in the high-energy triphosphate form (Atkinson, 1968):

$$\text{energy charge} = 1/2\{(2[ATP]+[ADP])/([AMP]+[ADP]+[ATP])\}$$

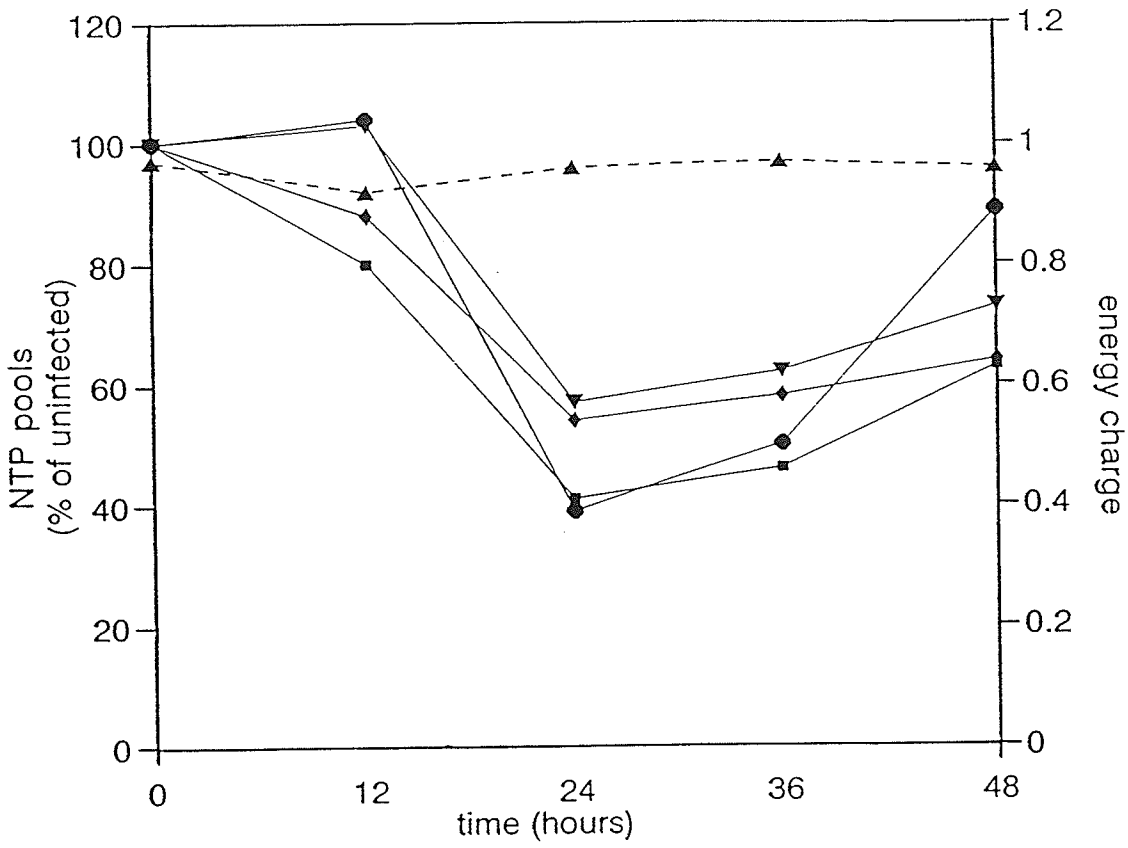


Figure 4: Energy charge and nucleoside triphosphate pools in *C. trachomatis*-infected logarithmically growing HeLa cells over the course of chlamydial infection. Parallel flasks of uninfected logarithmically growing and *C. trachomatis*-infected logarithmically growing HeLa cells were seeded and cultured as described in the *Methods*. At each of the indicated times duplicate flasks were removed and acid-soluble nucleotide pools were extracted. The acid-soluble ribonucleotides were then separated and analyzed by HPLC. Data for pools are expressed as a percentage of uninfected logarithmically growing culture: CTP (♥), UTP (◆), ATP (●), GTP (■). The pool sizes (pmoles NTP/μg DNA) for the uninfected logarithmically growing control cultures are: CTP, 27.3; UTP, 65.2; ATP, 133.5; GTP, 49.3. The energy charge (▲) was also calculated for infected cultures at each time point using the following formula: energy charge = $1/2\{(2[ATP]+[ADP])/([ATP]+[ADP]+[AMP])\}$.

Figure 4 shows that the energy charge of the HeLa cell was essentially unaffected by the presence of *C. trachomatis* and that it remained stable at approximately 0.98 throughout the course of infection.

In order for the energy charge to remain unchanged when the absolute size of the ATP pool is decreasing, there must be a corresponding decrease in the size of the total adenylate pool (ATP + ADP + AMP). In keeping with this it was found that the total adenylate pool of a *C. trachomatis* L2-infected HeLa culture at 24 h p.i. was reduced by 43% compared to an uninfected HeLa control culture (200 pmoles nucleotide/μg DNA compared to 114 pmoles nucleotide/μg DNA).

b) NTP pools and energy charge in RBs and EBs

The nucleotide-pool data presented above are for *C. trachomatis*-infected HeLa cells, and the vast majority of these pools would be host cell derived. In order to determine the contribution of *C. trachomatis* L2 to the pools and to assess its energy charge, the nucleotide pools in highly purified RBs and EBs were quantified. Table 7 shows the size of the total adenylate-nucleotide pool, and the calculated energy charge for purified RBs and EBs. It is not surprising that, like in most cells, the ATP pool is the largest of the nucleoside-triphosphate pools in both the RB and the EB. It is interesting that UTP is the smallest pool in the RB, whereas CTP is the smallest pool in the EB. The calculated energy charge in the RB (0.58) is also much smaller than it is in the EB (0.80). Also shown in Table 7 is the predicted concentration of the total adenylate pool in RBs and EBs. These values are calculated using the assumption that both RBs and EBs are spherical and the diameter of an RB is three times that of an EB (0.9 μm versus 0.3 μm) (Matsumoto, 1988). Based on these assumptions the approximate concentration of the adenylate pool in the RB is 1 mM, a value similar to that calculated for the *C. trachomatis* L2-infected HeLa cell at 22 h p.i. (1.5 mM). In sharp contrast the estimated concentration of the adenylate pool in the EB is much higher (38 mM).

Table 7: Nucleotide pools and energy charge in *C. trachomatis* reticulate and elementary bodies

chlamydial form[a]	pmoles nucleotide per μg DNA				Total adenylate pool[b] (pmoles/μg DNA)	energy charge[c]	Estimated adenylate pool conc (mM)[d]
	CTP	UTP	ATP	GTP			
RB	9	7	47	13	120	0.58	1
EB	2	16	73	7	160	0.80	38

[a]Hypaque gradient purified reticulate (RB) and elementary bodies (EB).
[b]AMP + ADP + ATP
[c]Energy charge = ½{(2[ATP]+[ADP])/([AMP]+[ADP]+[ATP])}
[d]Calculated according to the procedure of Neidhardt (1987) and based on the assumption that the EB diameter is 1/3 the size of the RB diameter (Matsumoto, 1988).

3. The role of mitochondrial ATP in the growth of *C. trachomatis*

To directly test the hypothesis that mitochondrial generated ATP is essential for chlamydial survival *C. trachomatis* growth was measured in a mutant Chinese-hamster-lung-fibroblast cell line (CCL 16-B2) with severely compromised mitochondrial function (Ditta *et al*, 1976). The CCL 16-B2 cell line lacked the capacity to synthesize ATP by oxidative phosphorylation as a result of a defect in complex I of the electron-transport chain (Ditta *et al*, 1976). Chlamydial growth was assessed by measuring the incorporation of radiolabelled uridine into chlamydial specific DNA. As a control experiment the ability of *C. trachomatis* L2 to grow in the parental cell line (CCL 16-B1) was assessed. In duplicate experiments using CCL 16-B1 cells as host, *C. trachomatis* incorporated 141,898 ± 11,315 d.p.m./10^6 cells into DNA and with CCL 16-B2 cells as host *C. trachomatis* incorporated 146,896 ± 10,634 d.p.m./10^6 cells of uridine into DNA. These results indicate that *C. trachomatis* grows as well in the respiration deficient mutant as it does in its wild-type counterpart.

B. EVIDENCE FOR A CHLAMYDIAL RIBONUCLEOTIDE REDUCTASE

1. Studies using hydroxyurea - an inhibitor of ribonucleotide reductase

a) Hydroxyurea sensitivity of wild-type *C. trachomatis*

The effect of hydroxyurea (HU) on *C. trachomatis* inclusion development in wild-type mouse L cells was determined. To ensure that the host cells were in the logarithmic phase rather than the stationary phase, a state in which little or no ribonucleotide reductase activity can be detected and dNTP pools are small (Spyrou and Reichard, 1988), these experiments were done with subconfluent cultures in the absence of cycloheximide. Chlamydial inclusion development was assessed by fluorescent staining. The dose of HU required to inhibit *C.*

trachomatis inclusion development by 50% (ID_{50}) was about 0.70 mM (Table 8). Since chlamydiae are intracellular parasites which depend on their host for many metabolites, it can be difficult to distinguish between host cell- and chlamydia-specific effects of a drug. For example, the ID_{50} of HU for wild-type mouse L cells is 0.07 mM (McClarty *et al*, 1986), a value 10-fold lower than that required to inhibit *C. trachomatis* inclusion development by the same amount. As a result, it is difficult to determine whether HU inhibits chlamydiae directly or indirectly via an effect on the host cell line.

To determine whether HU directly affects *C. trachomatis* inclusion formation, a hydroxyurea-resistant mouse L cell line, hereafter called LHF, was used as a host to support chlamydial growth. Again, these experiments were conducted with subconfluent cultures in the absence of cycloheximide. The ID_{50} of HU on LHF was 3.5 mM, a 50-fold increase over that inhibiting wild-type mouse L cells. This cell line is resistant to hydroxyurea because of an overproduction of the tyrosine free radical-containing small subunit of ribonucleotide reductase, the target of the drug (Ehrenberg and Reichard, 1972). The ID_{50} of HU on *C. trachomatis*-infected LHF cells was 0.76 mM, a value similar to that determined in wild-type mouse L cells (Table 8). In addition, two other parameters, infectivity and DNA synthesis, were used to assess the effects of HU on *C. trachomatis* growth in wild-type mouse L cells and LHF cells. The concentrations of drug required to reduce the production of progeny EB by 50% as measured by infectivity titration, and *C. trachomatis*-specific nucleic acid synthesis as measured by [^{3}H] adenine incorporation into chlamydial DNA, in the two cell lines are shown in Table 8. The results of these experiments clearly show that the concentration of HU required to bring about 50% inhibition of all growth parameters studied was approximately the same in both cell lines.

Table 8: Effect of hydroxyurea on *C. trachomatis* growth in wild-type and hydroxyurea-resistant mouse L cells

Mouse cell line infected[a]	ID_{50} hydroxyurea (mM)[b]		
	Inclusion formation[c]	EB production[d]	DNA synthesis[e]
Wild type	0.70	0.15	0.20
Hydroxyurea resistant (LHF)	0.76	0.17	0.18

[a] Wild-type or hydroxyurea-resistant (LHF) mouse L cells were infected with *C. trachomatis* L2 EBs as described in *Methods*. All experiments were conducted with subconfluent logarithmically growing cultures without cycloheximide in the p.i. medium.

[b] Values represent the concentration of hydroxyurea required to reduce *C. trachomatis* growth, as measured by three separate parameters, to 50% of that obtained by an infected control culture in the absence of hydroxyurea.

[c] Effect of hydroxyurea on inclusion formation was assessed at 48 h p.i. by fluorescent staining.

[d] Effect of hydroxyurea on the production of infectious EBs at 48 h p.i. was assessed by infectivity titration.

[e] Effect of hydroxyurea on *C. trachomatis* DNA synthesis was assessed by measuring [^{3}H] adenine incorporation into chlamydia-specific DNA at 22 h p.i. Host cell and *C. trachomatis* DNA synthesis activities were differentiated by using aphidicolin and norfloxacin as specific inhibitors of eukaryotic and prokaryotic DNA synthesis, respectively.

b) Hydroxyurea sensitivity in a series of mutant *C. trachomatis* isolates

A series of *C. trachomatis* isolates were sequentially selected in the presence of increasing concentrations of HU for the ability to proliferate in normally cytotoxic drug concentrations. The effect of HU on *C. trachomatis*-specific DNA synthesis in the parental wild-type population and the various drug-selected L2 isolates is shown in Figure 5. The indicated amounts of HU were added to infected HU-resistant mouse cells at 2 h p.i. 1 μg/ml cycloheximide was also present in the culture medium. At 22 h p.i., a time when L2 DNA synthesis has reached its maximum, [^{3}H] adenine was added for 3 h and incorporation of this precursor into chlamydial DNA was determined. All drug-selected L2 isolates exhibited a reduced sensitivity to the inhibitory effects of HU when compared with parental wild-type *C. trachomatis* L2. For example, when the ID_{50} values for HU are considered (Table 9), L2 isolates selected in the lowest drug concentration (1 mM) exhibited approximately a 3-fold elevation in drug resistance, whereas L2 isolates selected at the highest concentration (25 mM) were about 64-fold more resistant to HU than the wild-type population. Furthermore, the drug resistance properties of the L2 isolates significantly increased at each of the drug selection steps tested (1.0, 2.0, 5.0, 10.0, 15.0, 20.0, and 25 mM HU).

c) Stability of hydroxyurea-resistant phenotype and cross-resistance to guanazole

To obtain more information about the mutant drug resistance phenotype, the *C. trachomatis* isolate which readily grew in the presence of 10 mM HU ($L2H^R$-10.0) was studied in more detail. Figure 6 shows the relative inclusion-forming abilities of parental wild-type L2 and drug-resistant isolates $L2H^R$-10.0 in the presence of various concentrations of HU. One μg/ml cycloheximide was also present in the culture medium. In agreement with their DNA synthesis sensitivities, the $L2H^R$-10.0 isolate was significantly more resistant to the cytotoxic effects of HU than was wild-type L2. The concentration of HU required to inhibit $L2H^R$-10.0

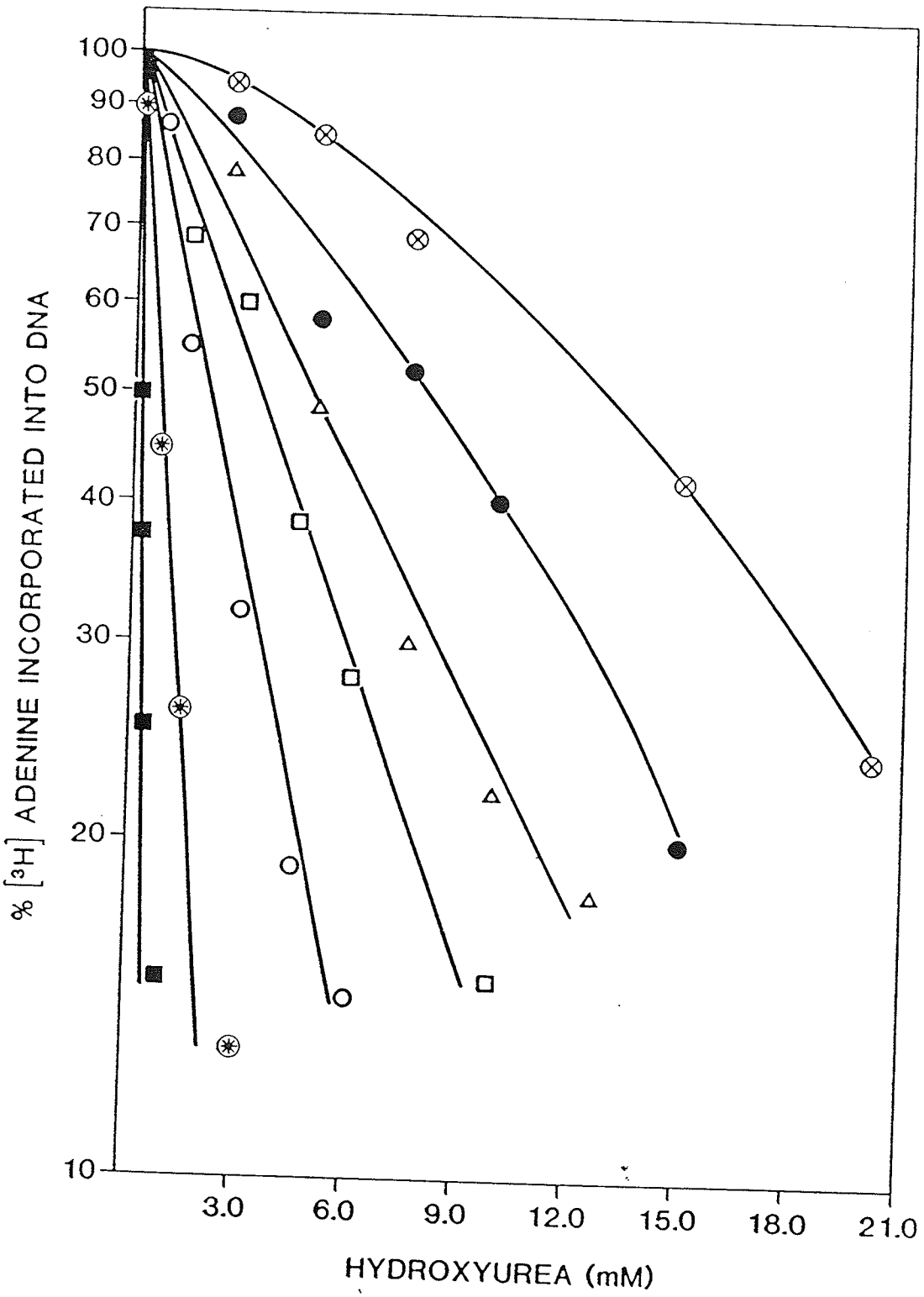

100
90
80
70
60
50
40
30
20
10
% [3H] ADENINE INCORPORATED INTO DNA
3.0
6.0
9.0
12.0
15.0
18.0
21.0
HYDROXYUREA (mM)

Figure 5: Effect of hydroxyurea on *in situ* DNA synthesis activity of wild-type and a series of hydroxyurea-resistant *C. trachomatis*. A confluent monolayer (5 cm dish) of hydroxyurea-resistant mouse L cells was inoculated with wild-type or any one of a series of hydroxyurea-resistant *C. trachomatis* EBs. After adsorption at 37°C for 2 h, the cell monolayers were washed and then incubated with MEM containing 10% FBS, 1 μg/ml cycloheximide, and the indicated concentration of hydroxyurea. After 22 h, [^{3}H] adenine was added to achieve a final concentration of 0.3 μM, and incubation was continued for 2 h at 37°C. After the monolayer was washed three times with ice-cold Tris-buffered saline, 0.3 M NaOH was added to degrade RNA. DNA was subsequently precipitated by the addition of 10% trichloroacetic acid. The resulting precipitate was collected by filtration and quantified by liquid scintillation counting. [^{3}H] adenine incorporation into *C. trachomatis* DNA is expressed as a percentage of [^{3}H] adenine incorporation into chlamydial DNA of infected controls in the absence of hydroxyurea. Wild-type (■); L2H^R-2.0 (⊗); L2H^R-5.0 (○); L2H^R-10.0 (□); L2H^R-15.0 (△); L2H^R-20.0 (●); L2H^R-25.0 (⊗).

Table 9: Summary of hydroxyurea ID_{50} values for DNA synthesis in a series of drug-resistant *C. trachomatis* isolates

C. trachomatis isolate[a]	Hydroxyurea ID_{50} (mM)[b]	Fold increase in resistance[c]
Wild-type L2	0.20	
$L2H^R$-1.0	0.55	2.8
$L2H^R$-2.0	1.0	5.0
$L2H^R$-5.0	2.2	11.0
$L2H^R$-10.0	3.5	17.5
$L2H^R$-15.0	5.0	25.0
$L2H^R$-20.0	8.1	40.5
$L2H^R$-25.0	12.9	64.5

[a] This series of hydroxyurea-resistant mutants was isolated from a wild-type population of *C. trachomatis* L2 by a stepwise procedure.

[b] The ID_{50} values given in this column represent the concentration of hydroxyurea required to inhibit *C. trachomatis*-specific DNA synthesis by 50%. All ID_{50} values are derived from the DNA synthesis curves (Figure 5) with the exception of $L2H^R$-1.0, which is not represented in Figure 5.

[c] Fold increase in hydroxyurea resistance is calculated by using the following equation: fold increase = ID_{50} hydroxyurea-resistant mutant EBs/ID_{50} wild-type L2 EBs.

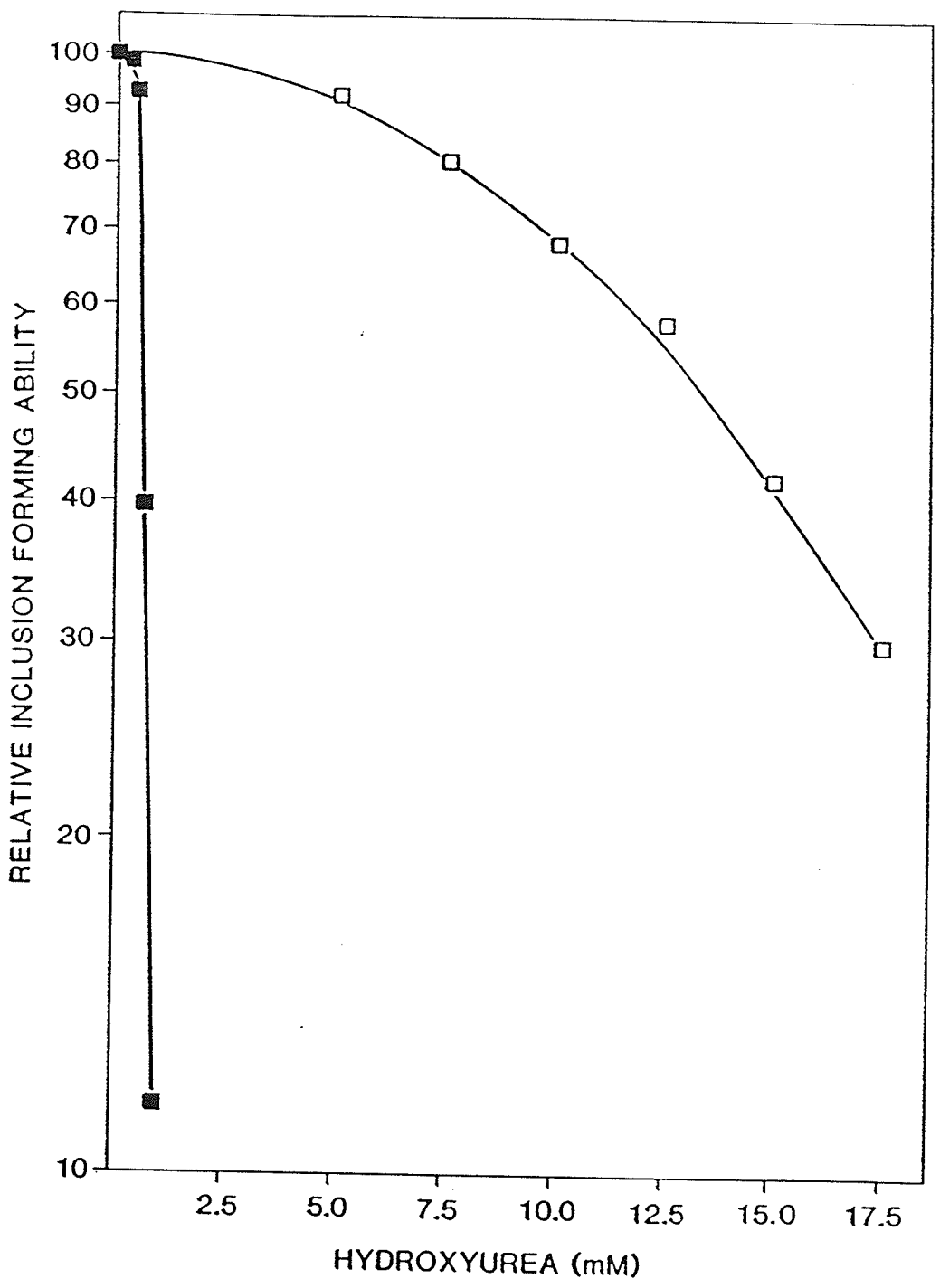

RELATIVE INCLUSION FORMING ABILITY
100
90
80
70
60
50
40
30
20
10
2.5
5.0
7.5
10.0
12.5
15.0
17.5
HYDROXYUREA (mM)

Figure 6: Effect of hydroxyurea on wild-type and L2H^R-10.0 hydroxyurea-resistant *C. trachomatis* inclusion formation in hydroxyurea-resistant mouse L cells. The hydroxyurea-resistant mouse L-cell line LHH was used as a host, and the chlamydial inoculum was either wild-type *C. trachomatis* L2 EBs (■) or hydroxyurea-resistant (L2H^R-10.0) *C. trachomatis* EBs (□). After adsorption at 37°C for 2 h, the cell monolayers were washed and then incubated with MEM-10% FCS containing 1 μg/ml cycloheximide and the indicated concentration of hydroxyurea. After 48 h, the chlamydial inclusions were stained with fluorescein-conjugated anti-*C. trachomatis* monoclonal antibody and then enumerated. Inclusion formation is expressed as a percentage of the inclusion formation of untreated controls.

inclusion formation by 50% was 13.0 mM a 17-fold increase over wild-type L2 (ID_{50} of 0.76 mM) (Table 10).

The stability of the $L2H^R$-10.0 drug resistance phenotype was assessed after passage in the absence of HU for 10 growth cycles. After 10 cycles, there was only a small decrease in the concentration of HU required to reduce either inclusion-forming ability or chlamydia-specific DNA synthesis by 50% (Table 10). These results suggest that the $L2H^R$-10.0 phenotype is quite stable.

Guanazole is another drug which inhibits DNA synthesis and has ribonucleotide reductase as its primary target (Wright and Lewis, 1974). Guanazole, like hydroxyurea, has been shown to inhibit deoxynucleotide synthesis by inactivating the tyrosine free radical of the small subunit of ribonucleotide reductase (Kjoller Larsen *et al*, 1982). Mammalian cell lines selected for resistance to HU have been shown to be cross resistant to guanazole and vice-versa (Wright and Lewis, 1974; Wright *et al*, 1989). The effect of guanazole on inclusion development and *C. trachomatis*-specific DNA synthesis in wild-type L2 and $L2H^R$-10.0 was determined (Table 10). The results indicated that the $L2H^R$-10.0 isolate is cross resistant to guanazole. The concentration of guanazole required to bring about a 50% reduction in inclusion development was 2.1 mM in wild-type L2 and 27.5 mM in $L2H^R$-10.0. Furthermore, concentrations of 0.55 and 8.3 mM guanazole were needed to reduce DNA synthesis by 50% in wild-type L2 and resistant $L2H^R$-10.0 isolates, respectively. The $L2H^R$-10.0 guanazole resistance phenotype was also stable, exhibiting little change in ID_{50} values after 10 passages in the absence of selective agent (Table 10).

d) Effect of hydroxyurea on wild-type and drug resistant *C. trachomatis* DNA synthesis

Aphidicolin and norfloxacin were used to distinguish between host and parasite specific DNA synthesis when the effect of HU on wild-type L2 and drug-resistant $L2H^R$-10.0 DNA synthesis in wild-type mouse L cells or mutant LHF cells was studied. These results are shown

Table 10: Stability and cross resistance properties of the *C. trachomatis* L2H^R-10.0 phenotype

C. trachomatis isolate[a]	ID_{50} of hydroxyurea (mM)[b]		ID_{50} of guanazole (mM)[b]	
	Inclusion formation	DNA synthesis	Inclusion formation	DNA synthesis
Wild-type L2	0.76	0.20	2.1	0.55
L2H^R-10.0$^+$	13.0	3.3	27.5	8.3
L2H^R-10.0$^-$	10.9	3.1	23.8	7.6

[a] *C. trachomatis* EBs used for infection of LHH cells were wild type and hydroxyurea-resistant L2H^R-10.0$^+$ and L2H^R-10.0$^-$. L2H^R-10.0$^+$ isolates were passaged in the presence of hydroxyurea, whereas L2H^R-10.0$^-$ isolates were passaged in the absence of selective agent for 10 growth cycles.

[b] Growth parameters used to determine ID_{50} values for both drugs are inclusion formation and DNA synthesis. All experiments were conducted with 1 μg/ml cycloheximide present in the p.i. culture medium.

in Table 11. Two different experimental conditions were used for all combinations of host cell line and *C. trachomatis* L2 isolate. Under one condition, no HU was added to the growth medium. Under the second condition, 1 mM HU was added to the cultures at 2 h p.i. Radiolabelled adenine was added at 22 h p.i. (or for uninfected cultures, [^{3}H] adenine was added 2 h after HU), incubation continued for 3 h, and then incorporation into DNA was determined. All experiments were done with subconfluent cultures in the absence of cycloheximide. [^{3}H] adenine was an efficient precursor for DNA synthesis in both logarithmically growing wild-type mouse L cells and HU-resistant LHF cells. As expected, this DNA synthesis activity was inhibited by the eukaryotic DNA polymerase inhibitor aphidicolin and unaffected by the prokaryotic DNA gyrase inhibitor norfloxacin. Addition of HU resulted in an almost complete inhibition of wild-type mouse L cell DNA synthesis. In keeping with their HU-resistant phenotype, LHF cells were still capable of synthesizing DNA in the presence of HU, and the activity was sensitive to aphidicolin but unaffected by norfloxacin. [^{3}H] adenine was also an efficient precursor for DNA replication in both host cell lines infected with wild-type *C. trachomatis*. In these cases, the detected DNA synthesis activity was inhibited by norfloxacin and aphidicolin. This indicated that both host cell and chlamydial DNA synthesis activities were being measured. When HU was added to wild-type mouse L cells infected with wild-type L2, [^{3}H] adenine incorporation was essentially abolished. In contrast, when HU was added to LHF cells infected with wild-type L2, [^{3}H] adenine was still incorporated into DNA. This incorporation was inhibited by aphidicolin but not by norfloxacin, indicating that it was host LHF-cell DNA synthesis activity. When L2H^R-10.0-infected logarithmically growing LHF cells were labelled with [^{3}H] adenine, both host- and parasite-specific DNA synthesis activities were detected. The differential effects of aphidicolin and norfloxacin indicate that the addition of HU to L2H^R-10.0-infected wild-type mouse L cells caused essentially complete inhibition of wild-type host but not mutant parasite DNA replication. However, when HU was added to L2H^R-10.0-infected LHF cells, the incorporation of [^{3}H] adenine into both host and parasite DNA continued.

Table 11: Effect of hydroxyurea on wild-type L2 and L2H^R-10.0 DNA synthesis in logarithmically growing host cells

Cell line[a]	Infection status[b]	Hydroxyurea addition[c]					
		No			Yes		
		Adenine incorporation (10^3 dpm/10^6 cells)[d]	% Incorporation remaining[e]		Adenine incorporation (10^3 dpm/10^6 cells)[d]	% Incorporation remaining[e]	
			Aph	Nor		Aph	Nor
Wild type	Uninfected	480	4	97	5.1	ND[f]	ND
	L2	300	38	60	4.2	ND	ND
	L2H^R-10.0	298	61	48	163	98	15
Hydroxyurea resistant	Uninfected	250	5	96	185	8	99
	L2	205	55	38	102	9	97
	L2H^R-10.0	233	65	29	129	58	47

[a] Cell lines used were logarithmically growing wild-type mouse L cells (1.0 x 10^6 cells per plate) or logarithmically growing hydroxyurea-resistant LHF cells (1.0 x 10^6 cells per plate).

[b] Logarithmically growing host cells were uninfected, infected with wild-type L2 *C. trachomatis* (L2), or infected with hydroxyurea-resistant L2 *C. trachomatis* (L2H^R-10.0). All experiments were conducted without cycloheximide in the p.i. culture medium.

[c] Experiments were conducted with parallel sets of cultures. To one set, no hydroxyurea was added. To the second set, 1 mM hydroxyurea was added at 2 h p.i.

[d] [^{3}H] adenine incorporation into DNA was determined after a 3 h labelling period as described in *Methods*. For infected cultures, [^{3}H] adenine was added at 22 h p.i. For uninfected cultures in the presence of hydroxyurea, [^{3}H] adenine was added 20 h after hydroxyurea. All analyses were made on duplicate dishes with values varying by less than 10%.

[e] Effect of aphidicolin (Aph, 10 μM) and norfloxacin (Nor, 200μM) on the incorporation of [^{3}H] adenine into DNA. Host cell and *C. trachomatis* DNA synthesis activities were differentiated by using aphidicolin and norfloxacin as specific inhibitors of eukaryotic and prokaryotic DNA synthesis, respectively. Inhibitors were added 2 h before the ^{3}H labelling period, and then incorporation of precursor into DNA was determined as described in *Methods*. The amount of radiolabel incorporated into DNA in the presence of inhibitor is expressed as a percentage of the uninhibited control.

[f] ND, not determined.

2. Ribonucleotide reductase activity in crude RB extracts

The results presented above provide strong evidence that *C. trachomatis* contained a class I ribonucleotide reductase. As a more direct test of this hypothesis, CDP reductase activity was assayed in crude extracts prepared from Hypaque-purified wild-type L2 and $L2H^R$-10.0 RBs. Ribonucleotide reductase activity has proved to be difficult to detect in crude extracts prepared from mammalian cells and bacteria mainly because the conditions required for the assay are also optimal for several other nucleotide-metabolizing enzymes (phosphatases, kinases, nucleotidases) that compete for the same substrate. Despite these limitations, low levels of CDP reductase activity in crude extracts, prepared from highly purified wild-type L2 RBs, was detected (Table 12).

Crude extracts prepared from MI mouse L cells did not contain a measurable level of CDP reductase activity. In contrast, activity was consistently observed in crude extracts prepared from logarithmically growing mouse L cells. The *C. trachomatis* RBs used for extract preparation were purified by Hypaque density gradient centrifugation. However, since chlamydiae are intracellular parasites, it is necessary to eliminate the possibility that the ribonucleotide reductase activity detected originated from host cell contamination. This was done by using specific monoclonal antibodies to both the large (R1) and small (R2) subunits of mammalian ribonucleotide reductase (Engstrom, 1982; Engstrom and Rozell, 1988). The anti-R1 monoclonal antibody has been shown to neutralize mouse ribonucleotide reductase activity when added to *in vitro* assays (Engstrom, 1982). Addition of these antibodies to the *in vitro* assays resulted in essentially complete inhibition of mouse L cell CDP reductase activity while having little or no effect on *C. trachomatis* activity (Table 12).

HU is an effective inhibitor of class I ribonucleotide reductases (Lammers and Follmann, 1983; Reichard, 1988; Stubbe, 1990; Reichard, 1993). When HU was added to our assay mixture, both host cell and *C. trachomatis* CDP reductase activities were inhibited. Also shown in Table 12 are the results of another series of CDP reductase assays conducted with

Table 12: Ribonucleotide reductase activity in crude extracts prepared from wild-type L2 and hydroxyurea-resistant L2H^R-10.0 *C. trachomatis* RBs

Source of crude extract[a]	Antibodies R1 and R2 (5μg each)[b]	Hydroxyurea (1.0 mM)[c]	CDP reductase activity (pmole/min/mg protein)[d]	% Activity
Logarithmically growing mouse cells	—	—	26.1 ± 3.6	100
	+	—	1.6 ± 0.9	5.9
	—	+	5.0 ± 1.8	19.0
Mock-infected mouse cells	—	—	<1.0	100
	+	—	ND[e]	ND
	—	+	ND	ND
Wild-type L2 RBs	—	—	5.1 ± 1.6	100
	+	—	4.7 ± 1.3	93
	—	+	1.7 ± 0.8	32
L2H^R-10.0 RBs	—	—	39.3 ± 5.9	100
	+	—	37.6 ± 6.1	96
	—	+	8.3 ± 2.2	21

[a] Crude extracts were prepared from the various sources as described in *Methods*.
[b] As indicated, crude extract (150 μg protein) preparations were preincubated with R1 and R2 monoclonal antibodies (15 μg of protein each for 30 min at 4°C). CDP reductase assays were initiated by the addition of complete reaction mix to the preincubated crude extract and then incubated at 37°C for 20 min.
[c] Hydroxyurea was present in the complete reaction mix as indicated.
[d] Ribonucleotide reductase assays were conducted as described in *Methods*. Each value represents the average of two experiments ± SE.
[e] ND, not determined.

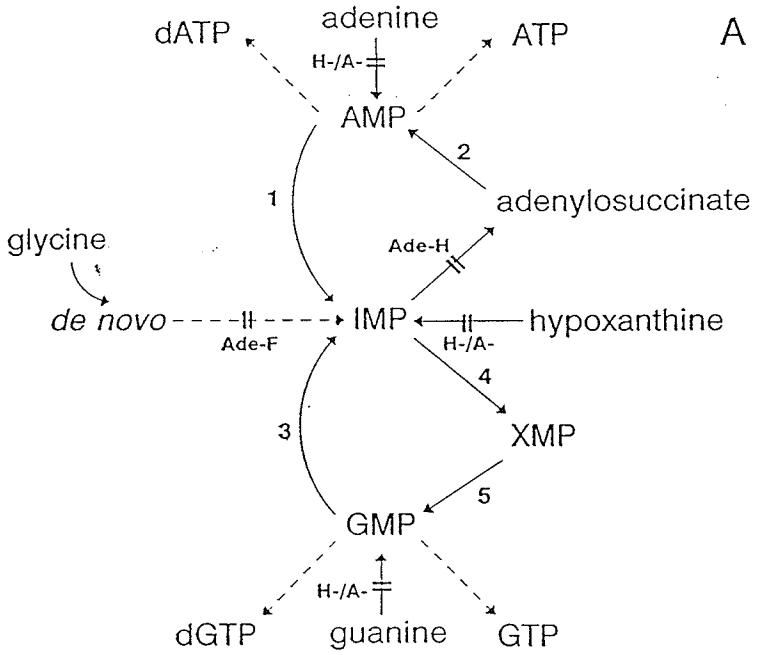
crude extract prepared from highly purified HU-resistant ($L2H^R$-10.0) RBs. The $L2H^R$-10.0 extract contained approximately eight-fold more activity than extract prepared from its wild-type L2 counterpart. Similar to the wild-type L2 activity, the $L2H^R$-10.0 CDP reductase activity was not affected by monoclonal antibodies to mammalian R1 and R2 and was inhibited by HU.

C. STUDIES OF NUCLEOTIDE METABOLISM PATHWAYS IN *C. TRACHOMATIS*

1. Effect of nucleotide deprivation on the growth of *C. trachomatis*

Work done by Hatch, and the studies presented here have indicated that *C. trachomatis* L2 and *C. psittaci* 6BC (Hatch, 1975) can, and do, draw on the nucleoside triphosphate pools of the host cell. However, these studies did not test if chlamydiae were absolutely dependent on their host for all four ribonucleoside triphosphates. To address this question the ability of *C. trachomatis* L2 to grow in a variety of mutant cell lines with well defined mutations in purine and pyrimidine biosynthesis (Figures 7A and 7B) was assessed. An initial experiment was done to determine if the size of a given nucleotide pool(s) in a particular mutant cell line could be manipulated by removing the required nucleobase(side) supplement from the culture medium.

The results presented in Table 13 indicate that the nucleoside triphosphate pool(s) in the various mutants could be decreased by depriving the cells of their required supplement for 8 h. The adenine auxotrophic Ade^-H cells, which have a mutation in adenylosuccinate synthetase, showed a reduction of the free ATP pool to 35% when cells were cultured in the absence of adenine. Similarly when the purine *de novo* synthesis deficient Ade^-F cells were starved of hypoxanthine, their total purine nucleoside triphosphate pools decreased (ATP to 42%, GTP to 35%). The pyrimidine *de novo* synthesis deficient *Urd-A* cells showed a marked decrease in pyrimidine nucleoside triphosphate pools upon starvation for uridine (CTP to 9%,


A
dATP
adenine
ATP
H-/A-
AMP
2
adenylosuccinate
1
glycine
Ade-H
de novo
Ade-F
IMP
H-/A-
hypoxanthine
4
3
XMP
5
GMP
H-/A-
dGTP
guanine
GTP

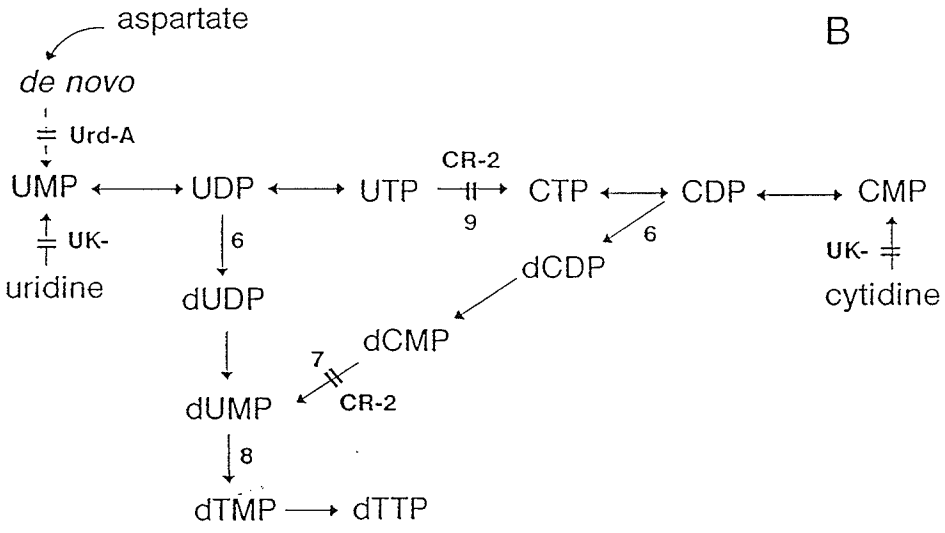

B
aspartate
de novo
Urd-A
UMP
UDP
UTP
CR-2
9
CTP
CDP
CMP
UK-
uridine
6
dUDP
6
dCDP
UK-
cytidine
dCMP
7
CR-2
dUMP
8
dTMP
dTTP

Figure 7: Major pathways of purine (A) and pyrimidine (B) nucleotide anabolic metabolism in animal cells. *De novo* synthesis and major salvage routes of anabolism are schematically represented. Not all possible routes of anabolism are included, just major routes relevant to this study. Single arrows do not necessarily imply one-step reactions; dashed arrows indicate multiple reaction pathways. Double slash represents the metabolic pathways disrupted in the particular mutant cell line. The defective enzymes in the various mutant cell lines are: AICAR formyltransferase, $Ade^{-}F$; adenylosuccinate synthetase, $Ade^{-}H$; CTP synthetase and dCMP deaminase, $CR^{-}2$; cytidine/uridine kinase, UK^{-}; HGPRT/APRT, H^{-}/A^{-}; and CAD enzyme complex, $Urd^{-}A$. The other enzymes are identified as follows: adenylate deaminase (1), adenylosuccinate lyase (2), GMP reductase (3), IMP dehydrogenase (4), GMP synthetase (5), ribonucleotide reductase (6), dCMP deaminase (7), thymidylate synthase (8), and CTP synthetase (9).

Table 13: Effect of supplement deprivation on nucleoside triphosphate pools in various CHO K1 mutant cell lines

cell line	mutation	required supplement	supplement present[a]	nucleoside triphosphate[b] (pmoles/μg DNA)			
				CTP	UTP	ATP	GTP
CHO K1	wild-type	—	—	79	187	509	80
Ade⁻H	adenylosuccinate synthetase	adenine	+	33	107	786	82
			—	73	277	275	120
Ade⁻F	purine *de novo* synthesis	hypoxanthine	+	107	220	1289	222
			—	186	452	541	77
Urd⁻A	pyrimidine *de novo* synthesis	uridine	+	217	335	1125	252
			—	19	17	1665	195
CR⁻2	CTP synthetase	cytidine	+	101	92	624	60
			—	22	116	993	88

[a]The various mutant cell lines were cultured in the presence of their required nucleobase(side) (+) supplement or were deprived of supplement (—) for 8 h prior to harvesting and nucleotide extraction.
[b]Acid soluble nucleotides were extracted, analyzed, and quantified as described in the *Methods*.

UTP to 5%). CTP synthetase deficient CR$^-$2 cells showed a decrease in CTP pool size to 22% when cytidine was not supplemented in the media. In all cases the mutant cell lines ceased growing within hours of nucleobase(side) supplement removal, however, the cell monolayers remained intact for several days.

These mutant cell lines were then used to directly assess the effect of nucleotide deprivation on *C. trachomatis* growth (Table 14). In the presence of their required supplements all of the mutant cell lines supported efficient *C. trachomatis* L2 growth, as indicated by the incorporation of nucleic acid precursor into DNA (Table 14) and by the production of a high titer of infectious EBs at the end of the 48 h growth cycle. Results from experiments with *C. trachomatis*-infected starved Ade$^-$H and Ade$^-$F cells indicate that chlamydial growth was reduced to approximately 40% when either the ATP pool or both the ATP and GTP pools were reduced. A dramatic reduction in *C. trachomatis* growth was seen when the host Urd$^-$A cells were cultured in the absence of uridine. In addition, when these infected mutant cells were cultured in the absence of nucleobase(side) supplement, host cell lysis usually occurred prior to the end of the 48 h *C. trachomatis* L2 growth cycle and few infectious EBs were recovered.

In contrast to the results presented above, *C. trachomatis* L2 grew as well in starved CR$^-$2 cells, which had a reduced CTP pool, as they did in CR$^-$2 cells cultured in the presence of cytidine (Table 14). Furthermore, infected-CR$^-$2 cultures yielded a high titer of EBs, regardless of whether they were cultured in the presence or absence of cytidine.

Table 14: Effect of nucleobase(side) deprivation on *C. trachomatis* growth in various mutant CHO K1 cell lines

host cell line[a]	required supplement	supplement present[b]	radiolabel incorporation	% of infected control[c]
Ade$^-$H	adenine	+	56.8	100
		—	22.9	40
Ade$^-$F	hypoxanthine	+	90.8	100
		—	36.5	40
Urd$^-$A	uridine	+	70.7	100
		—	9.1	13
CR$^-$2	cytidine	+	63.5	100
			63.0	99

[a]The various mutant cell lines were infected with *C. trachomatis* (3 x 10^6 cells per plate) cultured in the presence of 1 μg/ml cycloheximide.
[b]The various mutant cell lines were cultured in the presence or absence of their required nucleobase(side) supplement for 8 h prior to and during infection.
[c]The effect of nucleobase(side) deprivation on *C. trachomatis* growth was assessed by measuring incorporation of radiolabelled nucleic acid precursor into chlamydial DNA. To minimize specific activity changes, a purine nucleotide precursor ([^{3}H] adenine) was used for labelling of *C. trachomatis*-infected pyrimidine auxotrophs (Urd$^-$A and CR$^-$2), and a pyrimidine ([^{3}H] cytidine) was used for labelling infected purine auxotrophs (Ade$^-$H and Ade$^-$F). Radiolabelled precursor was added at 22 h p.i. and incubation was continued for 2 h. All analyses were made on duplicate dishes, with results varying by less than 10%. The effect of nucleobase(side) deprivation on the incorporation of radiolabel into DNA is expressed as a percentage of the non-deprived control.

2. Lack of nucleotide synthesis in *C. trachomatis*

a) *De novo* pathways

As a result of their mutant phenotypes Ade$^-$F and Urd$^-$A cells lack the ability to *de novo* synthesize purines and pyrimidines, respectively. To directly determine if *C. trachomatis* L2 can carry out *de novo* purine and/or pyrimidine nucleotide synthesis [^{14}C] glycine incorporation into purines in infected Ade$^-$F cells, and [^{14}C] aspartate incorporation into pyrimidines in infected-Urd$^-$A cells was monitored. As a control experiment the incorporation of [^{14}C] glycine and [^{14}C] aspartate into nucleic acids of logarithmically growing wild type CHO K1 cells was monitored. The results of these experiments clearly show that *C. trachomatis* L2 is incapable of *de novo* purine or pyrimidine synthesis (Figures 8A and 8B).

b) Nucleotide salvage and interconversion pathways

To test the possibility that *C. trachomatis* L2 may possess the ability to salvage certain nucleobase(side)s, we carried out a series of *in situ* radiolabelling experiments in various mutant host cell lines (Table 15). In agreement with the earlier experiments it was found that *C. trachomatis* L2 was able to utilize exogenously added adenine, guanine and hypoxanthine in wild type cells but not in the H$^-$/A$^-$ mutant mouse cell line. Similarly, *C. trachomatis* L2 could utilize cytidine and uridine when growing in wild type cells but could not utilize these precursors when growing in the mutant mouse cell line lacking cytidine/uridine kinase activity.

Using Ade$^-$H mutant host cells, which are unable to synthesize adenylate nucleotides, the ability of *C. trachomatis* to interconvert purine nucleotides was examined. When [^{14}C] guanine was added to chlamydial infected-Ade$^-$H cells, no labelled adenine appeared in *C. trachomatis* RNA or DNA, as determined by HPLC analysis of acid hydrolyzed nucleic acid.

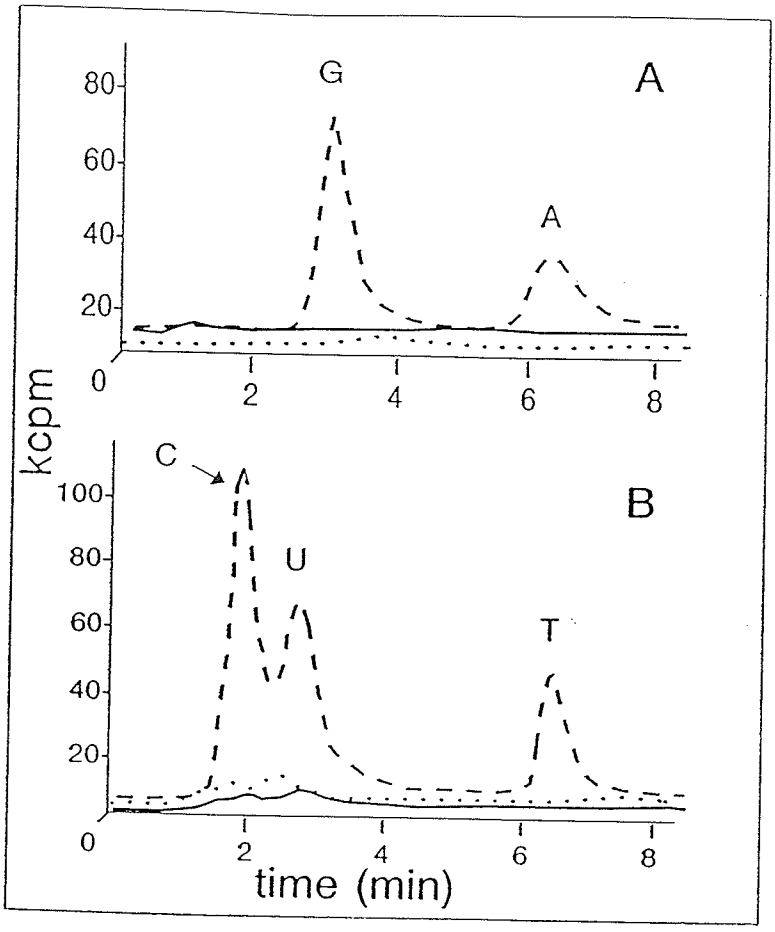


Figure 8: Incorporation of: (A) [^{14}C] glycine into nucleic acid bases of logarithmically growing CHO K1 (–), MI Ade$^-$F cells (....), and *C. trachomatis*-infected Ade$^-$F cells (—); and (B) [^{14}C] aspartic acid into nucleic acid bases of logarithmically growing CHO K1 cells (–), MI Urd$^-$A cells (....), and *C. trachomatis*-infected Urd$^-$A cells (—). Uninfected logarithmically growing CHO K1 cells, MI and *C. trachomatis*-infected Ade$^-$F and Urd$^-$A cells were cultured in the presence of radiolabel for 20 h. Nucleic acids were extracted and acid hydrolyzed to bases which were separated and analyzed by HPLC. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known adenine (A), guanine (G), cytosine (C), uracil (U), and thymine (T) standards. The positions of the free bases are indicated on the chromatogram.

Table 15: Incorporation of nucleic acid precursors into *C. trachomatis* L2 DNA in various mutant cell lines

Radiolabelled precursor added[a]	Incorporation of precursor into *C. trachomatis* DNA (10^3 dpm/10^6 cells)[b]		
	Wild type	HGPRT$^-$/ APRT$^-$	Cyt/Urd kinase$^-$
Adenine	152	4	78
Guanine	78	2	57
Hypoxanthine	133	5	66
Cytidine	82	91	2
Uridine	173	166	3

[a] The various radiolabelled precursors were added at 22 h p.i. to achieve a final concentration of 0.3 μM, then the incubation was continued for 2 h. The incorporation of ^{3}H-labelled precursor into DNA was determined as described in *Methods*. All analyses were made on duplicate dishes with results varying by less than 10%.

[b] Wild-type, HGPRT$^-$/APRT$^-$ mouse cells, and cytidine/uridine kinase deficient rat hepatoma cells were infected with *C. trachomatis* (3 x 10^6 cells/plate cultured in the presence of 1 μg/ml cycloheximide) as described in *Methods*.

Also, similar experiments using [^{14}C] hypoxanthine as precursor showed no radiolabelled adenine incorporation into *C. trachomatis* nucleic acid.

3. Evidence for cytidine nucleotide pathway enzymes in *C. trachomatis*

a) CTP synthetase

The fact that *C. trachomatis* grew efficiently in cytidine-starved CR-2 cells and lacked the ability to *de novo* synthesize pyrimidines suggested that they likely contain a CTP synthetase. To test if *C. trachomatis* L2 could convert uridine nucleotides to cytidine nucleotides, infected starved CR^{-}2 cells were labelled with [6-^{3}H] uridine (Figure 9A). Since the CR^{-}2 cell line lacks CTP synthetase activity, [6-^{3}H] uridine does not give rise to significant amounts of labelled cytosine in the nucleic acid. In contrast, when *C. trachomatis*-infected starved CR^{-}2 cells are labelled with [6-^{3}H] uridine a large amount of radiolabelled cytosine is found in the total nucleic acid suggesting the presence of a chlamydial CTP synthetase.

b) Deoxycytidine nucleotide deaminase

It has previously been shown that *C. trachomatis* L2 contains a thymidylate synthase (TS) (Fan *et al*, 1991). The dUMP required for the TS reaction can be synthesized from uridine or cytidine nucleotides, requiring ribonucleotide reduction, or ribonucleotide reduction and deoxycytidine nucleotide deamination, respectively. As shown earlier, ribonucleotide reductase is present in *C. trachomatis* L2. Since CR^{-}2 cells lack dCMP deaminase activity they cannot synthesize thymidine nucleotides from cytidine (Trudel *et al*, 1984). To determine if *C. trachomatis* L2 can obtain dUMP by deamination of deoxycytidine nucleotides the deoxynucleosides derived from the DNA of *C. trachomatis*-infected CR^{-}2 cells labelled with [^{14}C] cytidine were examined. The results of these experiments indicate that *C. trachomatis*

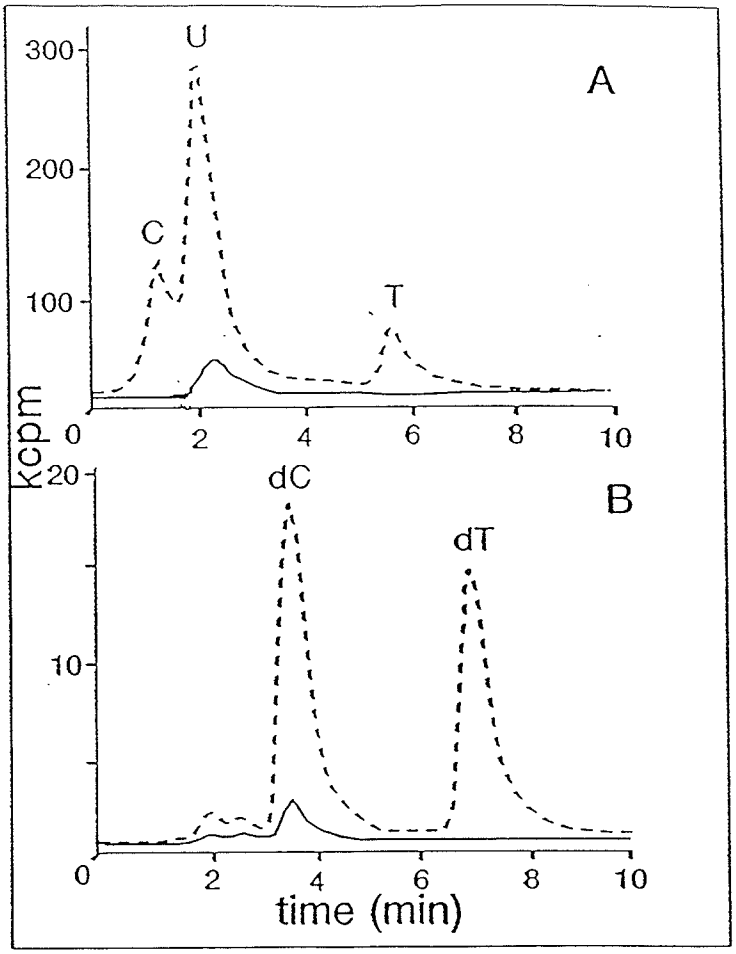


Figure 9: Incorporation of: (A) [6-^{3}H] uridine into nucleic acid bases of MI (—), and *C. trachomatis*-infected CHO CR^{-}2 cells (--); and (B) [^{14}C] cytidine into nucleic acid deoxynucleosides of MI (—), and *C. trachomatis*-infected (--) CHO CR^{-}2 cells. CHO CR^{-}2 cells were cultured in the presence of radiolabel for 20 h. Total nucleic acid was extracted and acid hydrolyzed to bases (A), or DNA was isolated and enzymatically hydrolyzed to deoxynucleosides (B) which were separated and analyzed by HPLC. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known cytosine (C), uracil (U), thymine (T), deoxycytidine (dC), and deoxythymidine (dT) standards. The identities of the peaks are indicated on the chromatograms.

infected CR^{-}2 cells do contain a substantial quantity of labelled thymidine in their DNA (Figure 9B), suggesting the presence of a *C. trachomatis* specific deoxycytidine nucleotide deaminase.

D. MOLECULAR CLONING AND BIOCHEMICAL STUDIES OF *C. TRACHOMATIS* CTP SYNTHETASE

1. Isolation of the *C. trachomatis* gene coding for CTP synthetase

a) Screening by functional complementation

C. trachomatis DNA was partially digested with Hind III and ligated into the pUC19 cloning vector. The resulting *C. trachomatis* Hind III partial digest library was then screened for CTP synthetase by functional complementation in *E. coli* JF646. JF646 is deficient in functional CTP synthetase activity (nitrosoguanidine mutagenesis) (Friesen *et al*, 1976; Friesen *et al*, 1978) and is therefore auxotrophic for cytidine. *E. coli* JF646 has previously been used to clone CTP synthetase (Weng *et al*, 1986). After transformation with the chlamydial library, two colonies were isolated which grew on selective media (containing ampicillin and lacking cytidine) and thus complemented the CTP synthetase activity deficiency of the host *E. coli* JF646. The plasmids from these complementing recombinant *E. coli* JF646 colonies were isolated and designated pH-1 and pH-2. The two plasmids appeared identical by restriction analysis. Therefore only one, pH-1, was further studied in detail. To confirm the complementation activity, pH-1 was used to transform *E. coli* JF646 and was again found to complement the CTP synthetase deficiency in the *E. coli* very well - this time giving confluent growth of recombinant colonies on the selective media (containing ampicillin and lacking cytidine). The plasmid pH-1 contained a 2.7 kbp DNA insert in the Hind III cloning site of pUC19.

b) *In vivo* CTP synthetase activity of recombinant *E. coli* JF646

In order to determine if the recombinant *E. coli* (containing pH-1) was capable of converting UTP to CTP, *in vivo* CTP synthetase activity was examined. [6-^{3}H] uracil was added to the selective growth medium (lacking cytidine) of cultures of recombinant *E. coli* JF646. The recombinant *E. coli* JF646 contained either pH-1 (containing the putative *C. trachomatis* CTP synthetase gene), pMW5 (containing the *E. coli pyrG* gene coding for CTP synthetase), or pUC19 (non-recombinant negative control). After 3 h incubation the nucleic acid was isolated and hydrolyzed to free bases and analyzed by HPLC. The results of the experiments are shown in Figure 10. Radiolabelled cytosine was found in nucleic acid derived free bases of the recombinant cultures (*E. coli* which contain either pMW5 or pH-1). The negative control culture which contained pUC19 showed no labelling of cytosine bases derived from nucleic acid. The recombinant plasmids confer the ability to convert uracil nucleotides to cytosine nucleotides in the CTP synthetase deficient *E. coli* JF646.

3. Nucleotide sequence and amino acid sequence analysis

a) Sequence analysis of pH-1

Nucleotide sequencing data was analyzed using PC/GENE software. The detection of protein coding regions in the chlamydial sequence was based on ATG or GTG start codons, and TAA, TGA, and TAG stop codons. All of the potential open reading frames (ORF) detected using this computer program are shown in Table 16.

The complete nucleotide sequence of the 2.7 kbp Hind III insert of pH-1 is shown in Figure 11. All nucleotide sequence shown was confirmed by sequencing both strands of the double stranded DNA. The GC ratio of this sequence is 41%. Analysis of this sequence for protein coding regions indicated the presence of two large overlapping ORFs, designated ORF1

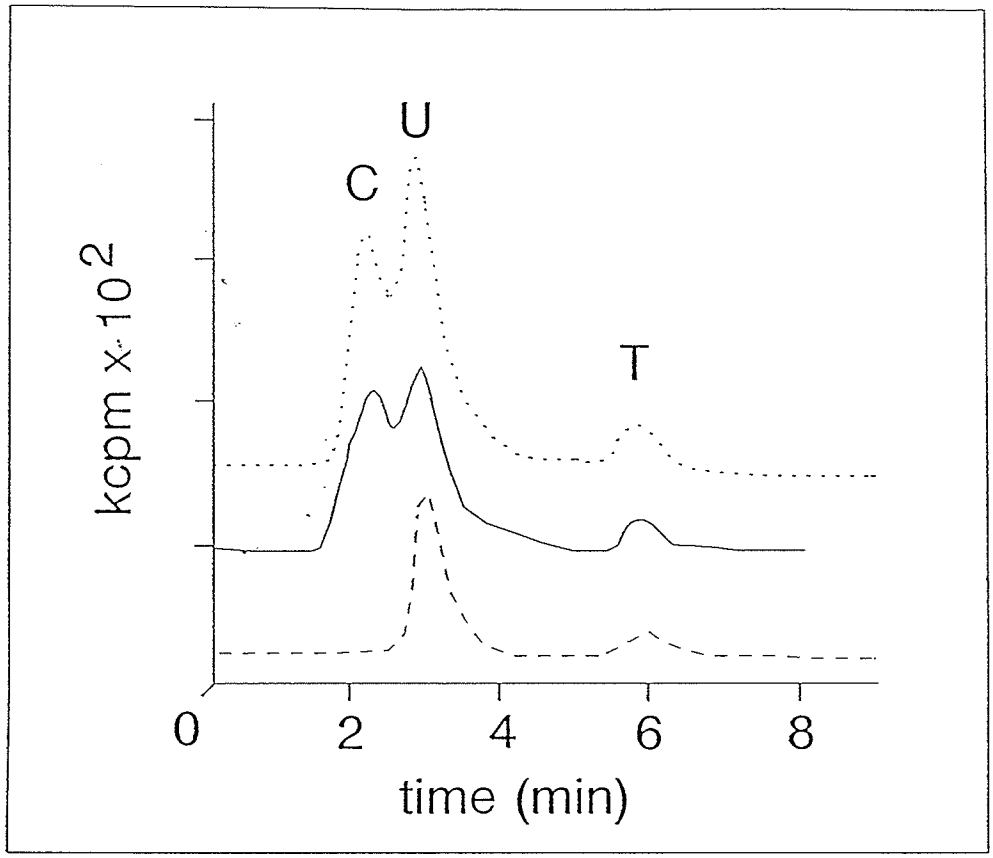


Figure 10: Incorporation of [6-^{3}H] uracil into nucleic acid of the CTP synthetase activity deficient *E. coli* JF646. *E. coli* JF646 has been transformed with pUC19 (—), pH-1 (plasmid contains chlamydial CTP synthetase) (—), or pMW5 (plasmid contains *E. coli* CTP synthetase) (....). Logarithmically growing *E. coli* JF646 were cultured in the presence of radiolabel for 3 h in minimal selective media lacking cytidine. Total nucleic acid was extracted and acid hydrolyzed to free nucleobases. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known cytosine (C), uracil (U), and thymine (T) standards as shown on the chromatograms.

Table 16: Detection of potential protein coding regions of *C. trachomatis* DNA sequenced from plasmids pH-1 and pH-11[a]

Source of DNA	Position (Base #)	Initiation Codon	Size (bp) of coding region	Size (aa) of coded peptide	Reading Frame
pH-1	801-920	GTG	120	40	3
	816-920	ATG	105	35	3
	350-1111	ATG	762	254	2
	650-1111	GTG	462	154	2
	704-1111	GTG	408	136	2
	794-1111	GTG	318	106	2
	1451-1537	ATG	87	29	2
	1463-1537	ATG	75	25	2
	2039-2104	ATG	66	22	2
	1090-2706	ATG	1617	539	1
	1273-2706	GTG	1434	478	1
	1933-2706	GTG	774	258	1
	2335-2706	ATG	372	124	1
	2374-2706	ATG	333	111	1
pH-1 and pH-11	2696-3088	ATG	393	131	2
	2906-3088	ATG	183	61	2

[a] PC/GENE software was used to detect potential open reading frames.

Figure 11: Nucleotide sequence of *C. trachomatis* obtained from pH-1 and pH-11. The deduced amino acid sequences of the three open reading frames are also shown. ORF 1 (350-1114) codes for CMP-KDO synthetase, ORF 2 (1090-2709) codes for CTP synthetase, and ORF 3 (2696-3091) codes for a protein of unknown function. Open reading frames were determined assuming the universal genetic code was followed: ATG start/methionine codon, and TAA, TGA, and TAG stop codons. The nucleotide sequence of the CTP synthetase gene for the CPEC-resistant mutant ($L2CP^R$-5.0) is identical to the wild-type sequence except for a T to G transversion point mutation at base 1536. As indicated in the figure, this mutation changes a GAT aspartic acid codon (D) for the wild-type to a GAG glutamic acid codon (E) for the mutant.

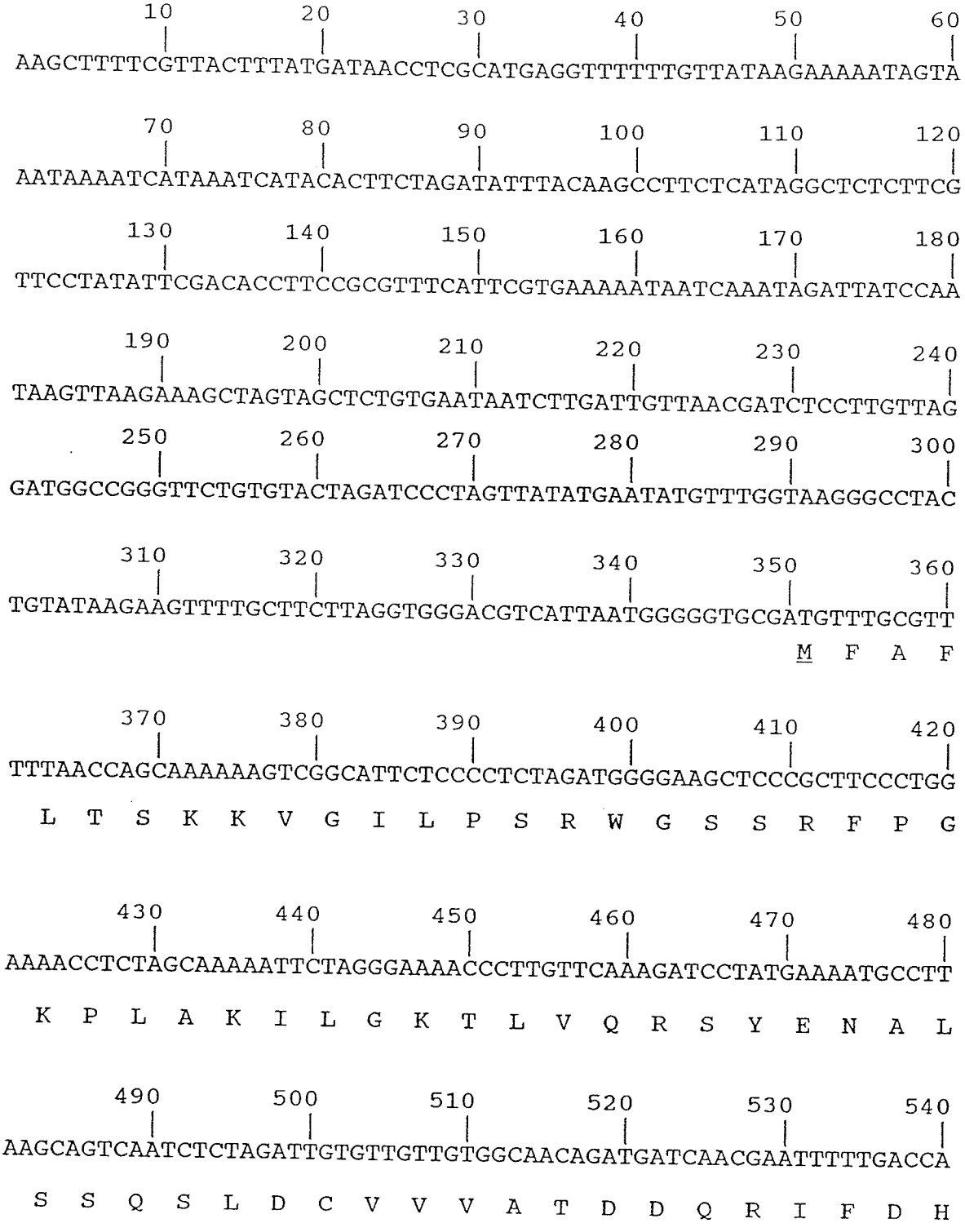

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fig 11

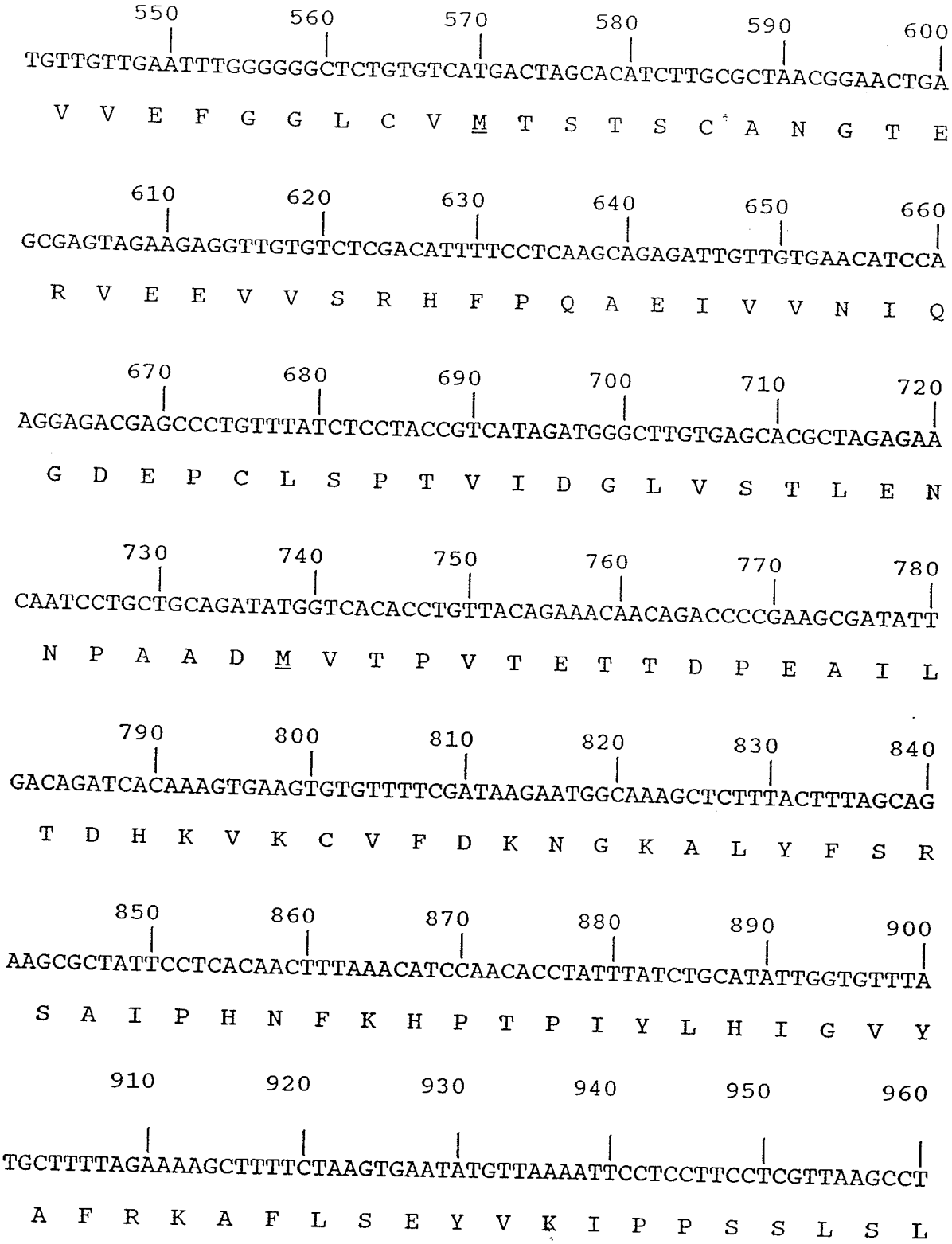
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fig 11

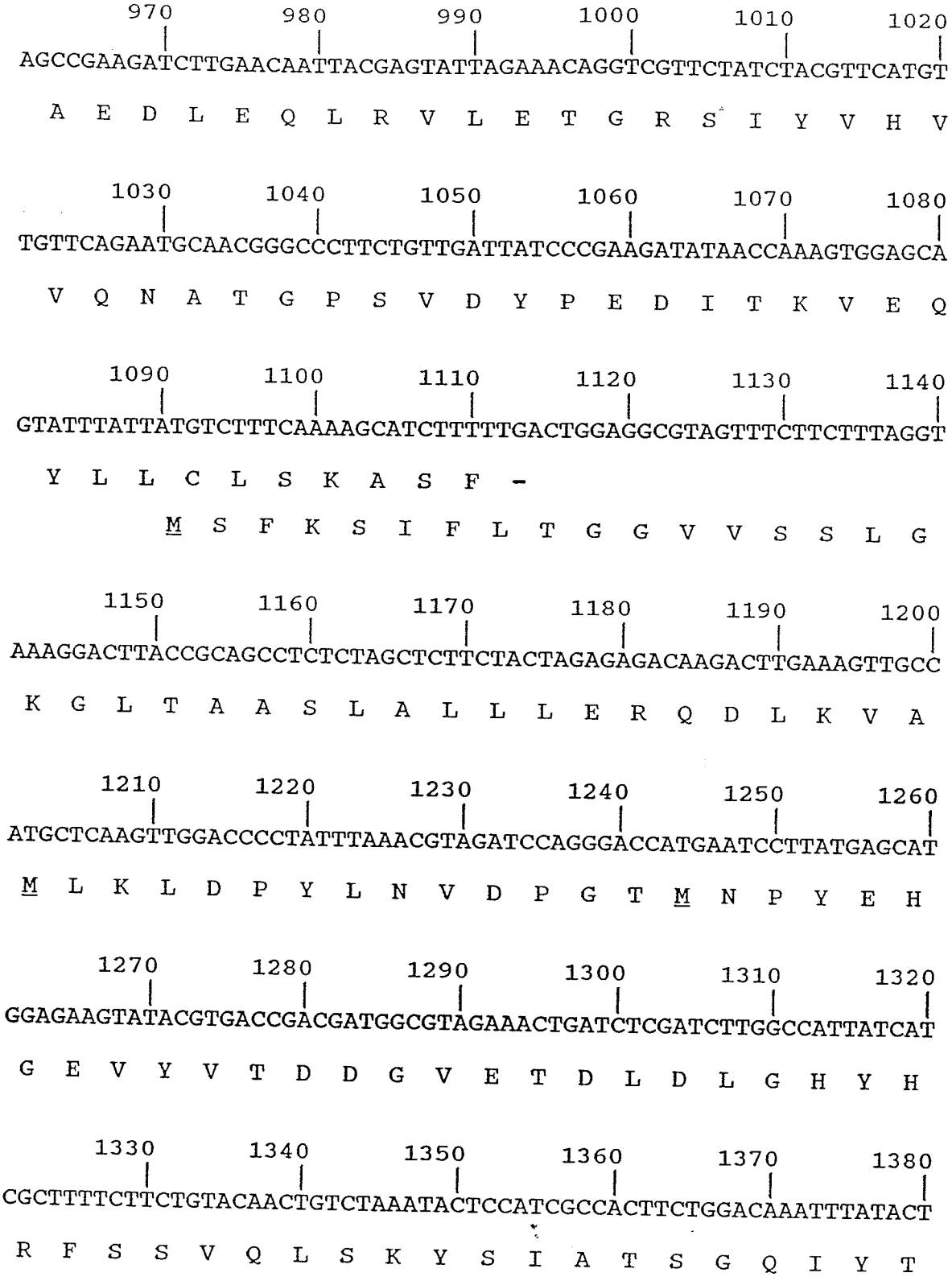
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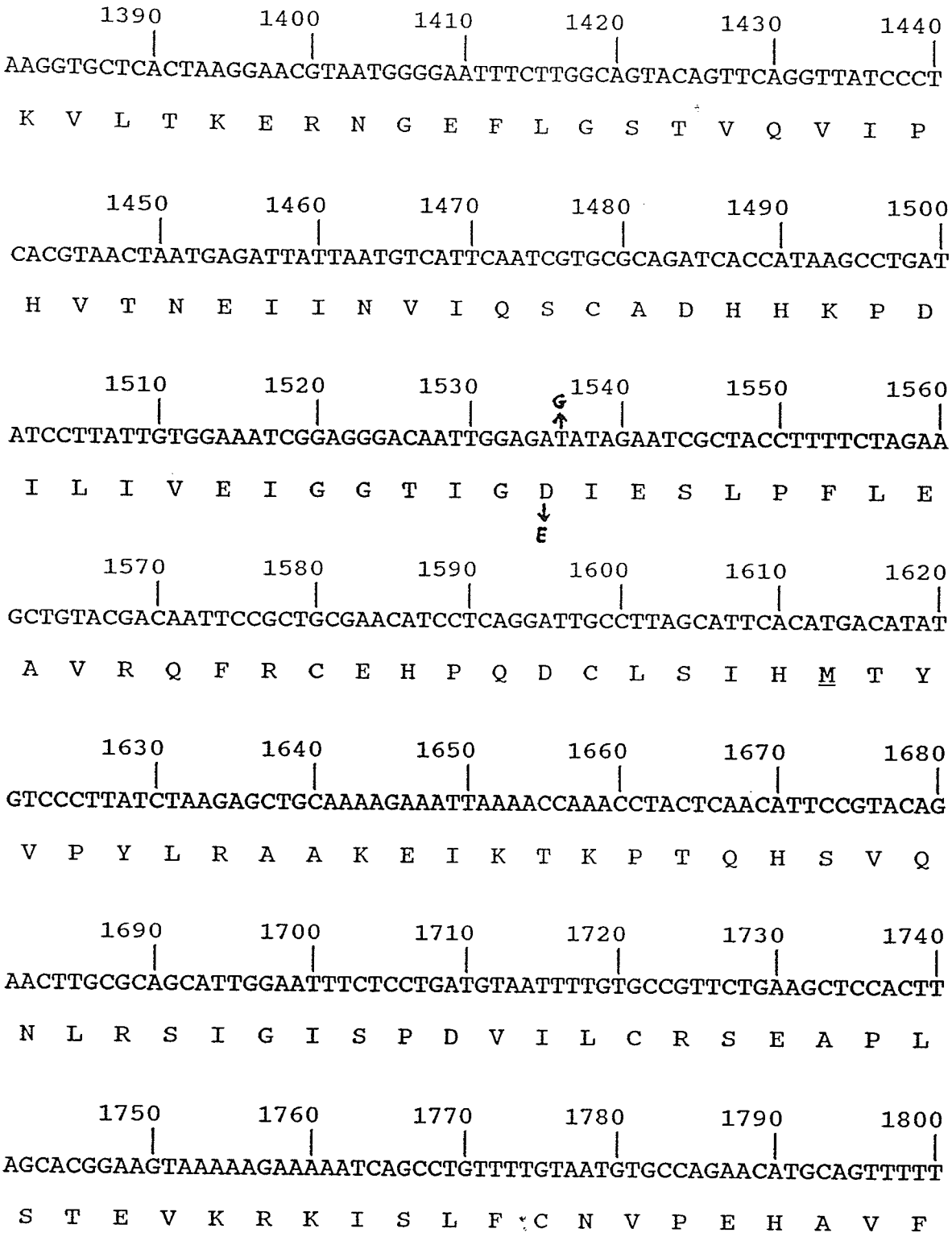
fig 11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G
E
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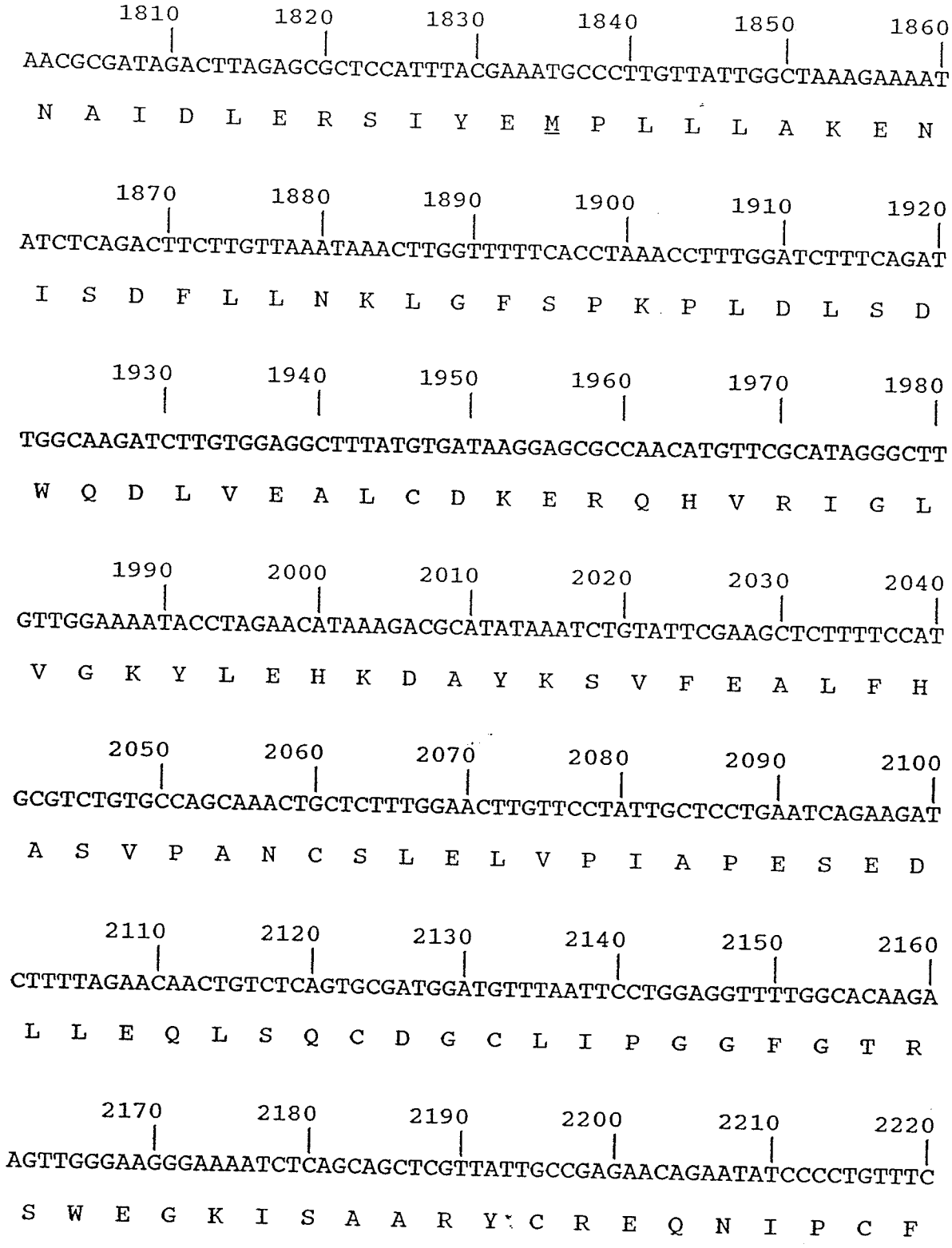
fig 11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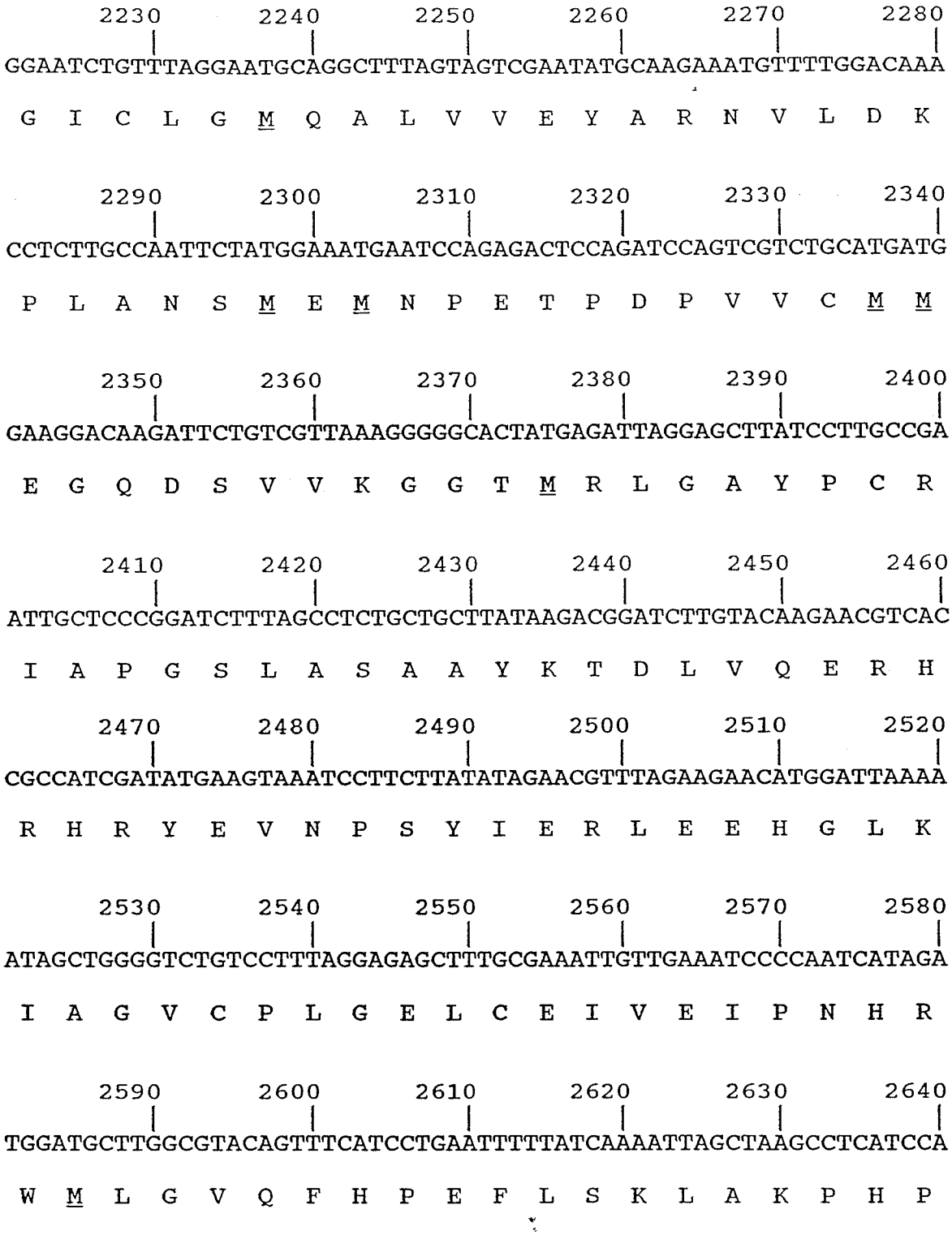
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fig 11

```
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       |          |          |          |          |          |
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                                                        M  N
 L  F  I  E  F  I  R  A  A  K  A  Y  S  L  E  K  A  N  H  E

      2710       2720       2730       2740       2750       2760
       |          |          |          |          |          |
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  I  A  K  Q  Q  Q  A  F  L  G  I  D  Y  G  K  K  R  I  G  L
H  R  -

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AGTAGTTTTCGGGAATCCATTACCTATGCAAAAAGCTTATGCTTCAAGCGTGCAATCAGA
  V  V  F  G  N  P  L  P  M  Q  K  A  Y  A  S  S  V  Q  S  E

      2950       2960       2970       2980       2990       3000
       |          |          |          |          |          |
AATTCAAGAACTAGCCGCACTCATCCAAGAAATGACTGCTATAGAAGTCATTCTTTGGGA
  I  Q  E  L  A  A  L  I  Q  E  M  T  A  I  E  V  I  L  W  D
```

fig 11

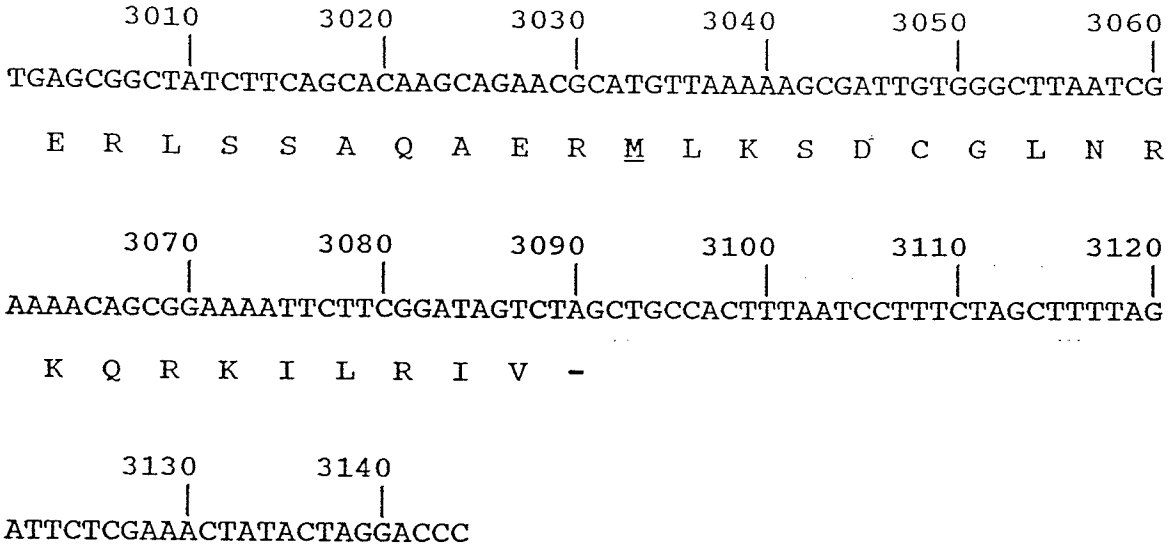

3010
3020
3030
3040
3050
3060
TGAGCGGCTATCTTCAGCACAAGCAGAACGCATGTTAAAAAGCGATTGTGGGCTTAATCG
E R L S S A Q A E R M L K S D C G L N R
3070
3080
3090
3100
3110
3120
AAAACAGCGGAAAATTCTTCGGATAGTCTAGCTGCCACTTTAATCCTTTCTAGCTTTTAG
K Q R K I L R I V -
3130
3140
ATTCTCGAAACTATACTAGGACCC

fig 11

and ORF2 (Figure 12). The amino acid sequence coded by these ORFs are shown under the corresponding nucleotide sequence in Figure 11.

The orientation of the 2.7 kbp DNA insert in pH-1 places the *lacZ* transcriptional promoter upstream of the ORFs (Figure 12). An experiment in which the orientation of the 2.7 kbp insert in the pUC19 vector was reversed failed to show complementation activity after transformation of *E. coli* JF646. This suggests that transcription of the chlamydial DNA was most likely driven by the *lacZ* promoter of pUC19.

Comparison of the amino acid sequences coded by ORF1 and ORF2 to known proteins in GenBank is shown in Table 17. The 765 bp ORF1 codes for a 254 amino acid protein with a deduced molecular weight of 28.2 kDa. This polypeptide shares 37.6% identity with the *E. coli kdsB* gene product and 34.6% identity with the *E. coli KpsU* gene product (Table 17). Both of these *E. coli* genes code for CMP-2-keto-3-deoxyoctulosonic acid synthetase (CMP-KDO synthetase). Alignment of the amino acid sequences of the two *E. coli* and *C. trachomatis* CMP-KDO synthetases is shown in Figure 13.

The 1620 bp ORF2 codes for a 539 amino acid protein with a deduced molecular weight of 59.8 kDa (Table 17). This polypeptide shares 48.1% identity with *E. coli pyrG*, 48.6% identity with *Bacillus subtilis ctrA*, 43.8% identity with the human gene coding for CTP synthetase, 40.8% identity with *Saccharomyces cerevisiae URA7*, 42.1% identity with *S. cerevisiae URA8*, 49.1% identity with the *Azospirillum brasilense* gene coding for CTP synthetase, and 44.5% identity with the *Spiroplasma citri* gene coding for CTP synthetase. All of these genes code for CTP synthetase. Alignment of the amino acid sequences of all known CTP synthetases is shown in Figure 14.

Analysis of the overlapping region between ORF1 and ORF2 indicates a 25 bp overlap between the ATG start codon of ORF2 and the TAA stop codon of ORF1 (Figure 12). A putative ribosome binding site, GGAG, is located 15 bases upstream of the ATG start of ORF2. ORF1 shows a putative ribosome binding site, GGAC, 25 bases upstream of the ATG

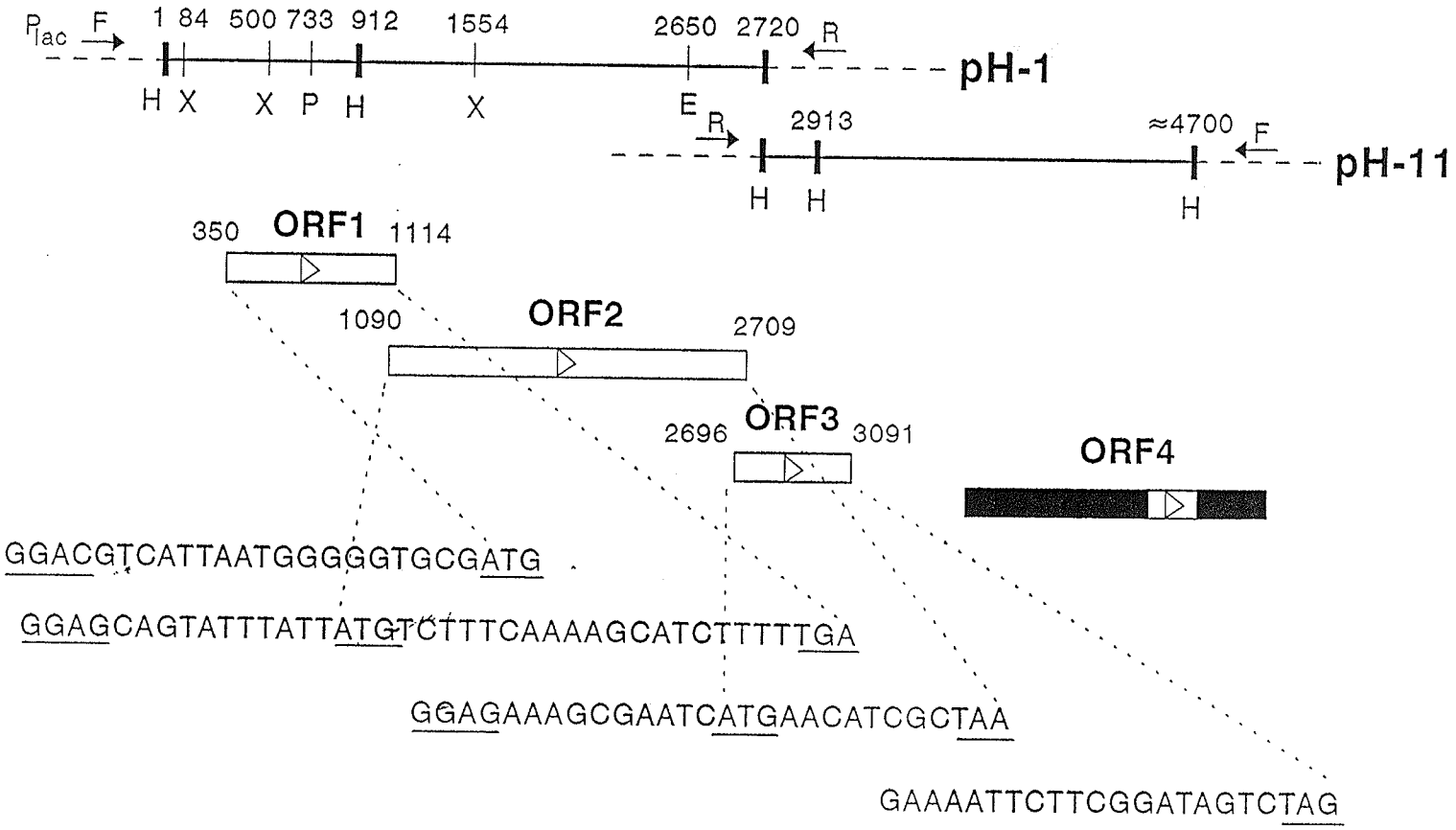

Plac F
1 84 500 733 912 1554 2650 2720 R
pH-1
H X X P H X E
R 2913 ≈4700 F
pH-11
H H H
350 ORF1 1114
1090 ORF2 2709
2696 ORF3 3091
ORF4
GGACGTCATTAATGGGGGTGCGATG
GGAGCAGTATTTATTATGTCTTTCAAAAGCATCTTTTTGA
GGAGAAAGCGAATCATGAACATCGCTAA
GAAAATTCTTCGGATAGTCTAG

Figure 12: Schematic outline of pH-1, pH-11 and overlapping open reading frames determined from the DNA sequence derived from these plasmids. pH-1 is a plasmid isolated by functional complementation using a partially digested Hind III *C. trachomatis* L2 genomic DNA library (cloned into the pUC19 cloning vector) to transform CTP synthetase deficient *E. coli* JF646. pH-11 was isolated by oligonucleotide probe hybridization screening of a partially digested Hind III *C. trachomatis* DNA library. The thicker solid line represents the chlamydial DNA insert and the thinner dashed line represents the pUC19 cloning vector. Selected restriction enzyme sites are marked: H, Hind III; X, Xba I; P, Pst I; and E, Eco R1. Forward (F) and reverse (R) primers, as well as the *lacZ* transcriptional promoter (P_{lac}) of the pUC19 vector are also shown. The open reading frames are represented by open rectangles and correspond to the genes for CMP-KDO synthetase (ORF 1), CTP synthetase (ORF 2), unknown (ORF 3), and glucose-6-phosphate dehydrogenase (ORF 4). ORF 4 has only been partially sequenced (open rectangle) - the expected size (i.e. not sequenced) of ORF 4 is shown by the solid rectangle. Direction of ORFs are indicated (5' ▷ 3'). The expanded regions of the gene boundaries are shown to indicate relative positions of the putative ribosome binding sites, GGA(G or C), the translational start sites (ATG), and the translational stop sites (TAG, TAA, or TGA).

Table 17: Comparisn of amino acid sequences coded by cloned open reading frames to known proteins

Open Reading Frame	# amino acids	Molecular Weight (kDa)	Enzyme	% Identity to known protein	Organism and gene	Size of known protein #amino acids (kDa)	Reference
ORF 1	254	28.2	CMP-KDO synthetase	37.6	*E. coli* kds B	248aa (27.4kDa)	Goldman *et al*, 1986
				34.6	*E. coli* KpsU	246aa (27.1kDa)	Pazzani *et al*, 1993
ORF2	539	59.8	CTP synthetase	48.1	*E. coli* pyr G	544aa (60.3kDa)	Weng *et al*, 1986
				48.6	*B. subtilis* ctr A	535aa (59.7kDa)	Trach *et al*, 1988
				43.8	human	591aa (66.7kDa)	Yamauchi *et al*, 1990
				40.8	*S. cerevesia* URA 7	570aa (63.6kDa)	Ozier-Kalogeropoulos *et al*, 1991
				42.1	*S. cerevisia* URA 8	577aa (64.6kDa)	Ozier-Kalogeropoulos *et al*, 1994
				49.1	*A. brasilense*	544aa (59.9kDa)	Zimmer and Hundeshagen, 1992
				44.5	*Spiroplasma citri*	535aa (59.9kDa)	Citti *et al*, 1993
ORF 3	131	14.5	?				
ORF 4[a]	NA		Glucose-6-phosphate 1-dehydrogenase	43.2	*E. coli* zwf	490aa (55.6kDa)	Rowley and Wolf, 1991

[a] This open reading frame was only partially sequenced. Therefore, the 43.2% identity of ORF 4 to *E. coli* glucose-6-phosphate 1-dehydrogenase is based on only a 125 amino acid overlap between the two sequences.

```
ECOKPSU     M-----SKAVIVIPARYGSSRLPGKPLLDIVGKPMIQHVYERALQVAGVA    45
ECOKDS      M-----S-FVVIIPARYASTRLPGKPLVDINGKPMIVHVLERA-RESGAE    43
L2LPS       MFAFLTSKKVGILPSRWGSSRFPGKPLAKILGKTLVQRSYENALSSQSLD    50
            *     *  * ..*.*..*.*.***** .* **... .  *.*    . .

ECOKPSU     EVWVATDDPRVEQAVQAFGGKAIMTRNDHESGTDRLVEVMHK---VEADI    92
ECOKDS      RIIVATDHEDVARAVEAAGGEVCMTRADHQSGTERLAEVVEKCAFSDDTV    93
L2LPS       CVVVATDDQRIFDHVVEFGGLCVMTSTSCANGTERVEEVVSR-HFPQAEI    99
             . ****.  .   * . **   **... ..**.*. **. .    ----

ECOKPSU     YINLQGDEPMIRPRDVETLLQGMRDDPALPVATLCHAISAAEAA-EPSTV   141
ECOKDS      IVNVQGDEPMIPATIIRQVADNL-AQRQVGMATLAVPIHNAEEAFNPNAV   142
L2LPS       VVNIQGDEPCLSPTVIDGLVSTLENNPAADMVTPVTETTDPEAILTDHKV   149
             .*.***** ...  .  .  .. ....  ..*    . ..*.  .   *

ECOKPSU     KVVVNTRQDALYFSRSPIPYPRN-------AEKARYLKHVGIYAYRRDVL   184
ECOKDS      KVVLDAEGYALYFSRATIPWDRDRFAEGLETVGDNFLRHLGIYGYRAGFI   192
L2LPS       KCVFDKNGKALYFSRSAIP-------HNFKHPTPIYL-HIGVYAFRKAFL   191
            * * .    ******..**                  .* *.*.*..* . .

ECOKPSU     QNYSQLPESMPEQAESLEQLRLMNAGINIR-TFEVAATGPGVDTPACLEK   233
ECOKDS      RRYVNWQPSPLEHIEMLEQLRVLWYGEKIHVAVAQEVPGTGVDTPEDLER   242
L2LPS       SEYVKIPPSSLSLAEDLEQLRVLETGRSIYVHVVQNATGPSVDYPEDITK   241
              * . . *  .  * *****..  * .*      ...*..** *. ...

ECOKPSU     VRALMAQELAENA   246
ECOKDS      VRAEMR-------   248
L2LPS       VEQYLLCLSKASF   254
            * . .
```

Figure 13: Comparison of deduced amino acid sequence of CMP-KDO synthetases of *E. coli KpsU*, ECOKPSU (Pazzani *et al*, 1993); *E. coli kdsB*, ECOKDS (Goldman *et al*, 1986); and *C. trachomatis* L2, L2LPS. Gaps are used to give the best alignment. Highly conserved amino acid residues (identical in all sequences) are indicated by (*), and related amino acids are shown by (.).

Figure 14: Comparison of deduced amino acid sequence of CTP synthetases of *Chlamydia trachomatis* L2, L2CTPS; *Bacillus subtilis*, BSCTPS (Trach *et al*, 1988); *Escherichia coli*, ECCTPS (Weng *et al*, 1986); *Azospirillum brasilense*, ABCTPS (Zimmer and Hundeshagen, 1992); *Spiroplasma citri*, SMECTPS (Citti *et al*, 1993); *Saccharomyces cerevisia URA7*, SC7CTPS (Ozier-Kalogeropoulos *et al*, 1991); *Saccharomyces cerevisia URA8*, SC8CTPS (Ozier-Kalogeropoulos *et al*, 1994); and human, HUCTPS (Yamauchi *et al*, 1990). Highly conserved amino acids identical in all sequences shown are indicated by (*). Related amino acids are indicated by (.). As indicated by □ on the alignment, the highly conserved aspartic acid (D) at position 149 of the chlamydial CTP synthetase is mutated to glutamic acid (E) in the CPEC-resistant *C. trachomatis* mutant. Seven mutation sites found by Whelan *et al* (1993), indicated by (↓), confer multidrug resistance to mammalian CTP synthetase. Also indicated on the alignment (▼) are the residues of the glutamine amidotransferase domain consensus sequence (Ozier-Kalogeropoulos *et al*, 1991):

(NH_2 terminus)—....G....G-C-G-Q....HPE....—(CO_2H terninus).

```
L2CTPS      MSFKSIFLTGGVVSSLGKGLTAASLALLLERQDLKVAMLKLDPYLNVDPG   50
BSCTPS      MT-KYIFVTGGVVSSLGKGIVAASLGRLLKNRGLNVTIQKFDPYINVDPG   49
ECCTPS      MTTNYIFVTGGVVSSLGKGIAAASLAAILEARGLNVTIMKLDPYINVDPG   50
ABCTPS      MT-RYIFITGGVVSSLGKGLASAALGALLQARGYKVRLAKLDPYLNVDPG   49
SMECTPS     MA-KYIFVTGGVVSGLGKGITASSIGVLLKASGLNVFMQKFDPYLNVDPG   49
SC7CTPS     M--KYVVVSGGVISGIGKGVLASSTGMLMKTLGLKVTSIKIDPYMNIDAG   48
SC8CTPS     M--KYVVVSGGVISGIGKGVLASSTGMLLKTLGLKVTSIKIDPYMNIDAG   48
HUCTPS      M--KYILVTGGVISGIGKGIIASSVGTILKSCGLHVTSIKIDPYINIDAG   48
            *  . . ..***.*..***. ... . ...  . .*   *.***.*.*.*

L2CTPS      TMNPYEHGEVYVTDDGVETDLDLGHYHRFSSVQLSKYSIATSGQIYTKVL  100
BSCTPS      TMSPYQHGEVFVTDDGAETDLDLGHYERFIDINLNKFSNVTTGKIYSTVL   99
ECCTPS      TMSPIQHGEVFVTEDGAETDLDLGHYERFIRTKMSRRNNFTTGRIYSDVL  100
ABCTPS      TMSPYQHGEVYVTDDGAETDLDLGHYERFTGVAARRGDNITTGRIYSNVI   99
SMECTPS     TMSPYQHGEVFVTTDGAETDLDLGHYERFIDENLTKESNITSGRMYKNVI   99
SC7CTPS     TMSPLEHGECFVLDDGGETDLDLGNYERYLGVTLTKDHNITTGKIYSHVI   98
SC8CTPS     TMSPLEHGECFVLDDGGETDLDLGNYERYLGITLSRDHNITTGKIYSQLI   98
HUCTPS      TFSPYEHGEVFVLDDGGEVDLDLGNYERFLDIRLTKDNNLTTGKIYQYVI   98
            *..* .*** .* .** *.*****.*.*.      .    *.*..*  ..

                        ↓
L2CTPS      TKERNGEFLGSTVQVIPHVTNEIINVIQSCA------DHHKPDILIVEIG  144
BSCTPS      KKERRGDYLGGTVQVIPHITNELKDRVYRAG------KETNADVVITEIG  143
ECCTPS      RKERRGDYLGATVQVIPHITNAIKERVLEGG------EGH--DVVLVEIG  142
ABCTPS      AKERRGDYLGATVQVIPHVTDQIKD--FIGA------ETTDEDFILCEIG  141
SMECTPS     EKERRGEYDGGTVQVVPHVTNEIKKKVYHAA------STSKADIIITEIG  143
SC7CTPS     AKERKGDYLGKTVQIVPHLTNAIQDWIERVAKIPVDDTGMEPDVCIIELG  148
SC8CTPS     SRERRGDYLGKTVQIVPHLTNAIQDWIQRVSKIPVDDTGLEPDVCIIELG  148
HUCTPS      NKERKGDYLGKTVQVVPHITDAIQEWVMRQALIPVDEDGLEPQVCVIELG  148
              .**.*.. * ***..**.*... .     .       .    . . *.*

            ↓ ↓  ↓  ↓  ↓
L2CTPS      GTIGDIESLPFLEAVRQFRCEH-PQDCLSIHMTYVPYLRAAKEIKTKPTQ  193
BSCTPS      GTVGDIESLPFLEAIRQMKSDIGRENVMYIHCTLVPYIKAAGELKTKPTQ  193
ECCTPS      GTVGDIESLPFLEAIRQMAVEIGREHTLFMHLTLVPYMAASGEVKTKPTQ  192
ABCTPS      GTVGDIESTPFLEAIRQFGNEVGPENALFIHLTLLPYIPTAGELKTKPTQ  191
SMECTPS     GTVGDIESLPFIEAIRQVRMEQGRENVIYMHVSLVPYIAASKESKTKPTQ  193
SC7CTPS     GTVGDIESAPFVEALRQFQFKVGKENFALIHVSLVP--VIHGEQKTKPTQ  196
SC8CTPS     GTVGDIESAPFVEALRQFQFEVGRENFALIHVSLVP--VIHGEQKTKPTQ  196
HUCTPS      GTVGDIESMPFIEAFRQFQFKVKRENFCNIHVSLVPQPSSTGEQKTKPTQ  198
            **.***** **.** **    .   ..   .* . .*       * ******
```

fig 14

```
                                                    ↓
L2CTPS      HSVQNLRSIGISPDVILCRSEAPLSTEVKRKISLFCNVPEHAVFNAIDLE   243
BSCTPS      HSVKELRSLGIQPNIIVVRTEMPISQDMKDKIALFCDIDTKAVIECEDAD   243
ECCTPS      HSVKELLSIGIQPDILICRSDRAVPANERAKIALFCNVPEKAVISLKDVD   242
ABCTPS      HSVKELLGMGIQANILLCRADRPIPENERKKIALFCNIRPERVIAALDVD   241
SMECTPS     HSVRETLSTGIQPDIVVARTEQATDDNVTEKIATFCNIEKSNVLVATDVA   243
SC7CTPS     AAIKGLRSLGLVPDMIACRCSETLDKPTIDKIAMFCHVGPEQVVNVHDVN   246
SC8CTPS     AAIKDLRLLGLIPDMIACRCSEELNRSTIDKIAMFCHVGPEQVVNVHDVN   246
HUCTPS      NSVRELRGLGLSPDLVVCRCSNPLDTSVKEKISMFCHVEPEQVICVHDVS   248
             ....    *. ....  * .          **. **..    *    * .

L2CTPS      RSIYEMPLLLAKENISDFLLNKLGFS----------PKPLDLSDWQDLVE   283
BSCTPS      -NLYSIPLELQKQGLDKLVCEHMKLAC---------KE-AEMSEWKELVN   282
ECCTPS      -SIYKIPGLLKSQGLDDYICKRFSLNC---------PE-ANLSEWEQVIF   281
ABCTPS      -SIYQVPVSYHEEGFDTQVLAYFGLPT---------EGKPDLSRWTSIVE   281
SMECTPS     -SILWSHKMY-EQNAQMVISKLLNLKS---------T-KTDMSEWKRFVE   281
SC7CTPS     -STYHVPLLLLEQKMIDYLHARLKLDEISLTEEEKQRGLELLSKWKATTG   295
SC8CTPS     -STYHVPLLLLKQHMIDYLHSRLKLGEVPLTLEDKERGSQLLTNWENMTK   295
HUCTPS      -SIYRVPLLLEEQGVVDYFLRRLDL-----PIERQPR--KMLMKWKEMAD   290
             .    .    ..          . .                .  *

L2CTPS      ALCDKERQHVRIGLVGKYLEHKDAYKSVFEALFHASVPANCSLELVPIAP   333
BSCTPS      KV-SNLSQTITIGLVGKYVELPDAYISVVESLRHAGYAFDTDVKVKWINA   331
ECCTPS      EE-ANPVSEVTIGMVGKYIELPDAYKSVIEALKHGGLKNRVSVNIKLIDS   330
ABCTPS      RV-RKPQGEVTIAVVGKYTSLLDSYKSLAEALTHGGIANNVKVKLDWIDS   330
SMECTPS     KI-NQSQQVIEIKLVGKYIETPDAYLSVSESLRIAGYENKVKIKIDWIKA   330
SC7CTPS     NF-DESMETVKIRLVGKYTNLKDSYLSVIKALEHSSMKCRRKLDIKWVEA   344
SC8CTPS     NL-DDSDDVVKIALVGKYTNLKDSYLSVTKSLEHASMKCRRQLEILWVEA   344
HUCTPS      RY-DRLLETCSIALVAKYTEFSDSYASVIKALEHSALAINHKLEIKYIDS   339
                      * .*.** .  *.* *. ..*  ..      ...  . .

                                                    ▼
L2CTPS      E---SEDLL----------EQLSQCDGCLIPGGFGTRS-----WEGKISA   365
BSCTPS      EEVTENNIA----------ELTSGTDGIIVPGGFGDRGVEG-----KIVA   366
ECCTPS      QDVETRGLE----------ILK-GLDAILVPGGFGYRGVEG-----MITT   364
ABCTPS      EIFEDESAV----------QRLENVHGILVPGGFGSRGTEG-----KIRA   365
SMECTPS     EDVNKKNDQ----------QLLKNAKGILVPGGFDERGFEGRGFEGKILA   370
SC7CTPS     TDLEPEAQESNKTKFHEAWNMVSTADGILIPGGFGVRGTEG-----MVLA   389
SC8CTPS     SNLEPETQEVDKNKFHDSWNKLSSADGILVPGGFGTRGIEG-----MILA   389
HUCTPS      ADLEPITSQEEPVRYHEAWQKLCSAHGVLVPGGFGVRGTEG-----KIQA   384
                   .                  .. ..****. *.         .. .

                                ▼  ▼  ▼  ▼
L2CTPS      ARYCREQNIPCFGICLGMQALVVEYARNVLDKPLANSMEMNPETPDPVVC   415
BSCTPS      TKYARENNIPFLGICLGMQVASIEYARNVLGLKGAHSAEIDPSTQYPIID   416
ECCTPS      ARFARENNIPYLGICLGMQVALIDYARHVANMENANSTEFVPDCKYPVVA   414
ABCTPS      AQFARERKVPYFGICFGMQMAVIESARNMAGIVDAGSTELGKPGN-PVVG   414
SMECTPS     CQFARENNIPFFGICFGMQAAVIEFARNVCHIQDANSSELT-ETKNAIID   419
SC7CTPS     ARWARENHIPFLGVCLGLQIATIEFTRSSLGRKDSHSAEFYPD-------   432
SC8CTPS     AKWARESGVPFLGVCLGLQVAAIEFARNVIGRPNSSSTEFLDET------   433
HUCTPS      IAWARNQKKPFLGVCLGMQLAVVEFSRNVLGWQDANSTEFDPTT------   428
              . *.   * .*.*.*.*   .. .*       . * *.
```

fig 14

```
L2CTPS      MM-------EGQDSVVK-------GGTMRLGAYPCRIAPGS---LASAAY   448
BSCTPS      LL---PE----------QKDVEDLGGTLRLGLYPCKLEEGT---KAFEVY   450
ECCTPS      LI---TEWRDENGNVEVRSEKSDLGGTMRLGAQQCQLVDDS---LVRQLY   458
ABCTPS      LLGLMTEWMRGNS-LEKRTEGTDVGGTMRLGTYPAKLVPGS---KVAEVY   460
SMECTPS     II-------------RGKDKTDALGGTLRLGNYKTTFVPNT---LAHKLY   453
SC7CTPS     ----IDE-KNHVVVFMPEIDKETMGGSMRLGLRPTFFQNETEWSQIKKLY   477
SC8CTPS     ---LLAP-EDPSSHIHAEIDKEHMGGTMRLGLRPTIFQPNSEWSNIRKLY   479
HUCTPS      --------SHPVVVDMPEHNPGQMGGTMRLGKRRTLFQTKN--SVMRKLY   468
                                  **..***     .   .        *

L2CTPS      KT-DLVQERHRHRYEVNPSYIERLEEHGLKIAGVCPLGELCEIVEIPNHR   497
BSCTPS      QD-EVVYERHRHRYEFNNEFRQQMEEQGFVFSGTSPDGRLVEIIELKDHP   499
ECCTPS      NA-PTIVERHRHRYEVNNSLLKQIEDAGLRVAGRSGDDQLVEIIEVPNHP   507
ABCTPS      GT-TDITERHRHRYEVNVYYKDRLEKVGLLFSGLSPT-QLPEIVEIPDHP   508
SMECTPS     GK-KWGFRTSRHRYEVNNDYREQLAQAGTVFSGTYVEKNLVEVIEIPKYP   502
SC7CTPS     GDVSEVHERHRHRYEINPKMVDELENNGLIFVGKDDTGKRCEILELKNHP   527
SC8CTPS     GEVNEVHERHRHRYEINPKIVNDMESRGFIFVGKDETGQRCEIFELKGHP   529
HUCTPS      GDADYLEERHRHRFEVNPVWKKCLEEQGLKFVGQDVEGERMEIVELEDHP   518
                     ***.* *    . ..  *    *         *. *.  ..

                    ▼▼▼
L2CTPS      WMLGVQFHPEFLSKLAKPHPLFIEFIRAAKA----Y--------------   529
BSCTPS      WFVASQFHPEFKSRPTRPQPLFKGFIGAS--------------------   528
ECCTPS      WFVACQFHPEFTSTPRDGHPLFAGFVKAA--------------------   536
ABCTPS      WFIGVQFHPELKSKPFDPHPLFTSFIKAA--------------------   537
SMECTPS     FYTAAQYHPEFTSRPNKPNPLFNGFVQAV--------------------   531
SC7CTPS     ---------EYTSKVLDPSKPFLGLVAASAGIL------------QDVIE   556
SC8CTPS     YYVGTQYHPEYTSKVLEPSRPFWGLVAQ----L------------RHTCE   563
HUCTPS      FFVGVQYHPEFLSRPIKPSPPYFGLLLASVGRLSHYLQKGCRLSPRDTYS   568
                     *  *         . ... .

L2CTPS      -------------SLEKANHEHR   539
BSCTPS      --------------VEAANQ--K   535
ECCTPS      --------------SEFQKRQAK   545
ABCTPS      --------------IE--QSRLV   544
SMECTPS     --------------I-----KNK   535
SC7CTPS     GKYDLEAGENKF---------NF   570
SC8CTPS     VIKDINLSEGNE---------NE   577
HUCTPS      DRSGSSSPDSEITELKFPSINHD   591
```

fig 14

start codon. There appeared to be an ATG start codon upstream of the ORF2 TAA stop codon creating a 14 bp overlap for a possible third ORF.

b) Isolation and sequence analysis of a Hind III fragment downstream of ORF2

The arrangement of the cloned genes in an operon seemed likely considering the overlapped arrangement of the genes. To determine whether a third ORF existed downstream from ORF2 it was necessary to screen the *C. trachomatis* Hind III partial digest library for the downstream DNA fragment.

The strategy used to obtain this fragment was to sequence *C. trachomatis* genomic DNA downstream of ORF2 using an oligonucleotide primer ("CKS12" 5' GCGAATCATGAA CATCG 3') running off of ORF2. From this sequence, an oligonucleotide primer was made ("RCKS13" 5' GGTAATGGATTCCCGAA 3') and subsequently used to generate a PCR product (≈350 bp) using purified *C. trachomatis* DNA as the template and one of the ORF2 sequencing primers ("CKS11" 5' CTTTAGGAGAGCTTTGCG 3') as the second PCR primer. This PCR product was then used as a probe to screen the *C. trachomatis* Hind III partial digest library by hybridization and the desired downstream fragment, designated pH-11 (≈1.7 kbp insert), was isolated.

The chlamydial DNA fragment contained in pH-11 is situated immediately downstream of the pH-1 chlamydial DNA fragment. There is a 6 bp overlap between the two sequences corresponding to the Hind III restriction site (both fragments were isolated from Hind III digested *C. trachomatis* libraries) - this was confirmed by sequencing *C. trachomatis* genomic DNA. Potential protein coding sequences for the chlamydial DNA contained in pH-11 are shown in Table 16.

Sequence analysis of pH-11 indicated a third ORF (ORF3) of 396 bp existed downstream of ORF2 (Figure 11). This putative ORF coded for a 131 amino acid polypeptide. No significant amino acid identity nor nucleotide sequence homology was found to any

sequence in GenBank (Table 17). PC/GENE analysis indicated that the deduced polypeptide would have molecular weight of 14.4 kDa, an isoelectric point of 10.27 (highly charged at pH 7.0), and may possibly be an integral membrane protein since it is predicted to contain one transmembrane segment (according to the method of Klein, Kanehisa and DeLisi in PC/GENE).

Analysis of sequence coming in from the other end of pH-11 (i.e. from the pUC19 forward primer end) indicated a possible fourth ORF (ORF4) had been partially sequenced (Figure 12). Amino acid sequence comparison to sequences in GenBank showed 43.2% identity in a 125 base overlap to *E. coli zwf* which codes for a 55.6 kDa glucose-6-phosphate 1-dehydrogenase (Table 17). As indicated in Figure 12, the orientation of ORF4 is in the same direction as ORFs 1 to 3. Although the 125 bases sequenced did not contain stop or start codons (both are required to define a particular ORF), this partially sequenced region was designated ORF4 for simplicity.

It appears that the putative operon consists of only ORF1, ORF2 and ORF3 for two reasons: (1) no ATG start codon, nor any putative ribosome binding site are evident either upstream or downstream of the TAG stop codon of ORF3, and (2) nucleotide sequence immediately upstream of ORF1 and immediately downstream of ORF3 indicate many stop codons are present in all reading frames.

3. Southern hybridization

Further evidence that the cloned CTP synthetase gene (ORF2) was *C. trachomatis* specific was obtained by doing a Southern blot hybridization. Flanking oligonucleotide primers to ORF2 were designed:

5'CTPS primer 5' GGGGAAGCTTGGAGCAGTATTTATTATGTC 3'

3'CTPS primer 5' CCCCGGATCCTGTTGTTGCTTAGCGATGT 3'

These primers were used to generate a CTP synthetase probe by PCR amplification using purified *C. trachomatis* DNA as the template. The PCR product was random primer α^{32}P-labelled and used to probe Southern blots of genomic DNA completely digested with a number of restriction enzymes. Hybridizations were done overnight at 65°C and then subjected to high stringency washing (the last of the washing steps being 0.1 x SSC, 0.1% SDS at 65°C). Figure 15 shows the resulting autoradiogram. Southern hybridizations of Bam H1 digested genomic DNA from *E. coli* XL1-blue, *Acholeplasma laidlawii* (mycoplasma), *C. psittaci* 6BC, and *C. psittaci* C10 were probed with the chlamydial CTP synthetase gene (PCR product) and showed no binding (Figure 15 lanes B, C, D, E). *C. trachomatis* L2 genomic DNA digested with Bam H1, Pst I, or Hind III show single bands (9 kb, 15 kb, and 1.6 kb respectively) upon hybridization (Figure 15 lanes F, H, J) as expected from the restriction sites determined from the CTP synthetase (ORF2) nucleotide sequence (Figure 12). The Hind III digestion of *C. trachomatis* L2 DNA showed the expected 1.6 kb band (Figure 15 lane J). Surprisingly, a single band (9 kb) resulted from hybridization to Eco R1 digested *C. trachomatis* L2 DNA (Figure 15 lane G) even though the nucleotide sequence indicated an Eco R1 cut site 70 bp upstream of the 3' end of ORF2 (CTP synthetase gene). There may be another downstream Eco R1 site within 500 bases of the Eco R1 site in ORF2 such that the resulting fragment of 500 bases or less would have run off the gel and would therefore not appear on the blot. As expected, two bands (approximately 1 kb and 4 kb) resulted from the hybridization with *C. trachomatis* L2 digested with Xba I as there was a single Xba I cut site (at position 1554) within the chlamydial CTP synthetase gene (Figure 15 lane I). Also as expected, there was strong hybridization of the wild-type *C. trachomatis* L2 CTP synthetase probe with Hind III digested CPEC-resistant mutant *C. trachomatis* (L2CP^{R}-5.0) genomic DNA (Figure 15 lane K).

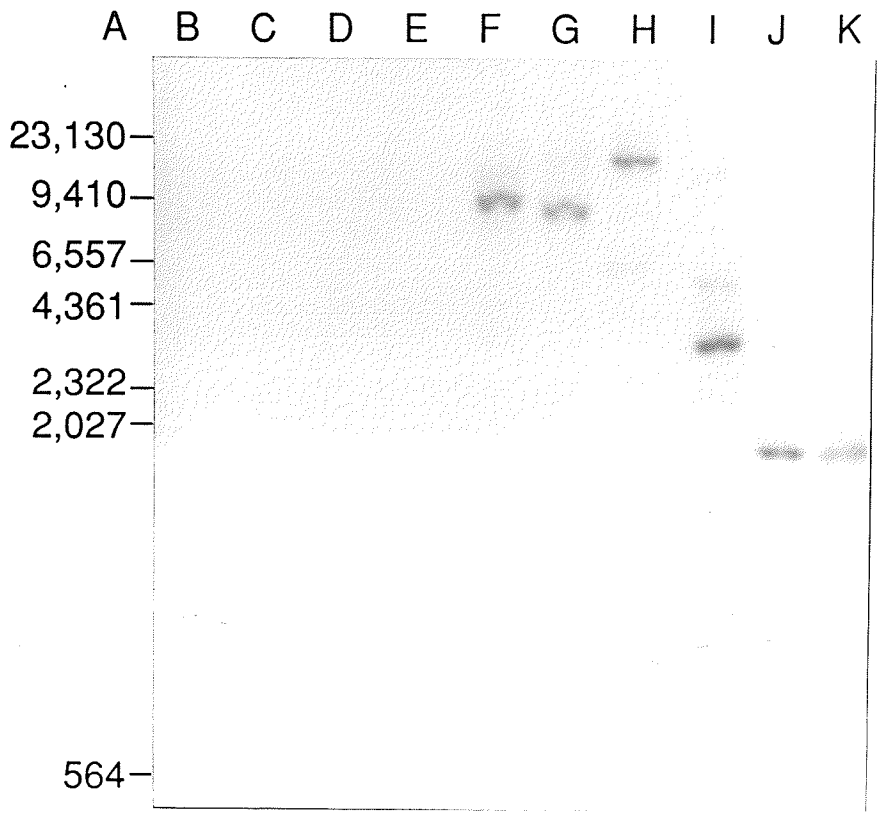


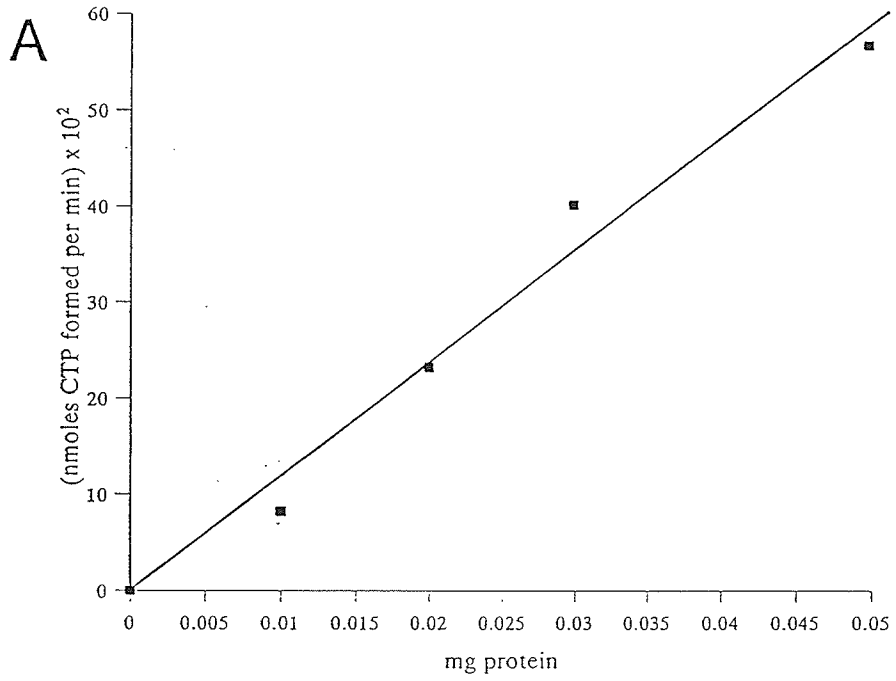
Figure 15: Southern hybridizations using wild-type *C. trachomatis* CTP synthetase gene sequence as a probe. Two oligonucleotide primers which flanked the chlamydial CTP synthetase gene were used to generate a 1.6 kbp DNA fragment by PCR amplification. This DNA fragment was random primer labelled with α-^{32}P-ATP and used as a probe for the southern blots. Equivalent amounts of restriction enzyme digested genomic DNA was present in each lane. Hybridization was carried out overnight at 65°C and then subjected to high stringency washing (last washing step was 0.1 x SSC, 0.1% SDS at 65°C). Lane A, molecular weight markers; lane B, *E. coli* XL1-blue DNA digested with BamH1; lane C, *Acholeplasma laidlawii* DNA digested with BamH1; lane D, *C. psittaci* C10 digested with BamH1; lane E, *C. psittaci* 6BC digested with BamH1; lanes F-J, wild-type *C. trachomatis* DNA digested with BamH1 (F), EcoR1 (G), PstI (H), XbaI (I), and HindIII (J); lane K, CPEC-resistant mutant *C. trachomatis* (L2CP^{R}-5.0) DNA digested with HindIII.

4. *In vitro* CTP synthetase assay

Extracts were prepared for *in vitro* CTP synthetase assays as outlined in *Methods*. The extracts of *E. coli* JF646 transformed with pH-1 (contains putative CTP synthetase gene from *C. trachomatis* L2), pMW5 (contains *E. coli pyrG* coding for CTP synthetase), or pUC19 (negative control cloning vector lacking insert) were subjected to streptomycin sulfate precipitation followed by ammonium sulfate precipitation. These extracts were then used for assaying CTP synthetase activity. This activity was monitored by measuring the amount of [^{3}H] CTP formed from [^{3}H] UTP by on-line radioactive flow detection after HPLC separation.

Preliminary experiments were carried out to optimize the assay for the extract amount and incubation time. The results presented in Figure 16 indicate that this assay is linear with respect to amount of protein added (Figure 16A), and time of incubation (Figure 16B). Optimal activity was estimated from these preliminary assays to occur using extract containing 30 μg protein, and an incubation time of 3 min. All subsequent assays were performed in triplicate using these optimal conditions.

The results of the *in vitro* CTP synthetase assays for extracts derived from recombinant *E. coli* JF646 expressing the gene for the *C. trachomatis* CTP synthetase are shown in Table 18. Using complete assay mix for the glutamine assay (2 mM glutamine, 10 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM UTP, 0.1 mM GTP, in 20 mM Tris-acetate buffer pH 7.2), CTP synthetase activity was found to be 11.6 $\pm$ 0.7 nmoles CTP produced/min/mg protein. Eliminating GTP (activator for the glutamine assay) or ATP (energy source) from the reaction mixture reduced enzyme activity to below the sensitivity of the assay. Reducing the concentration of $MgCl_2$ (to 0.2 mM), or the glutamine (to 0.4 mM) reduced enzyme activity by 54% (5.4 $\pm$ 0.4 nmoles CTP produced/min/mg protein) or 85% (1.8 $\pm$ 0.3 nmoles CTP produced/min/mg protein), respectively. The presence of 0.1 mM CTP (feedback inhibitor) in the reaction mixture decreased CTP synthetase activity by 95% (0.6 $\pm$ 0.2 nmoles CTP produced/min/mg protein). The ammonia assay (10 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM UTP, in 20 mM Tris-acetate


A
(nmoles CTP formed per min) x 10²
60
50
40
30
20
10
0
0
0.005
0.01
0.015
0.02
0.025
0.03
0.035
0.04
0.045
0.05
mg protein

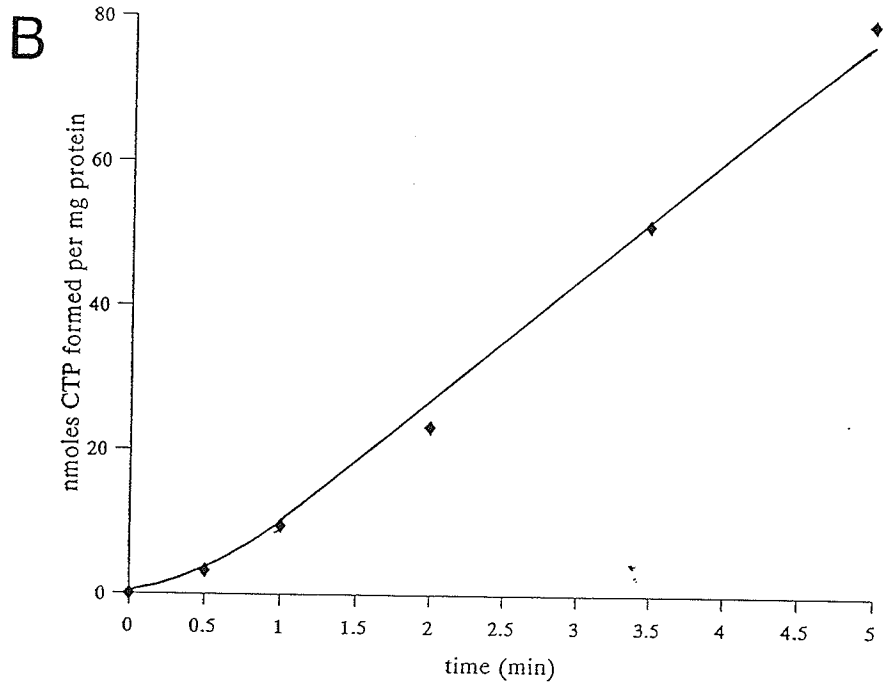

B
nmoles CTP formed per mg protein
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
time (min)

Figure 16: Optimization of *in vitro* CTP synthetase assay: (A) CTP formation in the presence of increasing protein concentrations, and (B) the increase of CTP formation with incubation time. The pre-reaction mixture contained: 2 mM glutamine, 10 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM UTP, 1 μCi [5,6-^{3}H] UTP, 0.1 mM GTP, in 20 mM Tris-acetate buffer pH 7.2 for a total assay mixture volume of 100 μl. (A) The indicated amount of protein extract was added and the reaction was terminated after 2 min by the addition of 4 N perchloric acid, or (B) 20 μg protein extract was added and the reaction was terminated at the indicated time by the addition of perchloric acid. The reaction product was neutralized using freon-tri-N-octylamine and 50 μl of the top layer was analyzed for radioactive-CTP by HPLC and on-line radioactive flow detection. Each assay condition was done in duplicate with results varying by less than 10%.

Table 18: *In vitro* CTP synthetase assay of extracts from recombinant *E. coli* expressing chlamydial DNA (pH-1) or *E. coli pyrG* (pMW5)

Extract	Assay	Conditions	Enzyme activity nmoles CTP produced/min/mg[c]
pH-1	glutamine assay	complete [a]	11.6 ± 0.7
		- GTP	< 0.1 [d]
		- ATP	< 0.1
		$[MgCl_2]$ = 0.2 mM	5.4 ± 0.4
		[glutamine] = 0.4 mM	1.8 ± 0.3
		[CTP] = 0.1 mM	0.6 ± 0.2
	ammonia assay	complete [b]	3.5 ± 0.5
pMW5	glutamine assay	complete [a]	24.9 ± 0.8
		- GTP	1.0 ± 0.6
		- ATP	< 0.1
		$[MgCl_2]$ = 0.2 mM	19.8 ± 1.6
		[glutamine] = 0.4 mM	15.9 ± 0.6
		[CTP] = 0.1 mM	8.1 ± 1.6
	ammonia assay	complete [b]	2.3 ± 0.2

[a] The glutamine assay uses glutamine as the amino group donor. The assay mixture contains 10 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM UTP, 0.1 mM GTP, 1 μCi [5,6-^{3}H] UTP, in 20 mM Tris-acetate buffer, pH 7.2. The reaction was started by addition of 30 μg protein, incubated at 37°C for 3 minutes, and stopped by the addition of 20 μl 4M $HClO_4$ and placed on ice. The mixture was extracted and neutralized with 1.1x volumes of freon-tri-N-octylamine. The top aqueous layer was analyzed for the formation of ^{3}H-CTP by HPLC using on-line radioactive flow detection.

[b] The ammonia assay uses $(NH_4)_2SO_4$ as the amino group donor. The assay mixture contains 10 mM $MgCl_2$, 0.5 mM ATP, 0.1 mM UTP, 1 μCi [5,6-^{3}H] UTP, in 2 mM Tris-acetate buffer, pH 8.1. The reaction was carried out as stated above.

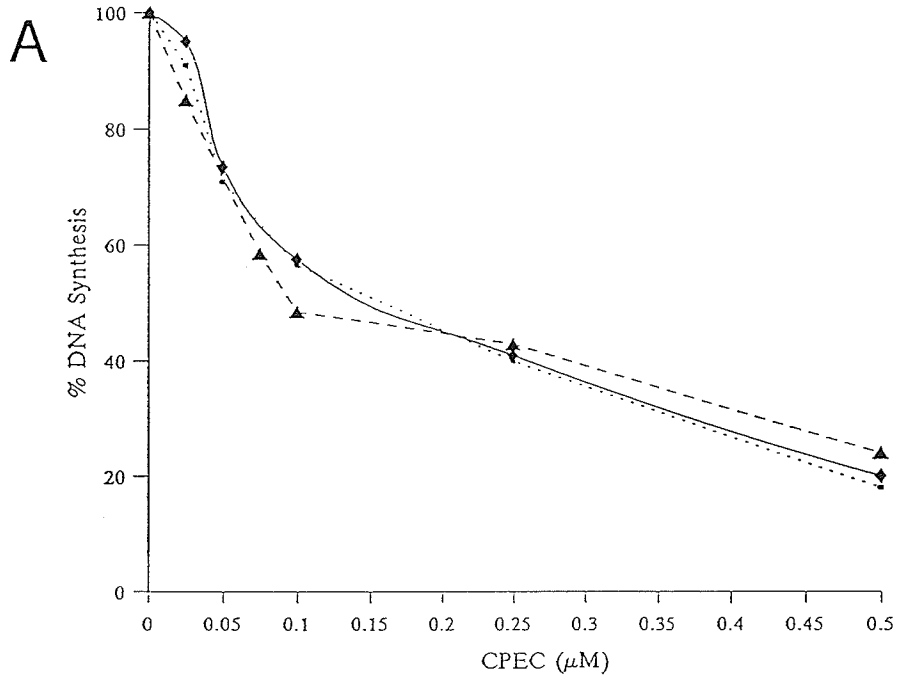
[c] Each assay was run in triplicate. The mean ± standard deviation is shown. Activity is expressed as nmoles CTP produced per min per mg protein.

[d] The sensitivity of the assay is 0.1 nmoles/min/mg.

buffer pH 8.1), which does not require the activator GTP, also showed enzyme activity (3.5 ± 0.5 nmoles CTP produced/min/mg protein). Negative control extracts derived from *E. coli* JF646 transformed using pUC19 showed no detectable activity under any conditions. Results for pMW5 (*E. coli* CTP synthetase) follow the same trends (i.e. requires ATP, GTP, $MgCl_2$, and glutamine for maximal activity and is feedback inhibited by CTP) as the *C. trachomatis* CTP synthetase activity but express relatively higher amounts of activity. For example, using complete assay conditions for the glutamine assay the activity was 24.9 ± 0.8 nmoles CTP produced/min/mg protein.

5. CPEC sensitivity in wild-type and mutant *C. trachomatis* isolates

C. trachomatis isolates were selected in the presence of increasing concentrations of cyclopentenyl cytosine (CPEC) for the ability to proliferate in the presence of normally cytotoxic drug concentrations. The effect of CPEC on wild-type *C. trachomatis* and host cell DNA synthesis was determined following the incorporation of [^{3}H] adenine into DNA and is shown in Figure 17A. The concentration of CPEC required to reduce DNA synthesis (as measured by ^{3}H-adenine incorporation into DNA) by 50% (ID_{50}) for uninfected logarithmically growing HeLa cells, wild-type *C. trachomatis* L2 in HeLa cells (in the presence of 1 μg/ml cycloheximide), and wild-type *C. trachomatis* L2 in cytidine-starved CR^-2 cells (in the presence of 1 μg/ml cycloheximide) was 0.17 μM, 0.16 μM and 0.09 μM CPEC respectively (Table 19). A CPEC-resistant *C. trachomatis* isolate was selected in the presence of 5 μM CPEC and was therefore designated $L2CP^R$-5.0. Growth of $L2CP^R$-5.0 in HeLa cells and cytidine-starved CR^-2 host cells in the presence of increasing concentrations of CPEC is shown in Figure 17B. The ID_{50} for $L2CP^R$-5.0 grown in HeLa cells and cytidine-starved CR^- 2 cells are 26 μM and 75 μM, respectively (Table 19). Both wild-type L2 and $L2CP^R$-5.0 are unaffected by CPEC when host cells deficient in uridine/cytidine kinase activity are used to


A
100
80
60
40
20
0
% DNA Synthesis
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
CPEC (μM)

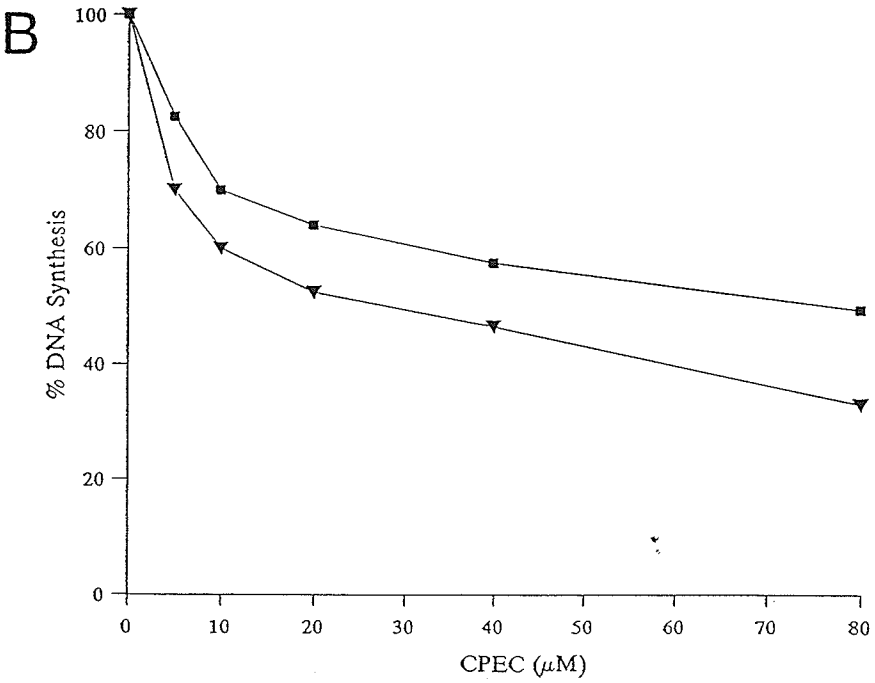

B
100
80
60
40
20
0
% DNA Synthesis
0
10
20
30
40
50
60
70
80
CPEC (μM)

Figure 17: Effect of cyclopentenyl cytosine (CPEC) on *in situ* DNA synthesis activity of (A) wild-type *C. trachomatis* L2 and (B) CPEC-resistant mutant *C. trachomatis*, L2CP^{R}-5.0. Sub-confluent (for uninfected logarithmically growing HeLa cells cutured in the absence of cycloheximide) or confluent (for *C. trachomatis*-infected HeLa cells or CR^{-}2 cells cultured in the presence of 1 μg/ml cycloheximide) monolayers (60 mm dish) were incubated in the indicated concentration of CPEC. Starved CR^{-}2 cells were cultured in the absence of cytidine for 8 h prior to and during infection. At 22 h p.i. [^{3}H] adenine was added to achieve a final concentration of 0.3 μM, and incubation was continued at 37°C for 2 h. The monolayer was washed three times with Tris-buffered saline and the RNA was degraded using 0.3 M NaOH. DNA was precipitated using 10% trichloroacetic acid, collected by filtration and subsequently quantified by liquid scintillation counting. [^{3}H] adenine incorporation into DNA is expressed as a percentage of [^{3}H] adenine incorporation in the absence of CPEC. (A) –◆– logarithmically growing HeLa cells, ..■.. wild-type *C. trachomatis* L2 in HeLa cells, --▲-- wild-type *C. trachomatis* L2 in cytidine starved CR^{-}2 cells; (B) ▼ L2CP^{R}-5.0 in HeLa cells, ■ L2CP^{R}-5.0 in cytidine starved CR^{-}2 cells.

TABLE 19: Summary of cyclopentenyl cytosine (CPEC) ID_{50} values for wild-type and drug-resistant *C. trachomatis* isolates

C. trachomatis isolate[a] and culture conditions[c]	CPEC ID_{50} (μM)[b]
logarithmically growing HeLa cells	0.17
wild-type L2 in HeLa cells	0.16
wild-type L2 in cytidine starved $CR^{-}2$ cells	0.09
$L2CP^{R}$-5.0 in HeLa cells	31.0
$L2CP^{R}$-5.0 in cytidine starved $CR^{-}2$ cells	75.0

[a] The CPEC-resistant isolate $L2CP^{R}$-5.0 was selected by a stepwise procedure in the presence of 5 μM CPEC.
[b] The ID_{50} values given in this column represent the concentration of CPEC required to inhibit *C. trachomatis*-specific DNA synthesis by 50%. All ID_{50} values are derived from the DNA synthesis curves (Figure 17A and 17B).
[c] Logarithmically growing HeLa cells were cultured in the absence of cycloheximide. *C. trachomatis*-infected HeLa cells and $CR^{-}2$ cells were cultured in the presence of 1μg/ml cycloheximide. Starved $CR^{-}2$ cells (cytidine auxotrophs) were cultured in the absence of cytidine for 8 h prior to and during infection. All cultures were incubated in the presence of the appropriate concentration of CPEC.

support chlamydial growth (data not shown) - presumably because the CPEC is not phosphorylated to its active triphosphate form (CPE-CTP) by either host or chlamydiae.

6. CPEC-resistant mutant *C. trachomatis* CTP synthetase gene

DNA from CPEC-resistant *C. trachomatis* EBs was isolated as described in *Methods*. PCR products were generated (using mutant chlamydial DNA as a template, and three sets of primers) such that any sequence of the CTP synthetase gene was present on at least two PCR products. The arrangement of the three sets of primers are as follows:

(i) 1074→ ←2700

(ii) 1309→ ←2191

(iii) 1835→ ←2554

(i) One PCR product (1626 bp) consisted of the entire CTP synthetase gene (bases 1074-2700). (ii) The second PCR product consisted of an 882 bp fragment amplified from a region toward the 5' end of the gene (bases 1309-2191). (iii) The third PCR product was a 719 bp fragment corresponding to a region toward the 3' end of the gene (bases 1835-2554). The PCR products were sequenced and compared to the wild-type *C. trachomatis* CTP synthetase gene sequence. The sequences were identical except for a single point mutation (T to G substitution) at base 1536 (see Figure 11 and Figure 14). This point mutation results in the GAT aspartic acid codon becoming a GAG glutamic acid codon. The mutation was confirmed by sequencing both strands of PCR products (i) and (ii), as well as by sequencing the CPEC-resistant mutant genomic DNA directly.

DISCUSSION

1. Interaction between *C. trachomatis* and the eukaryotic host cell

A major obstacle encountered when studying chlamydial DNA synthesis *in situ* is that it can be difficult to distinguish host- from parasite-specific activity. This is especially true when experiments are conducted in the absence of cycloheximide. However, *C. trachomatis* DNA synthesis studies were aided by the discovery that host and parasite DNA replication could be differentially inhibited with aphidicolin and norfloxacin, respectively (Figure 2). Using aphidicolin and norfloxacin, it was conclusively shown that both host and parasite are actively synthesizing DNA in *C. trachomatis*-infected logarithmically growing mouse L cells (Table 2).

dNTPs can be formed by *de novo* synthesis from small molecules or by salvage pathways. Salvage can involve direct phosphorylation of deoxynucleosides or phosphorylation of ribonucleosides followed by reduction of the ribose moiety (Reichard, 1988). The proper balance between dNTP pools is maintained by a network of biosynthetic and catabolic reactions. Especially important are the complicated allosteric controls regulating the activity and/or specificity of key enzymes of both *de novo* and salvage pathways (Reichard, 1988). The enzyme ribonucleotide reductase occupies a central position in the synthesis of dNTPs, since it is the only enzyme which catalyzes the *de novo* synthesis of dNTPs from NTPs (Lammers and Follmann, 1983; Reichard, 1988; Stubbe, 1989).

Given that chlamydiae do not synthesize purine and pyrimidine nucleotides *de novo* and lack deoxynucleoside kinases, three alternatives appear available to the parasite for obtaining dNTPs. One is that dNTPs could be obtained from the host in a manner similar to that used for ribonucleotides. Second, NTPs could be obtained from the host with subsequent conversion to dNTPs by a chlamydia-specific ribonucleotide reductase enzyme. The third possibility is that chlamydiae are flexible and both processes occur. The results presented in

this study support the hypothesis that *C. trachomatis* likely obtains dNTPs exclusively by way of the second alternative. All nucleobases and ribonucleosides that were utilized by the host cell as precursors for DNA synthesis could also be utilized by *C. trachomatis* (Table 3). These results are in agreement with those reported for *C. psittaci* (Tribby and Moulder, 1966). The only nucleobase that was not utilized by *C. trachomatis* was uracil, and it was also not utilized by the host mouse L cell. In keeping with its phenotype, the $HGPRT^-$ CHO cell line did not efficiently use adenosine, deoxyadenosine, guanosine, deoxyguanosine, or hypoxanthine as a precursor for DNA synthesis (Table 4). Interestingly, *C. trachomatis* also did not use any of the above precursors when growing in $HGPRT^-$ cells. These findings support the hypothesis that chlamydiae can utilize medium-supplied nucleic acid precursors only if the host cell is capable of elevating them to the nucleotide level (Cellabos and Hatch, 1979; Moulder, 1991).

C. trachomatis readily grows and carries out DNA replication in both cycloheximide-treated host cells which are resting and have depressed DNA synthesis activity and in logarithmically growing cells which are actively synthesizing DNA (Table 2). In either case, exogenously added thymidine was not utilized for *C. trachomatis* DNA synthesis. Since cells in the S phase contain much larger dNTP pools than resting cells (Spyrou and Reichard, 1988), the non-utilization of thymidine by chlamydiae is unlikely to be related to the absolute size of the dTTP pool present in the host cell.

Even though deoxycytidine and deoxyuridine were efficient precursors for host DNA synthesis, they were not utilized by *C. trachomatis* (Table 3). These findings are similar to those reported by Tribby and Moulder (1966) for *C. psittaci*. In addition, HPLC measurement of deoxynucleotide pools clearly indicated that exogenously supplied dC and dT are transported and subsequently phosphorylated to the triphosphate level, presumably by host-specified kinases, in both MI and infected cells (Table 6). Therefore, the inability of *C. trachomatis* to incorporate pyrimidine deoxynucleosides into DNA is not a result of the host cell being unable to make dCTP and dTTP available to the parasite. As a result, the most likely explanation for lack of utilization is that chlamydiae do not transport pyrimidine dNTPs.

The evaluation of utilization of purine deoxynucleosides by chlamydiae was more problematic because of the susceptibility of these precursors to degradation in most mammalian cells. To regulate purine deoxynucleoside metabolism, an HGPRT$^-$ cell line as well as inhibitors of (deoxy)adenosine deaminase (dCF) and purine nucleoside phosphorylase (8-AG), were used. The complexity of the system sets a limit to the level of interpretation. However, the results obtained support the hypothesis that *C. trachomatis* does not draw on host purine dNTP pools as a source of DNA precursors. In contrast, Tribby and Moulder (1966) concluded that dA and dG were efficiently utilized by *C. psittaci* as precursors for DNA synthesis. Examination of their results showed that a large portion of the radiolabelled dA and dG that was supplied was incorporated into RNA (Tribby and Moulder, 1966). Clearly, these purine precursors followed the catabolic pathways outlined in Figure 3 and were salvaged as ATP and/or GTP, the precursors for RNA polymerase. The results presented in Table 5 also showed that dA and dG were efficiently utilized by *C. trachomatis* for DNA synthesis. However, when precautions were taken to minimize the catabolic reactions by adding dCF and/or 8-AG and to prevent salvage by using an HGPRT$^-$ cell line, the incorporation of dA and dG into parasite DNA was essentially abolished (Table 5). The conclusion was that chlamydiae are unable to directly utilize purine deoxyribonucleotides from the host cell.

The results of experiments supporting this conclusion are presented in Table 5. A summary of these data is presented as the ratio of counts incorporated into DNA in the absence of dCF and/or 8-AG over the counts incorporated in the presence of inhibitors (Table 5, ratio columns). The control experiments measured cytidine incorporation into DNA by logarithmically growing, MI, and infected wild-type and HGPRT$^-$ cells under the two different conditions. Before cytidine nucleotides can be utilized for DNA synthesis, the ribose moiety must be reduced to its deoxy derivative, a reaction catalyzed by ribonucleotide reductase. The cytidine ratio is close to 1 for both wild-type and HGPRT$^-$ cells whether infected, MI, or logarithmically growing. These results suggest that dCF and/or 8-AG do not significantly alter the specific activity of the dCTP pool or directly affect host or parasite DNA replication.

Incorporation ratios above 1 for dA and dG could be interpreted as decreased DNA synthesis activity in the presence of dCF and/or 8-AG. However, since cytidine incorporation data suggest that these inhibitors do not directly affect DNA synthesis, the decreased incorporation of dA and dG is likely a reflection of a decreased specific activity of the corresponding dNTP pool. Similarly, although ratios below 1 could be interpreted as increased DNA synthesis activity in the presence of dCF and/or 8-AG, they more likely reflect the increased specific activity of the dATP and dGTP pools in the presence of inhibitors. The results obtained are in keeping with our current understanding of the regulation of enzymes involved in (deoxy)nucleoside metabolism in mammalian cells.

Calculated ratios were above 1 for logarithmically growing wild-type cells and *C. trachomatis*-infected wild-type cells when dA or dG were used as the precursor. In these two cases, addition of dCF and/or 8-AG likely leads to a decrease in the specific activity of the purine dNTP pools because host cell catabolism and salvage of labelled precursor to ribonucleotides is substantially reduced. For infected cultures at confluence in the presence of cycloheximide, the vast majority of host cells are in a resting state and host cell ribonucleotide reductase levels are extremely low (Reichard, 1988). As a result, the contribution to the dNTP pool from host cell *de novo* synthesis would be greatly reduced and the specific activity of the purine dNTP pools would increase. Since chlamydiae must draw on host NTP pools as their only source of nucleotides, they will be directly affected by the specific activity of the ribonucleotide pools. In the presence of dCF and/or 8-AG, the specific activities of the host purine ribonucleotide pools decrease. Taken together, these results suggest that a reduction in the specific activity of *C. trachomatis* purine dNTP pools would only occur if the parasite has the capacity to convert NTPs to dNTPs and at the same time lacks the ability to transport dNTPs directly from the host.

When $HGPRT^-$ cells are labelled with purine deoxynucleosides, very little radiolabel can enter the purine ribonucleotide pool because of the inability of these cells to salvage guanine and hypoxanthine. Therefore, the contribution of radiolabel to the dNTP pool from

the salvage pathway via ribonucleotide reductase is negligible. Despite the facts that chlamydiae are rapidly replicating their DNA, as indicated by labelling from cytidine, and that there is high specific activity of the MI host purine dNTP pool in the presence of dCF and/or 8-AG, as indicated by high levels of dA and dG incorporation into host DNA, *C. trachomatis*-infected HGPRT$^-$ cells do not incorporate significantly more dA and dG into DNA than do MI control cultures. These results again suggest that *C. trachomatis* does not rely on host cell purine dNTP pools as a source of DNA precursors.

In conclusion, the results reported in these studies support the hypothesis that *C. trachomatis* does not draw on host cell dNTP pools as a source of DNA precursors. As an alternative, *C. trachomatis* likely obtains substrates for DNA polymerase from the host cell as ribonucleotides, with subsequent conversion to deoxynucleotides being done by a chlamydial-specific ribonucleotide reductase activity. There are two main advantages to this later process of dNTP acquisition. It ensures that chlamydiae will have a supply of dNTPs even in non dividing host cells where concentrations of dNTPs, but not NTPs, could be limiting. Second, by being in control of their dNTP synthesis, chlamydiae can ensure that a balanced supply of dNTPs is generated, which will help preserve the fidelity of DNA replication.

The question whether *C. trachomatis* infection had an effect on the size and/or composition of the host cells' acid soluble nucleotide pools was of interest because of two unusual properties of chlamydiae which result in a total dependency on the host cell for NTPs. Firstly, chlamydiae are energy parasites, being incapable of synthesizing high energy intermediates, and therefore depend on the host cell for ATP (Moulder, 1991; Schachter, 1988; Schachter and Caldwell, 1980). Secondly, work by Hatch (1975), and the results presented above indicate that chlamydiae obtain NTPs directly from the host cell cytoplasm, by as yet undefined transport system(s). The results presented in Figure 4 indicate that the absolute size of the *C. trachomatis*-infected host cell NTP pools decrease by approximately 50% at mid-growth cycle. This is not unexpected given that this is the time during the growth cycle when the majority of organisms are in the metabolically active RB stage and RNA, DNA, and protein

synthesis activity are maximal. The fact that all four NTP pools respond to *C. trachomatis*-infection in an equivalent fashion, i.e., all decrease by approximately the same amount, suggests that there is a similar draw on all ribonucleotide pools. This result contrasts that of Hatch (1975) who showed that there was a much larger decrease in pyrimidine, as compared to purine, nucleotide pools in mouse L cells in response to infection with *C. psittaci* 6BC.

In spite of the decreased ATP pool size, the energy charge of the *C. trachomatis*-infected HeLa cell remained essentially constant during the growth cycle. As expected, given the above results, there was also a decrease in the total adenylate pool size of the chlamydial infected cell compared to its uninfected control. Taken together these results imply that *C. trachomatis* L2 infection imposes a significant draw on host cell nucleotide pools but does not impair the ability of the host to maintain its energy ratio. This is likely a key factor in the successful growth and survival of an energy parasite like chlamydiae. Clearly, it is in the best interest of chlamydiae not to harm the host cells' energy generating systems which they depend upon for survival.

Both highly purified RBs and EBs had measurable acid soluble nucleotide pools. Interestingly, there was a dramatic difference in the energy charge calculated for RBs (0.58) and EBs (0.80). Although measured by a different method, Hatch *et al* (1982) also found that *C. psittaci* 6BC RBs had a lower energy charge than EBs (0.17 vs. 0.84). They suggested that the low energy charge calculated for RBs may reflect continued metabolic activity by the RB in the absence of an exogenous source of ATP. In an attempt to overcome this, I tried to purify RBs in buffers containing a concentration of ATP (2.0 mM) similar to that found in the host cell cytoplasm. However, even under these conditions the calculated energy charge for RBs was less than 0.6. It is generally accepted that under normal conditions the bacterial energy charge is in the range of 0.87 to 0.95 and that growth ceases if the energy charge falls below 0.5 (Neidhardt *et al*, 1990). Although there is a possibility that the low energy charge is an artifact of the purification procedure, it may be that the low energy charge in an RB reflects its

limited capacity for ATP acquisition, by the ATP/ADP translocase, especially when energy demands are high and ATP utilization is maximal.

Even though the total number of moles of adenylate nucleotides was similar in the RB and the EB ($\approx 2 \times 10^{-10}$moles) the estimated concentration of the adenylate pool was dramatically different (1 mM for RBs vs. 38 mM for EBs). The lower concentration of the adenylate pool in the RB may be due to leakage of small molecules during purification of the fragile RB. However, it should be noted that the total adenylate pool in the RB is similar to that in the infected HeLa cell (1.0 versus 1.5 mM) and not that different from other bacteria ($\approx$3 mM) (Neuhard and Nygaard, 1987). More surprising is the high concentration of nucleotides in the EB. The calculation of these values is based on a variety of assumptions, thereby limiting our interpretation. However, in combination with the calculated energy charge of the EB, the results do suggest that the EB has a relatively large supply of high energy nucleotides available. Since the EB lacks ATP/ADP translocase activity (Hatch *et al*, 1982), this supply of nucleotides may be essential to fuel the early stages of EB to RB differentiation. This situation is in sharp contrast to endospores of *Bacillus sp.* which have undetectable NTP pools (Setlow, 1981). Production of much of the high-energy compounds needed early in Bacillus spore germination can be driven by metabolism of energy reserves stored in the dormant spore, a process which chlamydiae is presumably incapable of carrying out.

In the past there has been indirect biochemical (Becker and Asher, 1971; Gill and Stewart, 1970) and ultrastructural (Matsumoto, 1981; Matsumoto *et al*, 1991) evidence collected which may suggest that host cell mitochondrial generated ATP is essential for chlamydial survival. However, the results indicating that *C. trachomatis* grows as well in a host cell line with severely compromised mitochondrial function (CCL 16-B2) as it does in the wild-type counterpart (CCL 16-B1) suggest that oxidative phosphorylation is likely not essential for chlamydial survival. It was also found that *C. psittaci* Cal10 grew as well in either host cell line (data not shown). It has previously been shown that a 2-fold increase in the rate of glycolysis compensates for the defect in mitochondrial function in the CCL 16-B2 cell

line (Soderberg *et al*, 1980). In addition the CCL 16-B2 cell line has an energy charge and growth rate similar to its wild type counterpart (Soderberg *et al*, 1980). In light of these findings a reasonable interpretation of the results is that chlamydiae are dependent on host cell ATP for survival, however, it does not matter whether the ATP is generated via glycolysis or via mitochondrial respiration.

2. Ribonucleotide reductase in *C. trachomatis*

The work presented above showed that *C. trachomatis*-infected cells do not incorporate a significant amount of any of four medium-supplied deoxynucleosides (dC, dT, dA, dG) into chlamydial DNA. Importantly, these results indicated that in both mock-infected and infected cells, exogenously supplied deoxynucleosides were transported and subsequently phosphorylated to the triphosphate level, presumably by host-specified kinases. Therefore, the inability of *C. trachomatis* to utilize deoxynucleosides as precursors for DNA synthesis is not a result of the host cell not making dNTPs available. When taken together, the above results strongly suggest that chlamydiae lack a specific transport system(s) for dNTPs. As a result, it was hypothesized that chlamydiae likely obtain precursors for DNA synthesis as NTPs, with subsequent conversion to dNTPs being done by a chlamydia-specific ribonucleotide reductase.

We indeed found that *C. trachomatis* does contain a ribonucleotide reductase. Ribonucleotide reductase is the only enzyme known to catalyze the direct conversion of a ribonucleotide to its corresponding deoxyribonucleotide (Lammers and Follmann, 1983; Reichard, 1988; Stubbe, 1990). To date, three classes of ribonucleotide reductases have been described (Reichard, 1993). Interestingly, despite their differences, it appears that all ribonucleotide reductases function via a similar mechanism involving a radical (Eliasson *et al*, 1990; Stubbe, 1989; Reichard, 1993). Hydroxyurea rapidly inhibits DNA synthesis by acting as a radical scavenger (Ehrenberg and Reichard, 1972). Class I ribonucleotide reductases are susceptible to hydroxyurea.

Previous results from two independent laboratories showed that hydroxyurea blocks the synthesis of chlamydia-specific DNA; however, no connection was made to ribonucleotide reductase in either study (Rosenkranz *et al*, 1973; Sardinia *et al*, 1988). Furthermore, the results of these studies are difficult to interpret because it is well established that concentrations of hydroxyurea lower than those used are sufficient to inhibit host cell ribonucleotide reductase activity, thereby causing a rapid decrease in cellular dNTP pools (Reichard, 1988). Thus, the observed inhibition of chlamydial DNA synthesis could have resulted from a depletion of the supply of dNTPs available to the parasite from the host cell rather than a direct effect of a chlamydial-specific ribonucleotide reductase. Even though this was considered unlikely since dNTPs are not transported into *C. trachomatis*, the question was reexamined by using a hydroxyurea-resistant cell line (LHF) as a host to support *C. trachomatis* growth. Even in the presence of hydroxyurea, this cell line retained sufficient enzyme activity to provide an adequate supply of dNTPs for DNA replication. The results clearly indicated that *C. trachomatis* growth, as assessed by three independent parameters (DNA synthesis, inclusion formation and EB production), is inhibited by hydroxyurea at concentrations that have little or no effect on the growth of the LHF cell line (Table 8). These data certainly provide strong evidence that hydroxyurea has a direct effect on chlamydiae and that *C. trachomatis* contains a hydroxyurea-sensitive ribonucleotide reductase.

The initial attempts to isolate hydroxyurea-resistant *C. trachomatis* were unsuccessful. However, drug-resistant isolates were obtained with relative ease once the selection protocol was modified. With the original procedure, hydroxyurea was added at 2 h p.i., when internalized EBs had not yet differentiated to RBs. With the modified protocol, hydroxyurea was added at 22 h p.i., when RB growth was logarithmic. The reason why only the one selection protocol was successful in this case may simply reflect the greater opportunity for emergence of resistant mutants from the larger population of organisms present at the time of drug addition in the latter protocol. However, it is also possible that the difference reflects the

fact that hydroxyurea is added to a nondividing population of EBs in the first protocol and to a rapidly growing population of RBs in the second.

A series of drug-resistant *C. trachomatis* L2 isolates were selected by a stepwise procedure for increasing levels of resistance to the cytotoxic effects of hydroxyurea. Each successive drug selection step leading to the isolation of highly resistant *C. trachomatis* isolates was accompanied by elevations in resistance as assessed by DNA-synthesizing ability (Figure 5, Table 9). Additional studies conducted on one of the isolates, $L2H^R$-10.0, indicated that the drug resistance phenotype remained stable after 10 passages in the absence of hydroxyurea (Table 10).

Although the development of resistance could be due to alterations in membrane permeability and drug penetration, it more likely represents the selection of mutants with an alteration in ribonucleotide reductase expression. This view is supported by several lines of evidence. Hydroxyurea is a small molecule which has been shown, at least in mammalian tissue culture systems, to enter cells by a diffusion mechanism (Morgan *et al*, 1986). In addition, there is overwhelming evidence which indicates that the tyrosine free radical-containing small subunit of ribonucleotide reductase is the primary target of hydroxyurea (Ehrenberg and Reichard, 1972; Reichard, 1988). The finding that the $L2H^R$-10.0 isolate is cross resistant to guanazole (Table 10) also supports the view that a radical-containing ribonucleotide reductase is the target in chlamydiae. Results from electron paramagnetic resonance spectroscopy studies indicate that guanazole inactivates the tyrosine free radical of class I ribonucleotide reductases (Ehrenberg and Reichard, 1972; Kjoller Larsen *et al*, 1982). In addition, mammalian cell lines selected for resistance to hydroxyurea have been shown to be highly cross resistant to guanazole and *vice versa* (Wright, 1989; Wright and Lewis, 1974; Wright *et al*, 1989).

Since both wild-type and drug-resistant host cell lines as well as wild-type and drug-resistant *C. trachomatis* isolates were available, the effects of hydroxyurea on DNA synthesis activity in various host-parasite combinations were assessed (Table 11). The results obtained

from these experiments clearly indicated that inhibition of host cell and *C. trachomatis* DNA synthesis by hydroxyurea can occur but need not occur simultaneously. For example, 1 mM hydroxyurea inhibited both host and parasite DNA synthesis activity when wild-type host cells were infected with wild-type *C. trachomatis*. However, when wild-type host cells were infected with drug-resistant chlamydiae, the effect of hydroxyurea was almost exclusively on host cell DNA synthesis. Similarly, when drug-resistant host cells were infected with wild-type chlamydiae, the effect of hydroxyurea was only seen on parasite activity. In the presence of 1 mM hydroxyurea, the resistant host cell line had sufficient dNTP pools to support DNA synthesis, but wild-type *C. trachomatis* did not grow. The opposite result occurred when wild-type host cells were infected with drug-resistant chlamydiae in the presence of hydroxyurea. These results certainly suggest that host cell dNTP pools are not utilized by the parasite and are in complete agreement with the previous observations indicating that *C. trachomatis* cannot use exogenous deoxynucleosides as precursors for DNA synthesis.

Additional evidence for the existence of a ribonucleotide reductase in chlamydiae is provided by the demonstration of CDP reductase activity in crude extracts prepared from Hypaque-purified *C. trachomatis* RBs (Table 12). The possibility that the detected ribonucleotide reductase activity resulted from host cell contamination was eliminated by using monoclonal antibodies against both subunits of mammalian ribonucleotide reductase. The mouse L cell CDP reductase activity was almost completely inhibited by the monoclonal antibodies, whereas the chlamydial ribonucleotide reductase activity was essentially unaffected. As expected, both the mouse L-cell and *C. trachomatis* activities were inhibited by hydroxyurea.

By far the most common mechanism for the development of hydroxyurea resistance in mammalian cells, bacteria, and viruses is the overproduction of ribonucleotide reductase activity (Reichard, 1988; Wright, 1989; Wright *et al*, 1989). In keeping with these results, we found that crude extracts prepared from hydroxyurea-resistant $L2H^R$-10.0 RBs contained elevated (approximately eight-fold) levels of CDP reductase activity. This 8-fold elevation in

CDP reductase activity is significantly less than the 17.5-fold increase in hydroxyurea-resistance displayed by this same L2H^R-10.0 mutant. Therefore, the possibility that other factors have a role in the establishment of hydroxyurea resistance in this mutant cannot be ruled out. Hydroxyurea sensitivity is a property displayed by class I ribonucleotide reductases; however, definitive characterization and class establishment for the chlamydial enzyme await its further purification and/or molecular cloning. Similar to wild-type L2 activity, the L2H^R-10.0 ribonucleotide reductase activity was inhibited by hydroxyurea and unaffected by monoclonal antibodies which neutralize mouse cell enzyme activity.

3. Absence of most *de novo* and salvage pathway enzymes in *C. trachomatis*

From the data presented above it is evident that *C. trachomatis* L2 draws on the host cell's NTP pools and subsequently reduces the ribonucleotides to dNTPs using a chlamydia-specific ribonucleotide reductase. The next question asked was whether chlamydiae also have the capability to salvage and/or *de novo* synthesize nucleotides. The study of metabolic processes in chlamydiae is greatly complicated by the fact that they are obligate intracellular parasites. To aid the search for nucleotide metabolizing enzymes in *C. trachomatis* L2, an *in situ* approach with a variety of mutant host cell lines and several different radiolabelled nucleic acid precursors was used. By starving the various mutant cell lines for their required nucleobase(side) supplement, the effects of specific nucleotide deprivation on *C. trachomatis* growth was assessed. Under conditions of limiting adenine nucleotides (starved Ade$^-$H cells) or adenine and guanine nucleotides (starved Ade$^-$F cells), *C. trachomatis* L2 growth was reduced and few infectious EBs were produced. Similarly, *C. trachomatis* growth was inhibited by limiting uridine and cytidine nucleotides (starved Urd$^-$A cells). The limited chlamydial growth that did occur under these conditions is likely supported by host cell acid soluble nucleotide pools that are at least partially maintained, for a short period of time, by host cell RNA turnover.

Experiments using the purine auxotroph, Ade$^-$F, and the pyrimidine auxotroph, Urd$^-$A, as host cell lines, clearly show that *C. trachomatis* is unable to synthesize purines or pyrimidines *de novo* (Figure 8). It has been speculated, although not demonstrated, that members of the genus *Rickettsia*, another obligate intracellular bacterium, are also incapable of *de novo* nucleotide biosynthesis (Winkler, 1990). In addition the majority of eukaryotic intracellular parasites lack the ability to *de novo* synthesize purine nucleotides, however, most have retained the capacity for *de novo* pyrimidine biosynthesis (Hassan and Coombs, 1988).

The results also indicate that *C. trachomatis* L2 is incapable of salvaging purine nucleobases and pyrimidine nucleosides in mutant host cell lines that are incapable of raising these precursors to the nucleotide level. This is in sharp contrast to most eukaryotes and prokaryotes which possess numerous salvage pathways for the reutilization of purine and/or pyrimidine nucleobase(sides) that arise either endogenously or exogenously (Hassan and Coombs, 1988). In many eukaryotic intracellular parasites purine salvage enzymes are essential for survival (Hassan and Coombs, 1988). In addition, using *C. trachomatis*-infected Ade$^-$H cells and hypoxanthine or guanine as precursor, no labelled adenine in acid hydrolyzed nucleic acids could be detected. These results suggest that *C. trachomatis* lacks the common purine interconverting enzymes adenylosuccinate lyase, adenylosuccinate synthase, and GMP reductase (Figure 7). Taken together the above results emphasize the limited capacity *C. trachomatis* has for metabolizing nucleotides.

The fact that *C. trachomatis* grew as well in starved CR$^-$2 cells, which have a substantially reduced CTP pool, as they did in the cytidine supplemented counterpart suggested that chlamydiae may encode a CTP synthetase (Table 14). Further support for the presence of CTP synthetase comes from the results which show that exogenously added radiolabelled uridine is readily incorporated into nucleic acid cytosine by *C. trachomatis*-infected starved CR$^-$2 cells but not by the MI control culture (Figure 9A). Since it has previously been shown that both *C. psittaci* 6BC (Hatch, 1975) and *C. trachomatis* L2 (results presented earlier) can take CTP directly from the host cell it was initially unclear why chlamydiae would encode their

own CTP synthetase. One possibility would be that the chlamydial transporter responsible for acquiring CTP from the host is incapable of obtaining sufficient CTP to meet the parasite's demands. It is interesting that the smallest NTP pool in the EB is CTP, whereas in the RB the CTP pool is larger than the UTP pool. This change could be a reflection of RB CTP synthetase activity.

The ability of *C. trachomatis*-infected CR$^-$2 cells to incorporate exogenous cytidine into thymine of DNA suggests that chlamydiae also encodes its own deoxycytidine nucleotide deaminase (Figure 9B). Deoxycytidine nucleotide deamination can occur either at the monophosphate or triphosphate level (Mollgaard and Neuhard, 1983). Currently the substrate of the chlamydial enzyme is not known. dCMP deaminases are found in most eukaryotes and gram-positive bacteria, whereas dCTP deaminases are often found in gram-negative bacteria (Mollgaard and Neuhard, 1983). It has recently been reported that *Rickettsia prowazekii* encodes a dCTP deaminase (Speed and Winkler, 1991). The primary function of deoxycytidine nucleotide deamination is to provide the dUMP required for thymidine nucleotide synthesis. In *E. coli* and mammalian cells the majority of cellular dTTP is derived from cytidine nucleotides (Reichard, 1988). Fan *et al* (1991) have shown that *C. trachomatis* L2 encodes a thymidylate synthase, therefore, it is likely that a deoxycytidine nucleotide deaminase is required to supply a portion of the dUMP substrate.

4. CTP synthetase of *C. trachomatis* is in an operon involved in LPS biosynthesis

As mentioned above, the finding that *C. trachomatis* L2 could convert UTP to CTP suggested that chlamydiae encode a CTP synthetase; an intriguing observation since CTP can also be obtained directly from the host cell. The final series of experiments presented focused on the cloning and initial characterization of the CTP synthetase.

The existence of a *C. trachomatis*-specific CTP synthetase was confirmed by a number of experiments: (i) A *C. trachomatis*-specific DNA fragment cloned into pUC19 (pH-1) was

capable of complementing the deficiency of CTP synthetase activity in *E. coli* JF646. (ii) *E. coli* JF646 transformed with pH-1 was capable of converting radiolabelled uracil nucleotides to cytosine nucleotides (CTP synthetase activity) as shown by *in vivo* radiolabelling experiments (Figure 10). *E. coli* JF646 which had been transformed with pUC19 (nonrecombinant plasmid) were unable to convert uracil nucleotides to cytosine nucleotides suggesting that this activity was recombinant plasmid-specific (pH-1). (iii) The derived amino acid sequence of a portion of the *C. trachomatis*-specific DNA fragment was shown to share high sequence identity (42% to 49% overall) with known CTP synthetases (Table 17 and Figure 14). The amidotransferase amino acid consensus sequence common to all CTP synthetases sequenced so far was completely conserved in the chlamydial enzyme. (iv) Southern hybridizations with genomic DNA preparations indicated that the gene was chlamydial-specific - there was no cross hybridization with *E. coli* or mycoplasma DNA (Figure 15). Under high stringency conditions, there was no cross hybridization with *C. psittaci*. This is not surprising since *C. trachomatis* and *C. psittaci* share only 10% total DNA homology (Fukoshi and Hirai, 1992). In addition, the Southern hybridizations indicated that the CTP synthetase gene is present as a single copy gene on the chlamydial genome (Figure 15). (v) Finally, *in vitro* CTP synthetase activity was detected in extracts prepared from *E. coli* JF646 transformed with pH-1 (which contained the putative chlamydial CTP synthetase gene) (Table 17). Taken together, these results strongly support the hypothesis that *C. trachomatis* possesses a CTP synthetase.

CTP synthetase of *E. coli* has been thoroughly studied and is known to carry out the conversion of UTP to CTP using both glutamine (in the presence of GTP) and ammonia as the amino-group donor (Long and Koshland, 1978). In addition, the *E. coli* CTP synthetase also requires Mg^{2+} and ATP for enzymatic activity (Long and Koshland, 1978). Although the *in vitro C. trachomatis* CTP synthetase assays were performed with only minimally purified enzyme (streptomycin sulfate and ammonium sulfate precipitations), it was evident that there was an absolute requirement for ATP and GTP in the glutamine-donor assay (Table 17). Sufficient concentrations of $MgCl_2$ and glutamine are also required for maximal activity. In

addition, like CTP synthetases of *E. coli* and mammalian cells (Long and Koshland, 1978; Weinfeld *et al*, 1978), the *C. trachomatis* CTP synthetase is inhibited by CTP (Table 17). Like *E. coli*, the *C. trachomatis* CTP synthetase reaction can use ammonium sulfate (ammonia) as the amino-donor in the absence of the allosteric effector, GTP. These results suggest that the CTP synthetase of *C. trachomatis* may share similar properties with the well-studied *E. coli* CTP synthetase. CTP synthetase activity was not detected in crude extracts (no streptomycin or ammonium sulfate precipitations) prepared from Hypaque-gradient purified RBs. Possibly the combination of low CTP synthetase enzyme amounts, competition for UTP substrate, and/or the presence of inhibitors (such as CTP) may explain the lack of activity detected from the crude RB extract. Further enzymatic characterization of the CTP synthetase of *C. trachomatis* will require much larger amounts of highly purified recombinant protein.

The overlapping arrangement of the chlamydial CTP synthetase gene with two other open reading frames (the gene for CMP-KDO synthetase, and another gene of unknown function) suggests that these genes may be in an operon (Figure 12). This is the first report of an overlapping gene arrangement of this sort in chlamydiae. This overlapping arrangement is similar to that described for a number of *E. coli* and *B. subtilis* operons and may be suggestive of translational coupling (Zalkin and Ebbole, 1988). Translational coupling is a proposed mechanism of regulation (translational coupling has only been demonstrated *in vitro*) whereby gene translation from a polycistronic mRNA is at least partially dependent on translation of an upstream gene. The function of translational coupling would be to allow the proportionate synthesis of functionally related proteins. The two requirements of translational coupling are slightly overlapping genes, and the presence of a ribosome binding site (in the vicinity of the ATG start codon) for each gene of the operon (Zalkin and Ebbole, 1988). Both of these conditions are present for the chlamydial operon. However, the translation stop-translation start overlaps (14 and 25 bp overlaps) (Figure 12) are larger for this chlamydial operon than the 1 to 8 bp overlaps suggested by Zalkin and Ebbole (1988) to favor translational coupling.

The first gene in this operon is the CMP-KDO synthetase gene. CMP-KDO synthetase is responsible for activating KDO, an 8-carbon sugar, for its subsequent incorporation into gram-negative lipopolysaccharide (LPS) (Unger, 1981). This reaction requires CTP and Mg^{2+}. The CTP could be supplied from the activity of CTP synthetase. Thus, it appears that the CTP synthetase of *C. trachomatis* is contained in an LPS biosynthesis related operon. The enzymes encoded by the operon are responsible for synthesizing CMP-KDO, the substrate for 3-deoxy-D-manno-octulosonic acid transferase (KDO transferase). KDO transferase transfers the KDO sugars onto the lipid A moiety during LPS biosynthesis. The gene (*gseA*) for KDO transferase has recently been cloned in *C. trachomatis* (Belunis *et al*, 1992) and *C. psittaci* (Mamat *et al*, 1993).

It may be that there is a certain period in the chlamydial life cycle when there is a high demand for LPS biosynthesis (for example during maximum RB replication). At this time of maximal LPS biosynthesis, there may be a higher than usual draw on the CTP pools in the RB such that the CTP taken up directly from the host cell is not sufficient for both LPS biosynthesis as well as nucleic acid synthesis. Alternately, there may be a local draw on the CTP pool during LPS biosynthesis such that it is more efficient to channel the CTP directly to the CMP-KDO synthetase via the action of an associated CTP synthetase than to rely on the general intracellular CTP pools of the RB. One example of channelling is the close association of dihydrofolate reductase and thymidylate synthase (thymidylate synthesis cycle), either physically linked as in parasitic protozoans (Ivanetich and Santi, 1990), or closely related (overlapping coding regions) as in bacteriophage T4 (Chu *et al*, 1984) and *Bacillus subtillus* (Iwakura *et al*, 1988).

The gene product of ORF3 may possibly have some role in localizing the CMP-KDO synthetase and the CTP synthetase near the cytoplasmic face of the inner membrane where LPS biosynthesis occurs. This is supported by the prediction of the ORF3 gene product as an integral membrane protein as determined by PC/GENE computer analysis (method of Klein, Kanehisa and DeLisi). In addition, CMP-KDO is unstable (*in vitro* KDO transferase activity

requires the presence of active CMP-KDO synthetase (Belunis *et al*, 1992)) therefore the localization of the CMP-KDO generating system close to the KDO transferase may be advantageous. Although there is no net use of cytidine nucleotides during LPS biosynthesis, there is a net loss of high energy phosphates. Chlamydiae may not be able to resynthesize CTP via kinases, or may not be able to salvage them quickly enough back to the triphosphate level during LPS biosynthesis. This is the first report of a CTP synthetase possibly being encoded as part of an operon, although the *E. coli pyrG* (CTP synthetase gene) may be transcribed with the enolase gene (*eno*) as a *pyrG eno* polycistronic mRNA (Weng *et al*, 1986). More definitive evidence (such as northern blot analysis, reverse transcriptase-PCR, and/or S1 nuclease mapping) for the chlamydial CTP synthetase being encoded as part of an operon are needed. These experiments are made quite difficult due to the instability of prokaryotic mRNA (half-life of seconds to minutes). Northern blot analysis of chlamydial mRNA has been attempted by others in the lab and elsewhere (Tan *et al*, 1993) with minimal success - most likely due to the low concentration of the particular mRNA species being studied as well as the instability of the bacterial mRNA.

Cyclopentenyl cytosine (CPEC) triphosphate (CPE-CTP) is a known inhibitor of CTP synthetase (Kang *et al*, 1989). With the hope of gaining insight into the regulation of chlamydial CTP synthetase, mutant *C. trachomatis* were isolated which were resistant to CPEC. The fact that the development of resistance to high levels of CPEC (resistant up to 100 μM CPEC) occurred during the selection procedure at low (between 2 and 5 μM) CPEC concentrations suggested that this resistance may be the result of some structural mutation in the CTP synthetase rather than an overexpression of the enzyme (like that seen for hydroxyurea resistance of ribonucleotide reductase). This was confirmed by sequencing of the CTP synthetase gene from CPEC-resistant *C. trachomatis* which showed a single (T to G) point mutation. The mutation causes an aspartate to glutamate substitution at a highly conserved amino acid (all known CTP synthetases have an aspartic acid at this residue) (Figure 14). Although this would not affect the overall charge of the protein, the structure may be altered

such that CPE-CTP is no longer able inhibit the CTP synthetase activity. Recent studies of CPEC resistance in Molt-4 lymphoblasts indicated that the primary mechanism of resistance to CPEC in this particular case was a decreased uridine/cytidine kinase activity which thereby blocked the phosphorylation of CPEC to its cytotoxic triphosphate form (CPE-CTP) (Blaney *et al*, 1993). They suggested, however, that another mechanism such as increased or altered CTP synthetase activity could also be involved in order to account for the high level of CPEC resistance (Blaney *et al*, 1993). Other studies using mammalian CTP synthetase showed that CTP synthetase mutants resistant to arabinosyl cytosine and 5-fluorouracil are also cross resistant to CPEC (Whelan *et al*, 1993). The mutations in CTP synthetase responsible for multi-drug resistance are clustered on three conserved regions of the polypeptide and all mutations in these regions resulted in the loss of CTP feedback inhibition (Whelan *et al*, 1993). Although the aspartate to glutamate substitution found in the CPEC-resistant *C. trachomatis* L2 mutant (L2CP^{R}-5.0) is not one of the mutations found by Whelan *et al* (1993), the L2CP^{R}-5.0 mutation is located in one of the above mentioned conserved regions which is predicted to be the CTP binding domain (Whelan *et al*, 1993). I predict that further studies with L2CP^{R}-5.0 will show multiple drug resistance of the CTP synthetase, and that CTP feedback inhibition has been lost.

5. Summary

The metabolism of nucleotides in *C. trachomatis* is summarized in Figure 18. The host cell's intracellular environment provides NTPs which are taken up by the chlamydiae, supposedly via specific transport systems. Chlamydiae convert the NTPs to dNTPs, the precursors for DNA synthesis, using a chlamydial specific ribonucleotide reductase. This chlamydial enzyme resembles class I ribonucleotide reductases since it is sensitive to the class I reductase inhibitor hydroxyurea. The draw on NTPs from the host cell by chlamydiae is significant but not detrimental to the host cell in terms of the host cell's energy charge.

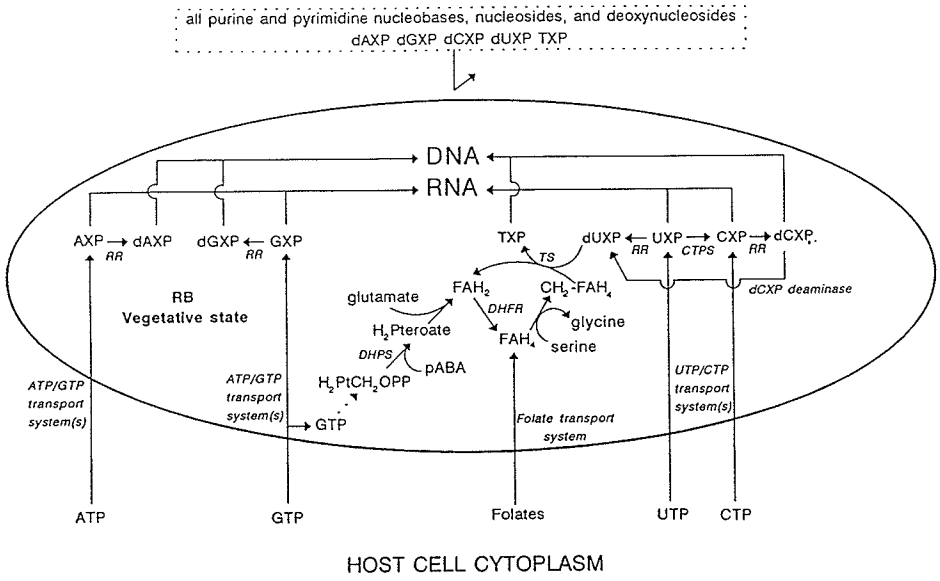


Figure 18: Summary of nucleotide metabolism in *C. trachomatis*. This model, borrowed from a review by Grant McClarty (1994), outlines the strategy *C. trachomatis* uses to obtain nucleotides. These pathways are thought to be present during RB growth. The chlamydial inclusion membrane is not shown for simplicity. The data in this thesis shows that nucleobases, nucleosides, deoxyribonucleosides, and deoxyribonucleotides (dNTP) are not taken up or are not metabolized by chlamydiae. It appears that transport system(s) for ribonucleotides (NTP) are present and are responsible for the direct acquisition of NTPs from the host cell cytoplasm. Data in this thesis also shows that the dNTPs, required for DNA synthesis, are generated by a chlamydial ribonucleotide reductase (RR). The presence of a chlamydial CTP synthetase (CTPS) indicates that *C. trachomatis* is auxotrophic for only three of the four NTPs. Fan *et al* (1991) have shown that dihydrofolate reductase (DHFR) and thymidylate synthase (TS) are present for the generation of the required thymidine deoxynucleotides. The appropriate (deoxy)ribonucleoside phosphates are indicated by (d)NXP.

Although CTP can be taken up by chlamydiae directly from the host cell, it can also be synthesized by a chlamydial specific CTP synthetase. The coding of the chlamydial CTP synthetase on an operon involved in lipopolysaccharide biosynthesis suggests that CTP synthetase mainly functions to provide CTP for the "charging" of KDO sugars (CMP-KDO). Other ways of obtaining (d)NTPs such as salvage and *de novo* pathways appear to be absent in *C. trachomatis*. However, the enzyme deoxycytidine nucleotide deaminase is present for conversion of deoxycytidine nucleotides to deoxyuridine nucleotides. In addition, Fan *et al* have (1991) shown that thymidylate synthase and dihydrofolate reductase are also present in *C. trachomatis*.

The data presented in this thesis provide considerable insight into the dynamic process of intracellular parasitism with respect to nucleotide metabolism. This work provides a basis for continued studies in a number of different directions. For example: What is the nature of the NTP transport system which must be present on the cytoplasmic membrane of *C. trachomatis* to allow for the net gain of NTPs? Is CTP synthetase an essential enzyme for LPS biosynthesis? Is the CTP synthetase/CMP-KDO synthetase/ORF3 encoded in an operon, and if so, how is it regulated throughout the chlamydial life cycle (translational coupling?)? Is the site of the point mutation in the CPEC-resistant CTP synthetase a critical residue in the active site and/or allosteric site of this enzyme?

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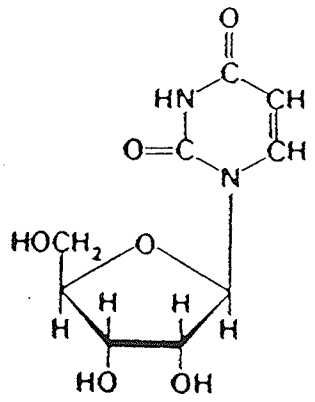
APPENDIX

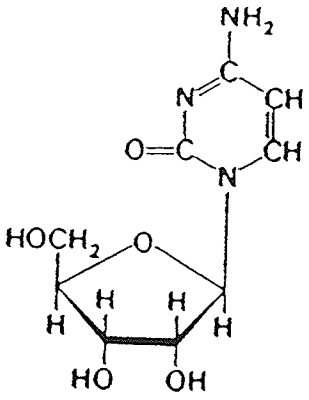
1. Abbreviations:

p.i.	post infection
MI	mock-infected
HU	hydroxyurea
RR	ribonucleotide reductase
NTP	ribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
CPEC	cyclopentenyl cytosine
KDO	3-deoxy-D-*manno*-octulosonic acid
RB	reticulate body
EB	elementary body
MOMP	major outer membrane protein
LPS	lipopolysaccharide
M_r	molecular weight
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
APRT	adenine phosphoribosyltransferase
MEM	minimal essential media
HPLC	high performance (pressure) liquid chromatography
MOI	multiplicity of infection
PCR	polymerase chain reaction

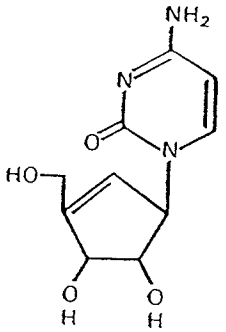
2. Structures



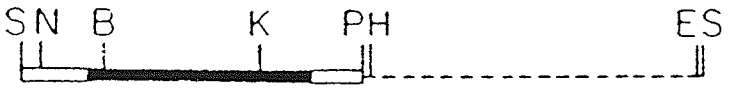
Uridine



Cytidine



CPEC (cyclopentenyl cytosine)



pMW5 : 2.6 kb insert in pUC8 vector (dashed line), *pyrG* (solid box), *E. coli* flanking DNA (open box), SalI (S), NruI (N), BamH1 (B), KpnI (K), PstI (P), HindIII (H).

From Weng *et al*, 1986.