Thymidylate Synthesis and Folate Metabolism by the Obligate Intracellular Parasite *Chlamydiae* - Metabolic Studies and Molecular Cloning

By

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A Thesis

Submitted to the

Faculty of Graduate Studies

in Partial Fulfillment of the

Requirement for the Degree of

Doctor of Philosophy

Department of Medical Microbiology

University of Manitoba

Winnipeg, Manitoba

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THYMIDYLATE SYNTHESIS AND FOLATE METABOLISM BY THE OBLIGATE INTRACELLULAR PARASITE *Chlamydiae* -METABOLIC STUDIES AND MOLECULAR CLONING

BY

HUIZHOU FAN

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

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ACKNOWLEDGEMENT

I sincerely thank Bob Brunham and Grant McClarty for their guidance, support and encouragement. I appreciate the assistance from the people in Bob's and Grant's laboratories. I am grateful to Guangming Zhong, Theresa Birkholz and Chunlin Yang for their help and support. I acknowledge Kevin Coombs and the staff in his laboratory for providing me with liters of cells. Finally, but most importantly, a big thank you goes to my wife and my family members for their love, support and sacrifice.

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ABSTRACT

Since host cell-derived thymidine is not incorporated into *Chlamydia trachomatis* DNA, we hypothesized that chlamydiae must synthesize dTMP de novo for DNA replication. The only known enzyme performing de novo dTMP synthesis is thymidylate synthase (TS). The goals of this thesis were to provide biochemical evidence for the existence of TS in chlamydiae, to investigate the mechanism by which the parasite obtains folate, a necessary cofactor for TS, and to provisionally characterize chlamydial TS. Results of a series of in situ experiments using a mutant cell line as chlamydial host which is incapable of de novo dTMP synthesis suggest that *C. trachomatis* converts dUMP into dTXP. In vitro experiments conclusively establish these findings by the demonstration of TS activity in extracts prepared from host-free chlamydial reticulate bodies. Furthermore it was found that both sulfa-sensitive and sulfa-resistant chlamydial strains can synthesize folates de novo; however strains vary significantly in their ability to transport preformed folates from the host cell. A *C. trachomatis* gene which is capable of complementing thymidine auxotrophy in *Escherichia coli* deficient in TS was cloned. Auxotrophic *E. coli* containing the complementing chlamydial DNA sequence converts dUMP to dTMP, using methylene tetrahydrofolate as the cofactor. The complementing DNA fragment contains an open reading frame of 1587 bp. Surprisingly this open reading frame shows absence of sequence homology to known TS. Unique in vitro characteristics shared by the enzyme activities from both chlamydial extract and recombinant *E. coli* extract suggest that *C. trachomatis* might encode a novel TS.

INTRODUCTION

Chlamydiae are obligate intracellular eubacteria; because of their unique developmental cycle, they are classified in their own order *Chalmydiales*, which is composed of one family *Chlamydiaceae*, with a single genus *Chlamydia* currently consisting of four recognized species: *C. trachomatis, C. psittaci, C. pneumoniae* and *C. pecorum* (Page, 1966; Page, 1968; Storz and Page, 1971; Moulder et al, 1984; Grayston et al, 1989; and Fukushi and Hirai, 1992). *C. trachomatis* and *C. psittaci*, the two most well studied species, are differentiated from each other on the basis of: 1) morphology of the inclusion; 2) accumulation of glycogen within the inclusion (as determined by iodine staining); 3) the ability to synthesize folates de novo (as determined by susceptibility to growth inhibition by sulfa drugs); and 4) natural host range (human vs non-human) (Page, 1966; Page, 1968; Storz and Page, 1971; Moulder et al, 1984; and Moulder, 1988). *C. trachomatis* naturally infects humans and mice and forms diffuse inclusions in which glycogen accumulates. The growth of *C. trachomatis* is susceptible to the action of sulfonamides. In contrast, *C. psittaci* naturally infects many birds and non-human mammals and forms dense inclusions lacking glycogen. With very few exceptions, members of *C. psittaci* are resistant to sulfa drugs. Some members which were formerly grouped under *C. psittaci* have recently been assigned to form two new species, *C. pneumoniae* (Grayston et al, 1989) and *C. pecorum* (Fukushi and Hirai, 1992). *C. pneumoniae* is a human pathogen although it resembles *C. psittaci* in other aspects, the bacterium is resistant to sulfa drugs, and they also develop dense inclusions

containing no glycogen (Grayston et al, 1989). *C. pecorum* is normally isolated from ruminants and also yields compact inclusions which do not contain glycogen, and they are resistant to sulfa action (Fukushi and Hirai, 1992).

The use of modern techniques for examining the relatedness of DNA from different sources has played a central role in recognition and confirmation of the present chlamydial taxonomy (Table 1, as illustrated by Fukushi and Hirai, 1992). Members within *C. trachomatis* are divided into three biovars, trachoma, lymphogranuloma, and mouse (Moulder, 1988). The trachoma and lymphogranuloma biovars can be further grouped into 15 serovars based on serological analysis. Serovars A, B, Ba, and C are the agents of trachoma and are the leading cause of preventable blindness. Serovars D through K are a common cause of sexually transmitted genital infection world wide and serovars L1 through L3 are the causative agents of lymphogranuloma venereum, a sexually transmitted disease (Moulder, 1988). There is high DNA homology between biovars trachoma and lymphogranuloma both being pathogens of humans; while they share significantly less homology with the mouse biovar which cause pneumonitis (See footnote of Table 1) (Moulder, 1988; and Fukushi and Hirai, 1992). *C. psittaci* is not divided into biovars because it is so heterogeneous that rational subdivision is presently not possible. *C. pneumoniae* and *C. pecorum* are not subdivided.

The unique life cycle of chlamydiae consists of two cell types, elementary body (EB) and reticulate body (RB). EBs are metabolically inert and their biological role is to

Table 1 **Levels of DNA-DNA Homology for *C. trachomatis, C. psittaci, C. pneumonia* and *C. pecorum***

Species	% Homology with			
	C. trachomatis	*C. psittaci*	*C. pneumoniae*	*C. pecorum*
C. trachomatis	20-92[a]	1-33	1-7	1-10
C. psittaci		14-95	1-8	1-19
C. pneumoniae			94-96	10
C. pecorum				88-100

[a] The levels of DNA-DNA homology are more than 92% between strains belonging to biovars trachoma and lymphogranuloma and 20% between mouse biovar and strains belonging to other biovars (Fukushi and Hirai, 1992).

survive the hostile extracellular environment and transmit infection from host to host. RBs are the metabolically active but non-infectious form responsible for chlamydial replication within the host cell (Moulder, 1991). Chlamydiae undergo their life cycle within the confines of a membrane-bound vacuole called an inclusion. The life cycle commences with the attachment of one or more EBs to the host cell. Soon after entry, EB differentiates to yield an RB which replicates by binary fission. Near the end of the developmental cycle, RBs differentiate back to EBs which are then released from the cell and then can start another infection cycle. Under optimal conditions in vitro, a cycle takes about 40-50 hours in *C. trachmomatis* lymphogranuloma biovar and *C. psittaci* (Ward, 1988). At about 20-30 hours after infection, most intracellular parasites are RBs. However, the developmental cycle observed in vitro is asynchronous. Interestingly, it has been observed that *C. trachomatis* grows more synchronously in cultured polarized cells treated with specific growth factors (Wyrick et al, 1989). Whether this phenomenon has implications for chlamydial infection in vivo requires further study.

Since chlamydiae are a specialized group of pathogenic intracellular bacteria, it is of great interest to study the biological events contributing to their unique life cycle. Binding of chlamydiae to host cell is critical to initiate infection. However, how they attach and enter the host cell is poorly understood. Recently Zhang and Stephens (1992) have proposed a novel model in which the attachment of chlamydiae to the host cell is mediated by binding of a chlamydial synthesized glycosaminoglycan to complementary structures on the surfaces of both the parasite and the host cell. Interestingly, similar

mechanisms have been established for other intracellular parasites (Ortega-Barria and Pereira, 1991) and viruses (WuDunn and Spear, 1989; and Okazaki et al, 1991).

Several interesting events that directly relate to EB ⇄ RB transition during the development cycle of chlamydiae have been observed. Soon after entry, an EB develops into an RB which then multiplies in the inclusion. Hatch et al (1986) demonstrated the reduction of the disulfide-bond-cross-linked major outer membrane protein (MOMP) to its monomeric form within an hour after the entry of EB into cells. Reduction of MOMP probably allows for passive transport of nutrients necessary for early development. MOMP reduction itself requires chlamydial protein synthesis as demonstrated by the fact that MOMP reduction is inhibited by chloramphenicol (Hatch et al, 1986). Also at a very early stage of chlamydial development, Crenshaw et al (1990); and Plaunt and Hatch (1988) have detected RNA and proteins which are either not synthesized or synthesized at very reduced amounts compared to later stage, suggesting that early cycle-specific RNA and protein synthesis is required for establishment of further chlamydial activity following MOMP reduction. At the late stage of the development cycle, RB converts into EB by reduction in size, internal condensation, nucleoid condensation, and formation of rigid cell wall. This event resembles to some extent the reverse of the reorganization of the EB to the RB in the early stage of infection. In going from RB to EB, MOMP becomes cross-linked by disulfide bonds and three other outer membrane proteins (12, 59, and 62 kDa) exceptionally, rich in cysteine, are added to the outer membrane. The observtion that MOMP remains monomeric until late in the infection cycle and is cross-

linked at the moment of host cell lysis and release of infectious EBs (Bavoil et al, 1984; and Hatch et al, 1986) suggests that cross-linking of MOMP might be a spontaneous response to the exposure of chlamydiae to the oxidizing extracellular environment. However Newhall (1988) observed that MOMP becomes progressively cross-linked during the last 24 hr of the developmental cycle, suggesting that intracellular formation of disulfide bonds could be brought about by specific chlamydial enzyme(s). Nucleoid condensation possibly precedes MOMP cross-linking during the conversion of RB to EB and is accomplished by two or more distinct DNA-binding proteins. Biological properties of two such proteins (Hc1 and Hc2) have been carefully examined. Both are EB-specific and homologous to eukaryotic histone H1 (Hackstadt et al, 1991; Tao et al, 1991; Perara et al, 1992; and Brickman et al, 1993). Expression of the 18-kDa Hc1 in *E. coli* causes the formation of a condensed nucleoid structure similar to that of EBs (Barry III et al, 1992) and expression of the 24-kDA Hc2 in *E. coli* induces a compaction of bacterial chromatin that is distinct from that observed upon Hc1 expression (Brickman et al, 1993). Therefore, Hc1 and Hc2 may play discrete roles in chlamydial differentiation at late-stage in the developmental cycle.

Being obligate intracellular parasites of humans, *C. trachomatis* and *C. pneumoniae* cause diseases worldwide; *C. psittaci* strains are one of the major threats to agriculture for they infect hundreds of species of birds as well as many species of mammals (Ward, 1983). *C. pecorum* causes numerous diseases in cattle and sheep. Undoubtedly, related to their success as parasites, chlamydiae have evolved a spectrum

of strategies of parasitism. Once located inside the host cell, chlamydiae perform all the biological activities within an inclusion. In the host cell cytoplasm, the chlamydial inclusion does not fuse with cell organelle lysosome (Friis, 1972; Kordova et al, 1971; Lawn et al, 1973; and Taverne et al, 1974). This strategy has also been adopted by some other intracellular pathogenic organisms such as *Mycobacterium tuberculosis* (Armstrong and Hart, 1971), *Legionella pneumonophila* (Horwitz, 1983) and *Toxoplasma gondii* (Jones and Hirsch, 1972). The chlamydial inhibition of phagosome-lysosome fusion requires as-yet-undefined structure(s) present in EBs but does not seem to need gene expression since it is a property of purified outer membranes of chlamydial EBs (Eissenberg et al, 1983; Friis, 1972; and Tribby et al, 1973). Prevention of lysosome fusion might be a factor determining the infectivity of EBs since RBs are rapidly destroyed even though they can also attach to and be ingested by the host cell (Wyrick and Brownridge, 1978).

The doubling time of intracellular chlamydiae is estimated to be 2 hr and they are considered to be fast growing when compared to other intracellular microorganisms such as rickettsiae and intracellular mycobacterium (Moulder, 1991). Between hundreds, and thousands of chlamydiae can be seen in a cell about 20 hours after infection by a single EB. Due to the limited size of their genome (1.0×10^6 bp) (Bikelund and Stephens, 1992), which was estimated to encode some 600 proteins (Stephens, 1988), many of which are likely essential for the unique biphasic life cycle, it has been speculated that chlamydiae must rely on the host cell for supplying many metabolic intermediates (Hatch,

1988). Obtaining preformed nutrients from the host cell may be an advantage for an intracellular parasite. While growing in the host cell, chlamydiae have access to a constant nutrient supply from the host in the absence of competition from other organisms. In contrast, although extracellular microorganisms can utilize nutrients present in growth environment, their growth is much more vulnerable to nutrient changes in growth environment and competition from a variety of species.

Despite the general concept that chlamydiae obtain a wide variety of compounds from the host cell, knowledge about which nutrients/cofactors chlamydiae can and/or must obtain from their host, and how these factors are allocated into, and then metabolized inside the parasites is limited (Hatch, 1988). Nevertheless, it is of interest to address these questions, since studying chlamydial metabolism will be indispensable for uncovering insights into chlamydial parasitism and might possibly lead to discovery of unique pathways related to the specialized life cycle. From a more practical perspective, metabolism studies will also be useful in providing a rational basis for intervention (treatment and prevention) of chlamydial infection and in the development of live attenuated chlamydiae for use as vaccine.

The way in which chlamydiae obtain nucleotides as a form of energy for many biological activities and as precursors for DNA replication and RNA synthesis has drawn many scholars' attention. Currently, the hypothesis of chlamydiae being an energy parasite is widely accepted (Moulder, 1991; and Hatch, 1988). In cell culture, chlamydial

growth is dependent on the presence of ATP and other high-energy metabolites generated by host cells and blocking energy generation prevents chlamydial growth (Becker and Asher, 1971; and Gill and Stewart, 1970). More specifically, it has been demonstrated that chlamydiae directly obtains nucleoside triphosphates (NTPs) from the host-cell cytoplasm (Hatch, 1975; McClarty and Tipples, 1991; and Tipples and McClarty, 1993). Free chlamydiae do not consume oxygen (Weiss et al, 1964), and evolution of carbon dioxide by host-free RBs is absolutely dependent on exogenous ATP (Weiss, 1965). Although host-free chlamydiae do degrade glucose (Weiss et al, 1964) and glucose-6-phosphate (Weiss, 1965) and also carry out part of the tricarboxylic acid cycle (Weiss, 1967), none of the reactions leads to generation of energy (Weiss, 1967). Moreover, flavoprotein and other cytochrome respiratory enzymes are absent in chlamydiae (Allan and Borvarnick, 1957; and Alland Borvarnick, 1962). More positive evidence for the energy parasite hypothesis was provided by Hatch et al (1982) who identified an ATP-ADP translocase in chlamydiae. Using the translocase, chlamydiae exchange ADP for host-generated ATP (Hatch et al, 1982), a similar mechanism is found in mitochondria (but with opposite orientation of nucleotide translocation) and rickettsiae (Winkler, 1976). Inhibition of host mitochondrial function with ethidium bromide (Becker and Asher, 1971) and antimycin (Gill and Stewart, 1970) causes reduced chlamydial growth. In addition, chlamydial inclusions are closely associated with mitochondria (Byrne, 1988); however, chlamydiae can grow in cells deficient in oxidative phosphorylation, suggesting that mitochondria function might not be essential for chlamydial growth (Tipples and McClarty, 1993).

Exact mechanisms by which chlamydiae obtain a net gain of nucleotides to support DNA replication and RNA synthesis are not known. The ATP-ADP translocase mechanism, while supplying a source of high energy phosphate, does not result in a net gain of nucleotides. Chlamydiae cannot synthesize purines or pyrimidines de novo as demonstrated by the fact that chlamydiae fail to incorporate radiolabelled glycine and aspartic acid into nucleic acids in host cell lines deficient in purine and pyrimidine de novo synthesis, respectively (Tipples and McClarty, 1993; McClarty and Fan, 1993; and McClarty and Qin, 1993). As a consequence, chlamydiae rely solely on the host cell for the precursors to synthesize RNA and DNA. Both *C. psittaci* (Hatch, 1975) and *C. trachomatis* (McClarty and Tipples, 1991; and Tipples and McClarty, 1993) have been shown to draw on the total acid-soluble NTP pools of their host for biosynthesis of their own RNA. The size of the *C. trachomatis*-infected host-cell NTP pools decrease by approximately 50% at mid-growth cycle when chlamydial RNA, DNA, and protein synthesis activity are maximal (Tipples and McClarty, 1993). However, *C. trachomatis* is incapable of directly utilizing host-supplied nucleobases and nucleosides (McClarty and Tipples, 1991; Qin and McClarty, 1992; and Tipples and McClarty, 1993). Since most bacteria can utilize exogenous nucleobases and/or nucleosides and are usually impermeable to highly charged nucleotides, chlamydiae stand out as highly unusual.

By using appropriate mutant cell lines with defined deficiencies in nucleotide metabolic pathways, it has been concluded that *C. trachomatis* is incapable of interconverting purine nucleotides (Tipples and McClarty, 1993). However, as

demonstrated, by using a CTP synthase-deficient cell line, it can convert UTP to CTP (Tipples and McClarty, 1993). Therefore, *C. trachomatis* appears to be auxotrophic for three of the four ribonucleoside triphosphate (ATP, GTP and UTP).

Tribby and Moulder (1966) reported that purine deoxyribonucleosides were incorporated into *C. psittaci* DNA but pyrimidine deoxyribonucleosides were not. Work by McClarty and Tipples (1991) indicates that the previously described incorporation of purine deoxyribonucleoside into chlamydial DNA was due to incorporation of purine nucleotides generated by the host cell purine deoxyribonucleoside catabolism and purine nucleobase salvage, and not by chlamydial uptake of purine deoxyribonucleosides(tides). By employing inhibitors of the purine catabolic reactions and mutant cell lines deficient in purine salvage enzymes (adenine and hypoxanthine-guanine phosphoribosyltransferases, respectively), it was concluded that chlamydiae take up neither purine nor pyrimidine deoxyribonucleotides from the host cell (McClarty and Tipples, 1991).

Given that chlamydiae can not synthesize purines or pyrimidines de novo nor salvage purine or pyrimidine nucleobases or nucleosides, chlamydiae must synthesize their own deoxyribonucleotides using host cell-supplied ribonucleotides as substrates. Ribonucleotide reductase is the only enzyme known to catalyze this conversion. Tipples and McClarty (1991) have obtained mutant *C. trachomatis* isolates whose growth is resistant to high concentration of hydroxyurea, an inhibitor of class 1 ribonucleotide

reductases, suggesting that chlamydiae do obtain ribonucleotides and subsequently convert them to deoxyribonucleotides by a chlamydial ribonucleotide reductase. Several types of ribonucleotide reductase have so far been recognized. They differ from one another in primary structure (single subunit monomer vs homodimer vs heterodimer), number of phosphates in substrates (di- vs triphosphates), condition of expression (aerobic vs anaerobic) and radical generator (iron-tyrosine vs others) (Reichard, 1993).

The inability to incorporate exogenous thymidine into chlamydial DNA has been very well documented (Starr and Sharon, 1963; Tribby and Moulder, 1966; Lin, 1968; Hatch, 1976; and Bose and Liebhaber, 1979). Even when the host cell has a fully active thymidine kinase (TK) and there is extensive labelling of host cell DNA, thymidine is minimally incorporated into chlamydial DNA (Hatch, 1976; and Bose and Liebhaber, 1979). Furthermore, it has been shown that *C. psittaci* 6BC (Hatch, 1976) lacks detectable thymidine kinase (TK) and grows normally in TK-deficient cell lines (Hatch, 1976). All these findings suggest that chlamydiae are unable to utilize host-supplied thymidine and/or thymidine nucleotides. Ribonucleotide reductase alone is capable of directly supplying three (dATP, dCTP, and dGTP) of the four dNTPs required for DNA replication (Reichard, 1988; and Stubbe, 1990). To meet the requirement for dTTP, the deoxyuridine phosphate generated by ribonucleotide reductase must be converted into thymidine phosphate. In all cells studied, a single enzyme, thymidylate synthase (TS), catalyzes this conversion (Ivanitich and Santi, 1989; and Maley and Maley, 1990).

Experiments to supply biochemical evidence for the existence of TS in chlamydiae and to initially characterize this enzyme have been major goals of this thesis.

The thymidylate synthesis cycle refers to the three sequential reactions catalyzed by TS, dihydrofolate reductase (DHFR), and serine hydroxymethyltransferase that are required for de novo synthesis of dTMP (Fig. 1). TS catalyzes the conversion of dUMP and 5,10-methylene tetrahydrofolate (5,10-CH_2-H_4folate) into dTMP and dihydrofolate (H_2folate). To maintain the cellular level of TS cofactor, the generated H_2folate has to be recycled. H_2folate is first reduced by DHFR to give H_4folate which is then converted, by serine hydroxymethyltransferase, back to TS cofactor 5,10-CH_2-H_4folate by the addition of a one-carbon unit (Ivanitich and Santi, 1989). Blockade of this cycle causes depletion of dTMP, cessation of DNA synthesis and resultant "thymineless death" (Maley and Maley, 1990; and Santi and Danenberg, 1984). Therefore the TS cycle has been chosen as a popular target for chemotherapeutic agents (Hitching, 1983; Maley and Maley, 1990; and Santi and Danenberg, 1984).

Primary structures of TSs from seventeen species have been obtained by direct amino acid sequencing and/or deduction from cloned nucleotide sequences (Fig. 2, from Ivanetich and Santi, 1989). TS in bacterophages, bacteria, mammalian viruses, yeast and vertebrates is a dimer of identical subunits of about 35 kDa. The subunit of TS in

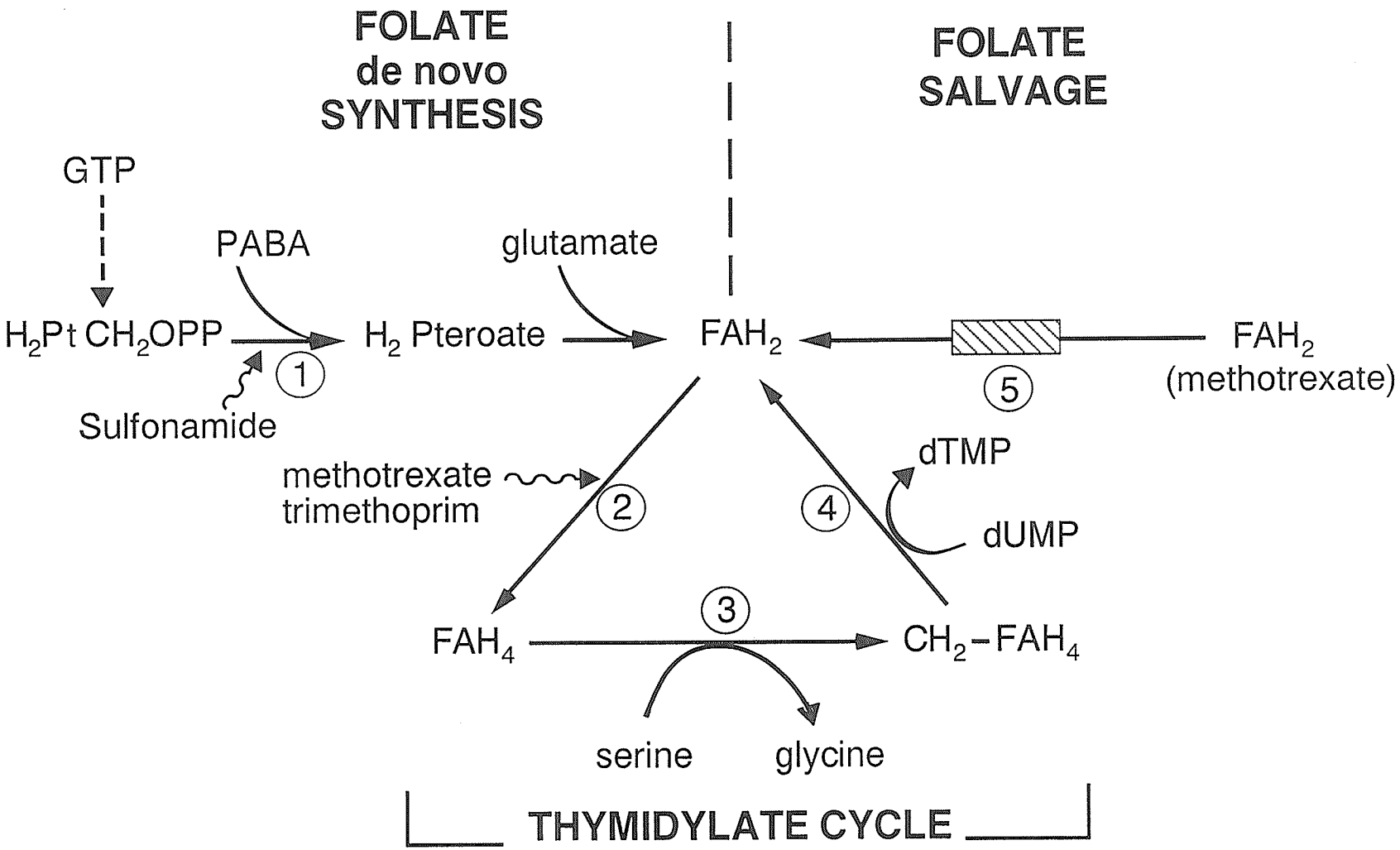
FOLATE
de novo
SYNTHESIS
FOLATE
SALVAGE
GTP
PABA
glutamate
$H_2Pt\ CH_2OPP$
H_2 Pteroate
FAH_2
FAH_2
(methotrexate)
1
5
Sulfonamide
methotrexate
trimethoprim
2
4
dTMP
dUMP
3
FAH_4
CH_2-FAH_4
serine
glycine
THYMIDYLATE CYCLE

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Figure 1. **Schematic Diagram of the Thymidylate Synthesis Cycle and its Relation to Folate *de novo* Synthesis and Salvage.** Not all possible routes of metabolism are included, just major routes relevant to this study. Squiggly arrows represent steps inhibited by sulfisoxazole, trimethoprim and methotrexate. Important enzymes are numbered as follows: 1, dihydropteroate synthase; 2, dihydrofolate reductase; 3, serine hydroxymethyltransferase; 4, thymidylate synthase; and a membrane transport system for folates. FAH_2, dihydrofolate; FAH_4, tetrahydrofolate; and CH_2-FAH_4, 5,10-methylene tetrahydrofolate.

parasitic protozoans is physically linked to DHFR, another enzyme in the TS cycle, as a single polypeptide. Depending on the species, the bifunctional TS-DHFRs have sizes ranging from 110 to 140 kDa, with subunit sizes of 55 to 70 kDa (Ivanetich and Santi, 1989; Ivanetich and Santi, 1990). Each TS-DHFR contains two domains, with DHFR at the amino terminus and TS at the carboxyl terminus, with the two domains separated by a junction peptide of varying size (Garret et al, 1984). The bifunctional TS-DHFR in protozoa probably resulted from fusion of the two independent genes in a more primitive organism because of putative biological advantages.

Although bifunctional TS-DHFR has been found from only protozoa so far, in a couple of cases, DHFR and TS genes are closely related to each other. In bacteriophage T4, the DHFR gene is placed upstream of the TS gene, with one base overlapped between the two open reading frames (Chu et al, 1984). In *Bacillus subtilis*, a TS gene and DHFR gene are also overlapped by one nucleotide; however, they are present in the order of TS gene-DHFR gene in 5′⇒3′ orientation, being the opposite to that in T4 phage (Iwakura et al, 1988).

It has been speculated that one of the biological advantages of a bifunctional DHFR-TS may be the metabolic channelling of H_2folate. TS is unique among enzymes that use folate cofactors in that for each mole of dTMP formed 1 mole of H_4folate is consumed; DHFR must rapidly reduce the H_2folate needed for continued dTMP

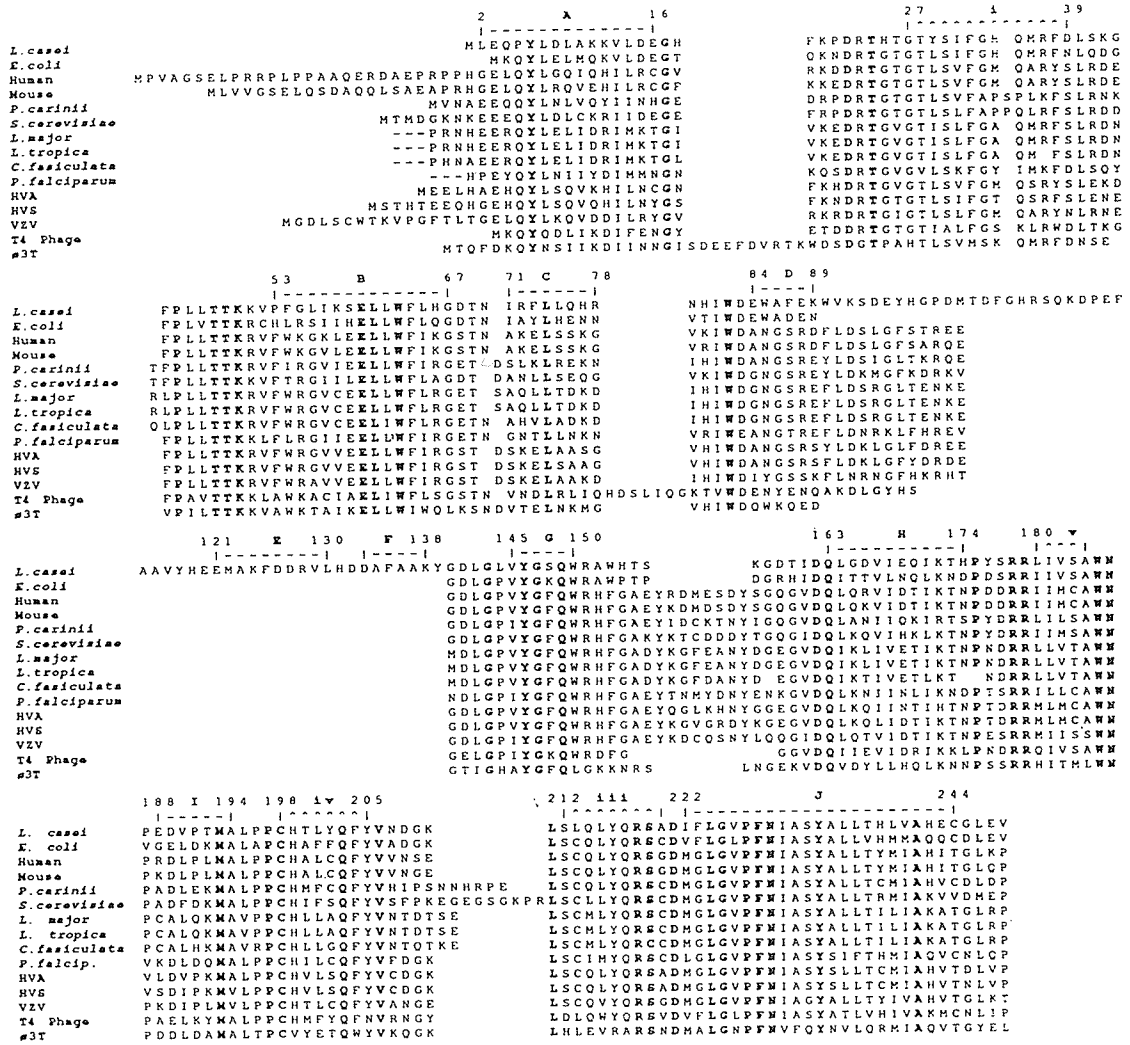

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Figure 2. **Amino Acid Sequences of TS from Different Species (Ivanetich and Santi, 1989).** Residues conserved among all species are shown in boldface. *L. casei, Lactobacillus casei; P. carinii, Pneumocystis carinii; S. cerevisiae, Saccharomyces cerevisiae; L. major, Leishmania major; L. tropica, Leishmania tropica; C. fasiculate, Crithidia fasiculate; P. falcip, Plasmodium falciperum;* HVA, *Herpesvirus ateles;* HVS, *Herpesvirus saimiri;* VZV, varicella-zoster virus; and ϕ3T, *Bacillus subtilis* phage ϕ3T.

synthesis. As the H_2folate produced by the TS of the bifunctional protein is channelled to and reduced by DHFR faster than it is released into the medium, the net rate of sequential reactions is governed by the rate of TS. There is no accumulation of inhibitory H_2folate, and there is no depletion of H_4folate; the bifunctional protein appears to be optimized for continued dTMP synthesis (Meek et al, 1985). In some other organisms, a similar effect may have been achieved by combining TS and DHFR within a multi-enzyme aggregate (Mathews et al, 1988).

Primary sequences from all sources studied have revealed that TS is one of the most conserved enzymes (Fig. 2). Nucleotide binding has been localized to cystinyl-SH group at residue 198 in *Lactobacillus casei* TS by peptide isolation of proteolysis of FdUMP-cofactor-enzyme ternary complex followed by peptide isolation (Maley and Maley, 1990). As determined similarly, folate-binding may involve either lysine-50 or lysine-51 (Maley and Maley, 1990). These residues are conserved in the enzyme from the species. In addition, X-ray structure of *L. casei* TS shows that all important secondary structural elements of TS are within highly conserved regions (Hardy et al, 1987), suggesting the core structure of *L. casei* TS will be similar to other TSs. Crystal structure of *Escherichia coli* TS containing the inhibitors, 5-fluorodeoxyuridylate (5-FdUMP) and 10-propagyl-5,8,-didazafolate, has also been resolved (Mathews et al, 1990a; and Mathews et al, 1990b). Amino acid residues from both subunits contribute to each active site (Hardy et al, 1987; and Mathews et al, 1990a and Mathews et al, 1990b). The substrate-binding pocket is a large funnel-shaped cleft surrounded by 30

amino acids, 28 from one subunit and two from the other. 5-FdUMP binds at the bottom of this pocket covalently linked through C-6 to cysteine. The folate derivative inhibitor binds in a partially folded conformation with its pABA-glutamate tail exposed at the entrance of the active cleft (Mathews et al, 1990a; and Mathews et al, 1990b). Ternary complex formation is associated with a large conformational change (Mathews et al, 1990; and Mathews et al, 1990b), confirming the transition-state intermediate proposed from kinetic studies (Maley and Maley, 1990).

Santi and Danenberg (1984) have described the TS catalytic mechanism in detail. Briefly, TS catalysis involves the initial formation of a covalent bond between the 6-position of dUMP and the -SH group of the conserved cysteine residue (number 198 in *L. casei* enzyme) of TS. Formation of this covalent adduct serves to activate the 5-position of dUMP for condensation of the one-carbon unit of the cofactor; this gives a covalent ternary complex in which the enzyme is linked to the 6-position of dUMP, and the cofactor to the 5-position. Subsequent proton removal, internal redox reaction and β-elimination provide the methylated pyrimidine nucleotide (dTMP), oxidized cofactor, and catalytically active enzyme (Santi, and Danenberg, 1984; and Ivanetich and Santi, 1989; and Maley and Maley, 1990).

Because of its central position in the pathway of DNA biosynthesis, TS is an important target in chemotherapy. A large number of analogues of nucleotide substrate dUMP and folate cofactor 5,10-CH_2-H_4folate have been synthesized and evaluated as

candidate inhibitors. Among inhibitors based on the structure of dUMP, 5-FdUMP is the one most extensively studied (Santi and Danenberg, 1984; and Maley and Maley, 1990). 5-fluorouracil which has been broadly used in cancer chemotherapy is known to inhibit TS after being converted to 5-FdUMP, although it has recently been suggested that the formation of nonfunctional fluorouracil-containing RNA might also contribute to fluorouracil cytotoxicity (Spiegelman et al, 1980). The mechanism of inhibition of 5-FdUMP has been proposed by Santi and Danenberg (1984) and supported by X-ray diffraction data (Mathews et al, 1990a; and Mathews et al, 1990b). Similar covalent bonds are formed between the inhibitor and the enzyme, resulting in a stable covalent adduct composed of TS, 5-FdUMP and the cofactor. The inhibition by 5-FdUMP is thus noncompetitive provided it is preincubated with the enzyme and CH_2-H_4folate prior to the addition of the substrate dUMP (Maley and Maley, 1990). Some of the dUMP analogues which have been modified in the pyrimidine ring or deoxyribosylphosphate group also have slight inhibition upon TS, but are of no practical value in chemotherapy (Santi and Danensberg, 1984).

In recent years more effort has been made to search for inhibitors based on folate rather than nucleotide derivatives for the folate molecule can be modified at numerous sites and a large number of derivatives can be synthesized. Practically any folate analogue will cause some inhibition of the enzyme reaction at high enough concentration (Santi and Danenberg, 1984). Analogues with very good inhibitory effect have been

obtained and some of them have been used for tumor therapy (Santi and Danenberg, 1984; and Jackman, 1993).

Recently, a molecular docking computer program was used to screen commercially available compounds for molecules that are complementary to TS (Shoichet et al, 1993). Besides retrieving the substrate and several known inhibitors, the computer program proposed putative inhibitors previously unknown to bind to the enzyme. Among the proposed chemicals, some have been shown to inhibit TS activity in vitro (Shoichet et al, 1993). Such inhibitors, having structures dissimilar to the substrate and cofactor, remain attractive because they are less likely to have the side effects that are produced by the nucleotide and folate analogues.

Folates are required for many other important biological reactions. The biologically active form of folate is H_4folate, which functions as a one-carbon unit carrier in the biosynthetic reactions, including methionine synthesis, purine nucleotide synthesis as well as dTMP synthesis (Shane and Stokstad, 1985; and Kisliuk, 1984). Folates also participate in the metabolism of serine, glycine and histidine (Schirck, 1984). In bacteria, folate is needed for the synthesis of pantothenic acid, a precursor for coenzyme A (Howell, 1988). Also in bacteria, folate plays a role in the formation of formylmethionyl-tRNA and initiation of protein synthesis (Staben and Rabinowitz, 1982). Furthermore, folates are necessary for DNA repair in bacteria (Johnson et al, 1988; and Payne et al, 1987).

As schematically shown in Fig. 1, folates can be obtained by living organisms by two different mechanisms: de novo synthesis and salvage. Most bacteria synthesize folates de novo since they are unable to take up exogenous folates (Hitching, 1983). In contrast, mammalian cells can obtain folates only by salvaging preformed folates from dietary sources (Shiota, 1984; and Hitching, 1983). The folate de novo synthesis pathway starts with the catalysis of dihydropteroate synthase (DHPS) which adds para-aminobenzoic acid (pABA) to 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (H_2PtCH_2OPP), and is accomplished by catalysis of dihydrofolate synthase which adds a glutamate onto the pABA residue in the dihydropteroate molecule to form H_2folate (Shiota, 1984). Sulfonamides are structural analogues and competitive antagonists of pABA, and thus prevent normal bacterial use of pABA for the de novo synthesis of folic acid (Anand, 1983). As such, microorganisms that are sensitive to sulfonamides must synthesize their own folates and those that can use preformed folates are resistant to sulfa drugs. For this reason, mammalian cells are not affected by sulfonamides (Hitching, 1983).

To enter the cell, exogenous folates have to be taken up by a folate transporter(s). All the folate transporters identified are energy-dependent and carrier-mediated (Henderson et al, 1977). A folate transport protein has been isolated and purified from the cell membrane of *L. casei*, one of the few bacteria auxotrophic for folates (Henderson et al, 1977). This transporter transports folates with broad specificity and high efficiency [e.g. folate, 5-methyl tetrahydrofolate (5-CH_3-H_4folate), 5-formyl tetrahydrofolate(5-CH0-

H_4folate) and folate-based chemotherapeutic agents such as methotrexate are each taken up with Kt values in the nanomolar range] (Henderson et al, 1977). *Pediococcus cerevisiae* also contains a transporter which, however, transports only reduced folates such as 5-CHO-H_4folate, 5-CH_3-H_4folate, and H_4folate, but not folate, H_2folate, methotrexate, and aminopterin (another derivative of folate) (Nandelbaum-Shavit and Grossowicz, 1970). Mammalian cells such as L1210 cells contain at least two types of folate transporters in the cell membrane, one primarily for reduced folates (Price and Freisheim, 1987; and Yang et al, 1988) and the other for folate (Henderson et al, 1988). The reduced folate transporter has a much higher efficiency. Methotrexate and aminopterin were transported by the high-efficiency transporter (Price and Freisheim, 1987; and Yang et al, 1988).

DHFR catalyzes the reduction of H_2folate, one of the products of thymidylate synthesis, to H_4folate by $NADPH_2$. From some sources, mainly vertebrates, the enzyme also catalyzes a much slower reduction of folate. Bacterial DHFRs have a higher specificity for H_2folate (Blakley, 1984). Compared to TS which has been a target primarily for tumor chemotherapy (Santi and Danenberg, 1984; and Jackman, 1993), DHFR has been a target for antibacterial, antiprotozoal, antifungal as well as antitumor chemotherapy (Burchall, 1983; and Blakley, 1984). This is because DHFRs from different species have relatively low sequence homology and structure similarity. Aminopterin and methotrexate, two analogues of folate, have extremely high affinity for DHFRs from any sources except the type 2 plasmid enzyme (Burchall, 1983; and

Blakley, 1984). In the presence of $NADPH_2$, covalent bonding occurs, forming a tight ternary complex (Burchall, 1983; and Blakley, 1984) and therefore they are lethal to any cell they can enter. However, they do not inhibit most bacteria because these drugs rely on active folate transporters for entry into the cell (Burchall, 1983; and Blakley, 1984). The second type of DHFR inhibitors do not structurally resemble folate. Being diaminopyrimidine derivatives, they are small molecules and generally get into cells by passive diffusion (Burchall, 1983). Trimethoprim is such a compound and strongly inhibits bacterial DHFR; and pyrimethamine is among those active against malarial DHFR (Burchall, 1983; and Rollo, 1983). The diaminopyrimidines such as trimethoprim are much less active against mammalian DHFR and have little or no side effect at the dose used clinically (Burchall, 1983). The reason for this difference in potency has been elucidated by X-ray crystallographic studies. Trimethoprim was found to fit the substrate-binding site of *E. coli* DHFR but not mammalian DHFR (Mathews et al, 1985).

The importance of folates to chlamydiae growth been knwon for nearly half a century. Morgan (1948) showed that the growth of *C. psittaci* 6BC is inhibited by sulfonamide. However, *C. psittaci* 6BC is the only well-characterized strain of *C. psittaci* that is sulfonamide sensitive, whereas all *C. trachomatis* strains are sensitive to sulfa drugs (Moulder, 1988; and Moulder, 1991). Although most *C. psittaci* are resistant to sulfa action, they also require folate for thymidylate synthase. A simple explanation for the selective action of sulfonamides has been that *C. trachomatis* is capable of de novo folate synthesis whereas *C. psittaci* is not and they likely have the capacity to transport

folates directly from the host cell cytoplasm (Moulder, 1988; Tribby and Moulder, 1966; Morgan, 1952; and Moulder, 1991). However, this simple speculation fails to explain numerous discrepancies observed in studies using antifolate compounds. The ability of *C. psittaci* to transport folate is supported by the observation that aminopterin causes growth inhibition of *C. psittaci* francis strain (sulfa resistant) (Morgan, 1948; and Morgan, 1952). Unexpectedly, it was also shown that sulfa sensitive *C. psittaci* 6BC was also sensitive to aminopterin growth inhibition and moreover this inhibition was reversed by 5-CHO-H_4folate (Morgan, 1948; and Morgan, 1952). In addition, Reeve et al (1968) demonstrated that trimethoprim, a non-substrate analogue DHFR inhibitor, inhibited the growth of sulfa sensitive *C. trachomatis*, and that this inhibition was also reversed by 5-CHO-H_4folate. Finally, Hortermann et al (1959), and Colon and Moulder (1955) detected a difference in the composition of chlamydial species folate pools when analyzed by using folate-requirering bacteria, *L. casei* and *P. cerevisiae*. Since folates are essential for chlamydial growth (Tribby and Moulder, 1966; Hatch, 1976; and Moulder, 1991) and important in taxonomic classification (Moulder, 1988), we wanted to clarify the inconsistencies in the existing literature concerning folate metabolism in chlamydiae (Morgan, 1948; Morgan, 1952; Reeve, 1968; and Moulder, 1991). Studying the folate metabolism and acquisition by chlamydiae has been another important component of this thesis.

In aggregate, the objectives of the research project comprising this thesis have been to: 1) obtain biochemical evidence for the existence of TS in chlamydiae; 2)

characterize chlamydial TS; and 3) investigate the mechanisms by which chlamydiae obtain folate required for TS.

The obligate intracellular growth of chlamydiae makes studying chlamydial metabolism difficult and challenging. In many cases, in order to study chlamydia, one has to deal with host cell metabolic properties. It is often difficult to differentiate metabolic activities of parasites from host. It is not easy to obtain sufficiently pure and metabolically active organism for enzyme assays (Hatch, 1988). In addition, there are no simple procedures to select, isolate, and characterize mutants; and currently no gene transfer system is available (Stephens, 1988). To overcome these difficulties, we have been studying chlamydial metabolism by using well defined mutant cell lines as hosts for chlamydial growth, employing specific metabolic inhibitors for differential blocking of the metabolic activities of the host and the parasite, growing chlamydiae in tissue culture with medium of defined composition, detecting enzyme activities using highly purified RB extract as the source of enzyme, and cloning and expressing chlamydial genes in a heterologous system. By using these strategies, we have confirmed the existence of TS in chlamydiae. Molecular cloning has been carried out for initial characterization of chlamydial TS. Sequence analysis of the complementing DNA suggests that chlamydiae might encode a novel TS. In addition, results also suggest that both *C. trachomatis* and *C. psittaci* can synthesize folates de novo; however there is a considerable difference in their ability to obtain preformed folates from the host cell.

MATERIALS AND METHODS

1. Materials:

[2-^{3}H]adenine (28 Ci/mmole), 5-[methyl-^{3}H]thymine (50 Ci/mmole), 5-[methyl-^{3}H]thymidine (65 Ci/mmole), [6-^{3}H]uracil (20 Ci/mmole), [5-^{3}H]uracil (20 Ci/mmole), [6-^{3}H]uridine (20 Ci/mmole), [5-^{3}H]uridine (21 Ci/mmole), [6-^{3}H]dUMP (15 Ci/mmole), [5-^{3}H]dUMP (20 Ci/mmole), [3,5-^{3}H]pABA (50 Ci/mmole), [3',5',7,9-^{3}H]H_2folate (38 Ci/mmole), and [3',5',7,9-^{3}H]folic acid (40 Ci/mmole) were purchased from Moravek Biochemicals (Brea, CA). [γ-^{32}P]ATP (7500 Ci/mmole) and [α-^{32}P]dCTP (3500 Ci/mmole) were purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Unlabelled pABA, folic acid, H_2folate, H_4folate, 5-CHO-H_4folate, 5-CH_3-H_4folate, 5-fluorouracil, 5-fluoro-uridine (5-FUR), 5-fluoro-deoxyuridine (5-FUdr), sulfisoxazole, methotrexate, aminopterin, trimethoprim and pyrimethamine were purchased from Sigma Chemical Co. (St. Louis, Mo). Eagle's minimum essential medium (MEM), RPMI 1640 medium, endonucleases, Taq DNA polymerase, double strand DNA sequencing kit, nick-translation kit, and random primer labelling kit were purchased from GIBCO (Grand Island, N.Y.). ATP, dNTP, and T4 DNA ligase were purchased from Pharmacia Fine Chemicals Inc. (Uppsala, Sweden).

Oligonucleotides were synthesized at the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta.

6-hydroxymethyl-7,8-dihydropterin pyrophosphate (H_2PtCh_2OPP) was kindly provided by C. Allegra at the Medicine Branch, National Cancer Institute, Bethesda, MD.

Purified homogeneous human TS was generously provided by B. Yates at Welcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC.

2. Synthesis of 5,10-CH_2-H_4folate and 10-CHO-H_4folate

5,10-CH_2-H_4folate was prepared essentially according to published procedures (Krungkrai et al, 1989). H_4folate was dissolved (final concentration: 1 mM) in pH7.5, 0.1M potassium phosphate buffer containing 200 mM 2-mercaptoethanol and 10 mM formaldehyde. The 5,10-CH_2-H_4folate synthesis reaction was allowed to proceed for 10 min at room temperature in the dark. Analysis of the resulting preparation by high performance liquid chromatography (HPLC) confirmed that >95% of H_4folate was converted to 5,10-CH_2-H_4folate. 5,10-CH_2-H_4folate was stored at -70°C for up to a year without significant decomposition.

10-formyl-tetrahydrofolic acid (10-CHO-H_4folate) was synthesized from 5-CHO-H_4folate by the method of Rabinowitz (1963). Fifty mg of 5-CHO-H_4folate was dissolved in 4 ml of 1 M 2-mercaptoethanol, and the solution was adjusted to pH 1.5 with 1 M

HCl and incubated at 4°C for 4 hr. The resulting suspension containing 5,10-methenyl-tetrahydrofolic acid was then neutralized with KOH to pH 7.0 and converted to 10-CHO-H_4folate by incubating in an evacuated vessel at 4°C for 4 hr.

3. Cell lines and Culture Conditions:

The wild-type Chinese hamster ovary (CHO) K1 cell line was obtained from American Type Culture Collection (ATCC; Rockville, MD). CHO DHFR$^-$ cells are a mutant CHO K1 subline deficient in DHFR activity (Urlaub and Chasin, 1980). GC_3TK^- cells which are deficient in thymidine kinase were derived from wild type human GC_3C_1 cell line (Rodparvar et al, 1990; and Houghton et al, 1989). Wild type L cells were kindly provided by K. Coombs at the Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba.

The mouse L cells were routinely grown at 37°C in suspension culture with minimum essential medium supplemented with 5% fetal bovine serum (FBS) (Intergen Company, NY) and 0.2 mM glutamine (for details see Chlamydiae Propagation). All other cell lines were maintained as monolayers on the surface of plastic tissue culture flasks (Corning Glass Works, NY) at 37°C in a humidified atmosphere containing 5% CO_2 in air. Since the CHO K1 cell line is auxotrophic for proline, it was maintained in MEM supplemented with 10% FBS, 0.2 mM glutamine and 0.3 mM proline. CHO DHFR$^-$ cells were grown in the same medium supplemented with 10% FBS, 0.2 mM

glutamine, 0.3 mM proline, 0.3 mM glycine, 30 μM hypoxanthine, and 30 μM of thymidine. GC_3TK^- cell line was maintained in RPMI 1640 supplemented with 10% FBS.

4. Chlamydiae strains

C. trachomatis strain L2/434/Bu was originally obtained from C.C. Kuo, University of Washington (Seattle, WA) and has been maintained in our laboratory since 1982. *C. psittaci* psittacosis strain 6BC (catalog No. ATCC VR-125) and meningopneumonitis strain francis (catalog No. ATCC VR-122) were purchased from ATCC. The authenticity of these strains was periodically confirmed by serologic typing with monoclonal antibodies kindly performed by A. Andersen, United States Department of Agriculture National Animal Disease Center (Ames, IA).

5. Chlamydiae Propagation:

1) Large scale suspension culture of mouse L cells

Mouse L cells were grown in suspension in 2-12 litre bottles with the medium as described above. An aliquots of cell suspension was taken daily and cells were counted for the determination of cell concentration. To maintain and propagate the cells in

culture, cells were diluted to 4-5 x 10^5 cells/ml with prewarmed fresh medium when the cell concentration reaches 1-1.5 x 10^6 cells/ml.

2) Preparation of EB stocks

Mouse L cells maintained in suspension were seeded into 150 cm^2 tissue culture flasks (about 4.0 X 10^7 cells per flask). After confluent cell monolayers formed (usually within several hours of incubation at 37°C), all but 5 ml of medium was removed. *C. trachomatis* or *C. psittaci* EBs at a multiplicity of infection (MOI) of 3 inclusion forming units per cell were added to the flask which was then returned to the 37°C incubator. After 1.5 hr absorption, 25 ml of medium containing 1 μg of cycloheximide per ml was added into each flask. Infected flasks were cultured at 37°C. At 40-44 hr after infection, approximately 50 glass beads with diameter of 2 mm were added to the flask and cells were rolled off the plastic. The cell suspension was transferred to a centrifuge tube and subjected to centrifugation at 30,000 x g for 20 min (Beckman, JA-20 rotor) at 4°C. The cell pellet was resuspended in cold, pH 7.0, sucrose-phosphate-glutamate solution (SPG) (1-20 ml per original flask as necessary) and sonicated using a Branson Sonifier (Danbury, Conneticut). Aliquots of sonicated EB preparation were stored at -70°C. For experiments of folate metabolism, EBs were further washed three times with SPG to remove folates which might be carried over from original culture medium or host cells.

3) Preparation of concentrated EB stocks of *C. trachomatis* L2

The procedure described below was used for the preparation of concentrated EB stock that was needed for large scale preparation of *C. trachomatis* EBs. When cell density reached 1-1.3 x 10^6 cells/ml, *C. trachomatis* EB stock and cycloheximide (final concentration, 1.0 μg/ml) were added into the cell suspension. The suspension was then returned to the 37°C incubator. After 40-44 hr incubation, infected cells were pelleted by centrifugation at 500 x g for 10 min at 4°C. The cell pellet was resuspended in cold SPG (about 10 ml SPG for 1 liter cell culture) and disrupted by sonication. EB aliquots were stored at -70°C.

4) Titration of the infectivity of EB stock

Infectious EBs in the stock used for metabolic labelling were quantitated by counting the number of inclusions formed in the host cells after infection. Mouse L cells were seeded onto 1 cm^2 round coverslips in dram vials in a similar manner to flasks as described above. A 100 μl sample of each of 10 fold serial dilutions of EB stocks were inoculated onto duplicate cell monolayers and allowed to absorb for 1.5 hr at 37°C. 1 ml of medium containing cycloheximide was added into each vial and the cultures were incubated at 37°C. At 24-30 hr after infection, the medium was aspirated and monolayers were fixed with 100% acetone for 5 min. Monolayers infected with *C. trachomatis* were stained for 30 min at 37°C with fluorescein isothiocyanate-conjugated monoclonal antibodies for *C. trachomatis* (Syva Microtrack, Syva Company, Palo Alto, CA). The number of fluorescent inclusions in 30 high-power fields (40 x objective) of a coverslip

were counted in a Leitz fluorescence microscope and the infectivity of the EB preparation was expressed as inclusion-forming units per ml. The *C. psittaci*-infected monolayers were first reacted with 1:100 diluted ascites of a monoclonal antibody specific for chlamydial LPS and then stained with 1:100 diluted fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Pierce Co.). IFUs were calculated as described above.

5) Preparation of RB extracts

Mouse L cells in suspension were infected with *C. trachomatis* or *C. psittaci* EBs as described above. At 20-24 hr after infection, cells were collected by centrifugation and resuspended in cold Hank's balanced salt solution (HBSS; 5 ml per 1 liter culture). The resulting cell suspension, bathed in ice-water, was sonicated at intensity 3 for 45 seconds. The preparation was clarified by centrifugation (500 X g for 10 min) at 4°C. The supernatant was collected and the pellet was resuspended in cold HBSS, and sonication and centrifugation were repeated. The two supernatants are combined and layered onto a Renografin density gradient consisting of 15 ml of 30% and 10 ml of 44% Renografin in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.0) containing 0.85% NaCl and then centrifuged for 60 min at 40,000 x g (16,000 rpm) at 4°C in a Beckman SW28 rotor. The RBs which migrated to the Renografin interface were collected and diluted with 2 volumes of cold 0.01 M potassium phosphate buffer (pH7.0) containing 0.1 M KCl followed by centrifugation. The RB pellet was resuspended in the same buffer containing 1 mM dithiothreitol, and the suspension was sonicated in an ice-

ethanol bath with ten 10 sec bursts with a microprobe at intensity of 5. The sonicated RB suspension was clarified by centrifugation at 10,000 x g for 15 min at 4°C. The RB lysate was immediately used for enzyme assays or was stored at -70°C for later use.

Purified sham extracts were prepared from mock-infected mouse L cells by the same procedure used to purify RBs from infected mouse L cells.

6. In situ Incorporation of Radiolabelled Precursors into Chlamydial Nucleic Acids

CHO K1 cells, CHO DHFR$^-$ cells, GC_3C_1 cells, and GC_3TK^- cells were seeded into 60-mm diameter disposable tissue disposable tissue culture dishes (Corning Glass Works) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Confluent monolayers formed within 24 hrs. Before infection, all but 0.4 ml of medium was removed from dishes and monolayers were inoculated with EBs in 0.1 ml volume and at an MOI of 5 IFUs per cell, which normally resulted in 90-100% of the cells infected. After 1.5 h absorption, 4.5 ml of medium containing 1 μg of cycloheximide per ml was added into each dish. Dishes containing infected cells were incubated at 37°C as described above. At specified times after infection, all but 2 ml of medium was removed. Radiolabelled precursor was added, without dilution, to yield a final concentration of 0.3 μM. Incubation in the presence of the isotope was continued for 2 hours at 37°C in the presence of 5% CO_2. In all cases, incorporation of label into nucleic acid remained linear

beyond 2 hours, at no time was the isotope limiting in the experiments. To terminate the incorporation, the dishes were transferred immediately to an ice bath, medium was aspirated, and monolayers were rinsed once with ice cold phosphate buffer saline (PBS). The cell monolayer was dissolved in 2 ml of 0.5 N NaOH and incubated at 37°C overnight to degrade RNA. To determine radiolabel incorporation into DNA, the DNA was precipitated from the NaOH solubilized sample by adding 5 ml of 10% trichloroacetic acid (TCA) prepared in 0.1 M tetrasodium pyrophosphate, and the samples were incubated at 4°C for 2 hours. The precipitated DNA was collected by filtration through Whatman GF/B glass microfiber filters, and, after sequential washing with 10% TCA and 95% ethanol, the radioactivity in the dried filters was counted in 5 ml of scintillation cocktail (Universol; ICN Biomedicals, Inc., Costs Mesa, CA) by a liquid scintillation counter (Beckman LS 5000). In all cases, the values obtained for mock-infected control cultures were subtracted from the values obtained for chlamydiae-infected cultures. Unless otherwise indicated, values are normalized to 10^6 cells.

7. Acid Hydrolysis of DNA and Subsequent Nucleobase Analysis

In order to determine which nucleobases were labelled in DNA isolated from cultures pulsed with [5-^{3}H]uridine or [6-^{3}H]uridine, the following procedure was carried out. CHO DHFR$^-$ cell monolayers in tissue culture dishes were prepared and infected as described above. At 22 hr after infection, [5-^{3}H]uridine or [6-^{3}H]uridine was added without dilution to achieve a final concentration of 0.3 μM, and incubation was continued

at 37°C for 2 hr. Reactions were terminated on ice, medium was aspirated, and the cell monolayer was washed 3 times with ice cold PBS. DNA was isolated essentially as described by Schwartzman and Pfefferkorn (1981). The washed monolayers were scraped off plastic in 0.5 ml of cell lysis buffer (pH 8.0, 100 mM tris-HCl containing 0.5% NP-40, 0.1% sodium dodecyl sulfate, 1mM EDTA and 10 μg/ml proteinase K) with help of a rubber policeman. The preparation was transferred into a test tube and incubated at 50°C for 1 hr and then phenol-chloroform extracted three times. Nucleic acids were ethanol-precipitated and reconstituted in 0.1 ml of RNase (10 mg/ml) prepared in TE buffer (10 mM tris-HCl/1 mM EDTA, pH 8.0). After 1 hr incubation at 37°C, DNA in the preparation was precipitated by addition of 50 μl of 100% TCA and stored at 4°C for 1 hr. The DNA precipitate was washed with 10% TCA three times and hydrolyzed to free bases by boiling in 100 μl of 11.3 N perchloric acid for 1 hr. The acid hydrolyzed samples were neutralized with 113 μl of 10 N NaOH and subjected to high performance liquid chromatography (HPLC) analysis after clarification by centrifugation. Isotope incorporation into nucleobases was measured by on-line radioactive flow detection (Beckman 171 radiodetector) after separation of the nucleobases by HPLC on a 12.5-cm μBondapak C-18 column (Waters Chromatography Division) under isocratic conditions (flow rate 1 ml/min with 100 mM ammonium acetate [pH 4.25] containing 0.5% acetonitrile buffer) (Eick et al, 1983). The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} (Beckman 1066 UV detector) of known nucleobase standards. All data were plotted and processed with an IBM PC50 and Beckman System Gold software.

Similar experiments were done using TS^- *E. coli* transformed with recombinant plasmids containing candidate *C. trachomatis* TS gene. The transformed bacteria were inoculated into 5 ml of enriched minimum medium (see bacteria media) containing ampicillin (final concentration, 100 μg/ml) and cultured at 37°C with constant shaking (200 rpm). When the OD value of the culture reached about 0.5, 1.0 ml of the bacteria suspension were transferred into a 15 ml culture tube and [6-^{3}H]uracil or [5-^{3}H]uracil (final concentration: 0.5 μM) was added into the bacteria suspension. To increase their utilization by the bacteria, 5 μM of unlabelled adenosine was also added in. The bacteria were cultured as above for 30 min and then collected by centrifugation. Hereafter the resulting bacteria pellet was treated in a similar fashion to chlamydiae-infected cells.

8. Preparation of Polyglutamate Hydrolase

Polyglutamate hydrolase was prepared and partially purified from hog kidneys according to the method of McMartin et al (1981). Two hog kidneys were defatted, sliced and homogenized with an electric homogenizer in 3 volumes of pH 6.0, 0.02 M potassium phosphate buffer. The preparation was centrifuged at 10,000 x g for 15 min (Beckman JA-21 centrifuger, JA-20 rotor). The supernatant was collected and solid ammonium sulfate was slowly added with stirring until 50% saturation was obtained. After stirring for an additional 20 min, the preparation was centrifuged at 30,000 X g for 15 min. The supernatant was treated with additional $(NH_4)_2SO_4$ to yield 75% saturation and the fraction containing polyglutamate hydrolase activity was pelleted down by

centrifugation (30,000 X g, 15 min) and reconstituted with 20 ml of pH 6.0, 0.2 M potassium phosphate. The preparation was dialyzed overnight against two 4 liters of the same phosphate buffer. After dialysis, the insoluble components were removed by centrifugation and the solution was heated at 60°C for 1 hr and precipitated protein was removed by centrifugation. Protein concentration of the supernatant was 17 mg/ml as determined by Bio-Rad standard assay and aliquots of the enzyme preparation were stored at -70°C.

9. Incorporation of [^{3}H]pABA into Chlamydial Folates in situ

Results from preliminary experiments indicated that [^{3}H]pABA incorporation by chlamydiae was greater if the host CHO K1 cells were depleted of intracellular folates. As a result, all [^{3}H]pABA-labelling experiments were done with CHO K1 cells that had been starved for folates before radiolabelling. To deplete CHO K1 cells of intracellular folates, cultures were grown first for one passenge in folate- and pABA-free Dulbecco modified Eagle medium (DME H-21), obtained from the Tissue Culture Facility, University of California San Francisco (San Francisco, CA), supplemented with 0.3 mM proline and 10% fetal bovine serum which had been extensively dialyzed against 10 mM PBS (pH7.4) and subsequently for 10 passages in the same medium containing 10% dialyzed serum, 0.3 mM proline, 0.3 mM glycine, 30 μM hypoxanthine and 30 μM thymidine. Since chlamydiae are auxotrophic for purine ribonucleotides, glycine, and

proline, it was necessary to keep these supplements in the culture medium after infection with chlamydiae and during the subsequent radiolabelling period.

In addition to depleting host cells of intracellular folates, it was also necessary to remove any folates in EB stock which was carried over from tissue culture medium and/or host cells by washing the EBs three times with SPG.

[^{3}H]pABA-labelling experiments were performed with parallel flasks (150 cm^2) of mock-infected and chlamydiae-infected folate-depleted CHO K1 cells (3-4 X 10^7 cells per flask). Immediately after 1.5 hr chlamydiae absorption, the cell monolayer was rinsed with HBSS, then 15 ml of DME H-21 medium supplemented with 10% dialyzed fetal bovine serum, 0.3 mM proline, 0.3 mM glycine, and 30 μM [^{3}H]pABA was added to each flask. After incubation at 37°C for 24 hr, the cells were harvested and intracellular folates were extracted as described previously (Allegra et al, 1986; and Boarman et al, 1990). Flasks were placed on ice, and the cell monolayer was rinsed 5 times with ice-cold PBS, then the cells were harvested into 1 ml of PBS by scraping the surface of the flask with a rubber policeman. The cell suspension was transferred to a 15 ml corex test tube and an equal volume of freshly-prepared solution containing 3% sodium ascorbate (pH6.0) and 3% 2-mercaptoethanol was added and the resulting mixture was heated at 100°C for 1 min. The cell debris were removed by centrifugation at 10,000 X g for 10 min (Beckman JA-20 rotor) at 4°C and the supernatant was treated with 0.5 ml of partially purified hog kidney polyglutamate hydrolase at 37°C for 30 min to convert all

folates to monoglutamates. Following hydrolase treatment, the preparation was again heated at 100°C for 1 min after addition of another 1 ml of 3% sodium ascorbate-3% 2-mercaptoethanol solution and the denatured protein was removed by centrifugation as described above. The folates in the preparation were concentrated and purified by using a C-18 cartridge (Sep-Pak; Waters Chromatography Division, Mioford, MA) as follows. The cartridge was prepared for the sample by initially washing with 10 ml of methanol followed by 20 ml of water, 2 ml of 5 mM PIC A (tetrabutyl ammonium phosphate; Waters Chromatography Division), and finally 10 ml of water. Elution of the sample was accomplished by using 3 ml of methanol, and the eluate was evaporated to dryness under a steady stream of N_2. The dried sample was dissolved in 100 μl of 5 mM PIC A. The solution was analyzed immediately or stored in liquid nitrogen. The individual folates were resolved by HPLC using a 12.5-cm μBondapak C-8 column (Waters Chromatography Division) under isocratic conditions; the mobile phase consisted of 22.5% methanol and 77.5% 5 mM PIC A, pH5.5 and had a flow rate of 1 ml/min. Isotope incorporation into individual folates was determined by on-line radioactive flow detection. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{290} of known unlabelled folate standards which were coinjected with sample. Data were collected and processed with an IBM PC 50 using Beckman System Gold software.

10. Determination of Protein Concentration

Protein content in RB extracts and other preparation such as hog kidney polyglutamate hydrolase and lysate of *E. coli* cells expressing (see below) cloned chlamydial thymidylate synthase were determined by using a Bio-Rad protein quantitation kit (Bio-Rad Laboratories, Richmond, CA).

11. Enzyme Assays

1) Assay of thymidylate synthase activity in situ.

Intracellular chlamydiae-specific TS activity was measured in infected CHO DHFR$^-$ cells by monitoring the amount of tritium transferred to water when uridine labelled in the 5 position of the pyrimidine ring was used as a precursor (Rode et al, 1980; and Nicander and Reichard, 1983). The amount of isotope that transferred to water was used to measure the synthesis of dTMP from uridine phosphates via dUMP. Cell monolayers were prepared and infected as described above. At specific times postinoculation, [5-^{3}H]uridine was added, without dilution, into 2 ml of medium to yield a final concentration of 0.3 μM. After incubation at 37°C for the specified times, 100 ul of the medium was removed and treated with 500-μl of a suspension of 10% (wt/vol) activated charcoal, containing 5% TCA, to remove the radiolabelled uridine and its derivatives (Rode et al, 1980; and Nicander and Reichard, 1983). The mixture was rocked in a LabGuard Rocker at room temperature for 1 hr and then centrifuged to pellet the charcoal. Radioactivity in a 120-μl sample of the supernatant was counted after 5 ml

of liquid scintillation cocktail was added. Thymidylate synthase activity was expressed as disintegrations per minute of ^3H_2O released per 10^6 host cells.

2) Assay of thymidylate synthase activity in vitro

We used two different techniques to measure the chlamydial thymidylate synthase activity. The first was to detect the formation of the product dTMP on HPLC; this method incorporated [6-^{3}H]dUMP in the substrate. The other was to detect the formation of tritiated water when [5-^{3}H]dUMP was used as substrate.

Method A---detecting the formation of dTMP.

This method was adapted from that described by Krungkrai et al (1989) with modifications. 5,10-CH_2-H_4folate was prepared as described above. Final TS assay mixture contained, in a total volume of 100 μl, 10 μM of 5,10-CH_2-H_4folate and 10 μM of dUMP (with 1 μCi of [6-^{3}H]dUMP). Reaction was initiated by the addition of 50 μl of enzyme (RB extract, or bacteria lysate) and was allowed to proceed at 37°C for a specified period of time. The reaction was terminated by adding ice-cold TCA (final concentration: 10%), and the tubes were then placed on ice for 30 min. After centrifugation at 14,000 X g for 10 min to remove precipitated material, the supernatant was neutralized by extraction with 1.1 volumes of tri-n-octylamine-freon (Khym, 1975). The neutralized extract was analyzed by HPLC. dTMP product was seperated from

dUMP substrate by HPLC on a 12.5-cm μBondapak C-18 column under isocratic conditions (flow rate, 1 ml/min, with 10 mM potassium phosphate buffer [pH4.0]). The identity of the radioactive peaks were confirmed by simultaneously monitoring the A_{254} of known dTMP and dUMP standards (10 μl of 50 μM solution for each). Data were plotted and analyzed as described above.

Method B---detecting the formation of tritiated water.

The composition of the assay mixture is the same as method A except that [6-^{3}H]dUMP was replaced by [5-^{3}H]dUMP (Roberts, 1966). The reaction was terminated by adding 500 μl of a suspension of 10% activated charcoal and the sample was processed the same way as described for the in situ assay.

3) 5-FdUMP.TS.5,10-CH_2-H_4folate complex assay

Covalent 5-FdUMP.TS.5,10-CH_2-H_4folate complex were detected by using published procedures (Honess et al, 1986; and Thompson et al, 1987) with modifications. Commercially purchased [6-^{3}H]5-FdUMP was dried by speed-vacuum and reconstituted in a small volume of H_2O to give a concentration of 500 μM. The binding assay mixture, in a total volume of 10 μl, contained 50 μM [^{3}H]FdUMP, 50 μM 5,10-CH_2-H_4folate and crude extract containing 5-10 μg protein or 10 pg purified human TS prepared in a solution containing 5 μg BSA. After the mixture was incubated at 37°C for 10 min, 1 μl

of sample from each reaction was denatured by mixing with 9 μl of sample buffer (pH 6.8, 0.1 M Tris-HCl containing 10% glycerol, 5% 2-mercaptoethanol, 2.5% SDS and 1% bromophenol blue) followed by heating at 90°C for 5 min. The denatured sample was subjected to SDS-polyacrylamide gel electrophoresis (gel concentration: 12%) using a Bio-Rad mini-gel apparatus. Low range prestained molecular markers (Bio-Rad) were used at each run. The gel was fixed with acetic acid/methanol/water (10%/50%/40%) for 30 min followed by soaking the gel with a flurographic agent Enlightening (DuPont New England Nuclear). The gel was dried and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at -70°C for 3-7 days.

4) Assay of dihydropteroate synthase activity in vitro

DHPS activity was assayed by the method described by Merali et al (1990) with modifications. The assay mixture contained, in a final volume of 100 μl, 100 mM Tris-HCl (pH8.5), 5 mM NaF, 10 mM $MgCl_2$, 10 μM H_2PtCH_2OPP, 1 μM [^{3}H]pABA (50 μCi/ml), and 5 mM dithiothreitol. The reaction was initiated by the addition of a predetermined amount of crude RB extract as a source of enzyme and was allowed to proceed at 37°C for 60 min and then terminated by the addition of 100 μl of 3% ascorbate/3% 2-mercaptoethanol followed by boiling for 1 min. The denatured protein was removed by centrifugation at 10,000 X g for 10 min at 4°C and a 50 μl sample of the supernatant was spotted onto 3 X 30 cm strips of 3MM chromatography paper (Whatman International). The strips were developed in a descending chromatography tank

using a mobile phase buffer of 0.1 M KH_2PO_4 at pH 7.0. Once the buffer front had travelled 20 cm, the paper strip was removed from the chromatography tank, the origin containing the labelled product was cut from the strip, dried, and placed in a scintillation vial containing 10 ml cocktail. The vial was left at room temperature for overnight and then counted in a liquid scintillation counter.

5) Assay of dihydrofolate reductase activity in vitro

DHFR was assayed essentially as described by Baccanari et al (1975). We used two methods to detect the reaction.

Method A---detecting the conversion of folates

The complete reaction mixture contained, in a total volume of 100 μl, 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 μM $NADPH_2$, 100 μM [^{3}H]H_2folate(10 μCi/ml) or[^{3}H]folate (10 μM/ml), and predetermined amount of enzyme (RB extract or *E. coli* lysate). The reaction was allowed to proceed at room temperature for 10 min and was then terminated by the addition of 100 μl of 3% ascorbate/3% 2-mercaptoethanol followed by boiling for 1 min. Precipitated protein was removed by centrifugation and the radiolabelled folic acid, H_2folate, and H_4folate present in the supernatant were resolved by HPLC using a 12.5-cm μBondapak C-18 column under isocratic conditions; the mobile phase consisted of 5 mM PIC A, 10 mM $(NH_4)H_2PO_4$ (pH 7.3), 20%

methanol, and 5% acetonitrile. The identity of the radioactive folate peaks was confirmed by simultaneously monitoring the A_{290} of known folate, H_2folate, and H_4folate standards coinjected with each sample. Data was collected and analyzed as described above.

Method B---detecting the conversion of $NADPH_2$ to $NADP^+$

This standard assay method was based on the reduction of A_{340} due to the conversion of $NADPH_2$ to $NADP^+$. The reaction was carried out in a 1-cm light path disposable cuvette (Beckman Instruments) which had a reaction mixture containing 50 mM Tris-HCl (pH7.5), 1 mM dithiothreitol, 60 μM $NADPH_2$, 50 μM H_2folate and enzyme in a final volume of 1 ml. The A_{340} value was measured immediately, after the initiation of the reaction by the addition of the enzyme, and was measured again after 10 min incubation at room temperature, in a Beckman DU-62 spectrophotometer. Corrections were made for any dihydrofolate-independent $NADPH_2$ oxidase activity.

12. *E. coli* Strains Used in Molecular Cloning

E. coli strains used in cloning and characterization of TS gene from chlamydiae are listed in Table 2.

Table 2. ***E. coli*** **Strains Used in Cloning TS Gene**

Designation	Genotype or relevant properties	Reference or sources
BL21 Δ*thyA*	Δ*thyA*	R. Thompson[a]
X2913	Δ*thyA*	R. Thompson
ATCC23851	*thy*$^-$	ATCC
D3-157	*guaB22 xyl-7 rpsL125 Str*^{r}	Singer 1985
XL1-blue	*endA1 hsdR17(rk*$^-$*mk*$^+$*) supE11 thi-1 lambda*$^-$ *recA1 gyrA96 relA1 [F' proAB lac1qZΔM15 Tn10(tet*^{r}*)*	Strategene

[a] R. Thompson, Institute of Virology, University of Glasgow, U.K..

13. Bacterial Culture Media

1) **LB broth** contained in one liter 10 g bacto-tryptone (Difco), 5 g bacto-yeast extract and 10 g NaCl. The solution was adjusted to pH7.0 and autoclaved.

2) **LB agar** was made by adding 15 g Difco agar per liter to LB broth and was poured into 10- or 15-cm disposable dishes (approx. 30 or 90 ml/dish) after autoclaving. When needed, ampicillin (final concentration: 100 μg/ml) was added.

3) **SOC medium** contained in a liter 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.2 g KCl, 10 ml of 1 M $MgCl_2$ and 20 ml of 1 M glucose and was adjusted to pH7.0. Medium containing no $MgCl_2$ and glucose was autoclaved. 1 M $MgCl_2$ and 1 M glucose solutions were prepared separately, filter-sterilized, and added into the autoclaved medium.

4) **Enriched minimum agar** was prepared from the following components:

10 X solution A (Miller, 1972) contained, in 1-liter volume, 105 g K_2HPO_4, 45 g KH_2PO_4, 10 g $(NH_4)_2SO_4$, and 5 g sodium acetate$\bullet 2H_2O$ and was autoclaved.

100 X solution B was prepared by dissolving 0.5 g each of leucine, arginine, histidine, threonine, proline, thiamine in 100 ml of water and sterilized by filtration.

100 X solution C contained 500 mg of tryptophan and 500 mg of tyrosine which were dissolved in water with the help of drop-wise addition of 1.0 N NaOH solution and was filter-sterilized.

20% (w/v) glucose solution and 20% (w/v) $MgSO_4$ solution were prepared and filter-sterilized separately.

To make up enriched minimal plates, 18 g bacto-agar was autoclaved in 850 ml of water. When the agar had cooled down to approximately 80°C, 100 ml of solution A, 10 ml of each of solutions B and C, and 20% glucose, and 1 ml of 20% $MgSO_4$ were added. When needed, ampicillin, methionine, glycine, pantothenic acid (hemi-calcium salt), thymine, thymidine, adenine and guanine were added in to give final concentrations of 100 μg, 50 μg, 50 μg, 100 μg, 200 μg, 50 μg, 50 μg, and 50 μg per ml respectively.

14. Cloning Vectors

Multiple-copy plasmids pUC19 and pUC18 (Sambrook et al, 1989) were obtained from N. Simonsen, Department of Medical Microbiology, University of Manitoba, and GIBCO, respectively. For library construction, large-scale of plasmids were prepared from XL1-blue *E. coli* cells and were polyethylene glycol-purified (Sambrook et al, 1989).

15. Preparation Chlamydial Genomic DNA

C. trachomatis L2 EBs grown in suspension culture were purified and used for extraction of genomic DNA as follows. Six liters of mouse L cell suspension culture was infected with *C. trachomatis* L2 EBs and collected by centrifugation at 40 hrs after infection as described above. The cell pellet was resuspended in 50 ml ice-cold HBSS and disrupted by sonication. Cell debris were removed by centrifugation at 500 X g for 10 min at 4°C and the resulting supernatant was layered onto two 8 ml cushions of 35% Renografin, prepared in 10 mM HEPES buffer (pH7.0), in an ultracentrige tube and then centrifuged for 60 min at 44,000 X g, in a Beckman SW28 rotor, at 4°C. The pellet was resuspended in 60 ml of HBSS containing DNase (10 μg/ml) and left at 37°C for 1 hr with gentle shaking to digest any contaminating host DNA. The DNase-treated EB suspension was layered onto six Renografin gradients each containing 5 ml of 52%, 8 ml of 44%, and 13 ml of 40% Renografin and then centrifuged for 90 min at 51,000 X g at 4°C in a Beckman SW 28 rotor. EBs at the 44%-52% Renografin interface were collected, and diluted with 3 volumes of 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 M potassium chloride, and then pelleted by centrifugation at 33,000 X g for 30 min. The EBs were resuspended in 2 ml of 10 mM Tris-HCl (pH8.0) containing proteinase K (final concentration, 20 μg/ml) and left at 37°C for 30 min to digest any DNase left in the preparation. The EB suspension was then mixed with 8 ml of EB lysis solution consisting of 10 mM Tris-HCl (pH8.0), 1 mM EDTA, 0.1% Triton X-100, 0.5% SDS, and 100 ug/ml of proteinase K. The preparation was incubated overnight at

50°C. Nucleic acids in the preparation were then extracted three times with phenol:chloroform, once by chloroform, and precipitated with ethanol. The precipitate containing chlamydial nucleic acids was dissolved in 5 ml of 10 mM Tris-HCl (pH8.0) containing 1.0 mM EDTA (TE buffer) and treated with DNase-free RNase (final concentration: 100 ug/ml) for 1 hr at 37°C. The chlamydial DNA left in the preparation was again phenol:chloroform- and chloroform-extracted and ethanol-precipitated as described above and finally the precipitated DNA was dissolved in 5 ml of TE buffer (pH8.0). The concentration of DNA was judged by its A_{260} (1 O.D. = 50 μg double-stranded DNA/ml) measured in a Beckman spectrophotometer and the purity by the ratio of A_{260}/A_{280} (1.8-1.95) as well as visualization under ultraviolet short wave lamp after electrophoresis on agarose gel stained with ethidium bromide. Chlamydial DNA prepared this way had essentially no RNA or host DNA contamination.

16. Construction of Chlamydial Genomic DNA Library

50 μg of chlamydial genomic DNA was partially digested with restriction endonuclease. At the end of digestion, the enzyme in the preparation was inactivated by heating 10 min at 75°C in the presence of 10 mM EDTA. The digestion was layered onto 10-25% (w/v) sucrose continuous density gradient and was centrifuged 20 hr at 110,000 X g at room temperature in a SW40 rotor. After centrifugation, fractions of 0.5 ml size were taken from top to bottom and a 25 μl sample from each fraction was electrophoresed on a 0.6% agarose gel for judging the size of the DNA fragments.

Fractions mainly containing 2-10 kb DNA fragments were pooled and dialyzed overnight against TE buffer (pH 8.0). The dialyzed DNA solution was then precipitated with ethanol and quantitated on agarose gel stained by ethidium bromide.

To construct the library, pUC19 plasmids were digested with the appropriate restriction enzyme and dephosphorylated.

A ligation reaction contained 100 ng of digested vector, 500 ng of genomic DNA fragments, 1 μl of ligation buffer, 1 μl of 10 mM ATP and 4 units of T4 DNA ligase in a final volume of 10 μl. The reaction was allowed to proceed at room temperature overnight. At the end of incubation, the reaction was diluted with 90 μl of water and extracted twice with phenol: chloroform, once with chloroform and finally precipitated with ethanol. The precipitated DNA was dissolved in 50 μl of TE (pH 7.6).

To measure the efficiency of ligation, 0.5 μl of library DNA was used to transform competent XL1-blue *E. coli* cells by electroporation. The transformed cells were plated onto LB/ampicillin agar containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, prepared in dimethylformamide). After 16-20 hr incubation at 37°C, ratio of white to blue colonies was about 90:10, indicating high efficiency of library construction.

17. Preparation of Competent *E. coli* Cells

Competent *E. coli* cells were prepared for electro-transformation by the method of Dower et al (1988). Cells from a single colony were inoculated into 3-5 ml of LB broth and were cultured on a shaker (200 rpm) at 37°C. After overnight growth, 1-2 ml of bacterial suspension was inoculated into 700 ml LB in a 2-liter flask which was vigorously shaken (200 rpm) at 37°C. When the A_{600} of the culture reached 0.5-1.0, the flask was chilled in ice water for 5 min and cells were collected by centrifugation at 4,000 X g in a Beckman JA-10 rotor for 15 min at 4°C. The cells were resuspended in and washed with 700 ml of ice cold water. Washing with cold water was repeated once and cells were resuspended in 20 ml of ice-cold sterile 10% glycerol and pelleted down by centrifugation at 3,000 X g for 20 min at 4°C in a Sorvall benchtop centrifuge. The cells were finally resuspended in 1-3 ml of 10% glycerol (the final cell concentration should be at least 2-4 X 10^{10} bacteria/ml, as judged by A_{600} value: 1 OD = 8 X 10^{8} cells/ml). Aliquots of 50- or 100 - μl size per vial were made and stored at -70°C.

18. Electro-transformation of *E. coli* cells and Library Screening for Chlamydial TS

Transformation of *E. coli* cells by electroporation (Dower et al, 1988) was done in 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 msec. Competent *E. coli* cells removed from -70°C freezer were gently thawed at room temperature and then immediately placed on ice. Plasmid DNA

in a volume of 1-2 μl was added into and mixed with the cells prior to electroporation. The cell/DNA mixture was transferred into a Bio-Rad 1-mm gap disposable cuvette which had been chilled on ice for at least two min. The cuvette was exposed to a pulse at the above settings. Immediately following the pulse, 1 ml of SOC media of room temperature was added into the cuvette and gently mixed with the cells. The bacterial suspension was transferred into a 15-ml polypropylene culture tube (Falcon) which was then cultured on a shaker (200 rpm) for 1 hr at 37°C. When TS^- cells were transformed for screening for plasmids capable of complementing thymidine auxotrophy, 10 μl of the transformed bacteria suspension was plated onto a LB containing ampicillin to measure the total number of transformants; and the rest of the suspension was chilled in ice-water and then centrifuged (3,000 x g) at 4°C for 15 min. The cells were washed once with enriched minimal medium and then resuspended in 100 μl of the same medium. The bacterial suspension was plated onto enriched minimal agar (thymidine-free) supplemented with ampicillin and the plates were incubated at 37°C.

19. DNA Sequencing

Sequencing of cloned recombinant plasmids was done based on the protocols supplied with the dsDNA cycle sequencing kit purchased from BRL Life Technologies Inc. (Gaithersburg, MD), with slight modifications at the step of primer labelling. Briefly, the 5′ end labelling reaction of sequencing primer contained 10 pmol of primer, 2 μl of 5 X kinase buffer, 1 μl of γ-[^{32}P]ATP, and 2 μl of T4 polynucleotide kinase (1

unit/μl), in a final volume of 10 μl. The phosphorylation reaction was allowed to proceed for 30 min at 37°C and terminated by incubating at 55°C for 5 min. The labelled primer was used immediately or stored at -20°C for up to 5 days before use.

6.5% [acrylamide: bis-acrylamide (29: 1, w/w)] gel was used for all sequencing determinations. At the end of the electrophoresis, the gel was fixed in 4 liters of fixation solution containing 5% (v/v) acetic acid and 15% (v/v) methanol for 30 min and dried on a Biorad gel drier and finally exposed to X-Omat AR film overnight at -70°C.

20. Computational Analysis of Sequencing Data

Computation searching for sequence-homology was performed at the National Center for Biotechnology Information (Bethesda, MD) using BLAST network service by comparing the obtained sequence to the data in Genbank. Comparison of the obtained sequence and known TS and other analysis of obtained sequence data were done by using a PC/GENE software purchased from IntelliGenetics, Inc (Mountain View, California).

21. Other Techniques in DNA Manipulation

Preparation and PEG-purification of plasmids, restriction endonuclease digestion, agarose gel electrophoresis, preparation of radiolabelled probe by random primer and nick-translation, Southern and Northern hybridizations were carried out essentially as

suggested by Sambrook et al (1989) or according to instructions supplied by manufacturers.

RESULTS

PART 1. BIOCHEMICAL EVICENCE FOR THE EXISTENCE OF THYMIDYLATE SYNTHASE IN CHLAMYDIAE

1. Failure of Medium-Supplied Thymine or Thymidine to Serve as a Precursor for *C. trachomatis* DNA

Results of experiments to measure the incorporation of exogenously added [^{3}H]thymidine into the DNA of logarithmically growing, mock- and *C. trachomatis* L2-infected wild-type and TK$^-$ human cells are shown in Table 3. Thymidine was readily incorporated into the DNA of logarithmically growing wild-type cells; however, in keeping with the phenotype, [^{3}H]thymidine did not label TK$^-$ cell DNA. Mock-infected wild-type cultures (confluent monolayers in the presence of 1 μg of cycloheximide per ml) are not synthesizing DNA (McClarty and Tipples, 1991; Bose and Liebhaber, 1979; and Stimac and Housman, 1963) and, as a result, do not incorporate significant amounts of [^{3}H]thymidine. *C. trachomatis* L2-infected cultures incorporated essentially the same amount of [^{3}H]thymidine as do mock-infected controls. [^{3}H]thymine was not utilized to any significant extent by logarithmically growing, mock- or *C. trachomatis* L2-infected wild-type or TK$^-$ cells. To verify that the cultures are infected and that *C. trachomatis* L2 was growing, we monitored [^{3}H]adenine incorporation into DNA. *C. trachomatis* L2-

Table 3. Incorporation of Thymine, Thymidine, and Adenine into Host and *C.trachomatis* L2 DNA in Wild-type and TK⁻ Cells

Cell line	Status[a]	^{3}H-labelled precursors[b]		
		Thymine	Thymidine	Adenine
$GC_3C_1(TK^+)$	Log growing	5.1	2912	365
	Mock-infected	1.7	81	10
	C. trachomatis L2-infected	1.7	69	180
GC_3 (TK^-)	Log growing	0.7	5.9	260
	Mock-infected	0.8	6.1	11
	C. trachomatis L2-infected	0.8	4.8	120

[a] Wild-type human $GC_3C_1(TK^+)$ and mutant TK^- cells were either logarithmically growing (1.0×10^6 cells per plate cultured in the absence of cycloheximide) or mock- or *C. trachomatis* L2-infected confluent monolayer (3.0×10^6 cells per plate, cultured in the presence of 1 μg cycloheximide per ml).

[b] The various ^{3}H-labelled precursors were added to achieve a final concentration of 0.3 μM. Then incubation was continued for 2 hr, and the incorporation of precursor into DNA was determined as described in Material and Method. *C. trachomatis* L2-infected cultures were labelled at 24 hr postinfection. Results are expressed in 10^3 dpm/10^6 cells.

infected cultures, both wild-type and TK^-, incorporate approximately 18 and 10 times more adenine, respectively, than do mock-infected controls.

2. Growth of *C. trachomatis* in $DHFR^-$ Cells

The $DHFR^-$ cell line is genetically deficient in the DHFR gene and, as a result, cannot reduce H_2folate to H_4folate (Urlaub and Wilson, 1969). Since 5,10-CH_2-H_4folate is a required cofactor for TS, $DHFR^-$ cells are unable to synthesize dTMP from dUMP and consequently, are auxotrophic for thymidine. In order to determine if exogenously added thymidine had an effect on *C. trachomatis* L2 growth in $DHFR^-$ cells, we monitored chlamydial multiplication by two parameters, inclusion development and *C. trachomatis* L2-specific DNA synthesis activity. In one case, $DHFR^-$ cells were infected with *C. trachomatis* L2 EBs and then incubated with minimum essential medium supplemented with 10% fetal bovine serum, 30 μM thymidine, 30 μM hypoxanthine, 0.3 mM glycine, 0.3 mM proline and 1 μg cycloheximide per ml (i.e., complete medium). Under the second condition, $DHFR^-$ cells were starved for thymidine for 24 hr prior to infection and then, following inoculation with EBs, the cultures were incubated in complete medium minus thymidine. For measurements of DNA synthesis activity, cultures were labelled with [^{3}H]adenine for 2 hr at 24 hr postinfection. Chlamydial growth was also assessed at 40 hr postinfection by staining infected monolayers for the presence of *C. trachomatis* L2 inclusions. Results from these experiments clearly show

Table 4. Effect of Exogenous Thymidine on the Growth of *C. trachomatis* L2 in DHFR⁻ Cells

Thymidine in medium[a]	DNA synthesis[b]		Inclusion per coverslip
	Mock-infected	Infected	
Yes	1.3	105	20,100
No	1.0	113	19,750

[a] CHO DHFR⁻ cells were cultured in either the presence or absence of 30 μM thymidine for 24 prior to and during infection.

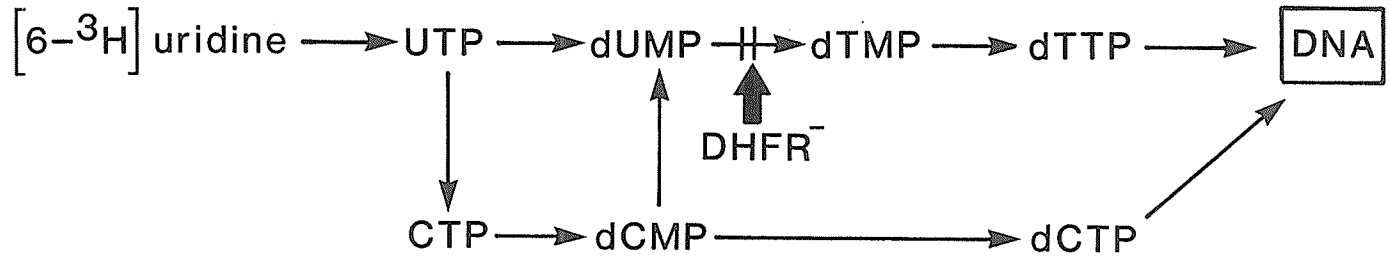
[b] The effect of exogenous thymidine on DNA synthesis was assessed by measuring [^{3}H]adenine (final concentration: 0.3 μM) incorporation into DNA. CHO DHFR⁻ cells were either mock- or *C. trachomatis*-infected confluent monolayers (3.0 X 10^6 cells per plate, cultured in the presence of 1 μg cycloheximide per ml). Results are expressed in 10^3 dpm/10^6 cells.

[c] The effect of exogenous thymidine on *C. trachomatis* L2 inclusion formation was assessed at 40 hr postinfection by fluorescent staining.

that exogenous thymidine has little or no effect on *C. trachomatis* L2 growth, as assessed by either parameter (Table 4).

3. Incorporation of Radiolabelled Uridine into *C. trachomatis* DNA

The results presented above indicate that *C. trachomatis* L2 does not utilize host-supplied thymidine or thymidine nucleotides and, therefore, suggest that the parasite must synthesize thymidine de novo from uridine. To address this question, we used [5-^{3}H]uridine and [6-^{3}H]uridine of approximately equal specific activity as precursor to label DNA of *C. trachomatis* L2. For these experiments, we again employed the CHO DHFR$^-$ cells as a host. The isotope was introduced as shown schematically in Fig. 3. Inside the cell, the nucleoside is rapidly phosphorylated to uridine phosphates and, subsequently, cytidine phosphates can be formed. Pyrimidine deoxyribonucleotides are formed by reduction of UDP and CDP. When [5-^{3}H]uridine is used as a precursor, deoxycytidine phosphates are labelled but thymidine phosphates are not, since isotope is lost from the pyrimidine ring and recovered as ^3H_2O in the medium during the formation of dTMP. In contrast, when [6-^{3}H]uridine is used as a precursor, all pyrimidine deoxyribotides are labelled, because the isotope is retained with pyrimidine ring. Logarithmically growing wild-type CHO K1 cells readily incorporate both [5-^{3}H]uridine and [6-^{3}H]uridine into DNA, with [6-^{3}H]uridine labelling more efficiently. Since DHFR$^-$ cells lack H_4folate and its derivatives (5,10-CH_2Hfolate, 5-CH_3-H_4folate, etc.), they are unable to synthesize


[6-3H] uridine
UTP
dUMP
dTMP
dTTP
DNA
DHFR-
CTP
dCMP
dCTP

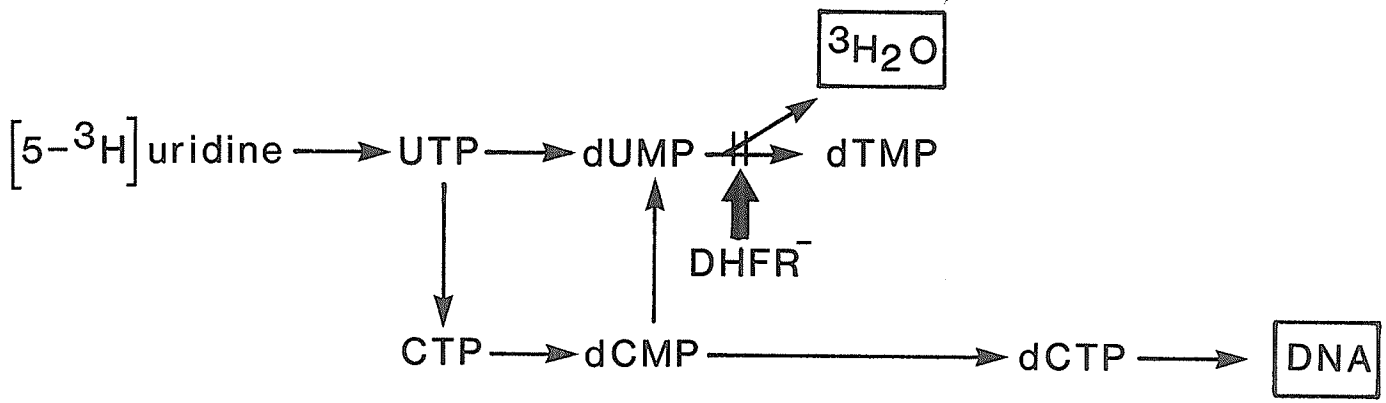

3H2O
[5-3H] uridine
UTP
dUMP
dTMP
DHFR-
CTP
dCMP
dCTP
DNA

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Figure 3. Schematic Diagram of the Metabolism of [6-^{3}H]uridine and [5-^{3}H]uridine in *C. trachomatis*-infected CHO DHFR$^-$ Cells. End products that accumulate the isotope are shown in boxes. Radiolabel is incorporated into DNA from [5-^{3}H]uridine via dCTP and from [6-^{3}H]uridine via both dCTP and dTTP. CHO DHFR$^-$ cells cannot convert dUMP to dTMP because they lack DHFR, which is required for the generation of CH_2-H_4folate, the necessary cofactor for the TS reaction. This metabolic deficiency is indicated by a heavy arrow.

Table 5. Incorporation of Radiolabelled Uridine into *C. trachomatis* L2 DNA in DHFR⁻ Cells

Cell line	Status[a]	Radiolabelled precursors[b]	
		[5-^{3}H]uridine	[6-^{3}H]uridine
CHO K1	Log growing	71	200
CHO DHFR⁻	Log growing	150	142
	mock-infected	0.3	0.7
	C. trachomatis L2-infected	41	120

[a] CHO K1 cells were logarithmically growing (1.0 X 10^6 cells per plate cultured in the absence of cycloheximide). DHFR⁻ cells were either logarithmically growing (1.0 X 10^6 per plate cultured in the absence of cycloheximide) or mock- or *C. trachomatis* L2-infected confluent monolayer (3.0 X 10^6 per plate cultured in the absence of cycloheximide).

[b] [5-^{3}H]uridine or [6-^{3}H]uridine was added to culture medium to achieve a final concentration of 0.3 μM, and then incorporation into DNA was determined as described in Material and Method. Results are expressed in 10^3 dpm per 10^6 cells.

thymidine from uridine. As a result, both [5-^{3}H]uridine and [6-^{3}H]uridine label the DNA of logarithmically growing DHFR$^-$ cells to about the same extent (Table 5). High levels (100 to 200 times that of mock-infected controls) of incorporation occurred when *C. trachomatis* L2-infected cultures were pulsed with [5-^{3}H]uridine or [6-^{3}H]uridine, with [6-^{3}H]uridine being more effective at labelling DNA. A possible explanation for this result is that *C. trachomatis* contains a TS, and, as a result [6-^{3}H]uridine gives rise to both labelled deoxycytidine and thymidine phosphates, whereas [5-^{3}H]uridine yields only labelled deoxycytidine phosphates.

We addressed this question by examining the base content of DNA isolated from *C. trachomatis*-infected and mock-infected DHFR$^-$ cells that had been labelled with either [5-^{3}H]uridine or [6-^{3}H]uridine. The isolated DNA was acid hydrolyzed and then the resulting free bases were separated by HPLC. The HPLC eluent was simultaneously monitored for UV absorbency and radioactivity. No radioactive peaks were detected from acid-hydrolyzed DNA isolated from [5-^{3}H]uridine or [6-^{3}H]uridine-labelled mock-infected DHFR$^-$ cells (Fig. 4 b and d, respectively). When [6-^{3}H]uridine was used as a precursor to label *C. trachomatis* L2-infected DHFR$^-$ cells, both radioactive cytosine and thymine were detected in the acid hydrolyzed DNA samples (Fig. 4 a). In contrast, when [5-^{3}H]uridine was used as a precursor, cytosine was the only radioactive nucleobase detected (Fig. 4 c).

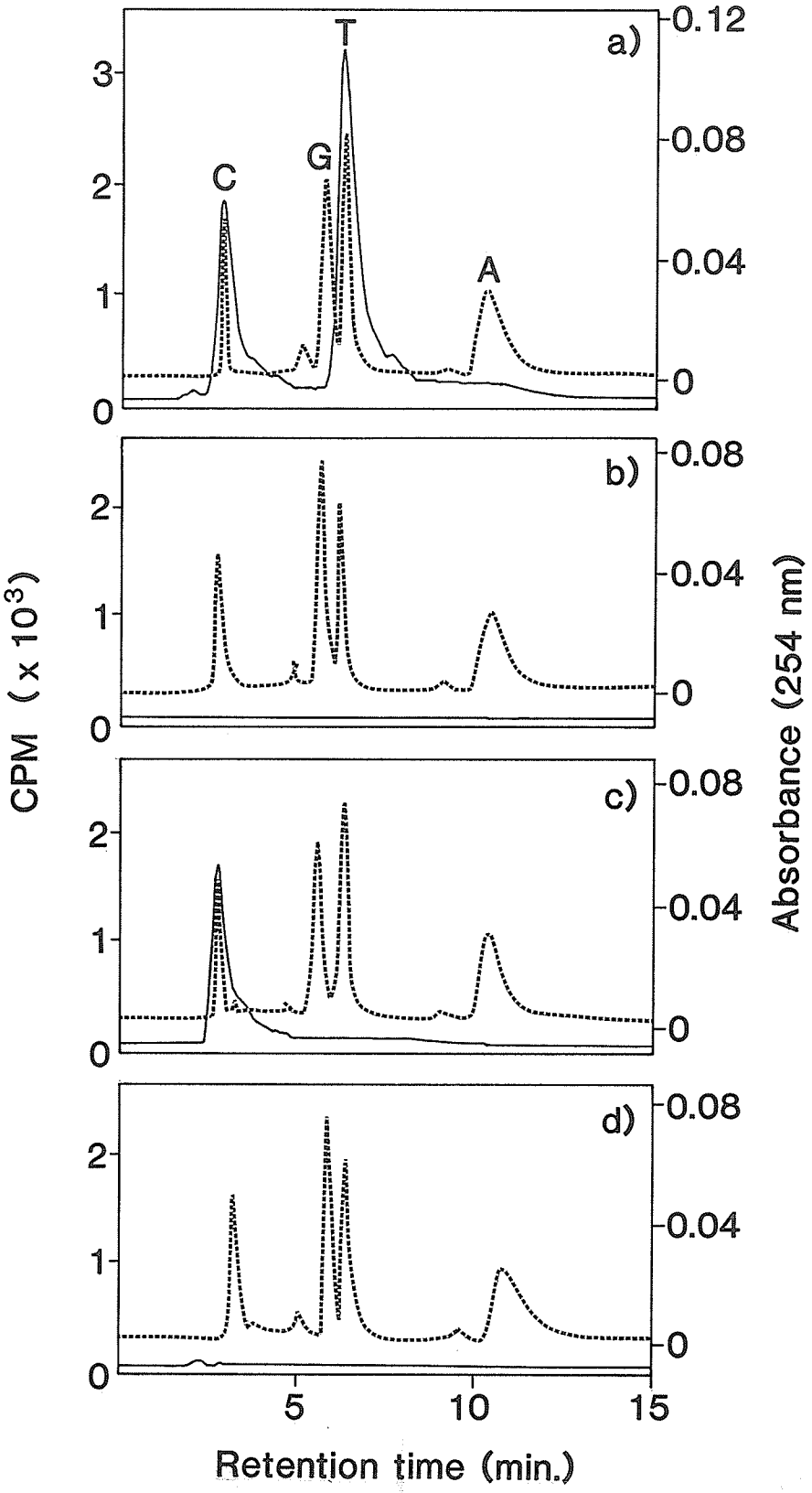
a)
b)
c)
d)
C
G
T
A
CPM (x 10³)
Absorbance (254 nm)
Retention time (min.)
0
1
2
3
5
10
15
0.04
0.08
0.12

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Figure 4. **Incorporation of [5-^{3}H]uridine and [6-^{3}H]uridine into DNA of Mock-infected and *C. trachomatis*-infected CHO DHFR$^-$ Cells.** Duplicate dishes of DHFR$^-$ cell monolayers (3.0 x 10^6 cells per plate) were infected with *C. trachomatis* L2 EBs and cultured in medium containing 1 μg/ml cycloheximide). At 24 hr postinfection, [6-^{3}H]uridine (a) or [5-^{3}H]uridine (c) (final concentration, 0.3 μM) was added and incubation was continued for 2 hr. DNA was isolated and hydrolyzed to free bases, which were separated and analyzed by HPLC. Chromatograms obtained from acid-hydrolyzed DNA of mock-infected DHFR$^-$ control cultures (3.0 x 10^6 cells per plate, cultured in the presence of 1 μg/ml cycloheximide) labelled with [6-^{3}H]uridine (b) or [5-^{3}H]uridine (d) are also shown. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known cytosine and thymine standards. The position of the free bases are indicated by letters: *A*, adenine; *G*, guanine; *C*, cytosine; *T*, thymine. A_{254} is shown by the dotted line; the radioactive detection of the ^{3}H label is shown by the solid line.

We also assessed the effects of 5-FUR and 5-FUdr on *C. trachomatis* L2 DNA synthesis activity. Both 5-FUR and 5-FUdr can be converted by host enzyme activities to 5-FdUMP, a specific inhibitor of TS (Ivanetich and Santi, 1989; and Maley and Maley, 1990). Our results indicate that 5-FUR is an effective inhibitor of *C. trachomatis* DNA synthesis and has an ID_{50} (concentration required to reduce incorporation of radiolabel into DNA by 50%) of 0.5 μM, whereas 5-FUdr had little or no effect, even at very high (100 μM) concentration (Fig. 5).

4. Demonstration of *C. trachomatis* TS Activity in situ

The presence of radioactive thymine in DNA of *C. trachomatis* L2-infected $DHFR^-$ cells labelled with [6-^{3}H]uridine certainly provides strong suggestive evidence for the presence of a parasite-specific TS. As a more direct test of this hypothesis, we conducted an in situ assay for TS. [5-^{3}H]deoxyuridine has been used to assay in situ TS activity in mammalian cells (Nicander and Reichard, 1983; and Rode et al, 1980). Since it has been shown that deoxyribonucleosides, including deoxyuridine, are very poorly incorporated into *C. trachomatis* DNA (McClarty and Tipples, 1991; and Tribby and Moulder, 1966), we had to use [5-^{3}H]uridine to monitor in situ TS activity in *C. trachomatis* L2-infected $DHFR^-$ cultures. The isotope was introduced from the medium as [5-^{3}H]uridine, as shown schematically in Fig. 3. Inside the cells, the nucleoside follows the metabolic pathway described above for labelling DNA with uridine. The basis of the in situ TS assay is the formation of ^3H_2O, with the isotope lost from the

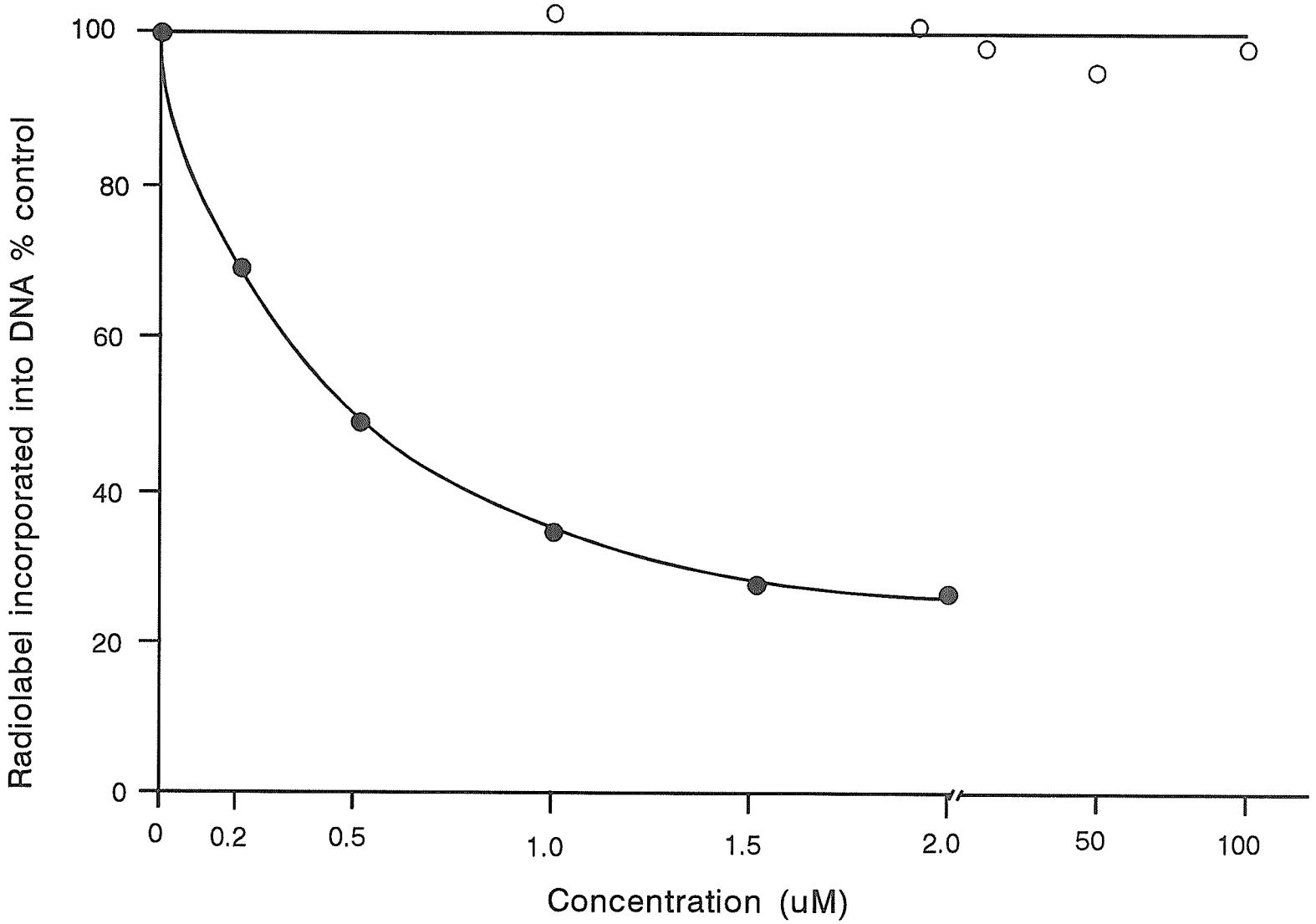
Radiolabel incorporated into DNA % control
100
80
60
40
20
0
0
0.2
0.5
1.0
1.5
2.0
50
100
Concentration (uM)

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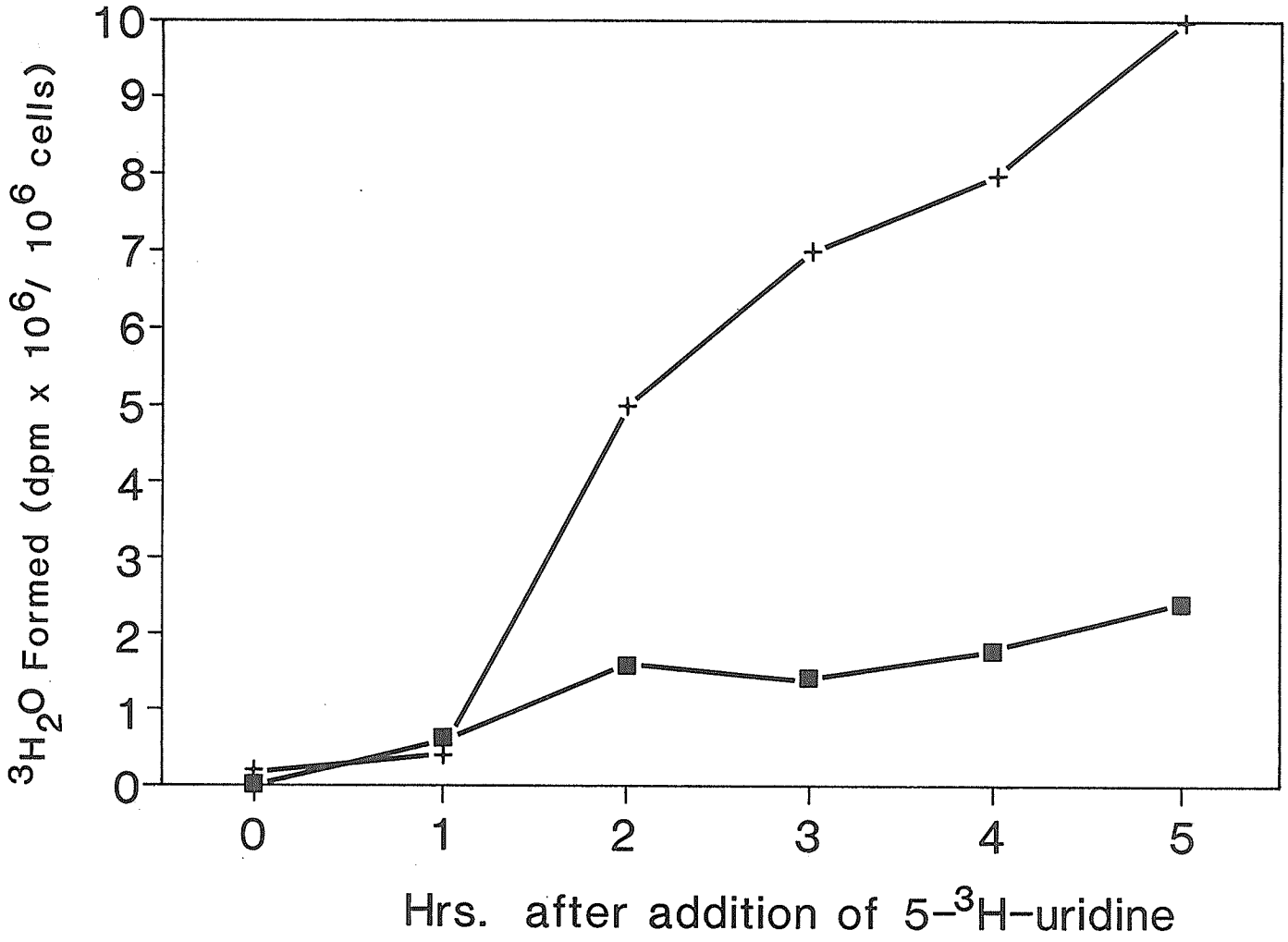
Figure 5. **Effect of 5-FUR (●) and 5-FUdr (○) on [6-^{3}H]uridine Incorporation into DNA in *C. trachomatis* L2-infected CHO DHFR$^-$ Cells.** Incorporation of the precursor into DNA was measured as described in the legend to Table 3. 5-FUR or 5-FUdr was added into culture medium to give indicated concentrations 2 hr prior to the addition of [5-^{3}H]uridine. The amount of radiolabel incorporated into DNA is expressed as a percentage of the uninhibited control (87,290 dpm/10^6 cells). All analyses were made with duplicate samples, and values varied by less than 10%.

pyrimidine ring during dUMP conversion to dTMP. Thus, the appearance of ^3H_2O in the medium is used to measure the TS reaction. Results from a time course in situ TS assay with [5-^{3}H]uridine labelled mock- and *C. trachomatis* L2-infected DHFR$^-$ cells are shown in Fig. 6. After a 5 hr labelling period, *C. trachomatis* L2-infected cultures had formed substantially more ^3H_2O than had mock-infected control cultures (Fig. 6). As expected, 5-FUR also effectively inhibited ^3H_2O formation (ID_{50}: 0.7 μM) (Fig. 7). Under similar conditions, 5-FUdr had no effect on isotope release.

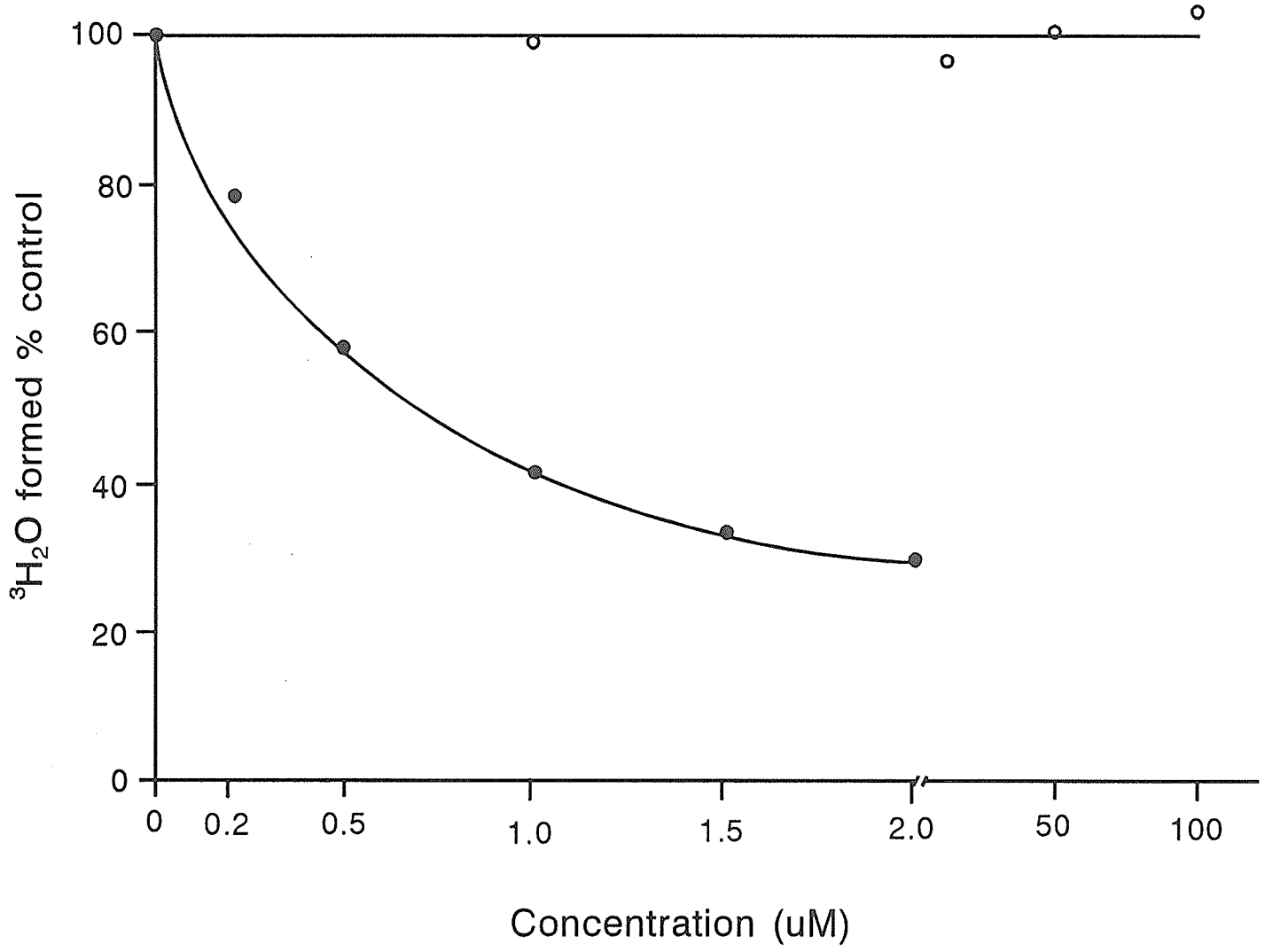
In order to determine whether in situ TS activity correlates with DNA synthesis activity during the chlamydial growth cycle, we monitored both activities at various times over the 48 hr developmental cycle (Fig. 8). In situ TS activity was monitored by measuring ^3H_2O formation from [5-^{3}H]uridine, and DNA synthesis was monitored by determining incorporation of [6-^{3}H]uridine into DNA. At the indicated times, [5-^{3}H]uridine was added to the medium of *C. trachomatis* L2-infected DHFR$^-$ cells. Then after 2 hr, the ^3H_2O formed and the amount of [6-^{3}H]uridine incorporated into DNA was determined. There is a good correlation between TS and DNA synthesis activities. Both activities first appear approximately 16 hr postinfection, peak at 24 to 32 hr postinfection, and rapidly decline thereafter.

5. Detection of in vitro TS Activity in Extracts Prepared from Highly Purified *C. trachomatis* RBs


^3H_2O Formed (dpm x 10^6/ 10^6 cells)
0
1
2
3
4
5
6
7
8
9
10
0
1
2
3
4
5
Hrs. after addition of 5-^{3}H-uridine

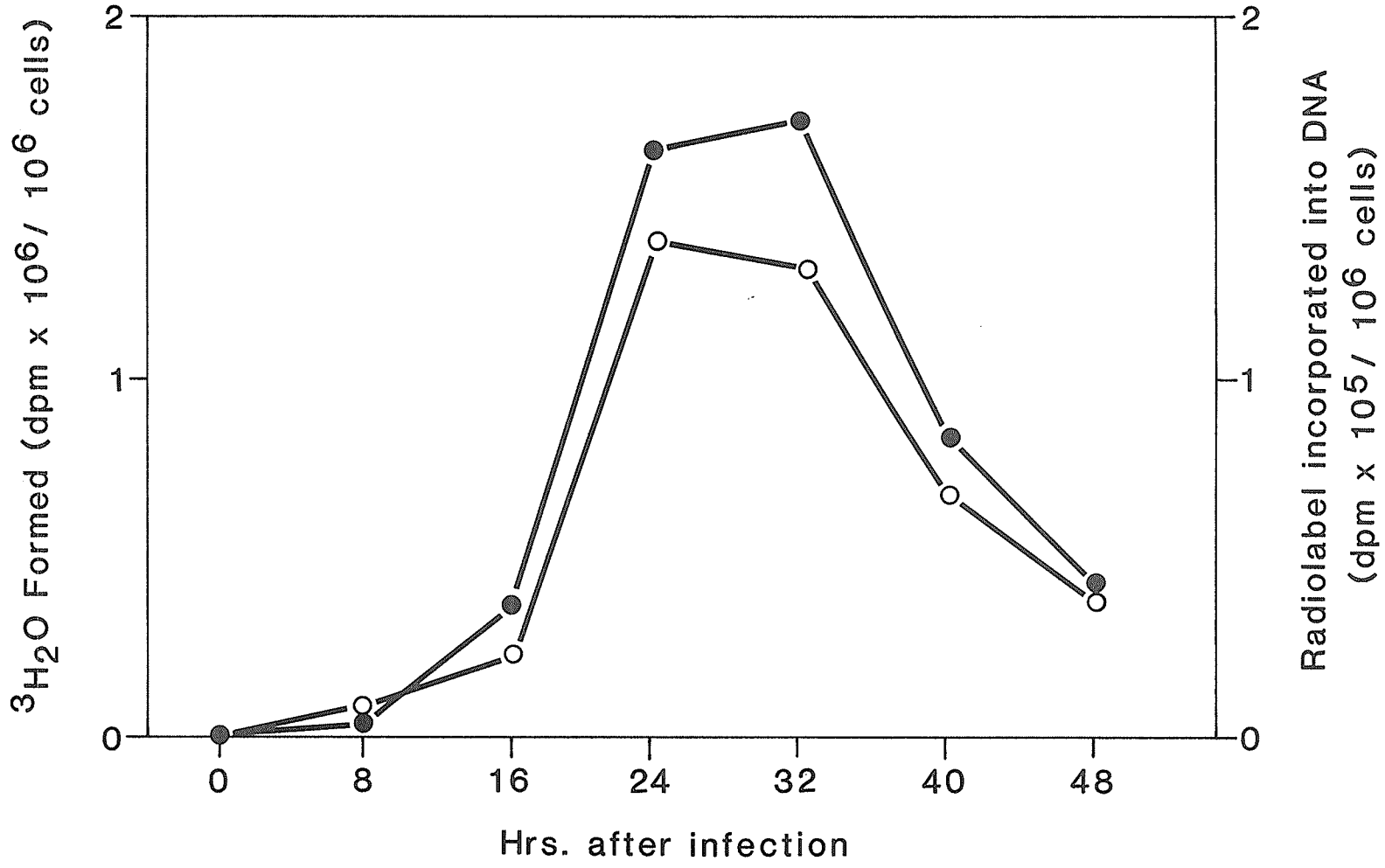
(Facing page)

Figure 6. **In situ TS Activity in *C. trachomatis* L2-infected CHO DHFR$^-$ Cells, Determined by Measuring the Amount of ^3H_2O Released into the Medium from Cells Labelled with [5-^{3}H]uridine.** Mock-infected DHFR$^-$ cells ( ), *C. trachomatis*-infected DHFR$^-$ cells (+) (both at 3.0 x 10^6 cells per plate, cultured in the presence of 1 μg/ml cycloheximide) were pulsed with [5-^{3}H]uridine (final concentration, 0.3 μM) at 24 hr postinfection. At the times indicated, an aliquot of the medium was removed and the amount of ^3H_2O present was determined. All analyses were made with duplicate samples, and values varied less than 10%.


100
80
60
40
20
0
^3H_2O formed % control
0
0.2
0.5
1.0
1.5
2.0
50
100
Concentration (uM)

(Facing page)

Figure 7. **Effect of 5-FUR (●) and 5-FUdr (○) on in situ TS Activity of *C. trachomatis* L2-infected CHO DHFR⁻ Cells.** In situ TS activity was measured as described in the legend to Figure 5. 5-FUR or 5-FUdr was added into culture medium to give indicated concentrations 2 hr prior to the addition of [5-^{3}H]uridine. ^3H_2O released into the medium was assayed 2 hr after the addition of the isotope. The amount of ^3H_2O released is expressed as a percentage of the uninhibited control. Values obtained, for mock-infected DHFR⁻ control cultures (3.0×10^6 cells per plate, cultured in the presence of 1 μg/ml cycloheximide) were subtracted from the values obtained for *C. trachomatis*-infected DHFR⁻ control cultures (3.0×10^6 cells per plate, cultured in the presence of 1 μg/ml cycloheximide). All analyses were made with duplicate samples, and values varied less than 10%.


^3H_2O Formed (dpm x 10^6/ 10^6 cells)
Radiolabel incorporated into DNA (dpm x 10^5/ 10^6 cells)
2
1
0
0
8
16
24
32
40
48
Hrs. after infection

(Facing page)

Figure 8. **In situ TS Activity and DNA Synthesis Activity during the *C. trachomatis* Growth Cycle.** Parallel dishes of $DHFR^-$ cells were infected with *C. trachomatis* L2 EBs. At each of the indicated times, one dish was labelled with [5-^{3}H]uridine for measurement of in situ TS activity (○) and the other with [6-^{3}H]uridine for measurement of DNA synthesis activity (●). Incubation in the presence of the isotope was continued for 2 hr, and then the amount of ^3H_2O present in the medium and the amount of radiolabel incorporated into DNA were determined. Values obtained, at each time, for mock-infected $DHFR^-$ control cultures (3.0×10^6 cells per plate, cultured in the presence of 1 μg/ml cycloheximide) were subtracted from the values obtained for *C. trachomatis*-infected $DHFR^-$ control cultures (3.0×10^6 cells per plate, cultured in the presence of 1 μg/ml cycloheximide). All analyses were made with duplicate samples, and values varied less than 10%.

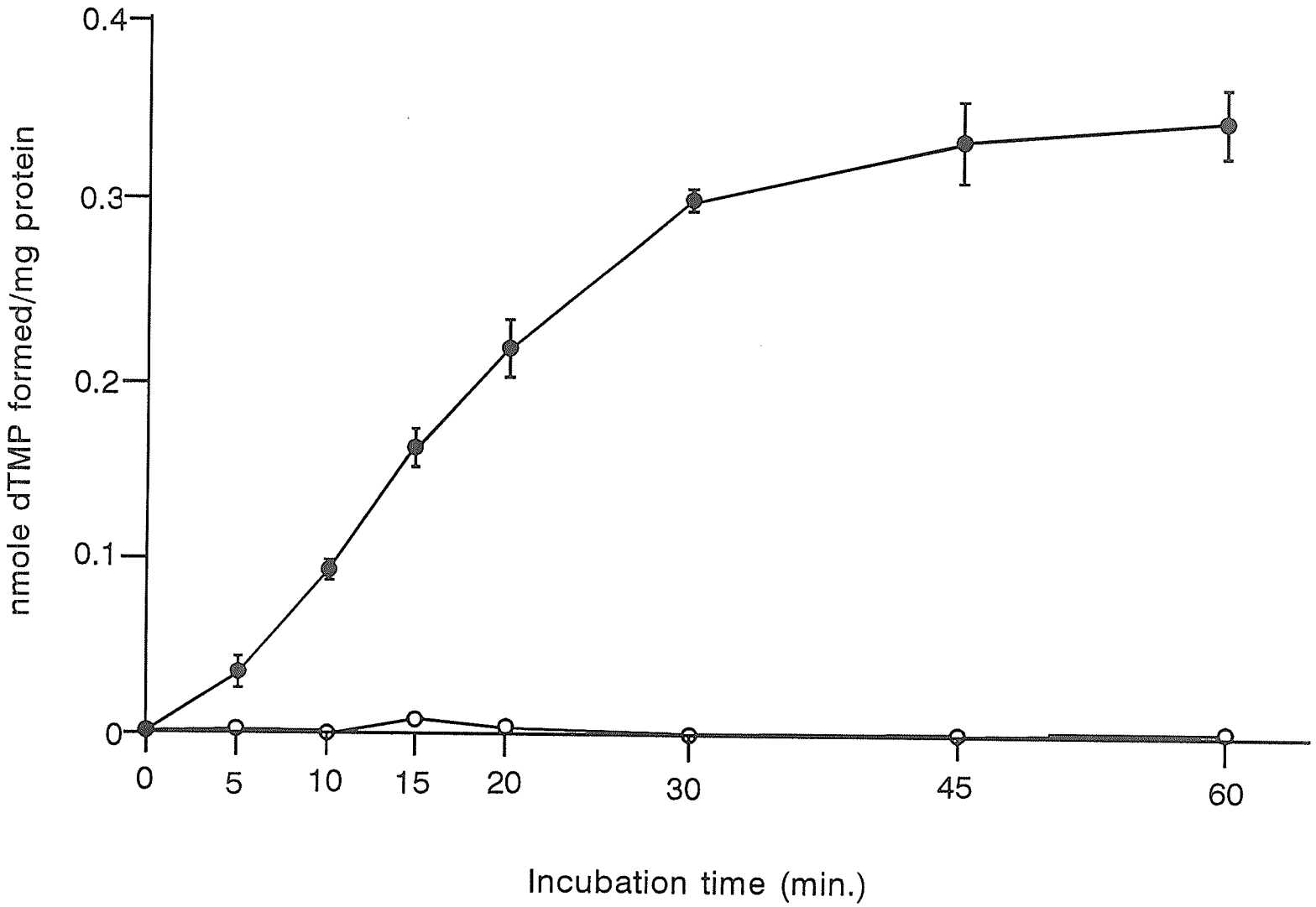
In order to conclusively show that *C. trachomatis* contains TS, we prepared extract from highly purified RBs and assayed for TS activity in vitro. We consistently detected radiolabelled dTMP formation from [6-^{3}H]dUMP by using RB extracts as a source of the enzyme (Table 6). Under our assay conditions, the TS activity remained linear for about 30 min (Fig. 9). The formation of dTMP was dependent on the presence of RB extract and 5,10-CH_2-H_4folate, the required cofactor for the TS reaction, and was inhibited by 5-FdUMP (Table 6). Mock-infected mouse L-cell extract and boiled RB extract had no measurable TS activities (Table 6).

Table 6. TS Activity in Crude Extracts Prepared from Purified *C. trachomatis* L2 Reticulate Bodies

Enzyme source	Cofactor or inhibitor[a]	TS activity[b]	%
RB extract	CH_2-H_4folate	15.6 ± 2.1	100
	CH_2-H_4folate + 5-FdUMP	1.4 ± 0.5	9
	5-CH_3-H_4folate	< 0.1	< 1
	5-CHO-H_4folate	< 0.1	< 1
	H_4folate	< 0.1	< 1
Boiled RB extract	CH_2-H_4folate	< 0.1	< 1
Mock-infected L-cell extract	CH_2-H_4folate	< 0.1	< 1

[a] Final concentration of each folate in the reaction mixture was 10 μM, same as substrate dUMP. CH_2-H_4folate was prepared by treating H_4folate with formaldehyde. To determine the effect of 5-FdUMP on TS activity, a complete reaction mix minus substrate was incubated in the presence of 100 μM 5-FdUMP for 30 min. The reaction was initiated by addition of dUMP substrate and incubation at 37°C.

[b] Reactions were carried out at 37°C for 20 min. Activity was expressed as pmole dTMP formed/mg protein/min and each value represents the mean ± standard deviation from two determinations.


0.4
0.3
0.2
0.1
0
nmole dTMP formed/mg protein
0
5
10
15
20
30
45
60
Incubation time (min.)

(Facing page)

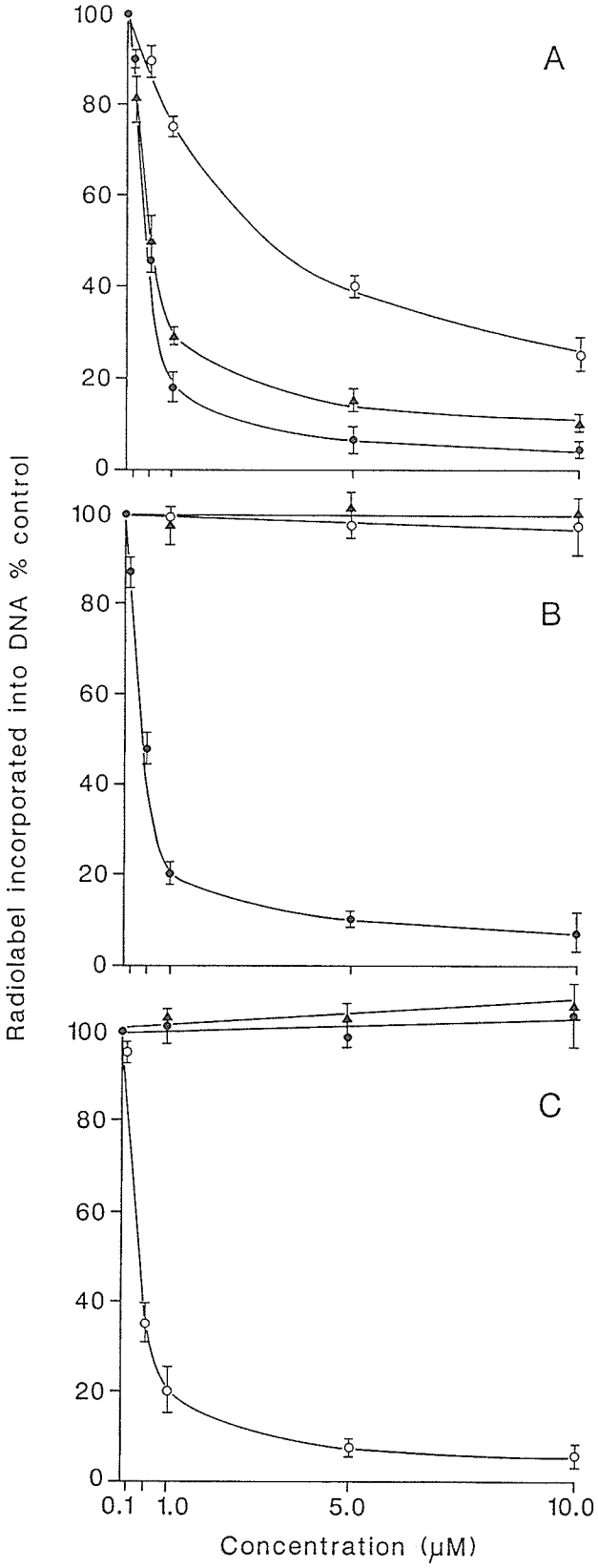
Figure 9. **Increase of dTMP Formation with Incubation Time.** Reaction mixture of TS assay contained 10 μM dUMP, 10 μM 5,10-CH_2-H_4folate, and 50 μl *C. trachomatis* L2 RB extract (●) or sham-purified mock-infected L-cell extract (○). Results represent mean $\pm$ standard deviation for duplicate samples.

PART 2. ACQUISITION AND SYNTHESIS OF FOLATES BY CHLAMYDIAE

1. Effect of Various Inhibitors of Folate Metabolism on Chlamydiae Growth

Initially we wanted to determine the effects of various inhibitors of folate metabolism on the growth of *C. trachomatis* and *C. psittaci*. Chlamydial growth was monitored by measuring the incorporation of [6-^{3}H]uridine into DNA in the presence of the eucaryotic protein synthesis inhibitor cycloheximide (McClarty and Tipples, 1991). For historical reasons, we used the commonly studied *C. trachomatis* strain L2 as well as *C. psittaci* psittacosis strain 6BC and *C. psittaci* meningopneumonitis strain francis (frequently referred to as *C. psittaci* Cal-10). Three drugs that target folate metabolism were tested. Sulfisoxazole, a competitive inhibitor of dihydropteroate synthase, inhibits de novo folate synthesis (Anand, 1983); trimethoprim, an inhibitor of bacterial DHFR that enters cells by simple diffusion (Wormser, 1983; and Buchall, 1983); and methotrexate, an aminopterin analogue that inhibits both mammalian and bacterial DHFR (Jackson and Grindey, 1984; and Flintoff, 1989). In vivo methotrexate is only effective against cells that have a transport system(s) for folates (Jackson and Grindey, 1984; and Buchall, 1983).

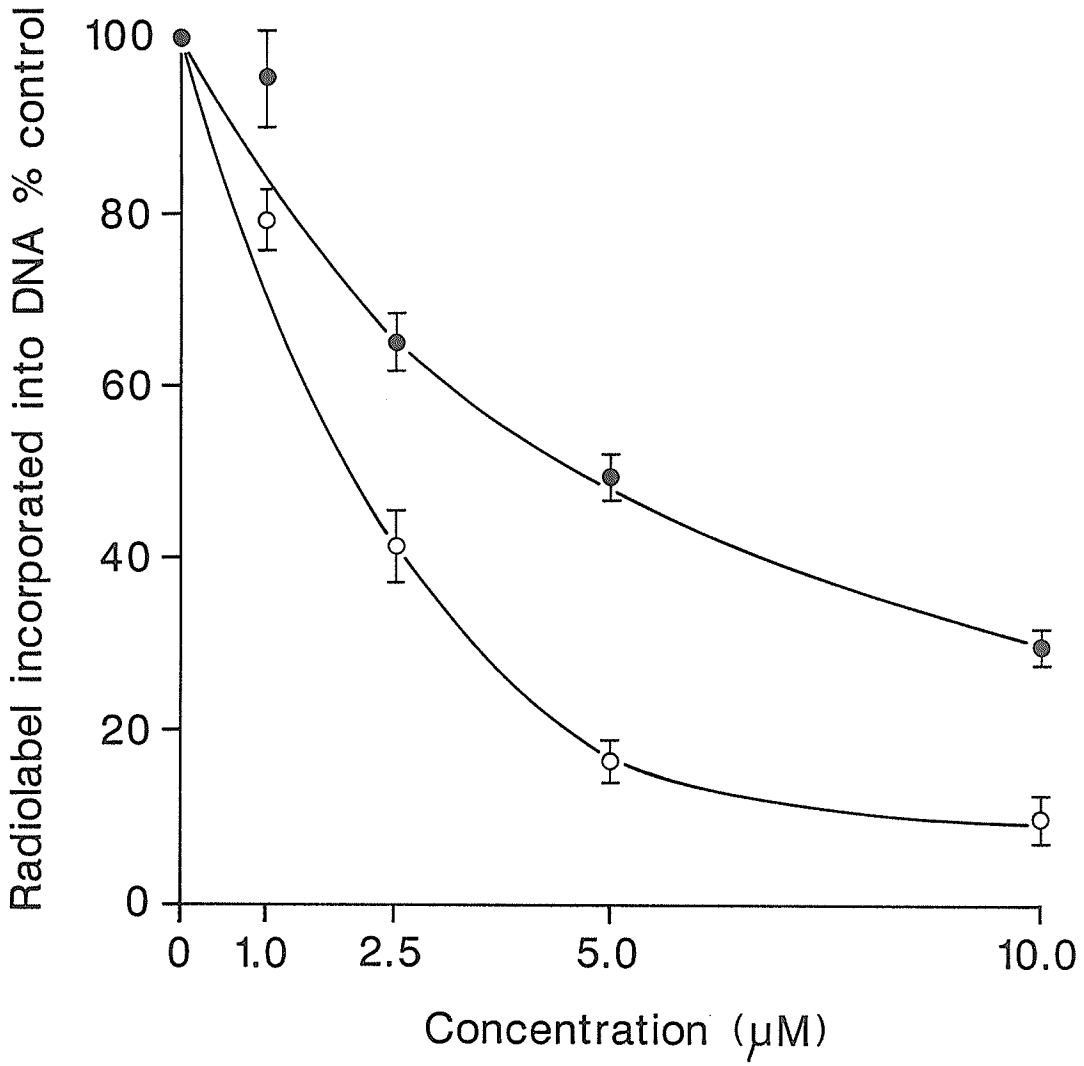
Results of experiments determining the effect of various concentrations of these three inhibitors on chlamydial growth in wild-type CHO K1 cells are shown in Fig. 10.


A
B
C
100
80
60
40
20
0
Radiolabel incorporated into DNA % control
0.1
1.0
5.0
10.0
Concentration (μM)

(Facing page)

Figure 10. Effect of Sulfisoxazole, Trimethoprim and Methotrexate on [6-^{3}H]uridine Incorporation into DNA in *C.trachomatis* L2 (A), *C.psittaci* 6BC (B) and *C.psittaci* francis (C)-infected Wild-type CHO K1 Cells. Confluent cell monolayers (4.0 x 10^6 cells per plate) were infected with chlamydial EBs and cultured in medium containing 1 μg/ml cycloheximide. The indicated concentrations of sulfisoxazole (●), trimethoprim (Δ) or methotrexate (○) were added immediately following infection with chlamydiae, i.e. 2 hr postinfection. Radiolabelled uridine (final concentration 0.3 μM) was added at 20 hr postinfection. The amount of radiolabel incorporated into DNA is expressed as a percentage of the uninhibited control. The following are 100% control values: *C. trachomatis* L2-infected cultures, 158,954 $\pm$ 17,963 dpm/10^6 cells; *C. psittaci* 6BC-infected cultures, 178,692 $\pm$ 22,756 dpm/10^6 cells; and *C. psittaci* francis-infected cultures, 143,650 $\pm$ 13,098 dpm/10^6 cells. The data represent the average of two determinations. Bars, standard deviation.

In keeping with earlier findings (Colon, 1962; Morgan, 1948; Morgan, 1952; and Huang and Eaton, 1949), sulfisoxazole was an effective inhibitor of both *C. trachomatis* strain L2 and *C. psittaci* strain 6BC growth. The concentration of sulfisoxazole required to inhibit DNA synthesis by 50% was 0.4 μM for *C. trachomatis* L2, 0.5 μM for *C. psittaci* 6BC. Also in agreement with previous reports (Moulder, 1991; Colon and Moulder, 1958) we found that sulfisoxazole had no effect on *C. psittaci* francis DNA synthesis. Trimethoprim was effective against *C. trachomatis* L2, having an ID_{50} value of 0.5 μM. In contrast neither of the *C. psittaci* strains was sensitive to trimethoprim. Both *C. trachomatis* L2 and *C. psittaci* francis were inhibited by methotrexate, having ID_{50} values of 3.2 and 0.3 μM, respectively. Growth of *C. psittaci* 6BC was unaffected by methotrexate even at concentration as high as 100 μM. Since the chlamydial ID_{50} values for methotrexate are much higher than the ID_{50} values for mammalian cell lines (Jackson and Grindey, 1984; and Flintoff, 1989), it is difficult to determine whether methotrexate inhibits chlamydiae directly or indirectly via an effect of the host cell line. This is particularly relevant when one considers that methotrexate inhibits de novo purine biosynthesis in mammalian cells (Jackson and Grindey, 1984; Buchall, 1983; and Flintoff, 1989) and chlamydiae are auxotrophic for purine ribonucleotides (Moulder, 1991; McClarty and Tipple, 1991; and Tipples and McClarty, 1993). To determine whether methotrexate directly affects chlamydiae replication, we used $DHFR^-$ cell line as a host to support parasite growth. As a result of the DHFR deficiency this cell line is unable to regenerate H_4folate from H_2folate and is unaffected by methotrexate (Urlaub


Radiolabel incorporated into DNA % control
100
80
60
40
20
0
0
1.0
2.5
5.0
10.0
Concentration (μM)

(Facing page)

Figure 11. Effect of Methotrexate on [6-^{3}H]uridine Incorporation into DNA in *C.trachomatis* L2 (●) and *C.psittaci* francis (○) -infected CHO DHFR$^-$ Cells. The indicated concentrations of methotrexate were added to the culture medium at 2 hr postinfection. Radiolabelled uridine was added at 20 hr postinfection. The amount of radiolabel incorporated into DNA is expressed as a percentage of the uninhibited control. The following are 100% control values: *C. trachomatis* L2-infected cultures, 124,763 ± 14,980 dpm/10^6 cells; and *C. psittaci* francis-infected cultures, 142,822 ± 16,587 dpm/10^6 cells. The data represent the average of two determinations. Bars, SD.

and Chasin, 1980). Methotrexate was an effective inhibitor of chlamydial growth in this cell line (Fig. 11). The concentration of methotrexate required to inhibit *C. trachomatis* L2 and *C. psittaci* francis DNA synthesis activity by 50% in this cell line was 4.8 and 2.0 μM respectively. As was the case with the wild-type cell line as host, *C. psittaci* 6BC growth was unaffected by methotrexate in the $DHFR^-$ cell line (data not shown).

2. Growth of Chlamydiae in Host Cells Depleted of Folates and pABA

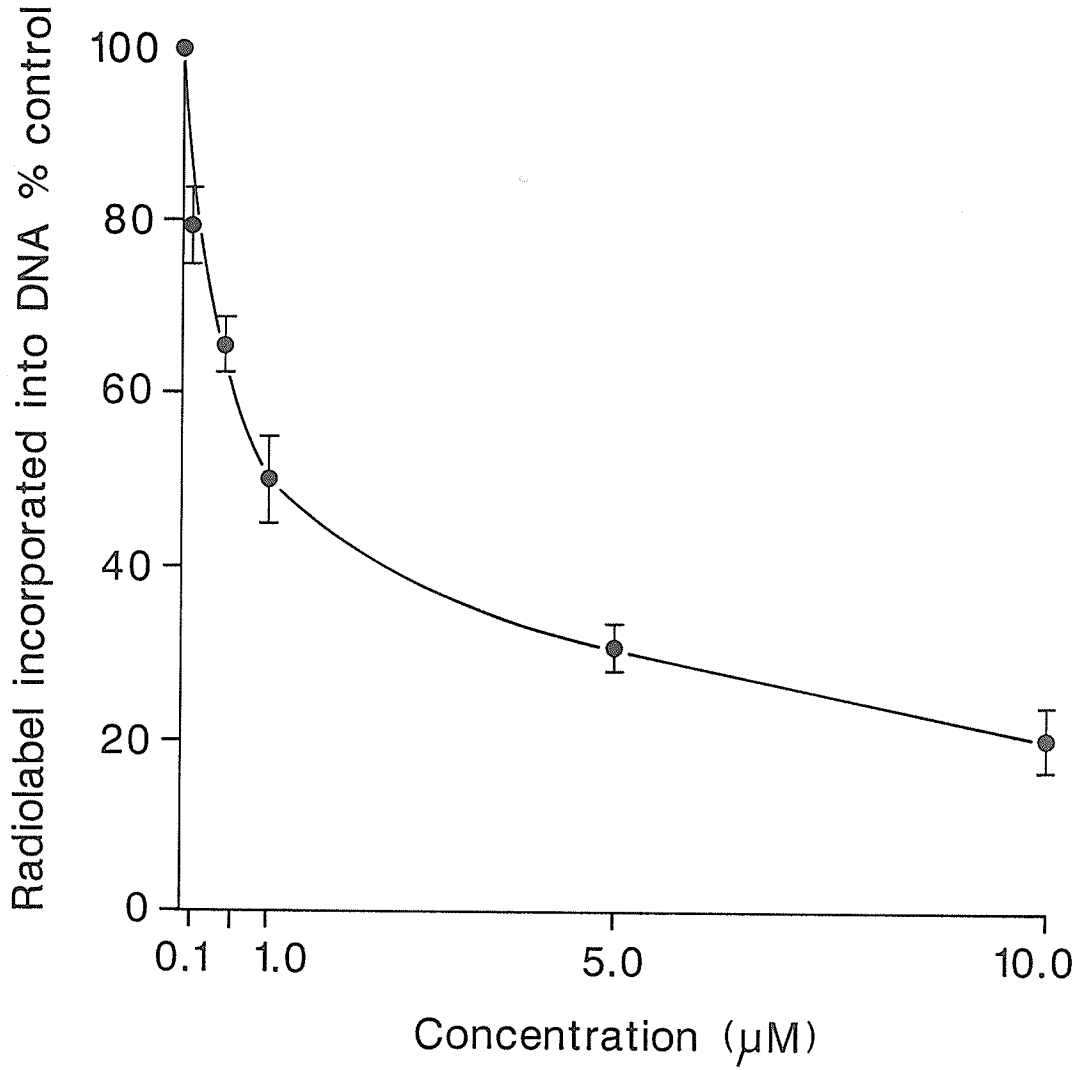
To evaluate the requirement of chlamydiae for exogenous folates we tested the ability of the parasite to grow in wild-type CHO K1 cells with depleted intracellular folate pools. To achieve maximal folate depletion, we grew the CHO K1 cells for 10 passages in folate- and pABA-free medium supplemented with hypoxanthine, proline, glycine, and thymidine. Results presented in Table 7 indicate that all three chlamydial strains grew as well in CHO K1 cells extensively starved for folates and pABA as they did in host cells that had been previously cultured in complete medium. The observation that *C. trachomatis* L2 and *C. psittaci* 6BC could grow in folate-depleted host cells is in keeping with their sulfa sensitivity and further supports the suggestion that these two strains can synthesize folates de novo. However, given that *C. psittaci* francis was resistant to sulfonamide (a result that suggested that it could obtain preformed folates from the host) we were surprised that it could grow so well in host cells depleted of folates. To help clarify this paradox we checked the sulfonamide sensitivity of *C. psittaci* francis growing in host cells depleted of folates. The results clearly showed that, in

Table 7. Effect of Exogenous Folate on the Growth of Chlamydiae in Chinese Hamster Ovary K1 Cells

Culture medium[a]	DNA synthesis[b]			
	Mock-infected	*C.trachomatis* L2	*C. psittaci* 6BC	*C. psittaci* francis
Folate-containing	0.9	162.6	197.9	189.4
Folate-free	0.7	151.2	191.0	197.4

[a] Before chlamydiae infection, CHO K1 cells were cultured in complete medium containing 2.2 μM folate or were depleted of intracellular folates by passage in folate- and pABA-free medium.

[b] The effect of exogenous folate on chlamydiae growth was assessed by measuring [6-^{3}H]uridine incorporation into DNA at 20 hr postinfection. Folate-replete or folate-depleted CHO K1 cells were either mock- or chlamydiae-infected confluent monolayers (3.0 X 10^6 cells per plate cultured in the presence of 1 μg cycloheximide/ml). Results are expressed in 10^3 dpm per 10^6 cells.


Radiolabel incorporated into DNA % control
100
80
60
40
20
0
0.1
1.0
5.0
10.0
Concentration (μM)

(Facing page)

Figure 12. **The Effect of Sulfisoxazole on [6-^{3}H]uridine Incorporation into DNA in *C. psittaci* francis-infected Folate- and pABA-depleted Wild-type CHO K1 Cells.** Confluent cell monolayers (4.0 x 10^6 cells per plate) cultured in folate- and pABA-free medium supplemented with proline, glycine, hypoxanthine and thymidine were infected with *C. psittaci* francis EBs. After infection, cells were cultured in the same medium minus thymidine and plus cycloheximide (final concentration, 1 μg/ml). The indicated concentrations of sulfisoxazole were added to the culture medium at 2 hr postinfection. [6-^{3}H]uridine was added at 20 hr postinfection. The amount of radiolabel incorporated into DNA is expressed as a percentage of the uninhibited control (169,094 $\pm$ 15,386 dpm/10^6 cells). The data represent the average of two determinations. Bars, SD.

contrast to the findings with cells rich in folate, *C. psittaci* francis was highly susceptible to sulfisoxazole inhibition when grown in folate-starved cells (Fig. 12). With *C. psittaci* francis-infected folate-starved cells the ID_{50} for sulfonamide was 1.0 μM.

3. Incorporation of [^{3}H]pABA into Chlamydial Folates

To directly test if chlamydiae could synthesize folates de novo, we determined the ability of all three strains to incorporate radiolabelled pABA into their folate pools. For these studies all chlamydial strains were grown in folate-depleted CHO K1 cells in the presence of [^{3}H]pABA. Fig. 13 shows typical elution profiles obtained after HPLC separation of radiolabelled folates extracted from *C. trachomatis* L2-, *C. psittaci* 6BC-, and *C. psittaci* francis-infected folate-starved CHO K1 cells. As expected, CHO K1 cells were unable to use [^{3}H]pABA for the synthesis of folates (data not shown). All three chlamydial strains incorporated [^{3}H]pABA into their folate pools; however, there are obvious differences in the elution profiles obtained for reduced folates when *C. trachomatis* and *C. psittaci* are compared. The major reduced folates produced by *C. trachomatis* L2 (Fig. 13 B) were H_4folate and 10-CHO-H_4folate; variable amounts of 5-CH_3-H_4folate or 5,10-CH_2-H_4folate (the two peaks coeluted) were also routinely detected. In contrast, the predominant reduced folate produced by *C. psittaci* strain 6BC (Fig. 13 C) was 10-CHO-H_4folate; with variable amounts of H_4folate, 5-CHO-H_4folate, and 5-CH_3-H_4folate/5,10-CH_2-H_4folate also being detected. *C. psittaci* strain francis (Fig. 13

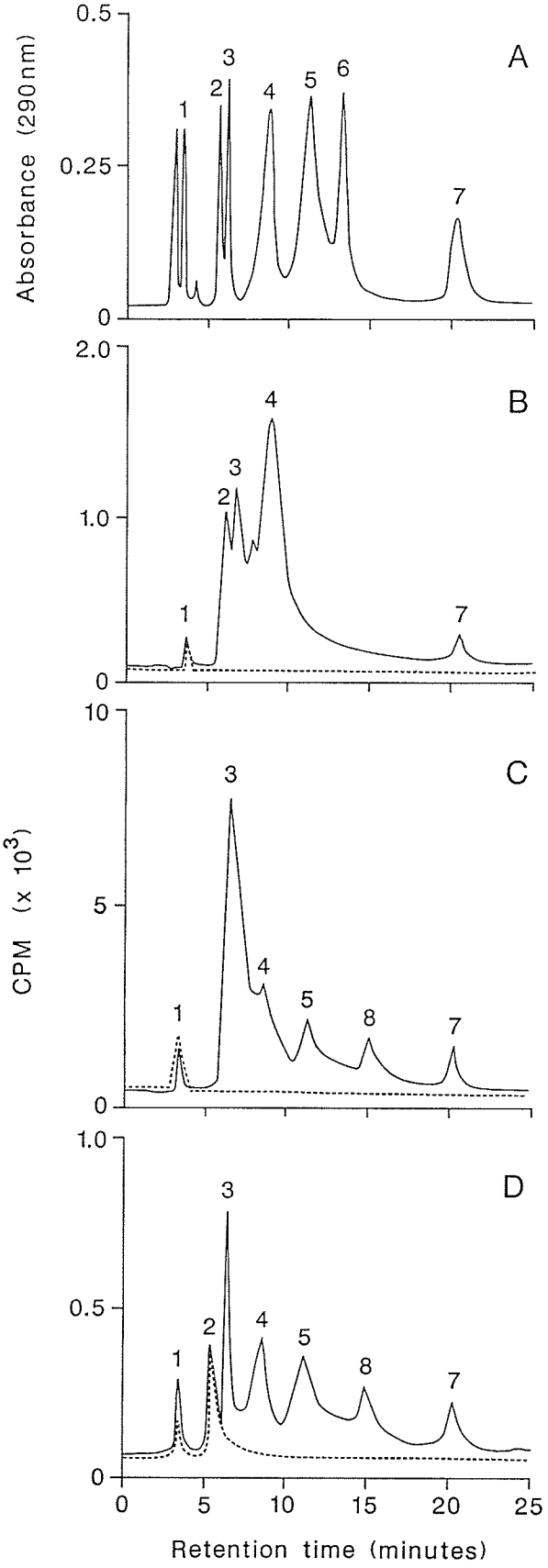

A
B
C
D
Absorbance (290nm)
CPM (x 10³)
Retention time (minutes)
0.5
0.25
0
2.0
1.0
10
5
1.0
0.5
0
0
5
10
15
20
25
1
2
3
4
5
6
7
8

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Figure 13. **Ultraviolet Absorption (A_{290}) profile of Folate Standards Separated by HPLC (A) and Radioactive Profile after Incorporation of [^{3}H]pABA into Folates by *C. trachomatis* L2 (B), *C. psittaci* 6BC (C), and *C. psittaci* francis (D) -infected Folate- and pABA-depleted Wild-type CHO K1 Cells.** Confluent cell monolayers (3.0 x 10^7 cells per plate) cultured in folate- and pABA-free medium supplemented with proline, glycine, hypoxanthine and thymidine were infected with chlamydial EBs. After infection, cells were cultured in the same medium supplemented with radiolabelled pABA and cycloheximide (final concentration, 1 μg/ml), but lacking thymidine. Intracellular folates were extracted and purified at 24 hr postinfection. Peaks identified were: 1, pABA; 2, pABA-glutamate; 3, 10-CHO-H_4folate; 4, H_4folate; 5, 5-CHO-H_4folate; 6, H_2folate; 7, 5-CH_3-H_4folate and/or 5,10-CH_2-H_4folate and 8, unidentified. Solid line represents radioactivity detected from folates isolated from chlamydiae-infected control cultures and the broken line represents radioactivity detected from folates extracted from chlamydiae-infected cultures treated with 10 μM sulfisoxazole.

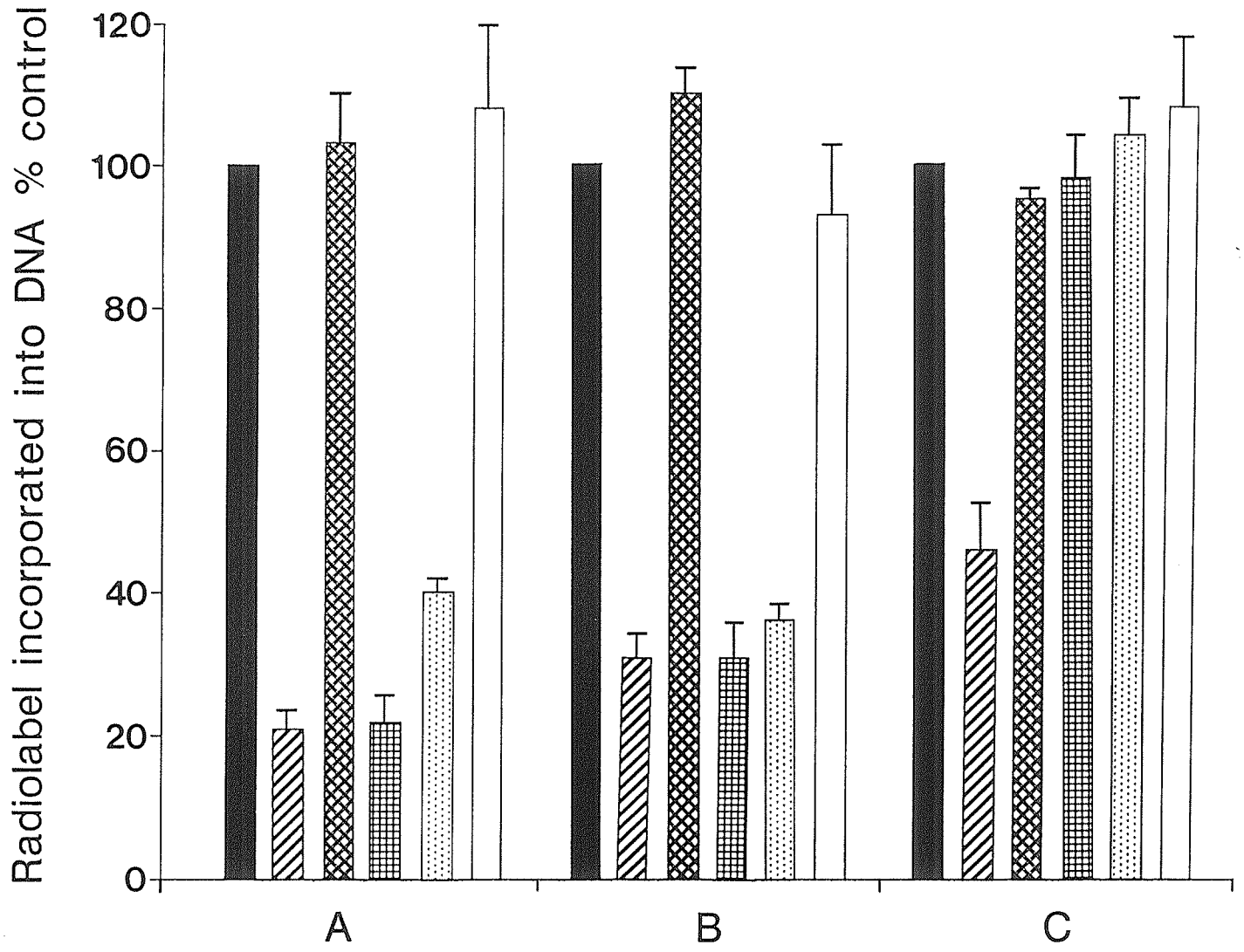
D) produced variableamounts 0f 10-CHO-H_4folate; with variable amounts of 10-CHO-H_4folate, H_4folate, 5-CHO-H_4folate, and 5-CH_3-H_4folate/5, 10-CH_2-H_4folate. Sulfisoxazole (10 μM) was effective in preventing the incorporation of [^{3}H]pABA into folates by all three chlamydial strains.

4. Detection of in vitro DHPS Activity in Chlamydial RB Extract.

To conclusively show that chlamydiae contain DHPS, we prepared extracts from highly purified *C. trachomatis* L2, *C. psittaci* 6BC and *C. psittaci* francis RBs and then assayed for DHPS activity in vitro. DHPS activity was measured by following the synthesis of dihydropteroate from [^{3}H]pABA and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate. We consistently detected DHPS activity using RB extracts prepared from any one of the three chlamydial strains as a source of enzyme. RB extracts prepared from *C. trachomatis* L2, *C. psittaci* 6BC and *C. psittaci* francis catalyzed the synthesis of 3.1 $\pm$ 0.5, 6.5 $\pm$ 1.6, and 2.8 $\pm$ 0.3 pmol dihydropteroate product/mg protein/min, respectively. The DHPS activity detected from all strains was inhibited > 90% by 10 μM sulfisoxazole.

5. Reversal of Sulfisoxazole Inhibition by pABA and Folates

It has been shown with numerous experimental systems that the inhibitory action of sulfa drugs can be antagonized by pABA (Anand, 1983). Results presented in Fig. 14


Radiolabel incorporated into DNA % control
120
100
80
60
40
20
0
A
B
C

(Facing page)

Figure 14. **Effect of Exogenous pABA and Folates on the Sulfisoxazole-Induced Inhibition of *C. trachomatis* L2 (A), *C. psittaci* 6BC (B), *C. psittaci* francis (C) DNA Synthesis.** Chlamydiae-infected folate and pABA-depleted wild-type CHO K1 cells (4.0×10^6 cells per plate cultured in folate- and pABA-free medium supplemented with proline, glycine, and hypoxanthine in the presence of 1 μg cycloheximide per ml) were incubated in the absence or presence of sulfisoxazole, pABA, and/or folates. The indicated components were added at 2 hr postinfection, and then at 20 hr postinfection, the cultures were pulsed with [6-^{3}H]uridine. The amount of radiolabel incorporated into DNA is expressed as a percentage of the uninhibited control. The following are 100% control values: *C. trachomatis* L2-infected cultures, 138,034 $\pm$ 15,984 dpm/10^6 cells; *C. psittaci* 6BC-infected cultures, 165,428 $\pm$ 19,683 dpm/10^6 cells; and *C. psittaci* francis-infected cultures, 167,592 $\pm$ 13,098 dpm/10^6 cells. The data represent the average of two determinations. Bars, SD. Chlamydiae-infected control cultures, (*solid bars*); chlamydiae-infected cultures plus 1.0 μM sulfisoxazole, (*hatched bars*); chlamydiae-infected cultures plus 1.0 μM sulfisoxazole and 1.0 μM pABA, (*cross-hatched bars*); chlamydiae-infected cultures plus 1.0 μM sulfisoxazole and 10.0 μM folic acid, (*square-checked bars*); chlamydiae-infected cultures plus 1.0 μM sulfisoxazole and 1.0 μM 5-CHO-H_4folate (*dotted bars*); and chlamydiae-infected cultures plus 1.0 μM sulfisoxazole and 10.0 μM 5-CHO-H_4folate (*open bars*).

indicate that, with folate-and pABA-depleted CHO K1 cells as host, the growth inhibition caused by 1 μM sulfonamide (Fig. 14 A-C, *hatched bars*) on all three strains of chlamydiae can be completely reversed by 0.1 μM pABA (Fig. 14 A-C, *cross-hatched bars*). With *C. psittaci* francis, 10 μM folic acid completely reversed the inhibition caused by 1 μM sulfisoxazole (Fig. 14 C, *square-checked* bar) and even 1 μM folic acid was sufficient to reverse 1 μM sulfisoxazole (161.233 $\pm$ 10.465 dpm/10^6 vs control values shown in Fig. 14 legend). In contrast, folic acid was much less effective at reversing the effects of sulfa on *C. trachomatis* L2 and *C. psittaci* 6BC, showing essentially no antagonism at 10 μM (Fig. 14 A and B, *square-checked* bars) and only partial (41.3% and 47.2%, respectively) reversion at 100 μM. We found that the inhibitory effects of 1 μM sulfisoxazole on *C. trachomatis* L2 and *C. psittaci* francis could be partially and completely reversed, respectively, by 1 μM 5-CHO-H_4folate (Fig. 14 A and C, *dotted bar*). At a concentration of 10 μM, 5-CHO-H_4folate completely reversed the inhibitory effects of 1 μM sulfisoxazole on *C. trachomatis* L2 (Fig. 14 A, *open bar*). Surprisingly, even though methotrexate did not inhibit the growth of *C. psittaci* 6BC (Fig. 11), we found that 10 μM 5-CHO-H_4folate could reverse the effects of 1 μM sulfisoxazole (Fig. 16 B, *open bar*).

Our commercial preparation of folic acid was ~98% pure; therefore it was possible that a small amount of contaminating pABA may have been present in our folate preparations. Since pABA was $\geq$ 100 times more effective at antagonizing sulfa activity compared with 5-CHO-H_4folate, it was possible that the reversion brought about by

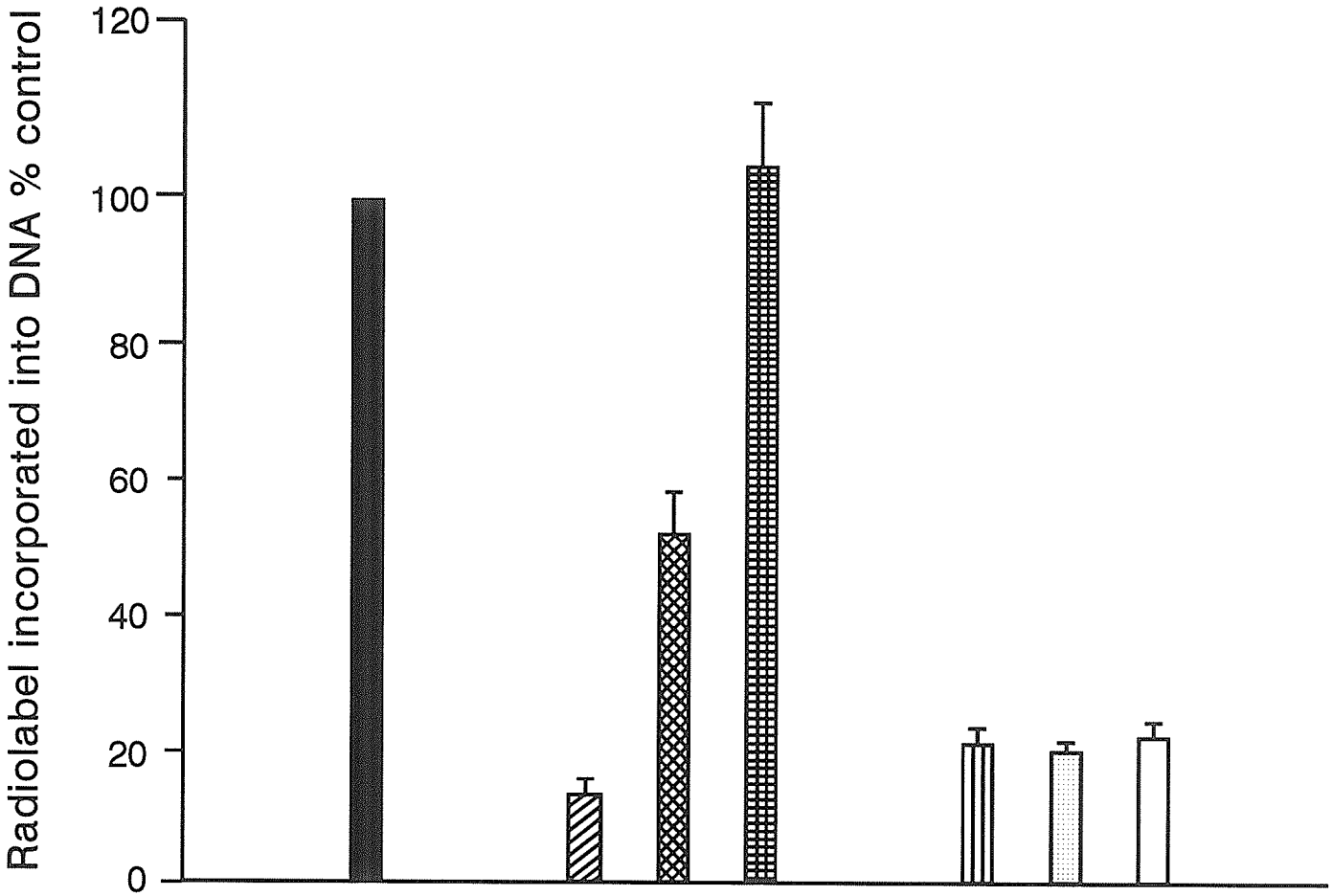

Radiolabel incorporated into DNA % control
120
100
80
60
40
20
0

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Figure 15. Effect of 5-CHO-H_4folate and Folic Acid on Trimethoprim and/or Sulfisoxazole-induced Inhibition of *C. trachomatis* L2 DNA Synthesis. Confluent monolayers of CHO K1 cells (4.0 x 10^6 cells per plate) were infected with *C. trachomatis* L2 and cultured in the presence of 1 μg cycloheximide. The indicated components were added at 2 hr postinfection, and then at 20 hr postinfection, the cultures were pulsed with [6-^{3}H]uridine. The amount of radiolabel incorporated into DNA is expressed as a percentage of the uninhibited control (100% control values: 150,320 $\pm$ 13,927 dpm/10^6 cells). The data represent the average of two determinations. Bars, SD. *C. trachomatis* L2-infected control cultures, (*solid bar*); infected cultures plus 1.0 μM sulfisoxazole and 1.0 μM trimethoprim, (*hatched bar*); infected cultures plus 1.0 μM sulfisoxazole/1.0 μM trimethoprim, and 1.0 μM 5-CHO-H_4folate, (*cross-hatched bar*); infected cultures plus 1.0 μM sulfisoxazole/1.0 μM trimethoprim, and 10.0 μM 5-CHO-H_4folate, (*square-checked bar*); infected cultures plus 1.0 μM sulfisoxazole, (*striped bar*); infected cultures plus 1.0 μM sulfisoxazole, and 50.0 μM folic acid, (*dotted bar*); and infected cultures plus 1.0 μM sulfisoxazole, and 100.0 μM folic acid (*open bar*).

folates was really caused by contaminating pABA. To eliminate this possibility we tested the ability of 5-CHO-H_4folate to reverse the inhibitory action of trimethoprim/sulfisoxazole against *C. trachomatis* L2. The results clearly show that 5-CHO-H_4folate can antagonize the combined activity (Fig. 15, *hatched bar*) of the DHFR inhibitor trimethoprim and the DHPS inhibitor sulfisoxazole (Fig. 15, *cross-hatched and square-checked bars*). As expected, folic acid (up to 100 μM) could not reverse 1.0 μM sulfisoxazole inhibition (Fig. 15, *striped bar*) of *C. trachomatis* L2 growth (*dotted and open bars*).

6. Detection of in vitro DHFR Activity in Chlamydial RB Extracts

To directly demonstrate that chlamydiae encode DHFR we conducted in vitro assays for DHFR using extract prepared from highly purified RBs as a source of enzyme (Table 8). As a control experiment, we conducted DHFR assays with crude extract prepared from logarithmically growing wild-type mouse L-cells. We consistently detected DHFR activity in extracts prepared from *C. trachomatis* L2 as well as *C. psittaci* 6BC and francis RBs. No activity was detected if folic acid was used as substrate. Purified sham extract prepared from mock-infected mouse cell extract had essentially no DHFR activity.

Similar to in situ results we found that trimethoprim was a highly effective inhibitor of *C. trachomatis* L2 DHFR activity in vitro, however, it was less effective

against *C. psittaci* 6BC and *C. psittaci* francis DHFR activity in vitro (Table 8). In agreement with previous observations that methotrexate is an effective in vitro inhibitor of DHFR activity from most bacterial and mammalian sources (Burchall, 1983), we found that it was active against in vitro activity of all three chlamydial strains.

Table 8. Dihydrofolate Reductase Activity in Crude Extracts Prepared from Logarithmically Growing Host Cells and Purified Chlamydiae Reticulate Bodies

Substrate[a]	Inhibitor[b]	Source of enzyme									
		Log growing L cells		Mock-infected L cells		*C.trachomatis* L2		*C.psittaci* 6BC		*C.psittaci* francis	
		DHFR activity[c]	%	DHFR activity	%	DHFR activity	%	DHFR activity	%	DHFR activity	%
H_2folate	--	3.65±0.56	100	<0.01	100	2.34±0.56	100	0.37±0.05	100	1.87±0.39	100
	TMP	4.18±0.17	114	ND[d]	ND	0.05±0.02	2	0.25±0.04	65	1.53±0.11	82
	MTX	0.45±0.06	12	ND	ND	1.26±0.15	54	0.03±0.02	8	1.03±0.12	55
Folic acid	--	0.25±0.05	7.1	ND	ND	<0.01	<1	<0.01	<1	<0.01	<1

[a] The complete DHFR reaction mix contained either 100 μM H_2folate or 100 μM folic acid as the substrate.

[b] To assess the effect of trimethoprim (TMP) and methotrexate (MTX) on DHFR activity, a complete reaction mix minus substrate was incubated in the presence of 10 nM TMP or 1 nM MTX for 10 min at 4°C. The reaction was initiated by the addition of H_2folate substrate and incubation at 30°C.

[c] Reactions were carried out at 30°C for 10 min. DHFR activity is expressed as nmol H_4folate formed/mg protein/min. Each value represents the mean ± standard deviation for two determinations.

[d] ND, not determined.

PAPR 3. MOLECULAR CLONING OF *C. TRACHOMATIS* TS GENE

1. Failure to Detect the *C. trachomatis* TS Gene Using Degenerate Oligonucleotides Reverse Translated from Known TS Protein Sequences

Since TS is a highly conserved protein, it was possible to screen a *C. trachomatis* genomic DNA library for the TS gene using degenerate oligonucleotides reverse translated from conserved regions of known TS proteins (Fig, 2). We synthesized two oligonucleotide mixtures. One of the oligonucleotide mixtures, designated TS-a (sequence: 5′ GG[A/T] GT[A/G/T] CC[A/T] TT[C/T] AA[C/T] AT[A/C/T] GC 3′) was designed from amino acid residues 224-230 (Leu-Gly-Val-Pro-Phe-Asn-Ile) of *L. casei* sequence. Leu-224, Gly-225, Pro-227, Phe-228, Asn-229 are found in all known TS sequences; and Val-226, and Ile-330 are variant in only three, and one out of 15 sequences, respectively. The other oligonucleotide mixture (sequence: 5′ GG[A/T CC[A/T] GT[A/G/T] TA[C/T] GG[A/T] TT[C/T] CA 3′) was based on residues 143-149 (Gly-Pro-Val-Tyr-Gly-Phe-Gln-Try) relative to the *L. casei* sequence; the Gly-143, Tyr-146, Gly-147 and Gln-149 residues are invariant in all known TS sequences, and Val-145, Pro-144 and Phe-148 are substituted in only one, two and three out of 15 sequences, respectively (Fig. 2). Among the conserved regions in known TS sequences, these two have lowest degree of degeneration. Also taken into account in the design of the sequences was the chlamydial codon preference (Wada et al, 1991). Previously, oligonucleotides designed for essentially the same regions have been used successfully

in library screening for TS cDNA from *Pneumocystis carii* and some protozoa (Invanetich and Santi, 1990).

When the synthetic oligonucleotides were end-labelled with ^{32}P and subjected to Southern hybridization, both were found to bind *C. trachomatis* L2 genomic DNA after overnight hybridization at 37°C in 6 x SSPE followed by one washing in 1 x SSC buffer at 37°C (Fig. 16, top). TS-a was, however, totally removed from the blotted genomic DNA after washing at a slightly higher stringency (42°C, 1 x SSC) (Fig. 16, bottom left). Under the same conditions, some of the TS-b binding was retained. We used TS-b as sequence primer to directly sequence *C. trachomatis* L2 genomic DNA (Fig. 16, bottom right). Sequence of a 82-base fragment (5′ TGTGCAAAGC TTGTGAATCA GGGTCATGGC GGACGGGTTT CGTGAATAGA TGCTACCAAA AATATTCTGC TAGTTTAGTA AG 3′) was obtained. The third reading frame of the nucleotide sequence can be deduced to a continuous 26 residue peptide (CKACESGSWRTGFVNRCYQKYSASLV). However, since this peptide did not shown any significant homology to the corresponding region of the known TS sequences, we decided to use an alternate approach for screening.

2. Library Screening by Genetic Complementation

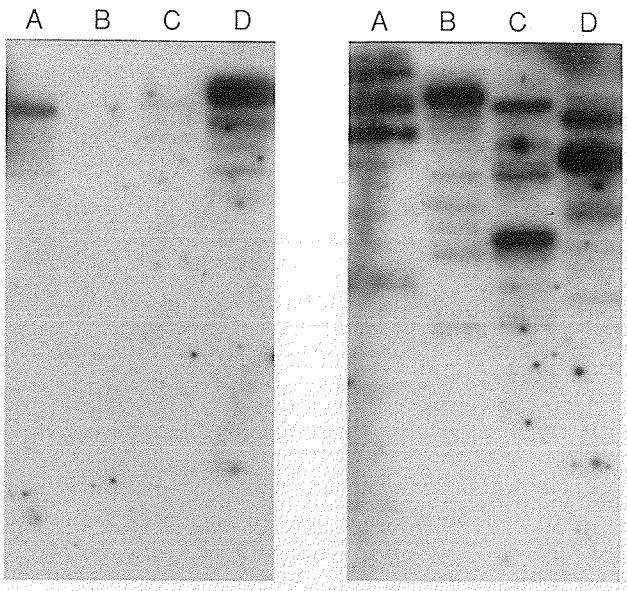


Figure 16. **Probing *C. trachomatis* L2 TS Gene with Degenerate Oligonucleotides.** *Bam*H1, *Eco*R1, *Hind*III, and *Pst*I-digested *C. trachomatis* genomic DNA was blotted onto nylon membrne (*Lanes A, B, C,* and *D*, respectively) and subjected to hybridization at 37°C using ^{32}P-labelled oligonucleotide mixtures TS-A (left) and TS-B (right) as probe. Top picture was obtained after wash at 37°C, and bottom was obtained after further washing at 42°C.

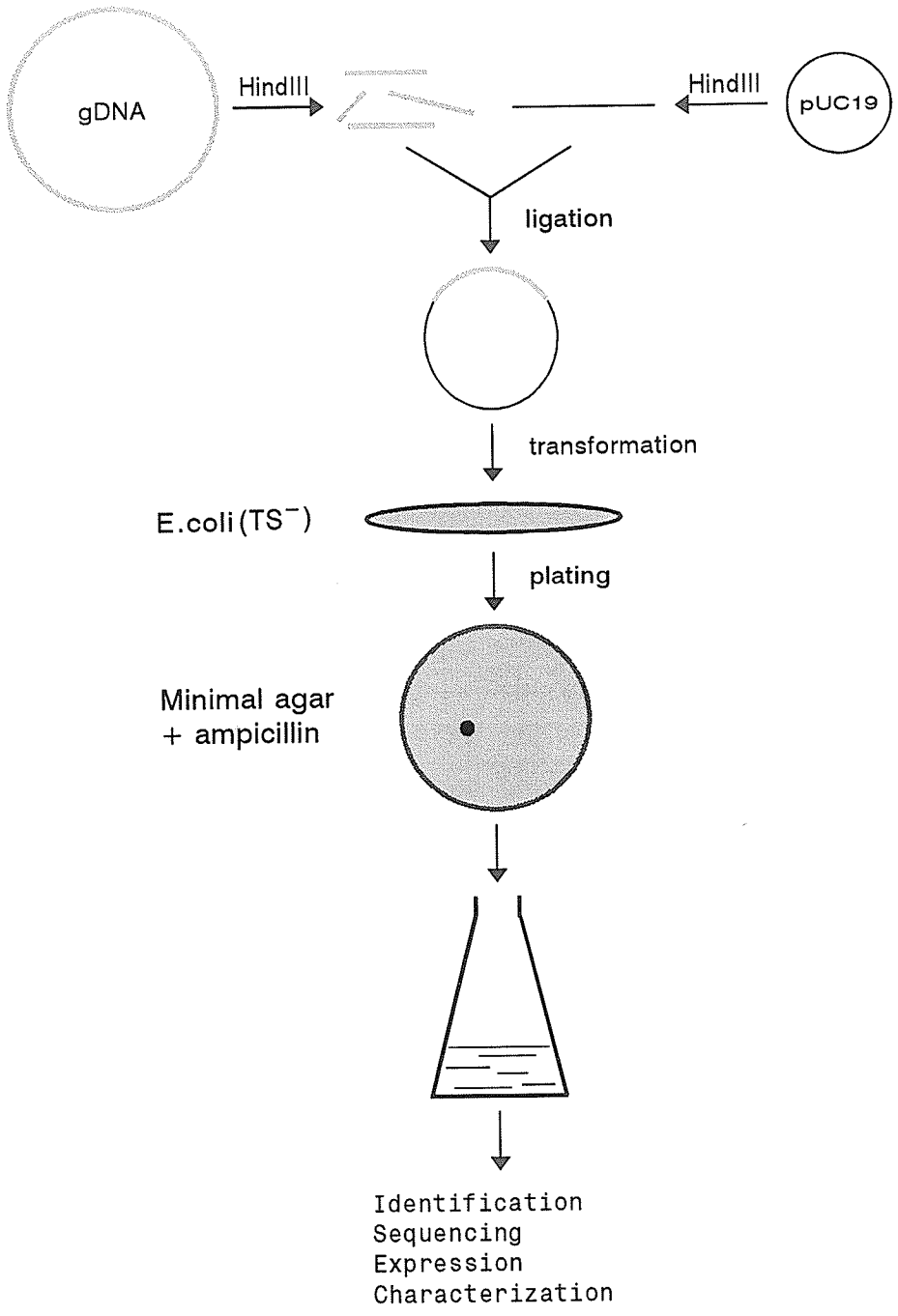


Figure 17. Strategy for cloning C.trachomatis TS gene

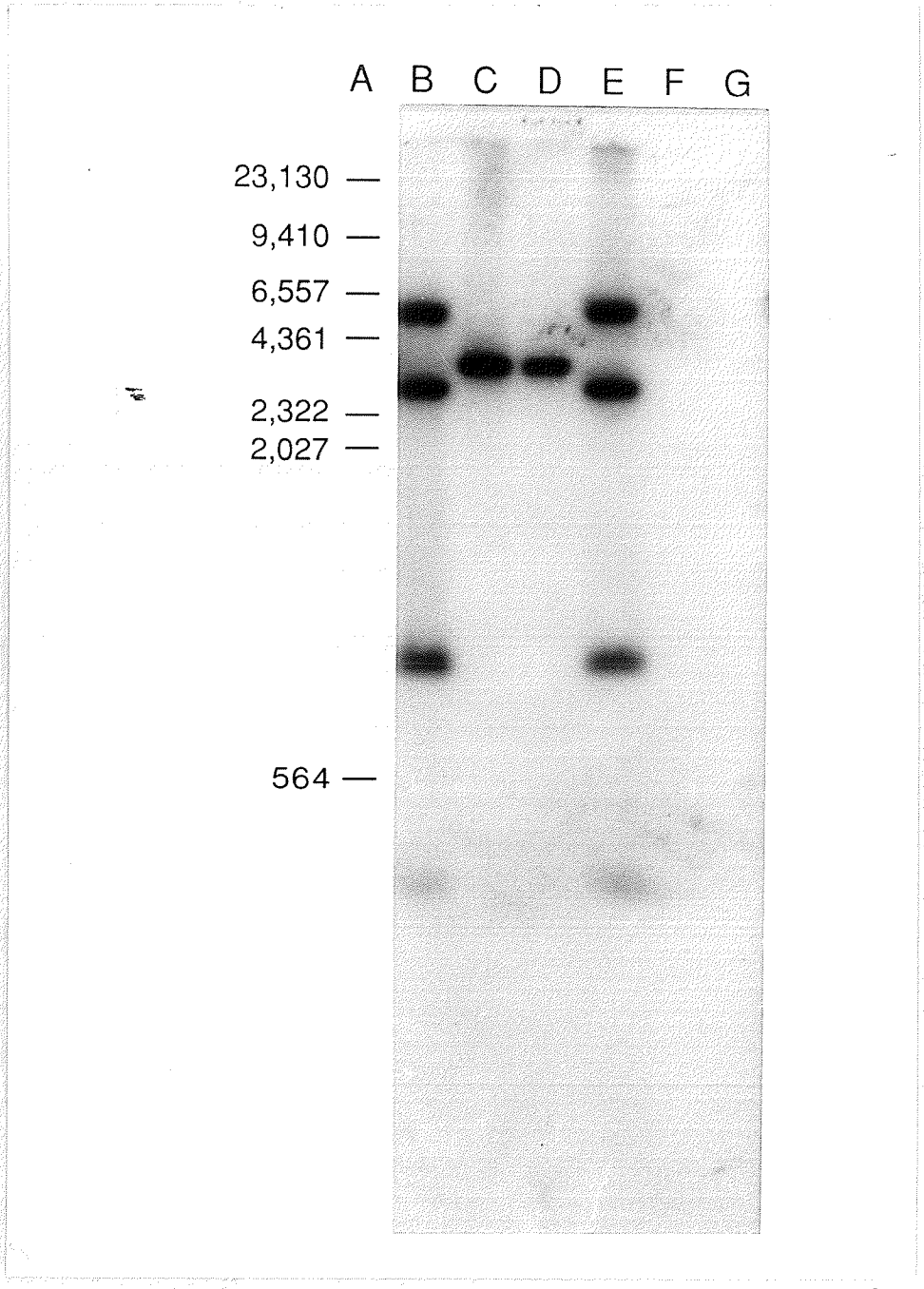
Interspecies complementation has proven to be a useful strategy for cloning metabolic pathway genes and cDNAs from bacteria, fungi, and mammals (Peoples and Sinskey, 1989; Dougherty et al, 1992; Williams and Kantrowitz, 1992; and Kaslow and Hill, 1990). Fig. 17 schematically presents our strategy for cloning the chlamydial TS gene. We constructed three *C. trachomatis* L2 genomic DNA libraries in the multi-copy plasmid pUC19 using *Hind*III, *Pst*I and *Xba*I, respectively and screened the libraries for complementation of the thymidine auxotrophy in TS$^-$ *E. coli* strains BL21 Δ*thyA* and ATCC23851. The medium that was used for screening had a defined composition and contained no thymidine; thus any clones which grow in the medium containing ampicillin should contain a recombinant plasmid expressing a potential chlamydial TS gene. When 4,000 recombinant *E. coli* containing *Hind*III-digested chlamydial DNA were screened, one (ATCC23851) and five (BL21 Δ*thyA*) colonies appeared on the ampicillin-containing enriched minimal agar after about 30 hr incubation at 37°C. Plasmids were prepared from all six clones. To ensure that the growth of the clones was the result of genetic complementation and to exclude the possibility of wild-type bacteria contamination or reverse mutation, the six plasmids were used for a second round of transformation of TS$^-$ *E. coli*. A third TS$^-$ *E. coli* strain, X2913 (genotype: Δ*thyA*) as well as ATCC23851 and BL21 Δ*thyA* were transformed by each of the clones and all the clones supported the growth of all three auxotrophic bacteria when grown in thymidine-free medium. As expected, no colonies appeared when the bacteria were transformed with pUC19 vector at 10^7 colony forming unit and plated on thymidine-free agar containing ampicillin. These results indicate that the thymidine protrophy displayed by the recombinant *E. coli* strains

was conferred by the recombinant plasmids. The plasmids were digested with *Hind*III and other restriction enzymes whose cutting sites exist within the pUC19 polylinker region and results showed that all clones shared the same restriction digestion pattern and contained a 3 kb *Hind*III insert, suggesting that all clones represented the same DNA fragment. One plasmid (designated pHF101) was used for further studies.

No positive clones were obtained using *Pst*I and *Xba*I libraries of chlamydial DNA after 6 x 10^5 and 1 x 10^6 colony forming units of recombinant plasmids were screened.

3. Identification of the Insert of pHF101 as *C. trachomatis* DNA

Southern hybridization was performed to confirm that the insert of pHF101 was from *C. trachomatis* genomic DNA. As shown in Fig. 18, [α-^{32}P]dCTP nick-translated pHF101 hybridized to *C. trachomatis* genomic DNA under high stringency conditions (in 6 x SSPE containing 0.1% SDS at 68°C). As expected, the probe bound to a single 3 kb DNA fragment, which is the same size as the insert in pHF101, in *Hind*III-restricted *C. trachomatis* L2 genomic DNA (which was used for library construction) at high stringent conditions (in 6 x SSPE at 68°C for hybridization) (Fig. 18 Lane C). *Pst*I-restricted *C. trachomatis* L2 DNA (Fig. 18 Lane B) showed four plasmid-bound fragments of different sizes; the number and size of fragments detected were in keeping with three *Pst*I cutting sites in the insert of pHF101 (see Fig. 19). The hybridizations


A B C D E F G
23,130
9,410
6,557
4,361
2,322
2,027
564

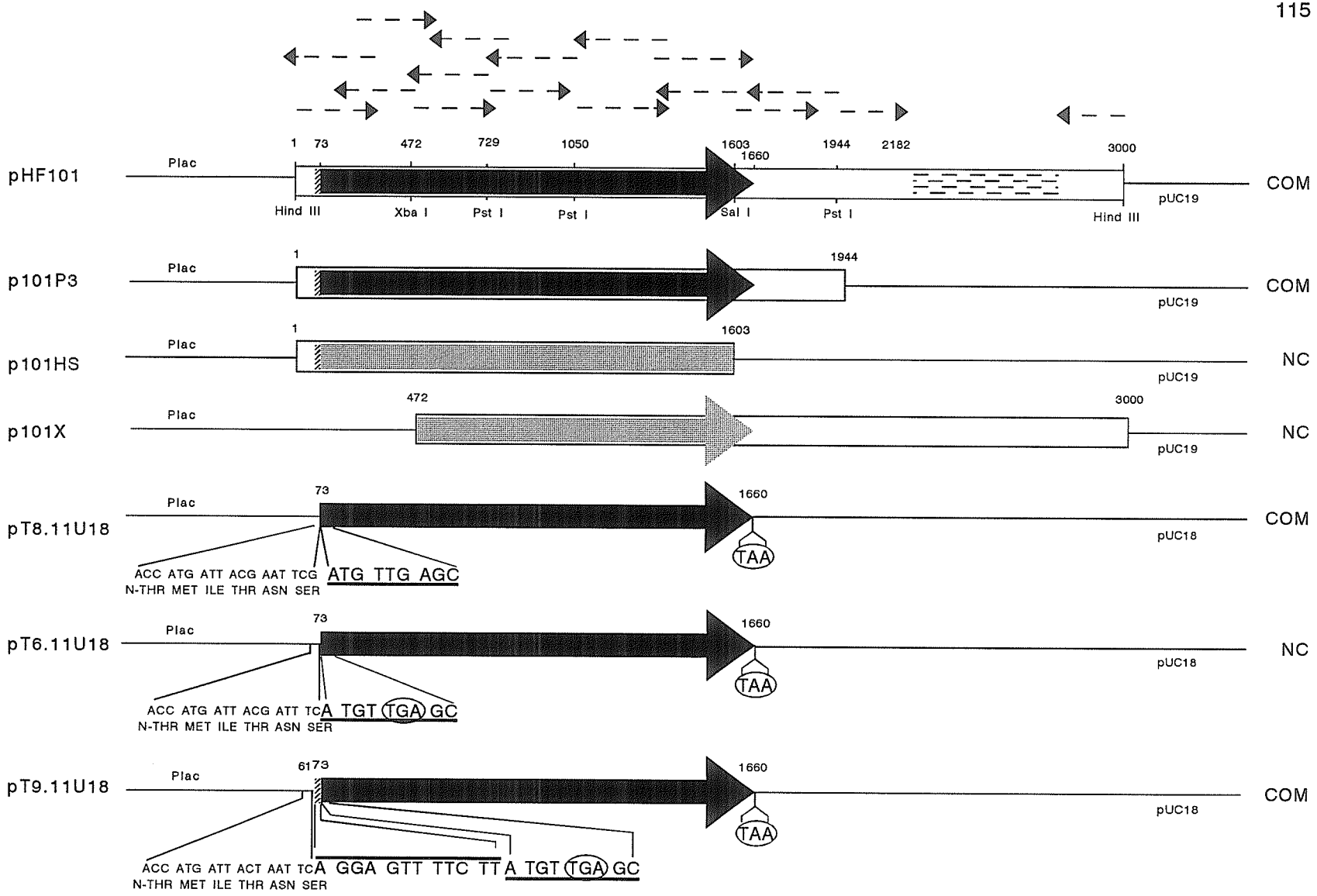
(Facing page)

Figure 18. **High Stringency Southern Hybridization Using pHF101 as Probe.** pHF101 was nick-translated with α-[^{32}P]dCTP and hybridization was carried out overnight at 68°C in 5 X SSPE containing 5 x Denhart's solution, 0.1% SDS and salmon sperm DNA. Washes were done at the same temperature in buffers with gradually decreased salt concentrations. Last washing was in 0.1 x SSC and 1% SDS for 30 min. *Lane A*, *Hind*III λDNA molecular markers (bp); *Lane B*, *Pst*I-restricted *C. trachomatis* L2 genomic DNA; *Lane C*, *Hind*III-restricted *C. trachomatis* L2 genomic DNA; *Lane D*, *Hind*III-restricted *C. trachomatis* A genomic DNA; *Lane E*, *Pst*I-restricted *C. trachomatis* A genomic DNA. *Lane F*, *Pst*I-restricted *C. psittaci* 6BC genomic DNA; and *Lane G*, *Pst*I-restricted *E. coli* XL1-blue genomic DNA.

were resistant to highly stringent washes (68°C in gradually decreasing salt concentrations [see Fig. 18 legend]). Under the same conditions, the radiolabelled plasmid also hybridized to *C. trachomatis* serovar A genomic DNA restricted with *Hind*III (Fig. 18 Lane D) or *Pst*I (Fig. 18 Lane E) in the same pattern as to *C. trachomatis* serovar L2 DNA. This is in agreement with the known high degree of homology (>92%) between these two serovars (Table 1). In contrast, the probe did not hybridize to *Hind*III-restricted genomic DNA of *C. psittaci* 6BC (Fig. 18 Lanes F). This is not surprising because there is very limited DNA homology (<33%) between the species *C. trachomatis* and *C. psittaci*. Importantly hybridization was not detected between pHF101 and the *Hind*III-restricted chromosomal DNA of *E. coli* XL1-blue, the recipient strain used for the preparation of vector plasmid pUC19 (Fig. 18 Lanes G). Collectively, the results of Southern hybridization suggests that pHF101 contains an insert originating from *C. trachomatis* DNA, likely to be a single copy conserved gene in the species *C. trachomatis*.

4. Subcloning and Sequencing of pHF101

The strategy used for subcloning and sequencing of pHF101 is shown in Fig. 19. The full length of the *Hind*III insert in pHF101 was about 3 kb. The plasmid was cut with enzymes whose cutting sites exist in the polylinker region of pUC19 plasmid. *Hind*III, *Xba*I, *Pst*I, and *Sal*I which were found to cut the insert were used for subcloning. Fragments resulted from restriction digestion with each of the above enzymes


pHF101
Plac
1
73
472
729
1050
1603
1660
1944
2182
3000
Hind III
Xba I
Pst I
Pst I
Sal I
Pst I
Hind III
pUC19
COM
p101P3
Plac
1
1944
pUC19
COM
p101HS
Plac
1
1603
pUC19
NC
p101X
Plac
472
3000
pUC19
NC
pT8.11U18
Plac
73
1660
TAA
pUC18
COM
ACC ATG ATT ACG AAT TCG ATG TTG AGC
N-THR MET ILE THR ASN SER
pT6.11U18
Plac
73
1660
TAA
pUC18
NC
ACC ATG ATT ACG ATT TCA TGT TGA GC
N-THR MET ILE THR ASN SER
pT9.11U18
Plac
61
73
1660
TAA
pUC18
COM
ACC ATG ATT ACT AAT TCA GGA GTT TTC TTA TGT TGA GC
N-THR MET ILE THR ASN SER

(Facing page)

Figure 19. Restriction Endonuclease Map of, Sequencing Strategy for and Construction for Confirmation of Functional Open Reading Frame in pHF101. On the left are designations of recombinant plasmids with corresponding structure in the center, and function (COM, complementing; or NC, non-complementing) on the right. The horizontal thin lines and long boxes represent DNA from vectors pUC19 or pUC18, and insert of chlamydial DNA, respectively. The broken arrows on the top of pHF101 indicate the position, direction and extent of sequence data which was obtained from each strand of the insert in this plasmid. Sequencing data of the arrows starting at enzyme cutting sites was obtained from subcloned plasmids using universal and reverse primers designed for M13/pUC vectors and otherwise from the original plasmid using primers with sequences obtained from subcloned fragments. The region filled with broken lines in the insert DNA was not sequenced. The base numbers are relative to the strand whose 5′ to 3′ direction correspond to the coding strand of the *LacZ* gene in the vector. Plac on the vectors represents *LacZ* gene promoter. The big solid arrow indicates the orientation and position of an intact open reading frame of 1587 bp (ORF1587) as determined by PC/GENE analysis. Incomplete ORF1587 in constructs p101X and p101HS are shown in dots. The small-size letters in constructs, pT8.11U18, T6.11U18 and T9.11U18 represent sequence from pUC18 vector and encoded amino acid residues at the N-terminus of *LacZ* protein α-peptide. Larger-size letters represents sequence from the inserts. Sequence from ORF1587 is underlined. The hatched area upstream of ORF1587 represents a hypothetical ribosomal binding site whose sequence is shown in pT9.11U18 is overscored. Stop codons are circled.

```
   1 AAGCTTTTTG CTGAAAAACT TTGAGTGTTT TGTTATGTGT GGTAGGCTAA TCTGCTTTAT
     TTCGAAAAAC GACTTTTTGA AACTCACAAA ACAATACACA CCATCCGATT AGACGAAATA

  61 AGGAGTTTTC TTATGTTGAG CAAAGAGGGT GGTTTTTCTG AGGAGCAAAG AGCGCGTTTA
     TCCTCAAAAG AATACAACTC GTTTCTCCCA CCAAAAAGAC TCCTCGTTTC TCGCGCAAAT

 121 TCGCATTTTG TGACGAATTT AGACTCGCCT ATATTTGCTT TGAAAAACCT TCCAGAAGTG
     AGCGTAAAAC ACTGCTTAAA TCTGAGCGGA TATAAACGAA ACTTTTTGGA AGGTCTTCAC

 181 GTTAAAGGCG CTTTATTTTC AAAATATTCC AGATCGACTC TGGGGTTGCG AACGCTTCTT
     CAATTTCCGC GAAATAAAAG TTTTATAAGG TCTAGCTGAG ACCCCAACGC TTGCGAAGAA

 241 TTGAAAGAAT TTTTAGATGG GGAAGGCGGT AATTTCCTTG ATGATGACCA ACAAGATTGT
     AACTTTCTTA AAAATCTACC CCTTCCGCCA TTAAAGGAAC TACTACTGGT TGTTCTAACA

 301 GAGTTGGGAA TCCAAAAAGC TGTGGACTTC TATCGTCGCG TTTTAGACAA CTTTGGTGAT
     CTCAACCCTT AGGTTTTTCG ACACCTGAAG ATAGCAGCGC AAAATCTGTT GAAACCACTA

 361 GATTCTGTTG GAGAGTTGGG AGGAGCGCAT CTTGCTCTGG AACAAGTATC CATGCTCGCA
     CTAAGACAAC CTCTCAACCC TCCTCGCGTA GAACGAGACC TTGTTCATAG GTACGAGCGT

 421 GCAAAAATTT TAGAAGATGC TCGGATTGGA GGGTCCCCCC TAGAAAAATC GTCTAGATAC
     CGTTTTTAAA ATCTTCTACG AGCCTAACCT CCCAGGGGGG ATCTTTTTAG CAGATCTATG

 481 GTTTATTTCG ATCAAAAAGT TAACGGGGAG TATTTATATT ACCGAGACCC TATTTTGATG
     CAAATAAAGC TAGTTTTTCA ATTGCCCCTC ATAAATATAA TGGCTCTGGG ATAAAACTAC

 541 ACCTCGGCCT TTAAAGACGT CTTTTTGGAT ACTTGTGATT TCCTATTCAA CACATACTCC
     TGGAGCCGGA AATTTCTGCA GAAAAACCTA TGAACACTAA AGGATAAGTT GTGTATGAGG

 601 GATCTTATCC CTCAGGTTCG TTCCCATTTC GAGAAACTAT ACCCTAAAGA TCCAGAAGTT
     CTAGAATAGG GAGTCCAAGC AAGGGTAAAG CTCTTTGATA TGGGATTTCT AGGTCTTCAA

 661 TCTCAATCAG CGTATACAGT TTCTTTACGA GCTAAAGTAT TAGACTGTTT ACGAGGTTTG
     AGAGTTAGTC GCATATGTCA AAGAAATGCT CGATTTCATA ATCTGACAAA TGCTCCAAAC

 721 TTACCTGCAG CGACACTCAC AAATTTAGGT TTTTTTGGTA ATGGCCGGTT TTGGCAGAAC
     AATGGACGTC GCTGTGAGTG TTTAAATCCA AAAAAACCAT TACCGGCCAA AACCGTCTTG

 781 TTGCTACACC GTTTGCAAGA CAATAGTTTG GTTGAGGTAC GCAATATTGG AGAGCAGTCC
     AACGATGTGG CAAACGTTCT GTTATCAAAC CAACTCCATG CGTTATAACC TCTCGTCAGG

 841 TTAACAGAAT TAATGAAAAT AATTCCCTCT TTTGTAAGCC GCGCAGAGTC TCATCATTAT
     AATTGTCTTA ATTACTTTTA TTAAGGGAGA AAACATTCGG CGCGTCTCAG AGTAGTAATA

 901 CATCACCAAG CTATGGTGGA TTACCGTCGG GCTTTAAAAG AACAATTAAA AAGTTTTGCA
     GTAGTGGTTC GATACCACCT AATGGCAGCC CGAAATTTTC TTGTTAATTT TTCAAAACGT

 961 CATCGTTACG GGGAAGAGAG AGAAATTTCG AAAGAGGCTG GTGTAAAATT AGTATACGGA
     GTAGCAATGC CCCTTCTCTC TCTTTAAAGC TTTCTCCGAC CACATTTTAA TCATATGCCT

1021 GATCCAGACG GGTTATACAA AATTGCTGCA GCCTACATGT TCCCCTACTC GGAACACACT
     CTAGGTCTGC CCAATATGTT TTAACGACGT CGGATGTACA AGGGGATGAG CCTTGTGTGA

1081 TATGCAGAGC TGTTAGATAT TTGTCGCAAT ATTCCTAATG AAGATCTCAT GCGTATCTTA
     ATACGTCTCG ACAATCTATA AACAGCGTTA TAAGGATTAC TTCTAGAGTA CGCATAGAAT

1141 GAGTCGGGAG CTTCTTTCCG AGAGAATCGG CGGCACAAAT CCCCTCGCGG ATTGGAATGT
     CTCAGCCCTC GAAGAAAGGC TCTCTTAGCC GCCGTGTTTA GGGGAGCGCC TAACCTTACA

1201 GCTGAGTTTG CTTTTGATAT TACAGCGGAT TTTGGAGCCT ATCGGGATTT ACAAAGACAT
     CGACTCAAAC GAAAACTATA ATGTCGCCTA AAACCTCGGA TAGCCCTAAA TGTTTCTGTA

1261 CGTATCCTAA CTCAAGAAAG ACAGCTTTTG ACGACAAAAT TGGGTTACAC GATGCCTTCA
     GCATAGGATT GAGTTCTTTC TGTCGAAAAC TGCTGTTTTA ACCCAATGTG CTACGGAAGT

1321 CAATTGATCG ACACTCCTAT GGAAGCTCCC TTCAGAGGAG CTATGGAAAA AGCTGATCAA
     GTTAACTAGC TGTGAGGATA CCTTCGAGGG AAGTCTCCTC GATACCTTTT TCGACTAGTT

1381 GCGTATCGTC TAATAGCAGA AGAGTTCCCA GAAGAAGCAC AATATGTGGT TCCTTTAGCT
     CGCATAGCAG ATTATCGTCT TCTCAAGGGT CTTCTTCGTG TTATACACCA AGGAAATCGA

1441 TACAATATTC GATGGCTTTT CCATATCAAC GCTAGAGGTT TGCAGTGGCT TTGTGAGTTA
     ATGTTATAAG CTACCGAAAA GGTATAGTTG CGATCTCCAA ACGTCACCGA AACACTCAAT

1501 CGCTCTCAAC CACAAGGGCA TGAAAGCTAT CGTAAAATTG CTATAGATAT GGCTAGAGAG
     GCGAGAGTTG GTGTTCCCGT ACTTTCGATA GCATTTTAAC GATATCTATA CCGATCTCTC

1561 GTTATTCAGT TTCATCCAGC TTACGAGCTG TTCTTGAAGT TTGTCGACTA CTCAGAGACT
     CAATAAGTCA AAGTAGGTCG AATGCTCGAC AAGAACTTCA AACAGCTGAT GAGTCTCTGA

1621 GACCTAGGAA GATTACAACA AGAATCGCGT AAAAAGTCTT AAAGAAAGTT CTGGATGAGA
     CTGGATCCTT CTAATGTTGT TCTTAGCGCA TTTTTCAGAA TTTCTTTCAA GACCTACTCT

1681 GGATTTGAAC CTCCGCCCCC TTGCACCCCA TGCAAGTGCG CTACCAGCTG CGCTACATCC
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1741 AGAAAATAGA AATAAGGGGT ATTAACCGAC CACAGTTTTA CAGTAAAAAC TTTCAAAGTC
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1801 AAGCTATTTA AATACAACGA ATATAAGTTT TTGGAAACCT GTTATAAACA AATAATTCCT
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1861 TCAAACTGAA ACTCGCATTT TTGAAATTCC CGAATACCAA TAAGAGCACA GCGTTCTATA
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1921 ATGGTTTCTA AAGAATCTCC TGCAGAAACA TCAGCTGCAT CGATATCAAC GGCTATAGAA
     TACCAAAGAT TTCTTAGAGG ACGTCTTTGT AGTCGACGTA GCTATAGTTG CCGATATCTT

1981 GCTGTAAAGG AAGCCCCCCC TCTCTCTCCT TTAACTTGAA AATTAACGAA AATACCCTGT
     CGACATTTCC TTCGGGGGGG AGAGAGAGGA AATTGAACTT TTAATTGCTT TTATGGGACA

2041 TTGGTTTTAG AAATATTTTT GAATCTGACG ATGTTGTTAT CAATTAACGT TTTCATTTTC
     AACCAAAATC TTTATAAAAA CTTAGACTGC TACAACAATA GTTAATTGCA AAAGTAAAAG

2101 TCTCAAATTT TTCGATCAAA AACTAGATAA AGCAGAGAAT GAGCTGTTTA TCACAGAACA
     AGAGTTTAAA AAGCTAGTTT TTGATCTATT TCGTCTCTTA CTCGACAAAT AGTGTCTTGT
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Figure 20. **Nucleotide Sequence Obtained from pHF101.** See Figure 19 for the relative position of the 2182 bp fragment in pHF101. The top and bottom strands are designated N2182A and N2182B, respectively. N2182A is in the same orientation as that of the coding strand of *LacZ* gene in pUC19 vector. Only base positions in N2182A are numbered.

alone or in combination (*Hind*III/*Xba*I, *Hind*III/*Pst*I, *Pst*I/*Xba*I, and *Pst*I/*Sal*I) were isolated and ligated into pUC19 vector restricted with the same enzyme(s). The subcloned plasmids were sequenced from both ends of the insert by using universal and reverse primers designed from M13/pUC vectors. Sequences obtained from the subcloned plasmids were aligned to give a 2,182 bp length continuous sequence at one end of the insert (Figs. 19 and 20). Furthermore pHF101 was digested with *Pst*I followed by direct ligation of the restricted pUC19 to form plasmids with deleted *Pst*I fragment(s). When TS^- *E. coli* (BL21 Δ*thyA*) was transformed with this ligation product and plated on thymidine-free selective medium, it was found that none of the 30 complementing clones tested contained the 1.05 kb *Pst*I/*Hind*III fragment, suggesting that this fragment is not required for the expression of TS function and therefore no further efforts were made to obtain the full sequence of the 1.05 kb *Pst*I/*Hind*III fragment (Fig. 19). At this stage, the sequencing data had been obtained from overlaping subcloned plasmids with some regions representing results generated from one strand only. Based on the primary sequencing data of the 1944 bp fragment (Fig. 19), five oligonucleotides were synthesized and used as sequencing primers to obtain the sequence of the complementary strand using the parental plasmid pHF101 as the sequencing template (Fig. 19). Results obtained from the second round of sequencing confirmed the data obtained from subcloned plasmids. The sequence of the continuous 2182 kb fragment is shown in Fig. 20.

5. Sequencing Analysis and Confirmation of Open Reading Frame

When the sequence of the 2182 bp fragment in pHF101 (Fig. 20) was subjected to analysis by PC/GENE, unexpected results were obtained. For the convenience of description, the strand which is in the same orientation as coding strand of vector *LacZ* gene is designated N2182A and the other N2182B. Searching for coding regions of prokaryote genes, the PC/GENE subprogram COD_PROK (which is based on sequence characteristics around translation initiation codon ATG in bacterial protein genes) (Kolaskar and Reddy, 1985) proposed a potential open reading frame starting from base 73 to base 1659 in N2182A (Table 9). This open reading frame (designated ORF1587) encodes a deduced protein (CTS529) of 529 amino acid residues. COD_PROK also indicated that in strand N2182A several other potential open reading frames were present although the longest was only 78 amino acid residues (Table 9). Analysis on strand N2182B indicated several potential short open reading frames as well (Table 9).

Assuming that any methionine residue can be the initiation site, more possible open reading frames are predicted in both N2182A (Table 10) and N2182B (Table 11).

Among the many potential open reading frames identified by both search methods (Tables 9-11), the small ones can easily be excluded as candidate open reading frames of the chlamydial TS gene. Only some of those on reading frame 1 of strand N2182A appear to encode polypeptides with comparable length to known TS. These longer ones stop at the same site (Table 10) and only the longest (ORF1587) was identified as potential open reading frame by PC/GENE COD_PROK subprogram. Thus ORF1587

Table 9. Potential Open Reading Frames Detected by Kolaskar and Reddy's Method[a] on Sequence of pHF101

Strand	Position (Base #)	Size (bp) of coding region	Size (aa) of coded peptide	Initiation codon
N2182A	73-1659	1587	529	ATG
	257-343	87	29	ATG
	356-430	75	25	GTG
	359-430	72	24	ATG
	1426-1659	234	78	GTG
	1675-1746	72	24	ATG
N2182B	87-338	252	84	ATG
	472-618	147	49	ATG
	608-682	75	25	ATG
	1443-1517	75	25	GTG

[a] Kolaskar and Reddy (1985).

Table 10. **Potential Open Reading Frames in Strand N2182A as Proposed by Starting with any ATG Codon**

Reading frame	Position (base #)	Size (bp) of coding region	Size (aa) of coded peptide
1	73-1659	1587	529
	412-1659	1248	416
	538-1659	1122	374
	853-1659	807	269
	913-1659	747	249
	1057-1659	603	201
	1129-1659	531	177
	1312-1659	348	116
	1339-1659	321	107
	1363-1659	297	99
	1549-1659	111	37
	1675-1746	72	24
	1921-2016	96	32
	2071-2127	57	19
2	35-76	42	14
	257-343	87	29
	281-343	63	21
	284-343	60	20
	359-430	72	24
	761-841	81	27
3	1710-1808	99	33
	2139-2180	42	14

[a] The minimal size for an ORF is set to 10 amino acids

Table 11. Potential Open Reading Frames in Strand N2182B as Purposed by Starting with any ATG Codon

Reading frame	Position (base #)	Size (bp) of coding region	Size (aa) of coded peptide
1	223-252	30	10
	472-618	147	49
2	608-682	75	25
	1280-1330	51	17
	1286-1330	45	15
	1289-1330	42	14
	1556-1597	42	14
	1793-1837	45	15
3	87-338	252	84
	1053-1100	48	16
	1770-1850	81	27

[a] The minimal size for an ORF is set to 10 amino acids

appears to have the greatest potential as a coding region for the chlamydial TS. As reviewed above, TS in most species studied is a homodimeric protein with molecular weights of about 35 KDa for each subunit. As shown in Fig. 2, among the isofunctional TSs, *L.casei* TS represents the largest protein with 316 residues (Maley et al, 1970). TSs in protozoa are in the form of bifunctional TS-DHFR exhibiting sizes ranging from 110 to 140 kDa, with subunit sizes of 55 to 70 kDa (Ivanetich and Santi, 1989; Ivanetich and Santi, 1990). No matter which form (isofunctional or bifunctional) the enzyme is, TS has a highly conserved primary structure (Ivanetich and Santi, 1989; Ivanetich and Santi, 1990). Comparison of amino acid sequences from any given two sources shows $\geq 50\%$ sequence identity. The amino acid sequence of CTS529 (Fig. 21) deduced from ORF1587 (Fig. 20) showed no significant homology to known TSs nor to any other protein in Genbank when analyzed by computation at the National Center for Biotechnology Information. The comparison of the primary structure of CTS529 to that of the *E. coli* TS and human TS as well as bifunctional *L. major* TS-DHFR is shown in Fig. 21. No regions in CTS529 have similar sequence to that of the nucleotide binding site which is highly conserved among known TSs (Figs. 2 and 21). A stretch of sequence (Q-L-L-T-T-K) in CTS529 was identified that resembles the folate-binding site (P-L-L-T-T-K) of conventional TSs and which is highly conserved in TSs from all species. The folate-binding site in the known TSs is close to the amino terminus (residues 45 through 50 in *L. casei* enzyme) (Fig. 2). However, the putative folate-binding site-like region in CTS529 is located toward the carboxy terminus (residues 404 through 409) (Fig. 21).

In attempt to directly associate the size of messenger RNA with the proposed open reading frames, Northern hybridization was performed. When ^{32}P-labelled pHF101 insert DNA prepared by random-primer labelling was used as a probe to detect specific chlamydial RNA blotted onto filters, no specific hybridization was obtained (Fig. 22 Lane A). As a control, PCR-amplified MOMP DNA probe detected two intense bands of the correct sizes (1.4 and 1.55 kb, respectively) (Stephens et al, 1986) in the same RNA sample (Fig. 22 Lane B).

The absence of homology between CTS529 and known TSs raises the possibility that the correct open reading frame in the fragment sequenced was not obtained because of sequencing errors even though both strands had been sequenced. To address this concern, pHF101 was further subcloned to give plasmids containing smaller DNA fragments and these subcloned fragments were tested for the capacity to genetically complement TS$^-$ *E. coli* (Fig. 19). As described above, plasmid (designated p101P3) with a 1.05 kb *Pst*I/*Hind*III fragment (at 3′ end relative to N2182A) deleted was capable of complementing as well as pHF101. However, p101HS constructed by further removal of a 341 bp *Sal*I/*Pst*I fragment, which carries a 3′ end 57 bp of ORF1587, from p101P3 was incapable of functional complementation in the auxotrophic *E. coli*. Removing a small *Hind*III/*Xba*I fragment (from base 1 to base 471, number as in strand N2182A) and retaining the other end of insert (in pHF101) intact resulted in p101X which was also inactive in correcting thymidine auxotrophy in the TS$^-$ *E. coli*. Taken together, the structure and function of plasmids p101P3, p101SH, p101X and pHF101 suggest that a

```
CTS529      MLSKEGGFSEEQRARLSHFV-TNLD--SPIFALKNLPEVVKGALFSKYSRSTLGL    52
LMTD        MSRAAARFKIPMPFTKADFAFPSLRAFSIVVALDMQHGIGDGESIPWRVPEDMTF    55
HUTS        MPVA-----------------------------------GSELPRR-------    11
ECTS        MK-------------------------------------------------------     2
            :

CTS529      ---RTLLLKEFLDGEGGNFLDDDQQDCELGIQK----AVDFYRRVLDNF-----G    95
LMTD        FKNQTTLLRNKKPPT-----EKKRNAVVMGRKTWESVPVKF-KPLKGRLNIVLSS   104
HUTS        -------------------------------------------------------    11
ECTS        -------------------------------------------------------     2

CTS529      DDSVGEL------GGAHLALEQVSMLAAKILEDARIGGSPLEKSSRYVYFDQKVN   144
LMTD        KATVEELLAPLPEGQRAAAAQDVVVVNGGLAEALRLLARPL--------YCSSIE   151
HUTS        -------------------------------------------------------    11
ECTS        -------------------------------------------------------     2

CTS529      GEYLYYRDPILMTSAFKDVFLDTC-DFLFNTYSDLIPQVRSHFEKLYPKDPEVSQ   198
LMTD        TAY-----CVGGAQVYADAMLSPCIEKLQEVYLTRIYATAPACTRFFPFPPENAA   201
HUTS        ----------------------------------------------PLPP----    15
ECTS        -------------------------------------------------------     2

CTS529      SAYTVSLRAKVLDCLRGLLPAATLTNLGFFGNGRFWQNLLHRLQDNSLVEVRNIG   253
LMTD        TAWDLASSQGRRKSEAEGLEFEICKYVPRNHEERQYLELIDRIMKTGIVKEDRTG   256
HUTS        -------AAGERDAEP-----------RPPHGELQYLGQIQHILRCGVRKDDRTG    52
ECTS        ---------------------------------QYLELMQKVLDEGTQKNDRTG    23
                                             ...       .  . ...:

CTS529      EQSLTELMKIIPSFVSRAESHHYHHQAMVDYRRALKEQLKSFAH-----RYGEER   303
LMTD        VGRIS-LFGAQMRFSLRDNRLPLLTTKRV-FWRGVCEELLWFLRGETSAQLLADK   309
HUTS        TGTLS-VFGMQARYSLRDE-FPLLTTKRV-FWKGKLEKLLWFIKGSTNAKELSSK   104
ECTS        TGTLS-IFGHQMRFNLQDG-FPLVTTKRC-HLRSIIHELLWFLQGDTNIAYLHEN    75
              .  .  .. . .  .  .. .,..          :..:  . .    .

CTS529      EISK-EAGVKLVYGDPDGLYKIAAAYMFP-YSEH------TYAELLDICRNIPNE   350
LMTD        DIHIWDGNCSREFLDSRGLTENKEMDLGPVYGFQWRHFGACYKGFEANYDGEGVD   364
HUTS        GVKIWDANGSRDFLDSLGFSTREEGDLGPVYFGQWRHFGAEYRDMESDYSGQGVD   159
ECTS        NVTIWDE----------WADENGDLGPVYGKQWRAWPTP--------DGRHID   110
               ...                  ...:.:  ...            .   .

CTS529      DLMRILESGASFRENRRHK----SPRGLECAEFAFDITADFGAYRDLQRHRILTQ   401
LMTD        QIKLIVETIKTNPNDRRLLVTAWNPCALQ--KMALPPCHLLAQF-------YVNT   410
HUTS        QLQRVIDTIKTNPDDRRIIMCAWNPRDLP--LMALPPCHALCQF-------YVVN   205
ECTS        QITTVLNQLKNDPDSRRIIVSAWNVGELD--KMALAPCHAFFQF-------YVAD   156
            .        .  .  ::    ...   :    .:. ...   ..       ..

CTS529      ERQLLTTKLGYTMPSQLIDTPMEAPFRGAMEKADQAYRLIAEEFPEE-----AQY   451
LMTD        DISELSCML-YQRSCDMG---LCVPFNIA------SYALLTILIAKATGLRPGEL   455
HUTS        --SELSCQL-YQRSGDMC---LGVPFNIA------SYALLTYMIAHITGLKPGDF   248
ECTS        --GKLSCQL-YQRSCDVF---LGLPFNIA------SYALLVHMMAQQCDLEVGDF   199
               :.. : :... .     .  ::..:      .:.:.    .   .  .

CTS529      VVPLAYNIRWLFHINARGLQWLCELRSQPQGHESYRKIAIDMAREVIQFHPAYEL   506
LMTD        VHTLGDAHVYRNHVDALKAQ----LERVPH---AFPTLIFKEERQYLE---DYEL   500
HUTS        IHTLGDAHIYLNHIEPLKIQ----LQREPR---PFPKLRILRKVEKID---DFKA   293
ECTS        VWTGGDTHLYSNHMDQTHLQ----LSREPR---PLPKLIIKRKPESIF---DYRF   244
              . .. . . .:      :    : . :      . .             .

CTS529      F-LKFVDYSETDLGRLQQESRKKS   529
LMTD        TDMEVIDYVPHPAIKMEMAV----   520
HUTS        EDFQIEGYNPHPTIKMEMAV----   313
ECTS        EDFEIEGYDPHPGIKAPVAI----   264
             .     : ... ..   .
```

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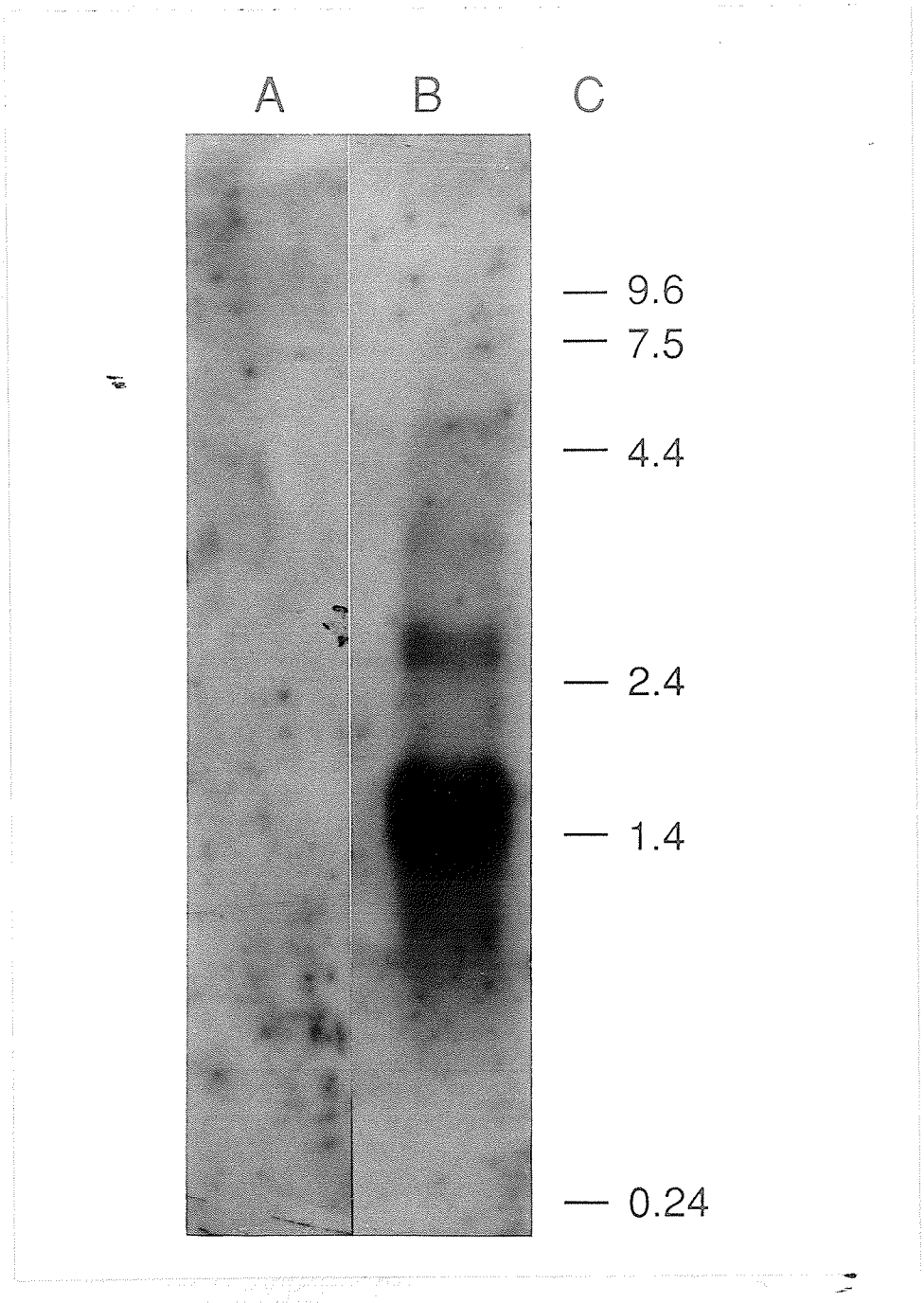
Figure 21. **Alignment of Deduced Amino Acid Sequence of CTS529 to those of *L. major* TS-DHFR (LMTD), Human TS (HUTS) and *E. coli* TS (ECTS).** The analysis was performed by PC/GENE software. Residues which are conserved among all four sequences are marked with : and those conserved among *L. major*, human and *E. coli* sequences are marked with .. Residues involved in nucleotide substrate binding and cofactor folate binding in *L. major, E. coli* and human TS are boxed with solid and broken lines, respectively. Sequence, in CTS529, which is highly homologous to that of folate binding site in other TS is overscored. Refer to Fig. 2 for more information of known TS sequences.


A
B
C
9.6
7.5
4.4
2.4
1.4
0.24

(Facing page)

Figure 22. **Northern Hybridizations.** *C. trachomatis* L2 RNA was prepared from cell cultures at 24 hr postinfection. *Hind*III fragment insert of pHF101 and PCR-amplified *C. trachomatis* L2 MOMP fragment were labelled with α-[^{32}P]dCTP by random primer labelling. Conditions for hybridization and wash were the same as Southern hybridization (Fig. 18). *Lane A*, probed with pHF101 insert; *Lane B*, probed with MOMP fragment; and *Lane C*, RNA molecular markers (kilobases).

DNA fragment longer than 1132 bp (bases 472 through 1603 in N2182A) is needed for the genetic complementation. However, loss of complementation might also be caused by the deletion of part of the noncoding region (such as promoter or ribosomal binding site) as well as coding region of the gene. To differentiate between these two possibilities, the following three oligonucleotides were synthesized and used for construction of recombinant plasmids with desired size, orientation and fine positioning of insert DNA fragment containing ORF1587 in vector pUC18:

T6, 5′ TATGAATTCATGTTGAGCAAAGAGGGTGGT 3′;

T8, 5′ TATGAATTCGATGTTGAGCAAAGAGGGTGGT 3′; and

T11, 5′ TTAGGATCCTTAAGACTTTTTACGCGATTC 3′.

T6 differs from T8 very slightly. Both incorporated the 5′ end 21 bases of ORF1587 (base 73 through base 93 in N2182A; Fig. 20) and an *Eco*R1 cut site (GAATTC). In T6 an ATG codon was placed immediately downstream of the *Eco*R1 cut site, while in T8 there is a G spacing the restriction site and the ATG. T11 incorporated a stop codon (TAA) in addition to the 3′ end sequence of ORF1587 and *Bam*H1 cutting site (GGATCC). Assuming that ORF1587 in N2182A is the correct open reading frame, when *Eco*R1/*Bam*H1 restricted PCR products which were amplified by using T8 and T11 were ligated to pUC18, a fusion protein with full length CTS529 and an extra 6 residues from α-peptide at its N-terminus should be generated. We reasoned that this fusion protein should probably complement thymidine auxotrophy in TS^- *E. coli*. However, since recombinant plasmid pUC18 containing T6 and T11 amplified fragment would contain an in-frame stop codon after only one residue of chlamydial DNA, we expected

that it would not complement TS⁻ *E. coli*. As expected, when TS⁻ *E. coli* strains were transformed with both constructs, pT8.11U18 (constructed by using primers T8 and T11) was able to complement all three TS⁻ *E. coli* strains in thymidine-free medium (Fig. 19). In contrast, pT6.11U18 failed to complement (Fig. 19). Furthermore to test if the first ATG triplex in ORF1587 is the translation starting site, another plasmid (designated pT9.11U18) was constructed by using oligonucleotide T9, 5′ TATGAATTCAGGAGTTTTCTTATGTTGAGC 3′, together with T11 in the same way as described above. In T9 there are 12 bases (AGGAGTTTTCTT) between the *Eco*R1 site and the initiation codon ATG of ORF1587. These 12 bases located from base 61 through base 72 (in strand N2182A) was considered to encode a ribosomal binding site in the RNA transcript. pT9.11U18 also complemented the TS⁻ *E. coli* strains (Fig. 19), suggesting that the transcript is able to direct synthesis of CTS529. The complementation by pT9.11U18 cannot be a result of a fusion protein since the same stop codon as described above in construct pT6.11U18 would terminate translation (Fig. 19). In aggregate, these results, together with those obtained with plasmids p101P3, p101HS and p101X, strongly suggest that ORF1587 represents a correct open reading frame encoding a protein (CTS529) which confers a thymidine prototrophic phenotype on thymidine auxotrophic *E. coli*.

6. Further Examination and Characterization of the Function of pHF101 and/or its Subclones

The fact that pHF101 can genetically complement the TS^- phenotype in mutant *E. coli*, and that ORF1587 and the encoded CTS529 lack homology to known TSs suggests that *C. trachomatis* may encode a novel protein which carries out de novo synthesis of dTMP. A series of in vivo and in vitro experiments were done to further examine and characterize the function of pHF101 and/or its subclones and to compare these characteristics to those with native chlamydial TS obtained from RB extracts.

1) In vivo incorporation of radiolabelled uracil into DNA

The design of these experiments was similar to the ones used to confirm the existence of TS in chlamydiae (see "Part 1" of "Results"). [^{3}H]thymine and [^{3}H]cytosine were detected from acid-hydrolyzed DNA preparation by HPLC when [6-^{3}H]uracil was added to the culture of pHF101-transformed BL21 Δ*thyA* growing in thymidine-free medium. When [5-^{3}H]uracil was used as a precursor, only radiolabelled cytosine was detected from acid-hydrolyzed bacterial DNA. For a control experiment, from acid-hydrolyzed DNA of pUC19-transformed BL21 Δ*thyA* (which had to be cultured in thymidine-containing medium), only radiolabelled cytosine was detected from hydrolyzed DNA when either [5-^{3}H] or [6-^{3}H]uracil was used as precursor for DNA synthesis of the bacteria. These results suggest that similar to the known TS, the protein encoded by pHF101 synthesizes thymidine nucleotides using uridine nucleotides as substrate.

2) Demonstration of TS activity in extracts prepared from TS⁻ *E. coli* transformed with complementing plasmids

TS activity can be detected consistently when extract prepared from TS⁻ *E. coli* transformed with complementing plasmids were used as sources of enzyme (Table 12). The data presented in Table 13 were obtained by measuring the formation of tritiated water using [5-^{3}H]dUMP as substrate. To verify the results, activity in pHF101-transformed BL21 Δ*thyA* and ATCC23851 extracts were also assayed with HPLC detecting the formation of [^{3}H]dTMP when [6-^{3}H]dUMP was used as substrate. Results obtained from the two methods were essentially identical.

3) Inhibition of TS activity in extracts prepared from TS⁻ *E. coli* transformed with complementing plasmids by folate derivatives

Since TS uses 5,10-CH_2-H_4folate as cofactor and our previous experiments showed that TS activity in the RB extract is dependent on the presence of this cofactor, we wanted to test if the TS activity expressed by the plasmids also requires it. When 5,10-CH_2-H_4folate was left out of the reaction mixture, surprisingly the TS activity in pHF101-transformed ATCC23851 and pT9.11-transformed BL21 Δ*thyA* and ATCC23851 remained essentially unchanged (Table 13). The most probable explanation would be that the crude bacterial extract contains enough cofactor. Several experiments were done to test this hypothesis. Firstly, TS activity in RB extract and purified human TS was

Table 12. **TS Activity in the Extracts of TS⁻ *E. coli* Strains Transformed with pHF101 and Subcloned Plasmids**

TS⁻ *E. coli* strain	Transformed with	TS activity[a]
BL21 Δ*thyA*	pHF101	129.8 ± 17.9
	pT8.11U18	77.3 ± 10.3
	pT9.11U18	164.2 ± 20.6
	pT6.11U18	<0.1
	pUC19	<0.1
X2913	pT9.11U18	85.6 ± 6.4
	pUC19	<0.1
ATCC23851	pHF101	125.7 ± 26.9
	pUC19	0.3 ± 0.2

[a] Reaction mixture contained, in a total volume of 100 μl, 10 μM [5-^{3}H]dUMP, 10 μM 5,10-CH_2H_4folate and 50 μl bacterial extract. Reaction was allowed to proceed at 37°C for 20 min. TS activity is expressed as pmole dTMP formed/mg protein/min and results represent mean ± standard deviation for two experiments.

assayed when the crude extract of pUC19-transformed ATCC23851, which had no TS activity, was used to replace exogenous 5,10-CH_2-H_4folate. We found that the extract prepared from pUC19-transformed ATCC23851 could supply enough 5,10-CH_2-H_4folate required for the TS activity in RB extract (Table 13). In the absence of exogenous 5,10-CH_2-H_4folate, the purified human TS, when assayed in reaction mixture containing crude extract of pUC19-transformed ATCC23851, showed about 9% activity compared to the normal assay conditions under which 5,10-CH_2-H_4folate was present (Table 13). The bacterial extract did not inhibit the activity of the purified human TS (Table 13). Secondly, we removed folates and all other small components from the crude bacterial extracts by dialysis. However, TS activity disappeared almost completely after overnight dialysis against 0.01 M potassium phosphate (pH7.0) containing 0.1 M KC1 (the same buffer used to prepare the crude bacterial extract) at 4°C (Table 14). Addition of EDTA and dithiothreitol, or DTT, $MgCl_2$ and $CaCl_2$ each up to a final concentration of 5 mM into dialysis buffer did not preserve the activity during the course of dialysis (Table 14). Crude extracts kept at the same temperature lost activity by about 20-30% (Table 14). Incorporation of non-dialysed pUC19-transformed *E. coli* extract into the reaction mixture did not restore the activity from the dialyzed extracts (Table 14). RB extracts also had undetectable level of TS activity after dialysis at the same conditions. However, since the activity also disappeared completely after being left overnight at 4°C, the effect of dialysis on the TS activity in RB extract is not certain (Table 14).

Table 13. Effect of Exogenous 5,10-CH_2-H_4folate on TS Activity in Extracts of TS^- *E. coli* Transformed with pHF101 and subcloned Plasmids, Chlamydial RBs and Human TS

Source of enzyme[a]	Addition of CH_2-H_4folate[b]	Addition of pUC19/ATCC23851	TS activity[d]	%
			pmole/mg protein/min	
pUC19/ATCC23851	Yes	-	0.1 ± 0.1	
pHF101/ATCC23851	Yes	No	125.7 ± 26.9	100
	No	No	148.0 ± 19.2	118.4
pT9.11U18/BL21 Δ*thyA*	Yes	No	150.6 ± 20.2	100
	No	No	169.5 ± 21.3	112.3
RB extract	Yes	No	7.80 ± 0.82	100
	No	No	<0.01	<0.1
	No	Yes	8.35 ± 0.61	107.1
			μmole/mg protein/min	
Human TS[c]	Yes	No	1.66 ± 0.12	100
	No	No	0	0
	Yes	Yes	1.63 ± 0.17	98.2
	No	Yes	0.15 ± 0.03	9.0

[a] pT9.11U18/BL21 Δ*thyA*, crude extract of pT9.11U18-transformed BL21 Δ*thyA*; pHF101/ATCC23851, crude extract of pHF101-transformed ATCC23851.

[b] Reaction mixture contained 10 μM dUMP (see legend of Table 12).

[c] Purified human TS was prepared in either 0.01 M potassium phosphate (pH7.0) containing 0.1 M KCl and 5 mg/ml bovine serum albumin or pUC19-transformed ATCC23851 extract (protein concentration: 5 mg/ml)

[d] Results in this column represent mean ± standard deviation for two experiments.

Table 14. Influence of Dialysis on TS Activity in the Extracts of Plasmids-transformed TS⁻ *E. coli* and RBs

Source of enzyme	Treatment	Addition of pUC19/23851	TS activity[c]	%
pHF101/ BL21 Δ*thyA*	Untreated control[a]	No	129.8 ± 17.9	100
	4°C, overnight	No	93.6 ± 16.1	72.1
	Overnight dialysis against buffer 1[b]	No	0.3 ± 0.2	0.2
	Overnight dialysis against buffer 1	Yes	0.2 ± 0.3	0.2
	Overnight dialysis against buffer 2[c]	No	<0.1	<0.1
	Overnight dialysis against buffer 3[d]	No	<0.1	<0.1
RB extract	Untreated control	No	9.67 ± 0.22	100
	4°C, overnight	No	0	0
	Overnight dialysis against buffer 1	No	0	0

[a] For untreated control, bacterial or RB extract was kept at -70°C until assay.

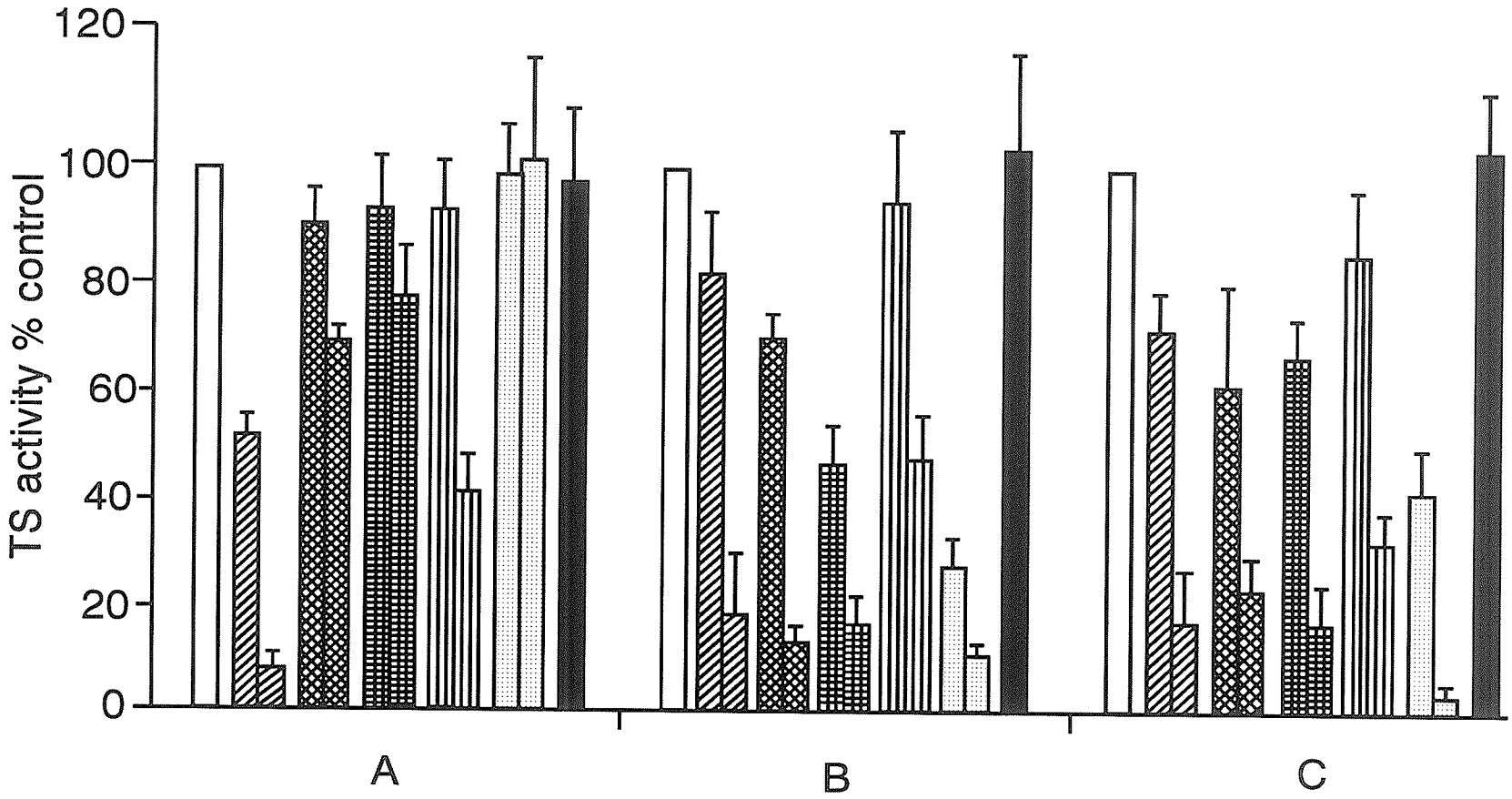
[b] Buffer 1 was 0.01 M potassium phosphate (pH7.0) containing 0.1 M KCl.

[c] Buffer 2 was made by adding dithiothreitol, $CaCl_2$ and $MgCl_2$ (final concentration: 5 mM each) to buffer 1.

[d] Buffer 3 was made by adding DTT and EDTA (final concentration: 5 mM each) to buffer 1.

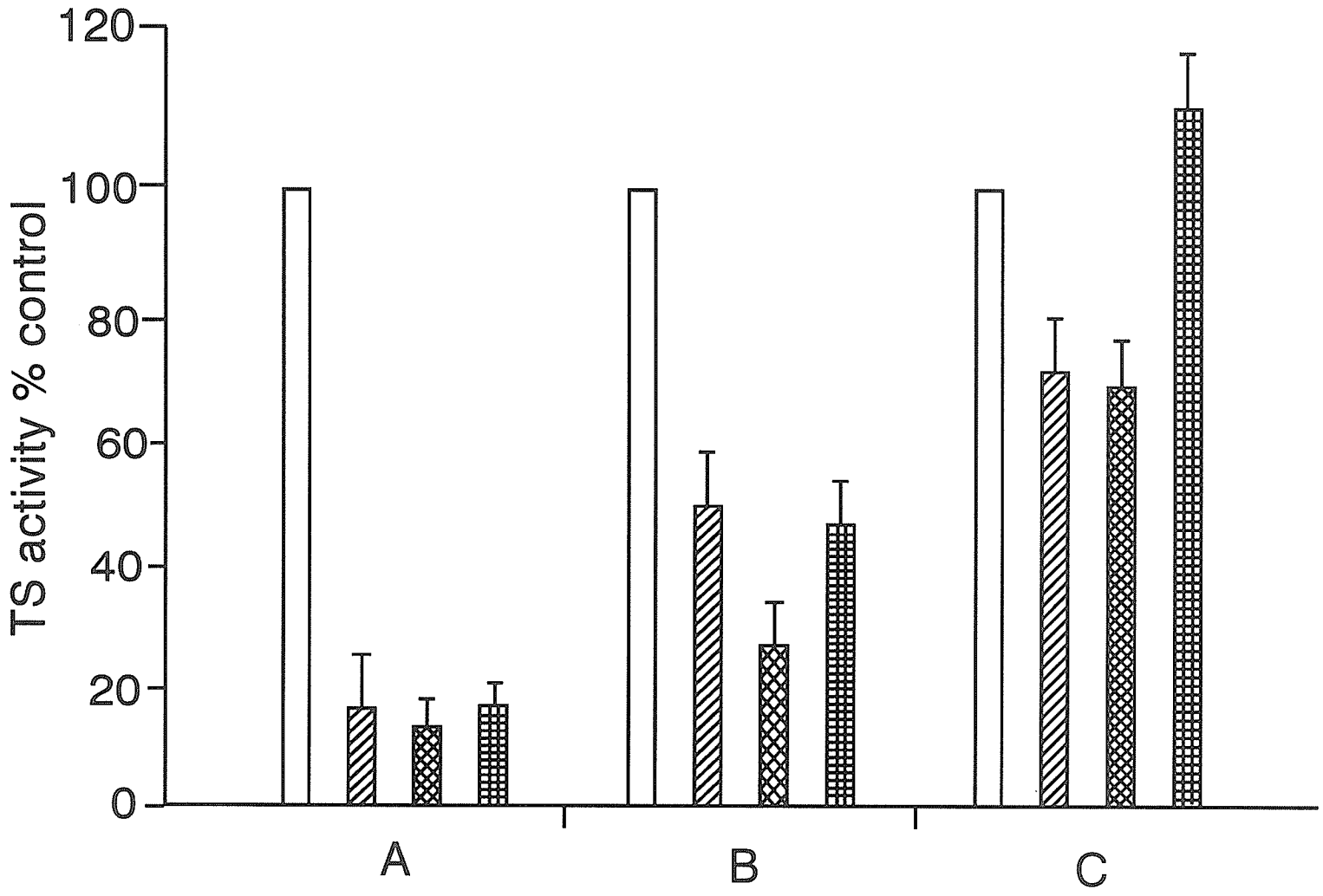
[e] TS activity was expressed as pmole dTMP formed/mg protein/min; results in this column represent mean ± standard deviation for two experiments.

The loss of activity after dialysis suggests that low molecular weight substance(s) might be required for the expressed protein to maintain its activity. This prevented us from directly evaluating the role of 5,10-CH_2-H_4folate in the catalysis of the recombinant protein. However, testing the effect of folate derivatives would be helpful to indirectly address this question, since TS activity is inhibitable with folate derivatives (Santi and Danenberg, 1984). In such experiments we tested the effects of five folate derivatives, 10-formyl-dideaza folic acid (10-FDDFA), 5-CHO-H_4folate, folate, methotrexate and aminopterin each at two different concentrations (0.1 mM and 1.0 mM) on TS activity in extracts prepared from pHF101-transformed BL21 Δ*thyA*. We found that at 1.0 mM all five derivatives are effective inhibitors of the TS activity present in the recombinant *E. coli* extract and at the lower concentration they also caused variable degrees of inhibition (Fig. 23 B). As shown in Fig. 23 C, *C. trachomatis* L2 RB extract displayed essentially the same profile of TS activity inhibition by the folate derivatives as did the extract of the recombinant *E. coli* extract. In contrast, the folate derivatives appeared to be much less effective in inhibiting human TS activity (Fig. 23 A). As a control, pABA showed no inhibitory effect on the activity from any of the materials tested at 1 mM (Fig. 23 A, B, and C). To further determine if inhibition by folate derivatives was based on substrate-analogue competition, we determined if increased concentrations of 5,10-CH_2-H_4folate could reverse the TS activity inhibition caused by the folate derivatives. When dealing with multi-substrate enzymes, unbalanced amounts of substrates will affect the enzyme reaction (Roberts, 1977); thus proper controls have to be set. For these


TS activity % control
120
100
80
60
40
20
0
A
B
C

(Facing page)

Figure 23. Effect of Folate Derivatives on in vitro TS Activity of Human TS (A), pHF101-transformed BL21 Δ*thyA* Extract (B) and RB Extract (C). TS activity in the control was assayed in mixture containing 10 μM dUMP, and 10 μM 5,10-CH_2-H_4folate. The indicated compounds were mixed with dUMP and 5,10-CH_2-H_4folate before the addition of enzyme. The enzyme activity is expressed as a percentage of the uninhibited controls. The 100% control values are: human TS, 1.47 $\pm$ 0.12 μmole dTMP formed/mg protein/min; pHF101-transformed BL21 Δ*thyA* extract, 133.2 $\pm$ 14.6 pmole/mg protein/min; and *C. trachomatis* L2 RB extract, 8.87 $\pm$ 1.01 pmole/mg protein/min. The data represent mean $\pm$ standard deviation for two determinations. No-drug control, (*open bars*); 10-formyl-dideaza folic acid, (*hatched bars*); 5-CHO-H_4folate, (*crossed hatched bars*); folic acid, (*square-checked bars*); methotrexate, (*striped bars*), aminopterin, (*dotted bars*); and pABA, (*solid bars*). For each compound, the left bar represents 0.1 mM, and the right 1.0 mM.


120
100
80
60
40
20
0
TS activity % control
A
B
C

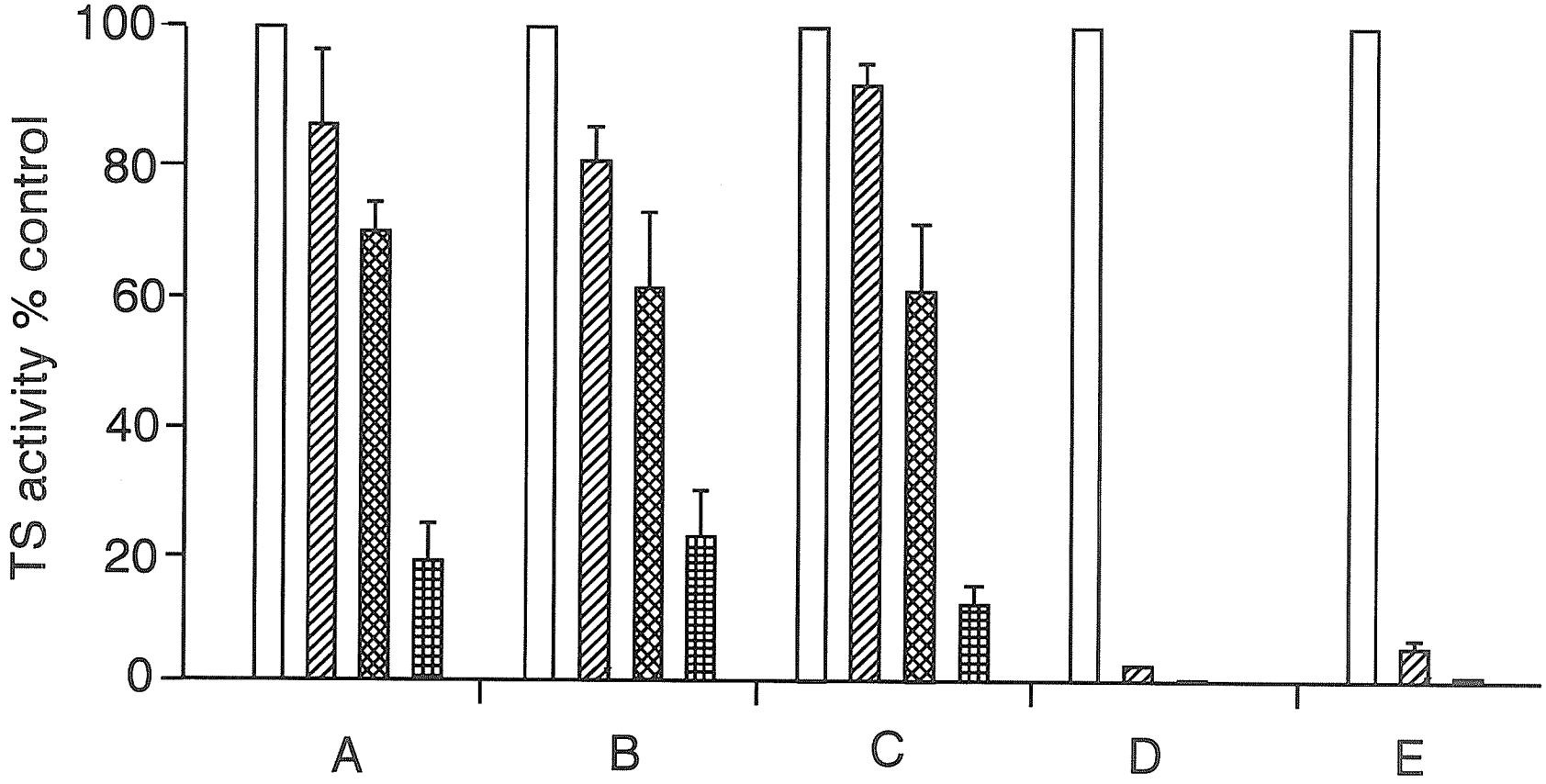
(Facing page)

Figure 24. Effect of 5,10-CH_2-H_4folate on the Inhibition of in vitro TS Activity in pHF101-transformed BL21 Δ*thyA* Extract Caused by 10-Formyl-dideazil-folic Acid (*Hatched Bars*), Folic Acid (*Cross-Hatched Bars*), and Methotrexate (*Square-checked Bars*). TS assay mixture contained 10 μM dUMP, 1 mM folate inhibitor, and 10 μM (A), or 100 μM (B), or 500 μM (C) 5,10-CH_2-H_4folate. The enzyme activity is expressed as a percentage of the controls containing only 5,10-CH_2-H_4folate (*open bars*). The 100% control values are: A, 127.9 pmole/mg protein/min; B, 101.5 pmole/mg protein/min; and C, 40.2 pmole/mg protein/min. The data represent mean ± standard deviation for two determinations.

experiments, the concentration of the substrate dUMP was fixed at 10 μM. In keeping with the results presented in Fig. 23, all three inhibitors (10-FDDFA, folate and methotrexate) at 1 mM concentration inhibited about 80% of the TS activity in the extract of pHF101-transformed BL21 Δ*thyA* by about 80%, when the cofactor 5,10-CH_2-H_4folate was present at the same concentration as the dUMP substrate (Fig. 24 A). When 5,10-CH_2-H_4folate is increased to 100 μM (Fig. 24 B) and 500 μM (Fig. 24 C), inhibition was partially or fully reversed, although excessive 5,10-CH_2-H_4folate itself also decreased the enzyme activity in the recombinant bacteria extract.

4) Effect of 5-FdUMP on the TS activity in extracts prepared from TS⁻ *E. coli*-transformed with complementing plasmids, and *C. trachomatis* L2 RB

It is very well known that 5-FdUMP is a potent TS inhibitor (Santi and Danenberg, 1984; Invanetich and Santi, 1989; and Maley and Maley, 1990). The TS activity in the extracts of pHF101- or pT9.11U18-transformed BL21 Δ*thyA* was inhibited with 5-FdUMP, but was much less sensitive than known TSs (Fig. 25 A and B, respectively). Effective inhibition was obtained only when high concentration (10 fold over substrate dUMP) was used even though the inhibitor had been preincubated with extract and cofactor 5,10-CH_2-H_4folate prior to the addition of substrate. TS activity in *C. trachomatis* L2 RB extract showed essentially the same response to 5-FdUMP (Fig. 25 C). However, in agreement with earlier findings (Santi and Danensberg, 1984; and

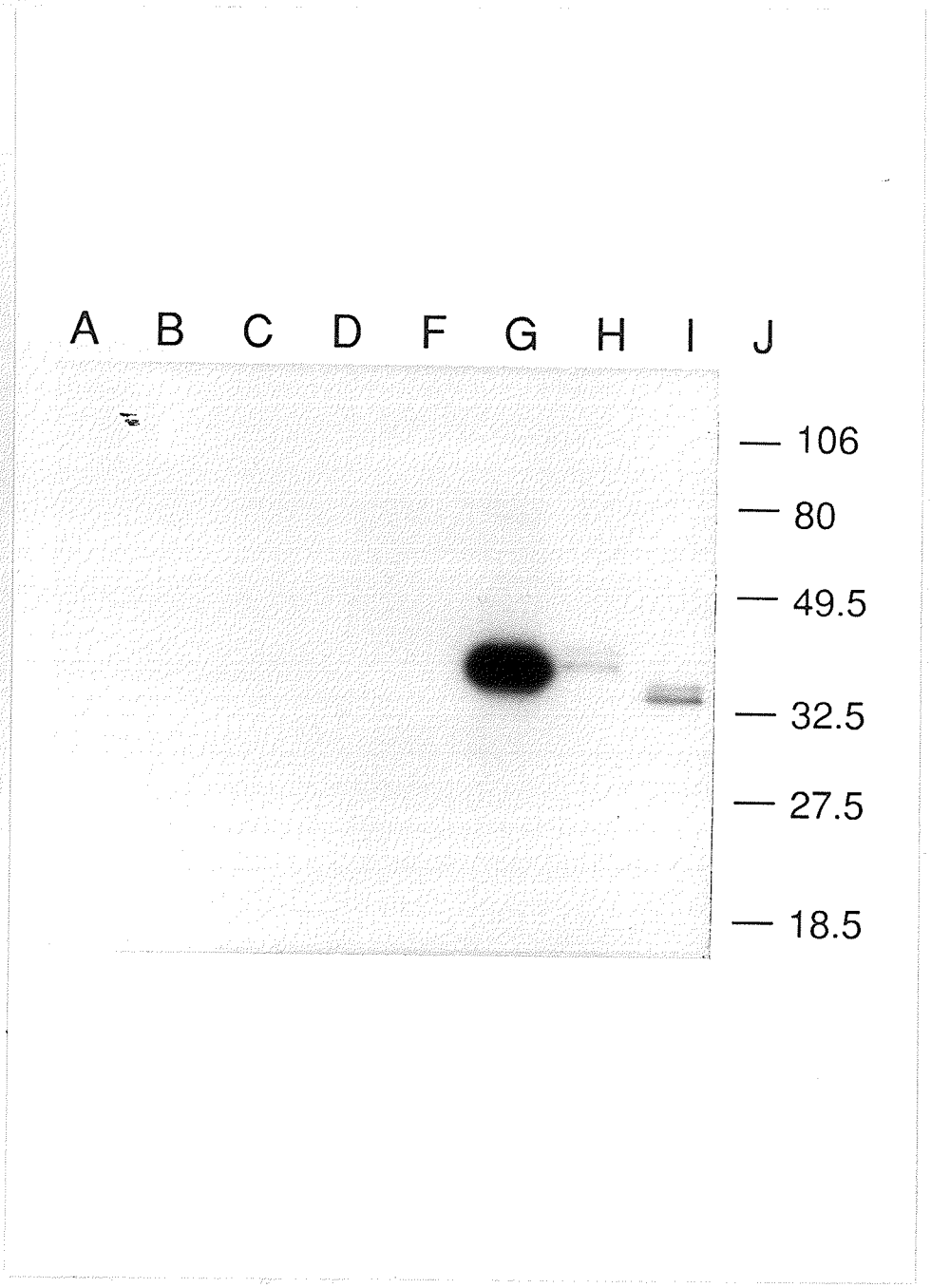

TS activity % control
100
80
60
40
20
0
A
B
C
D
E

(Facing page)

Figure 25. Effect of 5-FdUMP on in vitro TS Activity of pHF101-transformed BL21 Δ*thyA* extract (A), pT9.11U18-transformed BL21 Δ*thyA* Extract (B), *C. trachomatis* L2 RB Extract (C), and Human TS (D and E). The reaction mixture contained 10 μM dUMP and 10 μM 5,10-CH_2-H_4folate. 5-FdUMP was incubated with enzyme plus 5,10-CH_2-H_4folate for 30 min prior to the addition of substrate dUMP. The enzyme activity is expressed as a percentage of the uninhibited controls (*open bars*). The 100% control values are: A, 133.2 ± 14.6 pmole/mg protein/min; B, 172.0 ± 22.2 pmole/mg protein/min; and C, 8.87 ± 1.01 pmole/mg protein/min; D (human TS assayed in 0.01 M potassium phosphate-0.1 M potassium chloride buffer), 1.47 ± 0.12 μmole/mg protein/min; and E (human TS assayed in pUC19-transformed BL21 Δ*thyA* extract), 1.40 ± 0.15 μmole/mg protein/min. TS activity in pUC19-transformed BL21 Δ*thyA* extract was 0.1 ± 0.1 pmole/mg protein/min. The data represent mean ± standard deviation for two determinations. *Hatched bars, cross-hatched bars, square-checked bars* indicate 1.0 μM, 10.0 μM, and 100.0 μM 5-FdUMP, respectively.

Maley and Maley, 1990), human TS activity from the purified protein was inhibited by more than 95% when 5-FdUMP was present at 10 fold lower concentration than substrate dUMP (Fig. 25 D); while this concentration of 5-FdUMP inhibited the TS activity in transformed bacterial lysate and chlamydial RB extract by only 10-20% (Fig. 25 A, B, and C). To test if this poor inhibition in the crude extract was due to the degradation of the inhibitor, the effect of 5-FdUMP on human TS activity was examined when the crude extract of pUC19-transformed TS^- *E. coli* BL21, *ΔthyA* was incorporated into the reaction mixture. The results (Fig. 25 E) suggest that the crude bacterial extract had essentially no effect on 5-FdUMP inhibition.

The relatively low sensitivity of TS activity in the transformed bacterial and RB extracts to the inhibition of 5-FdUMP suggest that the interaction between the inhibitor and the enzyme may be different from that between inhibitor and known TSs. Previous studies (Santi and Danensberg, 1984; Mathews et al, 1990a; and Mathews et al, 1990b) suggested and confirmed that in the presence of 5,10-CH_2-H_4folate, 5-FdUMP covalently binds to TS to form a stable ternary complex. If radioactive 5-FdUMP is used, the complex can be detected following SDS-polyacrylamide gel electrophoresis and autoradiography (Thompson et al, 1987; and Honess et al, 1986). When [6-^{3}H]FdUMP was used, we could consistently demonstrate the ternary complex in crude extracts of wild-type *E. coli* (Fig. 26 Lane I), L929 cells (Lane H) as well as purified human TS (Lane G). However, we were unable to detect such a complex in extracts of pHF101-


A B C D F G H I J
106
80
49.5
32.5
27.5
18.5

(Facing page)

Figure 26. Detection of Covalent TS.FdUMP.folate Complex. The binding assay mixture contained 50 μM [^{3}H]FdUMP, 50 μM 5,10-CH_2-H_4folate and crude extract containing 5-10 μg protein or 5 μg bovine serum albumin containing 10 pg purified human TS. After the mixture was incubated at 37°C for 10 min, 1 μl of sample from each reaction was subjected to SDS-polyacrylamide gel electrophoresis (gel concentration: 12%). *Lane A*, pHF101-transformed X2913 extract; *Lane B*, pUC19-transformed X2913 extract; *Lane C*, pT9.11U18-transformed BL21 Δ*thyA* extract; *Lane D*, pUC19-transformed BL21 Δ*thyA* extract; *Lane F*, *C. trachomatis* L2 RB extract; *Lane G*, purified human TS; *Lane H*, mouse L929 cell extract; *Lane I*, Wild-type *E. coli* extract; and *Lane J*, low-range molecular markers (kDa).

transformed TS$^-$ X2913 (Fig. 26 Lane A) or pT9.11U18-transformed BL21 Δ*thyA* (Lane C). We also failed to detect ternary complex from *C. trachomatis* L2 RB extract (Fig. 26 Lane F). As expected, no radioactive ternary complex could be detected from extracts of vector pUC19-transformed X2913 (Fig. 26, Lane B) and BL21 Δ*thyA*.

5) Failure of pHF101 to Complement DHFR$^-$ *E. coli*

To directly test if pHF101 and/or its subclone containing ORF1587 also encodes DHFR, a DHFR$^-$ *E. coli* strain D3-157 (Singer et al, 1987) was transformed with pHF101 and pT9.11U18. Previously, it has been shown that the thymidine-, methionine-, glycine-, and pantothenic acid-auxotrophy in the mutant bacteria as result of DHFR deficiency could be corrected by transformation with plasmids containing either chromosomal or plasmid DHFR gene (Sanger, 1985). When the pHF101- or pT9.11U18-transformed D3-157 cells were cultured in the enriched minimal agar supplemented with adenine, guanine, thymidine, methionine, glycine, and pantothenic acid, colonies formed one day after plating; however, when thymidine, methionine, glycine, and pantothenic acid whose biosynthesis requires folates were absent in the medium, no colonies formed 5 days after plating, suggesting that pHF101 did not encode a DHFR.

7. Other Features of the Sequence in pHF101

The 2182 bp fragment sequence had a guanosine plus cytosine content of 40.1% which is very close to that (39.8) of *C. trachomatis* DNA (Hirai, 1992).

Analysis by PC/GENE software indicated there was no typical bacterial promoter sequence in either strand of the 2182 bp fragment. There was no rho-independent transcription termination signal sequence either. Bases 61 through 72 resembles a bacterial ribosomal binding site, in which AGGA was found 8 bases upstream of the ATG initiation codon of ORF1587. This is in agreement with findings with bacteria, in which most ribosomal binding sites involve either AGGA or GAGG at some 8 to 13 bases upstream from the initiator codon (Watson et al,1987).

DISCUSSION

The work presented in this thesis details some aspects of nucleotide metabolism in the obligate intracellular bacterial parasite, chlamydiae.

McClarty and Tipples (1991) reported that *C. trachomatis* does not utilize host cell dNTP pools as its source of DNA precursors. As an alternative, *C. trachomatis* obtains DNA precursors from the host cell as ribonucleotides, with subsequent reduction to deoxyribonucleotides being catalyzed by a chlamydiae-specific ribonucleotide reductase. Since ribonucleotide reductase is capable of supplying only three (dATP, dGTP, and dCTP) of the four precursors required, a question of how chlamydiae obtains the dTTP also required for DNA replication remained to be addressed. Based on the previous findings that host-derived thymidine cannot be incorporated into chlamydial DNA, we hypothesized that chlamydiae must de novo synthesize thymidine nucleotides through the TS cycle. The goal of this thesis was to supply biochemical evidence for the existence of TS in chlamydiae, to clone and express the chlamydial TS gene for characterization of its enzymmology, and to investigate the mechanisms by which chlamydiae obtain folates, the cofactor required by TS. The approaches of this project have been to probe chlamydial metabolic activities in situ, to detect enzymatic activities in vitro, and to molecularly clone and express genes encoding interesting chlamydial enzymes.

1. Metabolic Studies

1) Biochemical Evidence for Chlamydial TS

The nonincorporation of medium-supplied thymidine by intracellular chlamydiae has been thoroughly documented (Starr and Sharon, 1963; Tribby and Moulder, 1966; Lin, 1968; Hatch, 1976; and Bose and Liebhaber, 1979). Hatch (1976) showed that *C. psittaci* growing in either thymidine kinase (TK)-containing or TK-deficient mouse L cells is unable to incorporate medium-supplied thymidine into DNA. Our results confirm this finding for *C. trachomatis* (Table 3). In addition, our results show that the free-base thymine is also not utilized by *C. trachomatis*. The nonutilization of exogenous thymidine and thymine in both TK^+ and TK^- cells indicates that chlamydiae are unable to salvage any preformed thymine derivative from the host cell.

In this study, we made use of a mutant CHO cell line deficient in DHFR to assist our studies on thymidine metabolism in *C. trachomatis*. As a result of the DHFR deficiency, this cell line is unable to regenerate H_4folate from H_2folate and consequently loses the capacity to synthesize one-carbon H_4folate derivatives, which are required for many biochemical reactions, including thymidylate synthesis (Urlaub and Chasin, 1980). Using this mutant cell line, we were able to demonstrate that *C. trachomatis* L2 growth, as monitored by DNA synthesis activity and inclusion development, is essentially the same in the presence and absence of exogenous thymidine (Table 4). The combined

findings that *C. trachomatis* L2 is unable to salvage thymidine or thymine and is capable of normal growth in a thymidine-deficient host provide strong suggestive evidence that it is capable of de novo thymidine synthesis. TS is the only enzyme known to be capable of de novo thymidine synthesis (Santi, 1984; Ivanetich and Santi, 1989; and Maley and Maley, 1990). It is a ubiquitous enzyme present in almost all wild-type organisms. TS catalyzes an unusual reaction wherein 5,10-CH_2-H_4folate serves as both a one-carbon donor and a reductant in the conversion of dUMP to dTMP (Santi and Danenberg, 1984; Ivanetich and Santi, 1989; and Maley and Maley, 1990).

Further support for the presence of TS in chlamydiae comes from our results which show that intracellular *C. trachomatis* readily incorporates exogenously supplied uridine into DNA (Table 5). Uridine enters the host cell and is metabolized by the pathways shown schematically in Fig. 3. Uridine can label all pyrimidine ribonucleotides and deoxyribonucleotides, and, as a result, there are several pathways which the isotope could follow before finally being incorporated into chlamydial DNA. We believe that our results support the following pathway for uridine metabolism. Uridine enters the host cell and is sequentially phosphorylated to uridine phosphates by cellular uridine-cytidine kinase, uridylate kinase, and nucleoside diphosphate kinase. Some of the UTP formed will be converted to CTP by host cell CTP synthase. The UTP and CTP can then be taken into *C. trachomatis* by specific but as yet unidentified transport system(s). Within chlamydial RB, UTP can also be converted to CTP by *C. trachomatis*-specific CTP synthase (Tipples and McClarty, 1993). Inside the RB, the two pyrimidine

ribonucleotides are reduced to pyrimidine deoxyribonucleotides by a chlamydial ribonucleotide reductase. The dCTP thus formed is utilized directly by DNA polymerase. The deoxyuridine phosphate is further processed by chlamydial TS to thymidine phosphates, which are, in turn, incorporated into DNA.

This proposed pathway is supported by several independent lines of evidence. First, there would be few, if any, pyrimidine deoxyribonucleotides formed by the infected DHFR$^-$ host cell, since cellular ribonucleotide reductase levels are negligible in the presence of cycloheximide (Tipples and McClarty, 1991) and the DHFR$^-$ cell cannot synthesize thymidine phosphates (Urlaub and Chasin, 1980). Furthermore, even if some exogenously added uridine is converted to pyrimidine deoxyribonucleotides by host cell enzymes, these dNTPs cannot enter chlamydiae (McClarty and Tipples, 1991). Secondly, the work using both *C. psittaci* (Hatch, 1976) and *C. trachomatis* (McClarty and Tipples, 1991; and Tipples and McClarty, 1993) has shown that chlamydiae can and do draw on host cell ribonucleoside triphosphate pools as a source of precursors. Thirdly, Tipples and McClarty (1991) have demonstrated that *C. trachomatis* does contain a ribonucleotide reductase. Fourth, *C. trachomatis* DNA synthesis activity and in situ TS activity are inhibited by 5-UR, but not 5-FUdr. 5-FUdr is converted to 5-FdUMP in the host cell by thymidine kinase. The presence of 5-FdUMP in the host cell has no effect on *C. trachomatis* growth because it is a deoxyribonucleotide derivative, and as a result, it is not transported by chlamydiae. In contrast, 5-FUR is converted to 5-FUTP by host cell enzymes and then enters chlamydiae. Once inside the RB, the 5-FUTP is converted to

5-FdUMP by chlamydial ribonucleotide reductase. The resulting 5-FdUMP inhibits *C. trachomatis* TS activity, and as a result, the parasite is starved for thymidine nucleotides and DNA synthesis ceases. Fifth, the results presented in Fig. 4 indicate that exogenous [5-^{3}H]uridine gives rise to radiolabelled cytosine in *C. trachomatis* DNA and that exogenous [6-^{3}H]uridine yields both radiolabelled cytosine and thymine. In contrast, there is no radiolabel present in pyrimidine bases isolated from mock-infected DHFR$^-$ cells pulsed with either [5-^{3}H]uridine or [6-^{3}H]uridine. The only way to generate radioactive thymine under these conditions is by way of a *C. trachomatis*-specific TS.

Further evidence for the existence of TS in *C. trachomatis* comes from the demonstration of in situ TS activity when [5-^{3}H]uridine is used as precursor (Fig. 6). These studies were aided by the use of the DHFR$^-$ cell line, which has low background ^3H_2O formation. Similar to DNA synthesis activity, in situ TS activity was inhibited by 5-FUR but not by 5-FUdr. The in situ TS activity correlates very well with DNA synthesis activity, peaking at approximately 24 to 32 hr postinfection, which represent the times when the maximum number of RBs are undergoing binary fission. After this time, all metabolic activities rapidly decrease as RBs differentiate to EBs.

As a final line of evidence supporting the existence of TS, we were able to demonstrate in vitro TS activity as assayed by detection of dTMP formation from dUMP using extracts prepared from highly purified *C. trachomatis* RBs as a source of enzyme (Table 6). Similar to TS from other sources (Santi and Danenberg, 1984; Ivanetich and

Santi, 1989; and Maley and Maley, 1990), the *C. trachomatis* TS activity is dependent on 5,10-CH_2-H_4folate as a cofactor and was inhibited by 5-FdUMP.

2) Mechanisms for Chlamydiae Acquisition of Folate, the Essential TS Cofactor

The importance of folates for chlamydiae growth and taxonomic classification has long been recognized (Schachter, 1988; Schachter and Caldwell, 1980; Moulder, 1991; Colon, 1962; Morgan, 1948; Morgan, 1952; and Huang and Eaton, 1949). The work presented in this thesis confirmed the suggestion that chlamydiae require folates for the generation of thymidine nucleotides (Tribby and Moulder, 1966; and Moulder, 1991). It has generally been accepted that all *C. trachomatis* strains are sensitive to sulfonamides whereas all *C. psittaci* strains with the exception of 6BC, are resistant to sulfa action (Schachter, 1988; Schachter and Caldwell, 1980; Moulder, 1991; Colon, 1962; Morgan, 1948; Morgan, 1952; and Huang and Eaton, 1949). A reasonable explanation for these findings was that *C. trachomatis* strains and *C. psittaci* 6BC were capable of synthesizing folates de novo whereas the remainder of the *C. psittaci* strains were not. Unlike the simple interpretation required to explain the action of sulfonamides against chlamydiae it has proven difficult to interpret results obtained using antifols that target DHFR (Moulder, 1991; Morgan, 1952; and Reeve, 1968). One must be cautious when comparing various results obtained with DHFR inhibitors because many different host cell systems, i.e. chicken embryo and tissue culture cell lines from different mammalian species, have been employed and it has recently been shown that variations in

methodology markedly influence chlamydial antimicrobial susceptibility results (Ehret and Judson, 1988).

By working with a culture system as defined as possible and minimizing methodology variations, we have clarified some discrepancies concerning chlamydial antifolate susceptibility. In agreement with earlier in situ observations (Colon, 1962; and Morgan, 1948), we found that *C. trachomatis* L2 and *C. psittaci* 6BC were sensitive to sulfonamides, whereas *C. psittaci* francis was resistant. In addition our results indicate that trimethoprim was active against *C. trachomatis* L2, but had no effect against either *C. psittaci* strain. Methotrexate inhibited the growth of *C. trachomatis* L2 and *C. psittaci* francis but did not effect *C. psittaci* 6BC growth. Previously Morgan (1952) reported that *C. psittaci* 6BC was sensitive to aminopterin, we have found that the growth of *C. psittaci* 6BC is resistant to a wide variety of antifols, including aminopterin, methotrexate, and trimethoprim. The reason for the discrepancy between these results is not known but may relate to the different culture systems used or an inability to differentiate host and chlamydial toxicity. The methotrexate sensitive chlamydial strains were susceptible whether a wild type or a DHFR deficient cell line was used as host, a result which strongly suggests that methotrexate is directly affecting chlamydiae. The observation that exogenous thymidine could not reverse the inhibitory effect of methotrexate is in keeping with others and our own results which indicate that chlamydiae cannot salvage thymidine or thymidine nucleotides from the host and as a result rely on de novo synthesis of dTTP from dUMP.

The in situ methotrexate sensitivity observed with *C. trachomatis* L2 and *C. psittaci* francis implies that these strains can transport folates. This hypothesis is also supported by the finding that folinic acid could completely reverse trimethoprim/sulfisoxazole inhibition of *C. trachomatis* L2 growth since folinic acid can only enter the chlamydial cell through a folate transporter. It is surprising that *C. psittaci* 6BC was completely resistant to methotrexate especially since we found that 5-CHO-H_4folate could reverse sulfa inhibition of *C. psittaci* 6BC growth. One possible explanation for this finding is that *C. psittaci* 6BC may only be capable of transporting reduced folates. This would also explain why 5-CHO-H_4folate was much more effective at reversing sulfa action against *C. psittaci* 6BC than was folic acid. It is of interest to note that *P. cerevisiae* is resistant to aminopterin and methotrexate but it requires 5-CHO-H_4folate for growth and has a specific transport system for reduced folates (Mandelbaum-Slavit and Grossowicz, 1970).

Since *C. trachomatis* L2 and *C. psittaci* 6BC are sensitive to sulfa action, it has long been assumed that they must be capable of de novo folate synthesis. We have confirmed this by showing that both these strains readily grow in folate-depleted CHO K1 cells and more importantly and directly that both incorporate exogenous [^{3}H]pABA into folates. We consistently found that there was a difference in the composition of the intracellular folate pools between *C. trachomatis* and *C. psittaci* species. While reduced folates were predominant in both chlamydial species, the *C. trachomatis* L2 folate pool was dominated by H_4folate whereas the *C. psittaci* 6BC folate pool was dominated by

reduced folates carrying a one carbon unit (i.e. 10-CHO-H_4folate). At the present time the significance of this difference is not known. However, it is interesting that using the classical microbiological assay with *L. casei* and *P. cerevisiae*, Colon and Moulder (1958) also detected a difference in the composition of chlamydial species folate pools.

Because of its sulfonamide resistance and methotrexate sensitivity, two results which imply an absolute dependence on preformed folates, we were surprised to find that *C. psittaci* francis grew so well in CHO K1 cells depleted of intracellular folates. Although it might be argued that *C. psittaci* francis was able to grow because the host cell line was not completely depleted of intracellular folates, the switch from sulfonamide resistance to sulfonamide sensitivity argues against such a possibility. Furthermore our results clearly indicate that when growing in folate-depleted CHO K1 cells, *C. psittaci* francis incorporates exogenous [^{3}H]pABA into folates. As expected sulfisoxazole prevented the in situ incorporation of [^{3}H]pABA into folates in all three chlamydial strains.

Further support for the existence of a de novo synthesis pathway comes from our ability to detect DHPS activity in extracts prepared from highly purified RBs of all three chlamydial strains. Taken together these results indicate that *C. psittaci* francis, like *C. trachomatis* L2 and *C. psittaci* 6BC, can synthesize folates de novo. With folate starved CHO K1 cells as host, we found that sulfonamide inhibition of all chlamydial strains could be reversed by the addition of exogenous pABA. 5-CHO-H_4folate was able to

antagonize sulfonamide activity in all three strains, a result which supports the suggestion that all strains have the capacity to transport reduced folates. Interestingly, folic acid could also effectively reverse sulfa inhibition of *C. psittaci* francis growth but could not antagonize sulfa activity against *C. trachomatis* L2 or *C. psittaci* 6BC. Although many interpretations are possible we believe that this result likely reflects the fact that the host cell folate transporter has a lower affinity for folic acid than 5-CHO-H_4folate (Dembo and Sirotnak, 1984) and *C. psittaci* francis is more efficient at obtaining both reduced and non-reduced forms of folates from the host cell than are *C. trachomatis* L2 or *C. psittaci* 6BC. This hypothesis is also supported by the observation that when folates are present in the culture medium *C. psittaci* francis does not depend on de novo folates (as indicated by sulfa resistance) whereas *C. trachomatis* L2 and *C. psittaci* 6BC do (as indicated by sulfa sensitivity). At the present time we do not know why *C. psittaci* francis appears to be more efficient at obtaining folates from the host but it may be that it has a membrane transport system with greater activity or higher affinity for folates.

The results of studies evaluating the effectiveness of various antifols directed at DHFR implied that this enzyme exists in chlamydiae (Moulder, 1991; Colon, 1962; and Reeve et al, 1968). Our ability to detect DHFR activity in RB extracts from all three chlamydial strains confirm that the parasite genome does encode a DHFR. Results of in vitro DHFR assays indicate that the enzyme from all three strains is sensitive to methotrexate. This observation is in keeping with the finding that in vitro methotrexate is an effective inhibitor of DHFR from most sources examined to date (Burchall, 1983).

In agreement with in situ results we found that trimethoprim was a good inhibitor of *C. trachomatis* L2 DHFR activity in vitro. Both strains of *C. psittaci* were resistant to trimethoprim in situ and in vitro DHFR activities of these two strains were less sensitive to trimethoprim than was *C. trachomatis* L2 DHFR activity in vitro. However, the difference in the in vitro sensitivity between the species was not as great as might have been expected given the large difference in trimethoprim sensitivity in situ. This raises the possibility that there could be differences in the way *C. trachomatis* and *C. psittaci* metabolize trimethoprim or differences in their intrinsic permeability to the drug.

It is evident from the results presented that no simple concluding statement can be made in regards to folate metabolism in chlamydiae. The vast majority of free-living bacteria, both pathogenic and non-pathogenic, lack transport system(s) for preformed folates and thus depend on de novo synthesis. Recent studies on a variety of parasitic protozoa have shown that both de novo synthesis and salvage pathways for folates exist in eukaryotic intracellular parasites. For instance toxoplasma appear only capable of de novo folate biosynthesis (Allegra et al, 1987; and Kovacs et al, 1989), Leishmania appear totally dependent on salvage of preformed folates (Ellenberger and Beverley, 1987; and Kaur et al, 1988) and plasmodia appear capable of satisfying folate requirements by both pathways (Krungkrai et al, 1989). Intracellular parasites spend most of their lives within host cells rich in nutrients. In order to obtain non-diffusible nutrients from their host, intracellular parasites must evolve (or obtain) suitable transport systems. The advantage that a parasite has once it acquires the ability to obtain complex nutrients from its host

is that it can afford to loose the capability to synthesize the given nutrients de novo. This decrease in biosynthetic steps will be energetically favourable and may ultimately allow the parasite to decrease the size of its genome. In a nutrient rich environment auxotrophic microbes possess a growth advantage over wild-type strains (Zamenhof and Eichhorn, 1967). There would likely be a period of time when both capacities overlap and in some instances it may be necessary for the parasite to retain both pathways. We believe that folate metabolism in chlamydiae is currently at this stage of evolution. All strains have an absolute dependence on folates for de novo thymidine nucleotide synthesis. Originally this need was likely fulfilled via de novo folate synthesis as suggested by the ability of all strains tested to incorporate exogenous pABA into folates. More recently chlamydiae has obtained the necessary genetic information to allow them to acquire preformed folates from their host. The current status of the folate transport system(s) appears to vary from strain to strain. At one extreme *C. psittaci* francis fulfils its needs for folate strictly by transporting preformed host folates, but does retain the capacity to synthesize de novo. At the other extreme, *C. psittaci* 6BC appears to depend almost exclusively on its ability to synthesize folates de novo, however, it also has the capacity to transport reduced folate to a limited extent. Much of the discrepancy in the literature regarding the effectiveness of antifols against chlamydiae, both in situ and clinically probably results from the parasites variable dependence on the two folate acquisition pathways.

2. Molecular Cloning

1) Approaches for Enzyme Characterization and Screening Strategies for Gene Cloning

Almost all reactions in the cell are catalyzed by enzymes and thus enzymology is the core of biology (Dixon and Webb, 1979). Availability of a large quantity of pure biologically active protein is absolutely required for characterization of an enzyme. Classically this has been achieved by purifying native enzymes directly from living organisms (Scopes, 1982). However, this can be successful only when the enzyme of interest is abundant (Scopes, 1982) and/or a source rich in the enzyme can be easily obtained (Peterson et al, 1975). One strategy to increase its abundance is to select (or induce) mutant organism which overexpresses the enzyme (Dann et al, 1976; Newbold and Harding, 1971; and Gupta et al, 1977) and an example involving TS is that overepression of the enzyme in methotrexate-resistant *L. casei* (Maley et al, 1979) and *Leishmania tropica* (Meek et al, 1985). In the last two decades, more enzymologists have been working with recombinant enzymes which are expressed from a cell being the same as or different from its natural source and for many cases large quantities of pure and biologically active enzymes can be easily obtained this way (Invanetich and Santi, 1989; Invanetich and Santi, 1991; and Fersht, 1985). This new approach is extremely useful to studies of enzymes which are naturally expressed at low levels and/or whose source is limited (Invanetich and Santi, 1989; Invanetich and Santi, 1991; Clark et al, 1991; and

Fersht, 1985). However, to work with such an easily-obtained recombinant enzyme, its gene has first to be cloned. On the other hand, in order to express a large amount of recombinant enzyme, if possible, obtaining a small amount of purified native enzyme will facilitate gene cloning, for its provides the ability to obtain amino acid sequence which can be reverse-translated to make degenerate oligonucleotide useful screening for the corresoponding gene (Clark et al, 1991) as well as to verify the authenticity of the cloned gene (Clark et al, 1991; and Belfort, 1983). Even for enzymes available in large quantities, one may still want to clone their genes in order to study regulation of transcription, translation and interactions with other components and structures in the cell (Eastman et al, 1991; Chu et al, 1991; and Ayusawa et al, 1983). Also cloning the gene is a precondition for in vitro mutagenesis, a powerful approach for elucidation of structure-function studies in enzymatic catalysis (Fersht, 1985; Michaels et al, 1990; and Belfort and Petersen-Lane, 1984).

Since TS plays a central role in DNA synthesis (Santi and Danenberg, 1984; and Invanetich and Santi, 1989, Maley and Maley, 1990), we wanted to characterize chlamydial TS. Although we have been able to detect its activity consistently, efforts made to purify it from RB has been unsuccessful due to difficulties in obtaining enough RBs as starting material because of the obligate intracellular growth of chlamydiae and its low abundance. As an alternative, cloning its gene would be the place to start.

Because TS is a highly conserved protein, degenerate oligonucleotides with sequences reverse translated from conserved regions in the protein could be useful to screening a chlamydial library for TS gene-containing clones. TS genes from *Pneumocystis carinii* and some protozoa have been obtained this way (Edman et al, 1989; and Ivanetich and Santi, 1990). The two oligonucleotide mixtures we synthesized were based on amino acid sequences of highly conserved regions among known TS; and also amino acid residues in these two regions have relatively low codon degeneracy. As a matter of fact, oligonucleotides based on essentially the same regions have successfully been used in cloning TS cDNAs from *Pneumocystis carinii* (Edman et al, 1989), *Trypanosoma cruzi* and *Trypanosoma brucei* (Ivanetich and Santi, 1990). However, this approach was not successful in cloning the chlamydial TS gene.

Interspecies complementation has proven to be a useful strategy for cloning metabolic pathway genes from bacteria (Wardhan et al, 1989; and Peoples and Sinskey, 1989), fungi (Dougherty et al, 1992), protozoa (Kaslow and Hill, 1990), and mammals (Williams and Kantrowitz, 1992; Schild et al, 1990; Ayusawa et al, 1984; and Takeishi et al, 1985). A human TS cDNA, being the first mammalian TS cDNA isolated and sequenced, was obtained when a human fibroblast cDNA library was screened for complementation of thymidine auxotrophy in mouse TS^- cells (Ayusawa et al, 1984; and Takeishi et al, 1985). Provided a proper recipient cell with appropriate genetic mutation is available, screening a genomic DNA or cDNA library by genetic complementation has some advantages. First, this approach may provide a shortcut in molecular cloning (Chu,

1984; Ayusawa et al, 1984; and Takeishi et al, 1985); it can be done without any knowledge of protein sequence (corresponding enzyme from other species) and availability of antibodies (Peoples and Sinskey, 1989; Kaslow and Hill, 1990; and Banerjee et al, 1989). Second, screening procedures are usually simple, fast and inexpensive; no hybridization or antibody staining are required at this stage (Chu, 1984; Schild et al, 1989; Ayusawa et al, 1984; and Takeishi et al, 1985). Third, positive clones obtained by this approach must contain a whole intact gene, or at least the part required for function; multistep screening is thus unlikely to be needed as compared to screening by hybridization (Peoples and Sinskey, 1989; Dougherty et al, 1992; Banerjee et al, 1989, Williams and Kantrowitz, 1992; Kaslow and Hill, 1990, Ayusawa et al, 1984; and Takeishi et al, 1985). Fourth, it may provide a unique chance to find "unexpected" genes which encode functionally similar but structurally different enzymes (Schild et al, 1990); while screening by hybridization and antibody staining often results in obtaining the "expected" gene from different species.

Use of genetic complementation for library screening also has some limitations. Obviously, it is not suitable for cloning of intact genes encoding enzymes containing subunits of different primary structures unless the subunit coding regions are closely linked in an operon (Peoples and Sinkskey, 1989; and Schild et al, 1989). Functional complementation requires recognition of information stored in a non-coding region as well as in the coding region in the donor DNA by the recipient host cell, although this limitation can sometimes be overcome if an appropriate fusion cloning vector is used for

expressing the donor gene in the recipient cell (Wardhan, 1989). It is generally accepted that chlamydial promoters are either not recognized or recognized poorly by *E. coli* RNA polymerases (Palmer and Falkow; 1986; and Stephens, 1988). In most cases successful expression of chlamydial proteins is dependent on transcription promotion from the cloning vector (Palmer and Falkow; 1986; and Stephens, 1988; Hackstadt et al, 1991; Brickman et al, 1993; and Tao et al, 1991). In the sequence obtained from pHF101, computer analysis revealed no typical bacterial promoter regions. Clones which can successfully complement thymidine auxotrophy in TS^- *E. coli* were only obtained from a *Hind*III DNA library, one of three libraries that we constructed. Even after 10-20 fold more recombinants from *Pst*I and *Xba*I libraries had been screened, no complementing clones were found. ORF1587 in the complementing clone pHF101 is in the correct transcriptional orientation with respect to the betagalactodase (α-peptide) gene and the putative chlamydial initiation codon is only 72 bp downstream. These observations suggest that the expression of chlamydial protein CTS529 is dependent on the *E. coli Lac* promoter present in the vector pUC19.

When screening a library by genetic complementation, clones able to grow in selective medium could be a result of reversion mutation of the host cells as well as expression of foreign DNA carried by the cloning vector. Reversion mutation usually occurs when the defective gene carries a point mutation which has a relatively high chance of reversion (average being 10^{-6}) (David, 1990). Mutation induced by insertion has a much lower chance of reversion (David and Dulbecco, 1990; and Kleckner et al,

1977), or does not revert at all (de Bruijin and Lupski, 1984; and de Bruijin, 1987), and deletion mutations never revert (David and Dulbecco, 1990). Two of the three strains we used, BL21 Δ*thyA*, and X2913 are both deletion mutants and no TS^+ phenotype revertants have been isolated from either of these strains in the Institute of Virology, University of Glasgow (R. Thompson, personal communication) or in our laboratory. Although the mutation mechanism responsible for eaeating the TS^- phenotype of *E. coli* ATCC23851, the third TS^- strain we used, is not clear, we did not find any colonies formed after 2 X 10^{11} cells were plated onto thymidine-free agar, suggesting a low ($< 10^{-11}$) rate of reversion. In addition, transformation of TS^- *E. coli* strains with pHF101 and some of its subclones consistently resulted in growth of the bacteria in the absence of thymidine, while pUC19 vector-transformed TS^- *E. coli* have always been dependent on exogenous thymidine for growth. Therefore, we conclude the thymidine prototrophic phenotype must be specifically conferred by the chlamydial DNA contained in the recombinant plasmids.

2) Indication of a Novel TS in Chlamydiae

Using [^{32}P]-labelled plasmid pHF101 as probe, Southern hybridizations (Fig. 18) showed that the probe anneals to *C. trachomatis* L2 genomic DNA, the material used for construction of the library, under high stringency conditions. The plasmid also hybridizes to *C. trachomatis* A DNA which shares very high homology (>92) with *C. trachomatis* L2, but not to low homologous (<33%) *C. psittaci* DNA nor to *E. coli* DNA. In

addition, the sizes of hybridizing bands in *C. trachomatis* genomic DNA digested with *Hind*III and *Pst*I, respectively, are in agreement with the restriction map of the insert of pHF101 (Fig. 19). These results confirm that the plasmid pHF101 which complements the TS deficiency in mutant *E. coli* strains contains an insert of chlamydial DNA.

In most species TS is a homodimeric protein with subunit size of about 35 kDa. As shown in Fig. 2, among the isofunctional enzymes, *L. casei* TS and *E. coli* TS, represent the largest and the smallest proteins found to date, with open reading frames being 957 bp and 792 bp coding a 316 aa and a 264 aa polypeptide, respectively (Maley et al, 1970; and Belfort et al, 1983). Different from the enzyme in other species, TS in protozoan is physically linked to DHFR and the bifunctional TS-DHFRs have sizes that range from 110 to 140 kDA, with subunit sizes of 55 to 70 kDa (Ivanetich and Santi, 1989; Ivanetich and Santi, 1990). Given the surprisingly large size (529 aa protein with a molecular weight of 61 kDa) of CTS529 coded by ORF1587, it is highly unlikely that CTS529 is a bifunctional TS-DHFR. No matter which form (isofunctional or bifunctional) the enzyme is, TS has a highly conserved primary structure (Ivanetich and Santi, 1989; Ivanetich and Santi, 1990). Comparison of peptide sequences from any two sources show identity of $\geq 50\%$. The lack of sequence homology between CTS529 and bifunctional TS-DHFR (*L. major* as shown in Fig. 20) and the inability of pHF101 and its derivative pT9.11U18 to complement DHFR$^-$ *E. coli* suggest that pHF101 does not encode a bifunctional TS-DHFR.

Among the many potential open reading frames proposed by the computer program, or defined as starting at any ATG site and ending with a stop codon, most are too small and only those in reading frame 1 in strand N2182A appear to be comparable to the size of known TS. Comparison of the structure and function of plasmids constructs pT6.11U18, pT8.11U18, and pT9.11U18 leads to a conclusion that base 73 in N2182A is the likely initiation site of translation (Fig. 19), although this can only be confirmed by protein sequencing. If translation initiates at an ATG codon(s) further down stream (of base 73), one would expect pT6.11U18 to complement in TS^- *E. coli* as do pT8.11U18 and pT9.11U18. Furthermore any translations initiated upstream of base 73 as either a fusion or non-fusion protein results in translation termination because there are stop codon(s) in each of the three reading frames from base 1 through base 79 (Table 10 and Fig. 20). In addition, plasmid p101HS (Fig. 19) which is only 54 bp shorter at the 3′ end (in strand N2182A) than the functional pT8.11U18 and pT9.11U18 was unable to complement thymidine auxotrophy, indicating that the 51 bp before stop codon TAA (1660-1662 in N2182A) is also required. The results of functional mapping suggest the predicted ORF1587 is the open reading frame which is capable of genetically complementing thymidine auxotrophy in TS^- *E. coli*.

The unusually large size of open reading frame ORF 1587 and the facts that it is not homologous to known TS sequences and does not encode a DHFR (and therefore is not a bifunctional TS-DHFR) raise a interesting question as to whether the complementing CTS529 is capable of synthesizing thymidine nucleotides by a mechanism

different from that by known TSs. So far, TS has been found as the sole enzyme responsible for dTMP de novo synthesis in all the organisms studied (Santi and Danensberg, 1984; Ivanetich and Santi, 1989; and Maley and Maley, 1990). When [6-^{3}H]uracil was added to the growth medium of pHF101-transformed TS$^-$ *E. coli* strains BL21 Δ*thyA* and ATCC23851, [^{3}H]thymine can be detected from acid-hydrolyzed DNA, suggesting a conversion of uridine to thymidine nucleotides in vivo. Direct supportive evidence for this is provided by our ability to detect the formation of tritiated water and radiolabelled dTMP by using crude extract prepared from the TS$^-$ bacterial strains transformed with pHF101 and its derivatives, pT8.11U18 and pT9.11U18 (Table 12) when [5-^{3}H] and [6-^{3}H] dUMP were used as substrate, respectively.

TS catalysis is a folate-dependent reaction in which 5,10-CH_2-H_4folate serves as a methyl donor and reductant. Its activity can be inhibited by a broad spectrum of folate derivatives (Santi and Danenberg, 1984; and Jackman, 1993). The phenomenon that TS activity in the crude extract of TS$^-$ E. coli transformed with complementing plasmids does not require exogenous 5,10-CH_2-H_4folate can be explained by the presence of folates in the undialyzed bacterial extract since TS activity in *C. trachomatis* RB extract and the human purified protein can be also detected when extract of vector pUC19-transformed TS$^-$ *E. coli* was used to replace 5,10-CH_2-H_4folate (Table 13). The folate-dependence of the TS activity in the complementing plasmids-transformed bacteria extract is indirectly suggested by the demonstration of inhibition of enzyme activity with folate derivatives (Fig. 22) and further supported by the reversal of the inhibition by TS

cofactor 5,10-CH_2-H_4folate (Fig. 23). Interestingly, compared to the human enzyme, TS activity from the transformed bacteria extract is more susceptible to the folate derivatives tested (Fig. 22); however, definitive experiments will have to await further enzyme purification.

5-FdUMP, the structural analogue of substrate dUMP, strongly inhibits the catalysis of all known TSs by forming a stable covalent ternary complex (enzyme.FdUMP.folate) (Santi and Danenberg, 1984; Maley and Maley, 1990; Mathews et al, 1990a; and Mathews et al, 1990b). 5-FdUMP shows poor inhibitory effect on the enzyme activity in the crude extracts of complementing plasmids-transformed TS^- *E. coli,* however, even when 5-FdUMP has been preincubated with the extract in the presence of the folate cofactor (Fig. 24). Somewhat surprisingly we have been unable to detect the ternary complex (enzyme.FdUMP.folate) with our recombinant enzyme preparation whereas such a complex can consistently be detected from both extracts of wild-type *E. coli*, mouse L cells as well as purified human TS (Fig. 25)

Comparison of TS activity in RB extract and in extracts prepared from complementing plasmids-transformed TS^- *E. coli* provided evidence suggesting the CTS529 encoded by ORF1587 represents an authentic *C. trachomatis* TS. TS activity in both extracts is: a) fully active when assayed in crude bacterial extract without addition of exogenous 5,10-CH_2-H_4folate; b) highly sensitive to folate derivative inhibition; 3) however relatively insensitive to the nucleotide inhibitor, 5-FdUMP and incapable of

forming ternary covalent enzyme.FdUMP.folate complex; and 4) highly susceptible to denaturation (although activity in the RB extract appears more easily lost than that in the recombinant *E. coli* extract, this difference might be due to the lower activity, and lower protein concentration present in the RB extract).

Northern hybridization using ^{32}P-labelled pHF101 insert to probe RNA prepared from *C. trachomatis* L2 RB failed to provide useful information. However, the negative result should not be interpreted in a way to suggest that the gene is not expressed in *C. trachomatis*, since TS has a low copy number in most of the wild-type organisms (Ivanitich and Santi, 1984; and Maley and Maley, 1990) and in addition bacterial mRNA has an extremely short life with average of seconds to minutes as that others have also found it difficult to detect chlamydial mRNA (Tan et al, 1993). Furthermore, mRNAs for major structural proteins, like MOMP in chlamydiae, exist at higher concentration in the cell, probably because they are synthesized at a higher frequency and are more stable.

We have noted two characteristics of the TS activity in the extracts of complementing plasmids-transformed TS$^-$ *E. coli*: 1) activity loss upon dialysis; and 2) inability to form enzyme.FdUMP.folate complex. These characteristics have not been previously described with studied TSs. The results in Table 14 suggest an as yet unidentified small (dialysable) substance(s) is required for the stability (activity) of the enzyme. In addition, TS activity in the transformed bacteria extract appears to be more susceptible to action of folate

derivatives than human TS. Given the deduced primary structure of CTS529 which is almost completely different from known TS; that the conserved nucleotide binding site, was absent in CTS529, and a folate-binding site-like sequence (Q-L-L-T-T-K) in CTS529 is located at the C-terminus (residues 404 through 409 in CTS529), compared to the amino terminus in all known TSs (Figs. 2 and 20), it is not surprising that the TS activity shows unique characteristics. At the present stage of this study, the results we have obtained suggest that chlamydiae encode a novel TS. Despite the current supportive data, a definitive conclusion that ORF1587 encodes the authentic chlamydial TS awaits purification of the enzyme.

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APPENDIX

Abbreviations:

5,10-CH_2-H_4folate, 5,10-methylene-tetrahydrofolate;

5-CH_3-H_4folate, 5-methyl-tetrahydrofolate;

5-CHO-H_4folate, 5-formyl-tetrahydrofolate;

5-FdUMP, 5-fluoro-deoxyuridylate;

5-FUdr,5-fluoro-deoxyuridine;

5-FUR, 5-fluoro-uridine;

10-CHO-H_4folate, 10-formyl-tetrahydrofolate;

BSA, bovine serum albumin;

CHO, Chinese hamster ovary;

DHFR, dihydrofolate reductase;

DHPS, dihydropteroate synthase;

dNTP, deoxynucleoside triphosphate;

dTXP, thymidine nucleotide;

EB, elementary body;

HBSS, Hank's balanced salt solution;

H_2folate, dihydrofolate;

H_4folate, tetrahydrofolate;

HPLC, high performance liquid chromatography;

ID_{50}, antimetabolite concentration required to reduce incorporation of radiolabel into DNA by 50%;

IFU, inclusion forming unit;

MOMP, major outer membrane protein;

NTP, nucleoside triphosphate;

pABA, para-aminobenzoic acid;

PBS, phosphate buffer saline;

RB, reticulate body;

PCR, polymerase chain reaction;

SPG, sucrose-phosphate-glutamate solution;

TE, Tris-EDTA buffer;

TK, thymidine kinase;

TS, thymidylate synthase.