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STUDIES ON THE INTERACTION OF CHLAMYDIA
TRACHOMATIS WITH HOST CELLS

submitted by Alan Murray
for the degree of Doctor of Philosophy

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To Mum and Dad

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

MICROBIOLOGY

Doctor of Philosophy

STUDIES ON THE INTERACTION OF CHLAMYDIA TRACHOMATIS WITH HOST CELLS.

by Alan Murray

Chlamydia trachomatis is a pathogenic bacterium which replicates within and destroys eukaryotic cells. The mechanism by which chlamydiae are endocytosed is not understood. Preliminary work in this laboratory provided evidence that chlamydial uptake into HeLa 229 cells may be under bidirectional cyclic nucleotide control. Also, centrifugation of purified chlamydiae onto host cell surfaces was accompanied by an enhanced influx and efflux of $^{45}\text{Ca}^{2+}$ across the cell membrane. Highly sensitive ^{125}I -based cyclic nucleotide immunoassay systems were developed to monitor host cell cAMP and cGMP levels during chlamydial infection. A short term chlamydial dependent modulation of cyclic nucleotides was not observed, however, clear evidence was obtained of a significant elevation in host cell cAMP, cGMP and prostaglandin E detectable some 36 h after challenge. These responses were abolished by the anti-chlamydial drug rifampicin.

The association of a Ca^{2+} flux with chlamydial binding to the cell surface raised the possibility of interaction with a calcium dependent regulatory protein(s) (e.g. calmodulin) being involved in chlamydial uptake. A wide range of structurally diverse calmodulin inhibitors were all found to inhibit chlamydial infectivity for HeLa cells. Trifluoperazine was selected for detailed study. The inhibitory effect of trifluoperazine was reversible, dose-dependent and associated with impairment of chlamydial adhesion, uptake and development within the host cell.

Calmodulin might be implicated in chlamydial endocytosis via regulation of myosin light chain kinase, crucial to microfilament function. However the role of microfilaments in chlamydial endocytosis was uncertain. Endocytosis of chlamydial sized particles would be expected to be microfilament-dependent, but paradoxically a number of workers have reported that chlamydial infection of host cells is not affected by the microfilament inhibitor cytochalasin B. By measuring uptake of purified chlamydiae directly, it was possible to demonstrate that microfilaments and microtubules are important for the endocytosis of chlamydiae into host cells. However, cytochalasin B does not greatly reduce inclusion formation 48 h after challenge. Inhibitors of receptor mediated endocytosis did not inhibit chlamydial uptake, micropinocytosis of [^3H]-sucrose into host cells was unaffected, but specific chlamydial antibody bound distal to the site of chlamydial adhesion to the host cell did block chlamydial uptake. These findings together with electron microscopic observations were consistent with the hypothesis that chlamydiae enter cells by a microfilament-dependent zipper mechanism.

CHAPTER 1

INTRODUCTION

1.1 Historical background

A disease resembling trachoma was described by the Chinese in the 27th century B.C. Ebers papyrus of 1500 B.C. contains a description of an exudative cicatrizing eye disease, similar to the clinical entity known today as trachoma. Trachoma was common in ancient Greece, and was known to both Hippocrates and Heliodorus of Alexandria. The disease was also well known to the classical Romans. Pedanius Disorides (AD 40-91) a Sicilian physician coined the term 'Trachoma' and Celsus (AD 14) gave a good clinical description of roughness of the lids, and described treatment by rubbing and scarification (Duke-Elder 1965). Medieval Greek and Arab surgeons wrote about the disease and its treatment, and it was considered in lectures ascribed to Hanan ibn Ishaque (809-893) (Haddad 1975). The disease was introduced into Europe by returning crusaders from Palestine and again by the remnants of Napoleon's army returning from Egypt in 1798-99 (Duke-Elder 1965). Thus it can be seen that chlamydial infections have been a long-standing problem for man.

Research into the aetiology of chlamydial infection was initiated by Halberstaedter and von Prowazek who in 1907 found intracytoplasmic inclusions in the conjunctival scrapings from patients with trachoma. The psittacosis pandemic of 1929-30 in the United States prompted further research, resulting in the description by Bedson and Bland at the London Hospital in 1932 of the developmental cycle of psittacosis 'virus'. A further milestone occurred some 25 years later when T'ang and his associates in 1957 reported the isolation and growth of the agent of trachoma in the embryonated hens yolk-sac. Thus chlamydial infections have been the subject of research throughout the 20th century.

1.2 The Microbiology of Chlamydia

Chlamydiae show features which markedly distinguish them from all other groups of organisms. It is clear that chlamydiae are bacteria obligately parasitic within eukaryotic cells distinguished from the rickettsia by their unique growth cycle. Knowledge of the biology of chlamydiae is essential to an understanding of the pathogenesis of chlamydial infection. This section reviews some of the main features of chlamydial biology.

1.2.1 Taxonomy

Chlamydiae have the distinction of being placed in their own order, the Chlamydiales with one family Chlamydiaceae and one genus Chlamydia. Within this genus there are two species C.trachomatis and C.psittaci (table 1). These species are differentiated on the basis of their sulphonamide susceptibility and the accumulation of glycogen (which stains with iodine) within the inclusions.

C.trachomatis strains are susceptible to sulphonamide and their inclusions stain with iodine whereas C.psittaci strains are resistant to sulphonamide and iodine negative. C.psittaci causes infections in a wide variety of animals. In man, infection usually takes the form of an atypical pneumonia or endocarditis following the inhalation of dried excreta or secretions from birds. Preliminary evidence of serotypes within C.psittaci has been obtained (Eb and Orfila 1982; Schachter et al 1974; Schachter et al 1975) but these have not been characterized.

C.trachomatis strains include the mouse pneumonitis strain and 15 human pathogenic serotypes (Grayston and Wang 1975). Four of these serotypes are associated with hyperendemic trachoma (A, Ba, B and C); serotypes D-K cause genitourinary tract infections, conjunctivitis and neonatal pneumonia. Finally three serotypes (L1, L2 and L3) cause a more invasive sexually transmitted disease, lymphogranuloma venereum.

On the basis of DNA hybridisation (Kingsbury and Weiss 1968) and restriction endonuclease mapping (Peterson and de la Maza 1983) there

TABLE 1

Summary of the differences between *C.trachomatis* and *C.psittaci*

<u>Features</u>	<u><i>C.trachomatis</i></u>	<u><i>C.psittaci</i></u>
Natural infection:	Principally human ocular and urogenital diseases (e.g. Trachoma etc)	Respiratory, urogenital and systemic infections in a wide variety of animals. Man is incidentally infected. (e.g. Ornithosis)
Laboratory growth:	All strains, except LGV serotypes require centrifugation on to specially prepared tissue culture cells.	Grow readily in tissue culture without centrifugation or special cell treatment
Inclusions:	Compact, glycogen-containing stain with iodine	Diffuse, non-glycogen containing inclusions
Nucleic acid:	Guanosine plus cytosine 44%. Only 10% homology between <u><i>C.trachomatis</i></u> and <u><i>C.psittaci</i></u> DNA on hybridization.	Guanosine plus cytosine 41.2%.
Antibiotic sensitivity:	Sulphadiazine sensitive	Sulphadiazine resistant.

is little genetic homology between C.psittaci and C.trachomatis despite the apparently similar growth cycle. This suggests that the two species are genetically unrelated and are an example of convergent evolution from diverse ancestors. However, on Western blotting, antibody to C.trachomatis was found to cross react with antigens of a single strain of C.psittaci (Newhall et al 1982) showing that the two species share common proteins.

Chlamydial taxonomy may soon be expanded to accommodate other micro-organisms, including isolates from spiders, clams and fresh water coelenterates. The micro-organisms isolated from these phyla have morphological and developmental characteristics of chlamydiae and may represent new relatives in the family chlamydiaceae (Moulder, 1984).

1.2.2 The growth cycle

Chlamydiae are obligate intracellular parasites with a developmental cycle unique amongst prokaryotic organisms. Infection is initiated by the 200-300 nm elementary body (EB) which attaches to the host cell. Once attached the EB is endocytosed by an unknown mechanism. As would be expected for an organism which is an intracellular pathogen, endocytosis is efficient, chlamydiae are ingested by nonprofessional phagocytic cells more quickly than other bacteria (*E.coli*) or inert particles (polystyrene latex) (Byrne and Moulder 1978). Following endocytosis, the chlamydia-containing phagosome provides the organism with a protected intracellular environment as phagolysosomal fusion is prevented (Friis 1972; Lawn et al 1973; Wyrick and Brownridge 1978). This circumvention of phagolysosomal fusion is thought to be mediated through components on the EB cell surface (Friis 1972; Byrne and Moulder 1978; Eissenberg et al 1983), although again the mechanism is not known. Within 4-12 h post-infection the EBs enlarge and differentiate into 800-1000 nm reticulate bodies (RBs). The RBs are metabolically active, divide by binary fission and synthesise macromolecules such as RNA, DNA, proteins lipids and carbohydrates. Protein synthesis of C.trachomatis in emetine treated host cells is detected at about 12 h after infection and becomes maximum at about 42 h (Becker and Asher 1972).

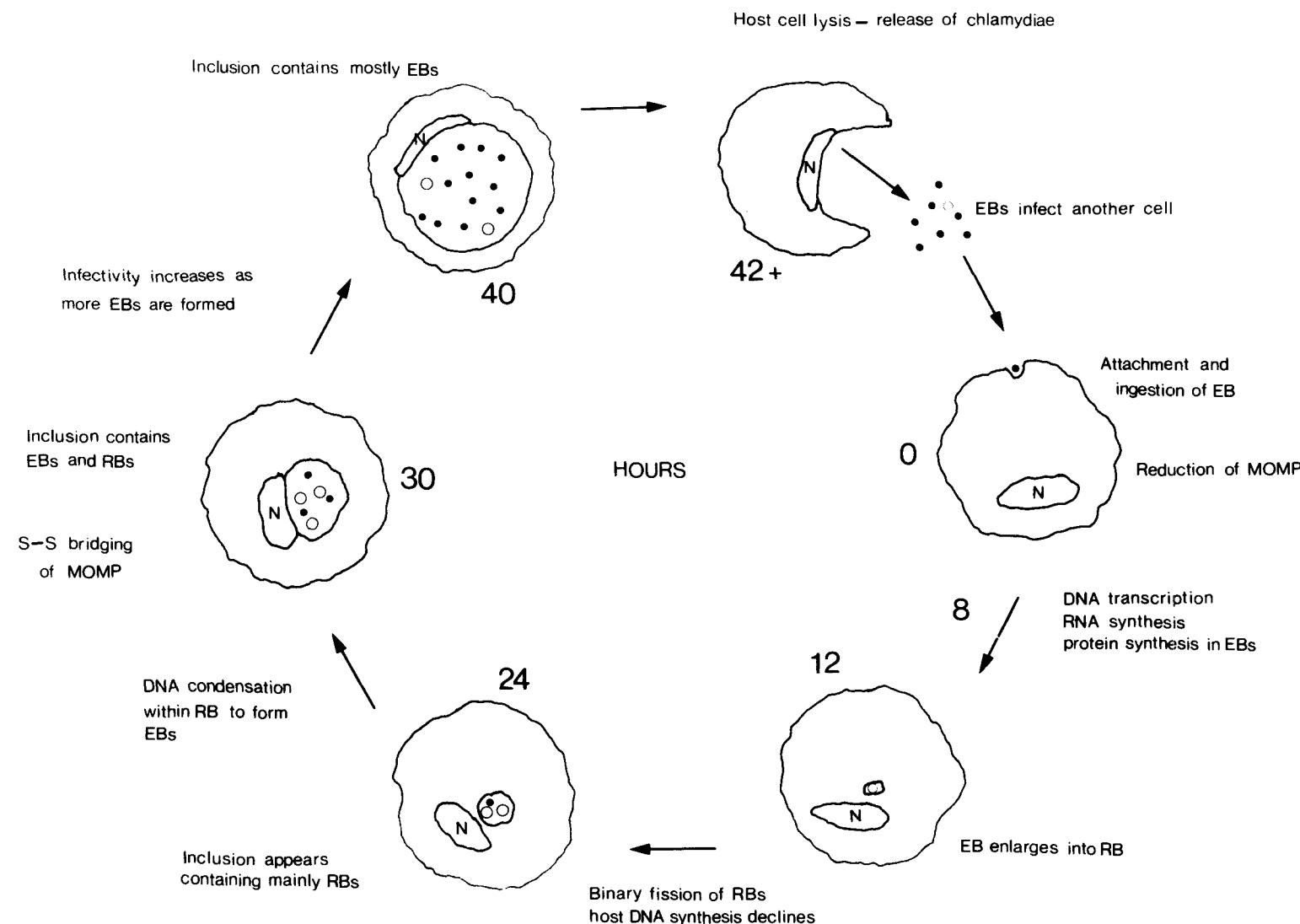
The synthesis of RNA in chlamydiae occurs between 15 and 30 h after infection (Gutter and Becker 1972; Schechter 1966). By 48 - 72 h the developmental cycle is complete and protein synthesis falls off (Becker and Asher 1972). The lipid metabolism of C.trachomatis LGV begins 6 - 12 h after infection, at the stage when the initial EBs are developing into RBs and increases continuously until the appearance of intracellular infectious EBs at 24 to 30 h (Reed, et al 1981). Chlamydiae contain fatty acids different from those found in host cells. Phosphatidylglycerol for example, is a component of the 6BC strain of C.psittaci, which is not found in the mammalian cells (monkey kidney cells) which supported its growth (Makino et al 1970). Glycogen accumulation in C.trachomatis inclusions peaks 48 - 60 h after infection and there is evidence that C.trachomatis contains genetic information for a specific glycogen synthetase. (Becker 1978). No chlamydial metabolic pathway capable of generating energy has been detected. Thus, chlamydiae have a requirement for ATP from the host to generate the proton motive force, necessary for the transportation of essential nutrients across the chlamydial cytoplasmic membrane (Hatch 1975). RBs are capable of incorporating and hydrolysis of ATP from the surrounding medium (Hatch et al 1982).

The cell envelopes of EBs and RBs resemble those of gram-negative bacteria in that both outer and inner trilaminar membranes are present (Tamara et al 1971). However, in contrast to the envelopes of gram-negative bacteria, both developmental forms of chlamydiae contain little or no muramic acid (Barbour et al 1982; Garrett et al 1974; Tamura and Manire 1967; Manire and Tamura 1967) and electron microscopy has failed to demonstrate any type of peptidoglycan layer between the inner and outer membranes (Caldwell et al 1981). Nonetheless, penicillin an antibiotic that inhibits bacterial growth by blocking cross-linking between the tetrapeptide side-chains of peptidoglycan molecules (Gale et al 1981), inhibits the growth and division of both chlamydial species (Barbour et al 1982; Matsumoto and Manire 1970). It has been suggested that chlamydial envelopes may have cross-linked tetrapeptides that are covalently bound to structures other than peptidoglycan (Garrett et al 1974), although

supportive evidence for this hypothesis is not yet available. Despite the lack of the usual stabilising peptidoglycan layer, the chlamydial EB is characteristically rigid and resistant to mechanical and osmotic shock. By contrast RBs are relatively fragile. These structural differences between the two developmental forms of chlamydiae may be due to the extent of S-S bridging in the cell envelopes. This concept was first suggested by Tamura and Manire who in 1967 demonstrated that C.psittaci RBs contained much less cysteine and methionine than EBs. Later studies have shown that sodium dodecyl sulphate (SDS) plus reducing agents such as mercaptoethanol were required for the complete solubilisation of C.psittaci EBs (Hatch et al 1981), whilst disulphide cross-linked protein complexes were described in the outer membranes of EBs of an oculogenital strain of C.trachomatis (Newhall and Jones 1983). The RB outer membranes of an LGV serotype of C.trachomatis has also been found to be deficient in cysteine-rich proteins and to be more susceptible to solubilisation in SDS than the outer membranes of EBs (Hatch et al 1984) confirming the initial observations of Tamura and colleagues. Cysteine deprivation markedly slows the conversion of RBs to EBs (Stirling et al 1983) perhaps reflecting the inability of RBs to synthesise cysteine-rich proteins and to form necessary disulphide-cross-linked complexes in the EB outer envelope. The morphological features of the chlamydial growth cycle are illustrated in Fig 1.

It has been shown that a single EB enlarges into a single RB. For this reason it is generally assumed that the DNA in a single RB condenses to give rise to a single EB. However, electron microscopy often shows the presence of more than one site of DNA condensation within reorganising RBs and these can divide so that 2 to 4 EBs are produced from a single RB (Ward 1983). Eventually a mature inclusion containing many EBs may occupy up to three quarters of the host cell volume. The mechanism of release from the host cell is unknown although it is assumed to result from cell lysis. Lysosomal enzymes and digested cell components are free in the host cell cytoplasm at the time of C.psittaci release (Todd and Storz 1975) although it is not clear whether this autodigestion is a cause of, or

Figure 1 Generalised growth cycle of C.trachomatis in tissue culture. The times refer to hours after chlamydial challenge. (N=nucleus)



a consequence of, cell lysis and chlamydial release. Clearly an enormous amount remains to be learnt concerning the control mechanisms governing the chlamydial growth cycle.

1.3 Chlamydial Infections

It is clear that infections resembling trachoma have been known for a very long time but the importance of chlamydiae in genital tract infection has only recently been appreciated following the advent of tissue culture techniques for growing chlamydiae. This section reviews the importance of chlamydiae to man in terms of the range and scale of human disease which these organisms cause.

1.3.1 Trachoma

Trachoma can be defined as chronic inflammation of the conjunctiva, the mucous lining covering the inside of the eyelid, and the surface of the eye ball and cornea, caused by Chlamydia trachomatis. Hyperendemic trachoma is a major cause of preventable blindness in rural communities of the developing world (Tarizzo 1972). It is estimated that approximately 500 million people are affected by trachoma worldwide with at least two million blinded, and a much larger number suffering from partial loss of vision (Tarizzo 1973). Clearly trachoma is a major problem in the third world. In the most heavily affected communities, most children are infected by age 1 to 2 years and starting at age 5 the prevalence of active disease declines steadily, although it may still occur in adults. Children therefore are the main reservoir of active trachoma in the community and are themselves a major proportion of the population in third world countries.

In the developing world, trachoma is primarily thought to spread from eye to eye (ocular promiscuity) with flies considered an important vector (Darougar and Jones 1983) and the disease is generally caused by C. trachomatis serotypes A, B, Ba or C (Jones 1974; Wang and Grayston 1974). In its early stages, trachoma presents as a follicular conjunctivitis, with papillary hypertrophy and inflammatory

infiltration involving the whole conjunctiva, particularly the upper tarsal conjunctiva. Later stages result in cicatrization of the conjunctiva appearing as fine linear and small stellate scars in mild cases or as broad confluent or synechial scars in more severe cases. Scarring and fibrous tissue of the tarsal conjunctiva distort the eyelid resulting in entropion and trichiasis. This may be followed by corneal ulceration with or without secondary bacterial infection leading to subsequent visual loss. Corneal lesions include inflammation of the epithelium and anterior stroma, with superficial neovascularization (Darougar 1983). Lymphoid follicles may form at the limbus which on resolution leave characteristic depressions known as Herbert's Pits. The pannus is usually more marked at the superior limbus, although it is present all round the limbus and may extend across the cornea. Several different systems have been proposed to classify the clinical stages and severity of trachoma (Assad and Maxwell-Lyons 1967; Jones 1974).

1.3.2 Adult Chlamydial Ophthalmia (Paratrachoma)

Paratrachoma is the term used to describe a group of ocular and genital tract infections caused by C.trachomatis serotypes D-K. The infection takes the form of inclusion conjunctivitis and produces a disease often indistinguishable from the acute inflammatory phase of trachoma although pannus formation is uncommon (Dawson et al 1970; Jones et al 1965). In adults, inclusion conjunctivitis usually occurs in sexually active people between 18 and 30 years of age in whom it is often possible to demonstrate chlamydial infection of the genital tract in both patient and their sexual consorts (Dawson and Schachter 1967). Eye-to-eye transmission is thought to be rare, but a few cases have been documented (Jones 1964). The main route of infection is thought to be from the genital tract to the eye, although further epidemiological studies are necessary.

1.3.3 Infection of the Male Genital Tract

In England and Wales, between 50 - 60% of cases of non-gonococcal urethritis (N.G.U.) are caused by C.trachomatis (Oriel 1982), the

aetiology of the remainder being uncertain. Thus chlamydiae are the major single cause of sexually transmitted disease in the UK. N.G.U. can occur as an acute symptomatic disease or as an asymptomatic infection, the presence of symptoms depending upon the degree of inflammatory response in the urethra. C.trachomatis is also recovered from the majority of cases of post-gonococcal urethritis (Richmond et al 1972) which frequently occurs following adequate penicillin treatment for gonorrhoea and presumably arises in patients double infected with both gonococci and chlamydia.

1.3.3.1 Epididymitis

Epididymitis is the most serious complication of male genital tract infection because it may result in infertility. Heap (1975) reported a significant rise in the titre of group reactive antibodies to chlamydia in two men with acute epididymitis suggesting that C.trachomatis might have caused the disease. Berger et al (1978) studied the role of C.trachomatis and other micro-organisms in 'idiopathic' epididymitis. Chlamydiae were isolated from 11 out of 13 men under the age of 35 but in only 2 out of 10 patients over the age of 35. It was therefore suggested that C.trachomatis was a major cause of epididymitis in younger men and coliform bacteria in older men.

1.3.4 Infection of the Female Genital Tract

In the female genital tract C.trachomatis may infect the cervix, urethra, the ducts of Bartholins glands and fallopian tubes with serious consequences to both mother and baby. Cervical infection by C.trachomatis is clearly common in women attending S.T.D. clinics in the U.K. with overall isolation rates of 12-31% reported (Burns et al 1975; Hilton et al 1974). Female partners of men with non-gonococcal urethritis contribute approximately one third of these cases (Oriel et al 1974). C.trachomatis is recovered from 45-68% of contacts of culture positive men (Alani et al 1977; Holmes et al 1975) showing that chlamydiae are highly infectious. Women with culture proven

chlamydial cervicitis show a marked inflammatory response which can be observed in the subepithelial connective tissue. This sometimes takes the form of follicular aggregates of lymphocytes (Hare et al 1981) perhaps analogous to those seen in the eye during acute conjunctivitis or trachoma. Primary cervical chlamydial infection is often asymptomatic but after repeated infection a progressively more severe inflammatory reaction occurs resulting in symptomatic disease (Swanson et al 1975).

1.3.4.1 Endometritis

Transient endometritis caused by C.trachomatis has been reported (Mårdh et al 1981). The condition is self limiting due to the monthly shedding of the epithelial lining and has generally been diagnosed in women who also had acute salpingitis. However, chlamydial infection confined to the cervix and endometrium have also been documented (Mårdh et al 1981). Chlamydial endometritis has also been produced in grivet monkeys (Møller et al 1980). The condition is important because it represents the main route of ascending infection from the cervix to the fallopian tubes.

1.3.4.2 Salpingitis

Acute non-tuberculous salpingitis is a disease that mainly afflicts women in their reproductive years. Approximately 83,000 cases of salpingitis in women aged 15-34 years occur each year in England and Wales with C.trachomatis responsible for around half of these (Weström and Mårdh 1983). The infection spreads canalicularly as a result of ascending infection through the uterine cavity (Weström and Mårdh 1983). The organism has been cultured from endometrial aspirates (Wölner-Hansen et al 1982) and demonstrated in fallopian tube biopsies by both histological and immunofluorescent staining (Møller et al 1979). In laparoscopically confirmed salpingitis cases it has been shown that 17% of women are rendered infertile because of post-infection tubal damage (Weström 1975; Weström and Mårdh 1980). The fertility prognosis worsens with repeated infections, increasing age of patient and severity of the disease episode (Weström and Mårdh

1980). In England and Wales it has been calculated that some 8600 women in each annual cohort will be rendered infertile as a result of tubal dysfunction following salpingitis and around 2700 women will suffer ectopic pregnancy (Weström 1982). These are the most serious complications of genital chlamydial infections.

1.3.4.3 Perihepatitis

Acute perihepatitis (Curtis-Fitz-Hugh syndrome) is characterised by the development of right sided upper abdominal pain with tenderness in the right upper quadrant. The syndrome is best known as a complication of gonococcal salpingitis although it is now clear that chlamydiae are also responsible. Müller-Schoop et al 1978 reported high chlamydial antibody levels in women with peritonitis from whom gonococci could not be isolated. Wood et al (1982) investigated 10 patients with clinically diagnosed Curtis-Fitz-Hugh syndrome, five had laparoscopic confirmation of perihepatitis and all 10 had raised antibodies to C.trachomatis within the range associated with active genital infection. These results suggest that perihepatitis may be an important manifestation of genital tract infection by C.trachomatis.

1.3.5 Chlamydial Infections of Infants

The source of neonatally acquired infection is the infected maternal cervix and transmission occurs during vaginal delivery (Hobson et al 1983) resulting in the involvement of multiple epithelial sites. (Schachter et al 1979a). The frequency of inclusion conjunctivitis developing in neonates born to women with chlamydial infection of the cervix has been reported as high as 50% with an even higher percentage, 67% developing serum antibodies against chlamydiae (Chandler et al 1977) indicating the high infectivity of C.trachomatis for the newborn. Chlamydiae in the birth canal aspirated by the infant can produce a distinctive atypical pneumonia characterised by a 'pertussoid' cough interstitial infiltration, eosinophilia and elevated serum immunoglobulins,

particularly IgM (Beem and Saxon 1977). Schachter et al 1979b examined 20 infants born through chlamydial infected cervices and showed that 20% developed chlamydial pneumonia, compared with none from a control group of unexposed infants.

1.3.6 Lymphogranuloma Venereum

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by C.trachomatis serotypes L1, L2, and L3 (Grayston and Wang 1975). The disease is endemic in the tropics. LGV organisms are more invasive both in vitro and in vivo than the oculogenital strains of C.trachomatis. LGV organisms are the only C.trachomatis strains which multiply readily in tissue culture without the need for centrifuge assisted infection. In man LGV infection is characterised by a transient insignificant local primary lesion followed by lymphangitis, lymphadenopathy and systemic complications. The primary lesion, a small evanescent vesicle sore, occurs 1-2 weeks after sexual contact. The second or bubonic stage with regional lymphadenopathy, develops 2-16 weeks after infection and is more commonly seen in men than women. The tertiary stage is characterised by fibrotic changes which, especially in women, may cause rectal strictures (Sigel, 1962; Sonck 1972). Proctitis with peri-rectal abscess or fistula-in-ano may be encountered in the male homosexual (Schachter and Osoba 1983). In both sexes constitutional symptoms, including fever, rigors, anorexia, nausea, loss of weight, backache, joint pains, lassitude, epistaxis and erythema nodosum may be noted.

1.3.7 Reiter's Syndrome

The classical triad of manifestations of Reiter's syndrome was urethritis, conjunctivitis and arthritis, (Reiter 1961) but today it is defined in terms of three of the following; urethritis/prostatitis, arthritis, conjunctivitis/uveitis, circinate balanitis/keratoderma blenorragica. Schachter et al 1966 reported that 30% of patients with Reiter's syndrome had evidence of chlamydial infection although chlamydiae are rarely recovered from the synovial fluid of affected joints. It seems probable that Reiter's syndrome is determined

immunologically. Brewerton et al 1973 reported the high association of a single histocompatibility antigen HLA B-27 with Reiter's syndrome; being found in 76% of patients. It may be that C.trachomatis is one of several infective agents which will precipitate Reiter's syndrome in a genetically predisposed individual. The mechanism is not clear, but it is conceivable that dissemination of chlamydial antigen from the genital site is followed by local immune responses which determine the clinical signs.

1.4 Immunology of C.trachomatis

The previous section has shown that chlamydiae are a major and important cause of human disease. Successful prevention of chlamydial infection by vaccination requires an understanding of chlamydial immunochemistry and of the human immune response to chlamydiae. The last 5 years has seen considerable progress in our understanding of chlamydial surface antigens which is reviewed in this section.

1.4.1 Chlamydial Antigens

1.4.1.1 Genus specific antigens

All members of the genus chlamydia share a common heat-stable (100°C for 30 min) group antigen (Schachter and Caldwell 1980). The antigen is soluble in ethyl ether, sodium lauryl sulphate and sodium deoxycholate and is inactivated by periodate oxidation suggesting that it is a lipopolysaccharide. The polysaccharide moiety with a molecular weight of 0.2 - 2.0 x 10⁶ daltons was isolated by the alkaline hydrolysis of partially purified ethyl ether-extracted antigen (Dhir et al 1972). The immunodominant determinant of this isolated component was identified as an acidic polysaccharide glycosidically bound to serologically inactive neutral carbohydrates. A strong immunological cross-reaction between this lipopolysaccharide antigen of C.trachomatis and the 2-keto-3-deoxyoctanoic core of the lipopolysaccharide of an Re mutant of Salmonella typhimurium has been reported (Nurminen et al 1983). The chlamydial LPS resembled the Re

LPS in molecular size, solubility and endotoxic properties. Three antigenic domains have recently been demonstrated with monoclonal antibodies on chlamydial LPS. Two of these epitopes were shared by other gram-negative bacteria including N.gonorrhoeae, S.typhimurium and Escherichia coli whilst the remaining epitope was specific to all members of the genus chlamydia (Caldwell and Hitchcock 1984).

The group lipopolysaccharide is probably located in the outer membrane of both EBs and RBs (Dhir and Boatman 1972). Studies using indirect immunofluorescence, has revealed group antigen associated with infected host cell cytoplasm independent of inclusions which appears to be released from infected cells (Richmond 1980). This observation is compatible with the hypothesis that replicating chlamydiae produce outer membrane blebs containing group antigen, which are excreted to the host cell surface during chlamydial replication (Richmond and Sterling 1981). Such exposed antigens on the cell surface may modulate the host defence against chlamydial infection (Eissenberg et al 1983; Lammert 1982) or serve as a target for immune attack.

1.4.1.2 Species-Specific Antigens

All members of the C.trachomatis species share common species-specific antigens (Schachter and Caldwell 1980). Crossed-immunoelectrophoresis of Triton X-100 solubilised extracts of C.trachomatis LGV 434 and C.psittaci Cal 10 strains resolved 18 species-specific antigens, 15 of which were probably proteins as they were heat-labile and susceptible to protease digestion (Caldwell et al 1975). One C.trachomatis species-specific antigen that was consistently immunoprecipitated by sera from patients with proven chlamydial infection was purified to homogeneity by immunoabsorption using monospecific antibody (Caldwell and Kuo 1977). This antigen was shown to be a highly immunogenic protein common to all C.trachomatis strains with a molecular weight of 155,000 daltons. Species-specific antigens have also been demonstrated in purified reticulate bodies by immunofluorescence (Yong et al 1979).

1.4.1.3 Type-Specific Antigens

Serotype-specific antigens are determinants shared only by certain chlamydial isolates within a species and have only been characterised for C.trachomatis. The micro-immunofluorescence (Micro-1F) test (section 1.4.2.2) resolves C.trachomatis isolates into 15 serotypes based on trypsin-labile proteins at the surface of the EB. Antisera to a given serotype show cross-reactions to a lesser titre in the Micro-1F test with other serotypes belonging to the same antigenic complex. Serotypes C, J, H, I and A are serologically related to the C complex, whilst serotypes B, E, D, L1 and L2 form the B complex. Serotypes G and F show cross-reactions with the B-complex, whilst type K and L3 are antigenically related to the C-complex, but also show some reactivity with the B-complex (Wang and Grayston 1982). These relationships are summarised in Table 2. The predominant polypeptide revealed following SDS polyacrylamide gel electrophoresis of C.trachomatis serotypes, has a molecular weight of 38000-42000 daltons depending on the serotype (Salari and Ward 1981). The molecular weight distribution of this major polypeptide, referred to as the major outer-membrane protein (MOMP), correspond closely with the predominant human infection caused by the serotype. Monoclonal antibodies produced in mice following immunization with sarkosyl extracted C.trachomatis L1 outer membrane complexes reacted with type specific epitopes on the MOMP (Terho et al 1982). Moreover SDS-denatured purified MOMP elicits a type specific immune response in rabbits demonstrated by rocket immunoelectrophoresis or Micro-1F (Caldwell and Schachter 1982). Thus, it is likely that epitopes on MOMP largely determine chlamydial serotype specificity. However, the so-called 'type specific' monoclonals reported at the time of writing had only been tested against single chlamydial strains representative of each serotype. Until these monoclonals are shown to react with all of a number of chlamydial strains of given serotype, their type specificity has not been proven; they may simply have strain or subtype specificity.

Other proteins may also determine chlamydial type specificity. A protein extracted from C.trachomatis infected BHK-21 cells in 0.2% Triton X-100 pH 10.0 and purified by liquid chromatography, formed

TABLE 2

Serotype relationships of *C.trachomatis*, described
using the microimmunofluorescence test

<u>C complex</u>	<u>B complex</u>	
C	B	
J	Ba	Senior
H	E	
I	D	G
A	L ₁	F
	L ₂	
K		
L ₃		Junior

Types G and F give occasional cross-reactions with the B complex.

Types K and L₃ are related to the C complex but bridge both C and B complexes.

Asymmetric cross reactions between the related types are common, e.g. type C antiserum reacts strongly with both C and A antigens whilst A antiserum reacts only with A antigen.

an immune complex with type-specific antibody and had a molecular weight of 30,000 - 32,000 daltons (Hourihan et al 1980). A type-specific protein of 27,000 daltons has been isolated from egg yolk infected with C.trachomatis serotype A (Sacks and MacDonald 1979). The type specificity of this antigen was demonstrated by its ability to bind only homologous antibody obtained from either hyperimmune rabbit antisera or serum from trachoma-infected owl monkeys.

1.4.2 Serological and Immunological Techniques

Methods of assessing the humoral immune response to chlamydial infection still rely on whole organisms or crude antigens due to the logistic difficulty of preparing large quantities of purified antigens. Recombinant DNA technology may ultimately offer a method of preparing the latter. This section reviews the serological techniques commonly used to demonstrate chlamydial antibody responses.

1.4.2.1 Complement Fixation Test

The complement fixation (CF) test measures the antibody response to the heat stable lipopolysaccharide antigen shared by all members of the genus chlamydia (Dhir et al 1972) (Section 1.4.1.1). The CF test is useful for the detection of the high LPS antibody titres in systemic chlamydial infections such as psittacosis and LGV but it is insufficiently sensitive to be of diagnostic value in localised chlamydial infections (Schachter 1976). Antigenic cross reactions have been reported in the CF test between chlamydiae and *Acinetobacter Calcoaceticus* (Brade and Bruner 1979).

1.4.2.2 Microimmunofluorescence test

The microimmunofluorescence (Micro-IF) test, developed by Wang and Grayston in 1970 is widely used to serotype chlamydial isolates and for seroepidemiology. The immunological relationships between the 15 different chlamydial serotypes revealed by Micro-IF have been outlined in Section 1.4.1.3. For this test the antigens are

partially purified chlamydial elementary bodies of appropriate serotype which may be used separately or pooled into appropriate serologically or epidemiologically related clusters (Wang et al 1975; Treharne et al 1977). Antibody bound to these antigens is detected using fluorescent anti-globulin. The availability of Ig-class specific immunoglobulin conjugates has enabled acute C.trachomatis antibody (IgA, IgM) to be differentiated from persistent antibody due to previous infection (Wang and Grayston 1982). Purified reticulate bodies may also be used as antigen in the Micro-IF test and react broadly with C.trachomatis and C.psittaci antibody (Young et al 1979). However, despite this genus specificity, the Micro-IF test based on RB antigen is limited, as like the genus specific CF test it typically does not react early in acute C.trachomatis infection (Wang and Grayston 1982). The Micro-IF test based on elementary body antigens is both more sensitive and specific than the complement fixation test and is capable of detecting antibody in 91% of patients with proven ocular chlamydial infections (Darougar et al 1978). However, it is time consuming and cumbersome to perform and the estimation of titre end points is difficult to standardise. The technique is therefore primarily applicable to chlamydial research laboratories doing epidemiological studies.

1.4.2.3 Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) used for the detection of antibodies to chlamydia is group specific (Treharne and Forsey 1983). The assay has been described using antigen derived from a C.psittaci agent (Lewis et al 1976), C.trachomatis LGV L2 (Levy and McCormack 1982) and LGV L1 serotypes (Mabey et al 1985). The antigen may be whole elementary bodies or an extracted group specific antigen (Evans and Taylor-Robinson 1982). The sensitivity of ELISA is greater than the CF or Micro-IF tests. Moreover the test is objective and readily automated so that large numbers of sera may be preliminarily screened for chlamydial antibody. However, the sensitivity of the test may be a disadvantage as low level 'natural' antibody to the core LPS component of gram negative bacteria cross reactive with chlamydiae, will be detected. Thus the main use of the test using crude chlamydial antigen, is the screening of sera for

antibody prior to using more specific but cumbersome methods like Micro-IF. Once purified chlamydial antigens become available in quantity free from antigens cross reactive with other bacteria, the ELISA test may become the method of choice for chlamydial antibody detection.

1.4.3 Immune Response to Chlamydial Infection

Complete understanding of the humoral immune response to chlamydial infection requires quantitation of the mass of antibody of each immunoglobulin class and subclass reaction with given chlamydial antigens. This ideal is far from being achieved although the Micro-IF test has given valuable seroepidemiological information. Even less is known about the role of cell mediated immunity in chlamydial infection despite its likely importance in the development of blinding pannus in trachoma. This section reviews the immune response to ocular, genital and respiratory infection.

1.4.3.1 Ocular infections

Both trachoma and paratrachoma evoke an immune response, resulting in chlamydial antibodies in both serum and tears. The presence of antichlamydial IgG in the tears of patients with trachoma or paratrachoma correlates well with the severity of conjunctival inflammation and the isolation of C. trachomatis from the eye. Clearly chlamydiae may actively replicate in ocular tissue despite the presence of local antibody. Serum IgG correlates less well, perhaps as a result of persisting antibody derived from previous infection (Treharne et al 1978a; Darougar et al 1978). A correlation between IgM and the clinical activity of trachoma has been shown (Holmes et al 1975). However, seroepidemiological studies indicate that reinfection often occurs with the same serotype when an IgM response is often not evident (Wang and Grayston 1971). The isolation of chlamydiae from patients with trachoma is only possible during acute exacerbation of the disease suggesting that the chronic inflammation may be due to residual chlamydial antigens.

The role of cell mediated responses to ocular infection is at

present uncertain. Skin tests using various antigens on a group of isolation positive children in The Gambia with clinically active trachoma have been compared with children who were isolation negative and had no clinical signs of trachoma. The trachoma group gave only 2 positive results and the control group gave 1 (Sowa et al 1965). A similar study carried out in South Africa on trachoma patients gave negative skin tests on the 5 children investigated, but it was found that approximately one third of adults had a positive reaction. One explanation for a disappointing correlation between chlamydial ocular infections and skin hypersensitivity reaction could be that optimal concentrations of antigen were not used for skin testing. A low dose of antigen as well as the absence of delayed hypersensitivity could give a negative result (Monnickendam et al 1981). Skin test positivity correlated better with severe upper tarsal inflammatory disease than with blinding damage (Ballard 1982).

Experimental ocular infection of human volunteers or sub human primates with trachoma or paratrachoma serotypes, produces a disease clinically resembling paratrachoma. In owl monkeys ocular infection is accompanied by mild to moderate inflammatory disease, antibodies in tears and blood, and a cell mediated immune response. Limitation of the infection was thought to be the result of cell mediated immunity as leukocyte migration inhibition tests became positive on day 9 whilst the number of infected conjunctival cells fell 5 to 9 days after challenge, before the appearance of chlamydial antibody (Sacks et al 1978). Experimental ocular infections in guinea-pigs with GPIC agent, also suggests that the cell mediated immune response is important in limiting chlamydial infection. Infected conjunctival epithelial cells were maximal 4 to 7 days after challenge whereas chlamydial antibodies did not appear in serum and tear fluid until day 10 (Monnickendam et al 1980). However, antibody may also play a role as treatment of infected guinea-pigs with cyclophosphamide to inhibit antibody production, considerably prolonged the infection (Modabber et al 1976). Evidence of protective immunity in the GPIC model stems from the finding that reinfection of guinea-pigs with chlamydiae 1 to 6 months after initial challenge required a higher dose than was originally necessary (Monnickendam et al 1981). The factors govern-

ing this immunity are probably the synthesis of chlamydial antibody, particularly secretory IgA in tear fluid for a short term protection (Pearce et al 1981) plus a cell mediated immune response which reduced the ability of GPIC agent to replicate on rechallenge (Monnickendam and Pearce 1983).

1.4.3.2 Genital Infections

Chlamydial genital infections do not always evoke a measurable humoral response (Treharne et al 1978b). Chlamydial infections in the female genital tract generally elicit a more pronounced antibody response than in the male, perhaps due to the potentially larger surface area of epithelium involved, the asymptomatic nature and consequent chronicity of the infection (Taylor-Robinson and Thomas 1980). The geometric mean titre (GMT) of IgG antibody in isolation positive women with cervical infection varies between 1:66 and 1:121 whereas the GMT of IgG in men with non gonococcal urethritis reaches only 1:8.6 to 1:45 (Treharne et al 1983). Several studies in the U.K. and overseas have reported raised chlamydial antibodies in women with past or current pelvic inflammatory disease compared with controls (Sweet et al 1980; Simmons et al 1979; Paavonen et al 1979). No in vitro studies have been performed to determine whether these antibodies might play a role in preventing reinfection.

Animal models of genital tract infection have been established in an attempt to elucidate the role of the immune response. Male and female sub-human primates are susceptible to genital infection with C.trachomatis serotypes D-K. In female marmosets partial protective immunity is acquired following such infection as subsequent vaginal infections are of a shorter duration. Antibody titres are boosted by reinfection with homologous or heterologous strains and there is some correlation between the serum antibody titre and the duration of infection, the latter being shortest in animals with the highest titres (Johnson et al 1980). The factors involved in recovery from genital tract infection in mice were investigated by Tuffrey et al 1982. These workers compared the course of infection in athymic nude mice with immunologically competent controls. The genital tracts of

these mice were infected with strain SA-2f, an LGV strain of C.trachomatis. Development of the infection was dependent on the subcutaneous injection of progesterone which was thought to function by preventing uterine shedding of target epithelial cells. The infection was self limiting in both progesterone treated CBA and congenic CBA/nu mice lasting about 60 days in both groups. This suggested that the recovery of CBA mice from genital infection with strain SA-2f is not strongly T-cell dependent. Unfortunately the infectivity of C.trachomatis serovars D to K which cause the majority of human infections have not been investigated by these workers in this mouse model.

1.4.3.3 Respiratory Infections

Pneumonitis in infants elicits high serum antibody levels, particularly IgM with values of >1:256 commonly found (Schachter et al 1979b). Serum IgG titres greater than 1:512 have been reported. Although the presence of antibody in serum, tears, and nasopharyngeal washings have been considered highly specific for the diagnosis of chlamydial pneumonitis (Harrison et al 1978), the possibility that it is derived from maternal serum antibody must be considered (Hammerschlag et al 1979) particularly during the first month of life.

Animal models have also been used to investigate the immune response to respiratory infections. Kuo and Chen 1980 infected mice with C.trachomatis of human origin and found the yield of organism from the lung reached a peak on the second and third day after inoculation. The infections were usually self limiting. Antibody to chlamydiae started to appear from day 7, during the decline of infection and was still present at the end of the experiment on day 35. Delayed hypersensitivity was detected from day 5 until day 21 and also coincided with the decline of the infection. Stephens et al 1982 reported that immunosuppression of mice with corticosteroids, which primarily eliminates the induction of cell-mediated immunity (Weston et al 1973), or with cyclophosphamide substantially prolonged pulmonary infection with a trachoma serotype of C.trachomatis. It was concluded that both humoral and cell mediated immunity was

important in terminating the infection. Experiments with the murine strain of C. trachomatis, by Williams et al 1981 provided evidence that thymus derived cells were necessary for the production of specific antibodies against this agent. Athymic (nu/nu) Balb/C mice were significantly more susceptible to MoPn than their heterozygous (nu/+) immunologically competent litter-mates. Unlike normal Balb/C mice the nude mice did not produce a significant antibody response to the MoPn agent, suggesting that T helper cells may be important for antibody production to chlamydial antigens. Intrauterine immunization protected the normal Balb/C mice but not nu/nu mice against intranasal challenge. Moreover high titre immune serum from heterozygous litter mates could transfer resistance to the nude mice suggesting that antibody was important (Williams et al 1982). It was concluded that T-cell dependent serum components, probably specific antibodies, are important in the host defence against pneumonia due to infection with MoPn agent.

1.4.3.4 Delayed Hypersensitivity

Cell mediated immunity in chlamydial infections has mainly been investigated by measuring delayed type dermal hypersensitivity. Delayed hypersensitivity reactions to lymphogranuloma venereum was first reported by Frei in 1925 using an intradermal test. Its sensitivity in patients with LGV varied from 36% to 95% (Schachter et al 1969; Abrams 1968). In animals, delayed hypersensitivity develops after reinfection with either homologous or heterologous strains, or following immunization with an inadequate vaccine (Kuo et al 1971a, b; Grayston 1967; Wang and Grayston 1967a, b). Delayed type hypersensitivity (skin test) reactions have been reported in trachoma patients (Bernkopf, et al 1966; Bietti et al 1967) the reactive antigen being a heat-stable protein (Kuo et al 1971a).

1.4.3.5 Immunization

Several laboratories have attempted vaccine trials aimed at preventing trachoma. In Taiwan, pre-school children were immunized

with C.trachomatis elementary bodies from locally isolated strains TW-1 or a mixture of TW-1 and TW-3 (Woolridge et al 1967 a,b,c). The partially purified vaccine was mixed with alum as an adjuvant prior to administration. The vaccine reduced trachoma infection by 60% after 2 years, however 2.5-3 years after the booster injection, the protection began to disappear and at the end of 6 years no protection at all was observed.

Dhir et al (1967) reported on the efficacy of two trachoma vaccines: a bivalent genetron (trifluoro trichlorethane) - purified vaccine containing 2.2×10^9 particles per ml of C.trachomatis strain TW-3 and TW-5 and a vaccine made of elementary bodies purified on sucrose-KCl gradients and containing 1.2×10^9 particles per ml. The vaccines were given to children in the Punjab (India). Both vaccines gave some protection from trachoma; the immunized group had a 10% incidence of trachoma after one year compared with 37% amongst the unimmunized.

Nichols et al 1969 performed a vaccine trial on children in Saudi Arabia using formalinized vaccine preparations which contained equal portions of two C.trachomatis strains most commonly isolated in Arabia. (TRIC/1/ET/Har-13/OT[Egypt-2] and TRIC/2/SAU/Har-2/OT[SA-2]). The vaccine had no effect on the trachoma attack rate over a period of 2 years, but a reduction in the number of inclusions was observed amongst vaccinated children.

Vaccination field trials in the Gambia were carried out by Sowa et al 1969 using elementary bodies from C.trachomatis strain MRC-187. The organism was isolated from a Gambian child suffering from early trachoma with micropannus, and was purified from infected egg yolk sacs. Prior to use, the vaccines and control preparations were emulsified in an equal volume of a mixture of 9 parts of mineral oil and 1 part mannide mono-oleate as adjuvant. Each vaccine dose contained $10^{9.0}$ to $10^{9.8}$ elementary bodies. The vaccine gave barely significant protection at 6 months after immunization and by 2 years the immunized children suffered from more severe infection than the unimmunized controls. Other vaccination studies have been performed

in Ethiopia (Guerra et al 1967; 1971) and the U.S.S.R. (Terskikh et al 1967).

Preventive vaccination of humans against trachoma is still regarded as experimental, as an effective vaccine has yet to be developed. Identification and separation of the antigens which induce protective immunity from those which evoke hypersensitivity and tissue damage is likely to be a pre-requisite of such a development.

1.5 Aims and Objectives of this Research

One of the major problems impeding the development of chlamydial vaccines is the difficulty of producing large quantities of chlamydiae. In part this is due to our lack of understanding of how chlamydiae infect cells. Clearly the mechanism of chlamydial invasion of cells must be of major pathogenic importance as chlamydiae cannot replicate outside cells. Moreover, understanding of the mechanisms of chlamydial penetration into cells and of the chlamydial components involved might permit the design of better vaccines.

Chlamydiae provide an ideal and important model for investigating how organisms enter cells because of their obligate intracellular parasitism. Unlike viruses chlamydial attachment, internalization and subsequent development can be readily monitored by radiolabelling the organism with non-essential amino acids in the presence of eukaryotic protein synthesis inhibitors (Becker and Asher 1972; Allen and Pearce 1982). Chlamydial survival depends on the efficient entry of chlamydiae into host cells. Indeed, chlamydial uptake into HeLa and L cells occurs at a rate 10-100 times greater than that observed for *Esherichia Coli* or polystyrene latex (Byrne and Moulder 1978) although it can be questioned whether this is a valid comparison. Nevertheless, the mechanism by which chlamydiae are endocytosed and the identity of any host or parasite receptors is confused.

One possibility is that chlamydiae enter cells by a process analogous to the 'zipper mechanism' proposed by Griffin et al 1976 for

immunoglobulin and complement mediated phagocytosis by macrophages. Following the adhesion of chlamydiae, microfilament mediated movement of the host cell surface binds the cell membrane sequentially to ligands on the chlamydial surface resulting in phagocytosis. However, evidence against a phagocytic uptake mechanism comes from the observation that the microfilament inhibitor cytochalasin B fails to decrease chlamydial infectivity for tissue culture cells (Stirling and Richmond 1977; Gregory et al 1979; Sompolinsky and Richmond 1974; Lee 1981; Kuo C.C. 1978).

An alternative hypothesis is that chlamydiae are ingested in a manner that is independent of microfilament function. Analysis of the binding of polyvalent ligands to cell membranes suggests that invagination of the host cell membrane following particle attachment may be the most thermodynamically stable configuration for the membrane to adopt (Patterson et al 1979). Again it is not clear whether this argument applies to a polyvalent ligand as large as chlamydiae although it has been discussed in relation to influenza virus entry.

A third possibility is that like certain viruses, ingestion is achieved by exploiting the normal mechanism of receptor-mediated endocytosis into clathrin coated vesicles (Schlegel et al 1982; Marsh and Helenius 1980). This process is used by the host cell for the internalization of β -lipoprotein and other physiologically important environmental proteins and is independent of host cell microfilament function (Goldstein et al 1979).

Previous work in this laboratory provided evidence supporting the concept that chlamydial infection of HeLa cells was under bidirectional cyclic nucleotide control (Ward and Salari 1982). A prime objective at the onset of this research was therefore to establish whether interaction of chlamydiae with the host cell membrane during endocytosis, induced alterations in the endogenous levels of host cell cyclic nucleotides and/or prostaglandins. Ward and Salari 1982 also demonstrated that centrifugation of C. trachomatis

onto HeLa 229 cells resulted in increased movement of Ca^{2+} across the host cell membrane. The possible involvement of Ca^{2+} in chlamydial endocytosis was therefore considered in this study. The possibility that Ca^{2+} might interact with host cell Ca^{2+} regulatory proteins to promote chlamydial infection was investigated.

This thesis describes experiments performed to establish the route of chlamydial endocytosis in a HeLa 229 tissue culture cell model.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chlamydiae

Chlamydial strains used were C. trachomatis: L1/440/LN and L2/434/BU. These strains were provided by the Institute of Ophthalmology, University of London, Judd Street, London WC1H 9QS.

2.2 Tissue Culture

The primary isolation of Chlamydia trachomatis organisms in a human cervical epithelium cell line, HeLa 229 cells, has been reported (Kuo et al 1972) and this cell line has proved very susceptible to chlamydial infection in this laboratory. HeLa 229 cells (Flow Laboratories Limited, Irvine, Ayrshire, Scotland, U.K.) were grown in Dulbecco's modified minimal essential medium (DMEM) containing 4 g per L glucose and supplemented with 10% (v/v) foetal calf serum, at 36°C in an atmosphere of 5% (v/v) CO_2 in air. Antibiotics were omitted from routine cell passage to ensure that any mycoplasma or bacterial contamination would rapidly become manifest.

2.3 Monitoring of Tissue Culture Cells for Mycoplasma Contamination

The method was based on the observation in this laboratory that mycoplasmas, when present as a contaminant in HeLa 229 cell tissue culture, grew more profusely in the presence of the alkaloid emetine hydrochloride (Sigma) than in the absence of the drug. Emetine inhibits the 80s ribosome dependent protein synthesis of eukaryotic cells without affecting the 70s ribosome dependent protein synthesis of prokaryotic cells. (Becker and Asher 1972). This selective inhibition favours mycoplasmas which posses 70s ribosomes, in their competition with the HeLa 229 cells for nutrients in the tissue culture medium.

HeLa 229 cells were grown in DMEM on 13 mm diameter No. 1 coverslips (Chance Propper Limited, Smethwick, Warley, England) to produce a confluent monolayer. Following 48 h incubation in DMEM containing 1 μ g per ml emetine hydrochloride the cells were washed with phosphate buffered saline (PBS) and fixed for 10 minutes with absolute methanol. The fixed cells were washed twice with PBS then incubated for 10 minutes at 36°C with a freshly prepared solution of 10 μ g per ml of the fluorochrome Hoechst 33258 (Hoechst U.K. Ltd., Salisbury Road, Hounslow, Middlesex, England) in PBS.

Hoechst 33258, a benzimidazole compound, specifically forms a fluorescent complex with DNA but not RNA by selective binding to the adenine-thymine rich regions of double stranded DNA (Arndt-Jovin and Jovin 1977). Stained material was washed twice with PBS and mounted in 50% (v/v) glycerol in PBS cell surface down on a microscope slide. The preparations were examined with an Ortholux II microscope (E Leitz, Wetzlar, W. Germany) fitted with an HBO 200 ultra violet source and Ploem I incident light illumination. The filters used were: a 4 mm BG 38 in the lamphouse, 2x2 mm UG1 filters for narrow band excitation at 365 nm, a TK 400/k 400 dichroic mirror/supression filter and a K 460 (460 nm) barrier filter. (Salari and Ward 1979).

2.4 Estimation of Chlamydial Particles in Suspension

The number of chlamydial particles per ml in a given inoculum were counted in a Thoma bacterial counting chamber using an Ortholux II microscope equipped with an oil immersion N.A. 1.2 dark ground condenser. Sample dilutions of the chlamydial suspension were prepared in PBS containing 1% (v/v) glutaraldehyde to inactivate the chlamydiae. The average number of chlamydiae per square was calculated from total counts in 100 squares. Two further preparations of the same suspension were made and the mean result taken. Inocula were standardised to 4-8 x 10¹⁰ particles per ml and stored frozen at -196°C.

2.5 Titration of Chlamydial Infectivity in Cell Culture

2.5.1 Quantitation by Inclusion Counting

HeLa 229 cells were grown in DMEM overnight to a confluent monolayer on 13 mm diameter coverslips in 24 well Costar R tissue culture trays (Costar, 205 Broadway, Cambridge, Mass. 02139 U.S.A.). After 18-22 h incubation, monolayers containing approximately 5×10^5 cells, were washed with HBSS (Hanks balanced salt solution). The washed cells were inoculated with 500 μ l of appropriate dilutions of chlamydiae in DMEM. The trays were then placed in microtitre centrifuge carriers (Dynatech) and centrifuged at 1000 g for 1 h, followed by incubation at 36°C. Two hours after centrifugation the infected monolayers were washed in HBSS and incubated in DMEM containing 1 μ g per ml emetine hydrochloride, for 40 to 48 h. The cell monolayers were either stained with Hoechst 33258 as described in section 2.3 or alternatively inclusions were examined by phase or interference microscopy. Chlamydial inclusions in unstained preparations could be identified unambiguously as early as 22 h after infection both by their characteristic morphology and by Brownian movement of chlamydial particles within the cell. Inclusions in stained preparations were counted using a Leitz Ortholux II fluorescent microscope fitted with a counting graticule. Generally, inclusions on replicate (quadruplicate) coverslips were examined and at least 10 fields counted on each coverslip.

2.5.2 Quantitation of Chlamydial Growth by DL-[3 H]-Threonine Incorporation

Chlamydial growth in HeLa 229 cells was assessed by measuring the chlamydia-dependent utilisation of DL-[3 H] threonine (Amersham International) (Allan and Pearce 1982) in threonine deficient DMEM (TD-DMEM); a modification of the method of Ward and Salari 1982. HeLa 229 cells were grown overnight to confluent monolayers in 1.0 ml volumes of DMEM in 24 well tissue culture trays. The monolayers were washed in HBSS and incubated in DMEM containing 1 μ g per ml emetine hydrochloride for 2h at 36°C. After a further wash in HBSS the cells

were inoculated with 500 μ l volumes of either DMEM (uninfected control) or appropriate dilutions of purified chlamydiae. Following centrifugation at 1000 g for 1 h the trays were incubated for 2 h at 36°C to permit chlamydial attachment and uptake. After washing in HBSS to remove non-adherent chlamydiae, 1.0 ml of TD-DMEM containing 1.0 μ g of emetine hydrochloride plus 2 μ Ci(74k Bq) DL-[³H] threonine was added to each well, and the cells incubated at 36°C for 42 h.

To determine DL-[³H] threonine incorporation, the cells were washed 4 times in 5ml HBSS then solubilised in 700 μ l 1% (w/v) sodium dodecyl sulphate (SDS) in 0.1M NaOH at 36°C overnight and the radioactivity determined by liquid scintillation counting. (Section 2.8). Threonine incorporation by uninfected cells in the presence of emetine was typically 10-20% of the incorporation of infected cells. This background was subtracted to give the chlamydial dependent threonine uptake. Preliminary experiments established that >90% of the radioactivity taken up by emetine-treated chlamydia-infected cells was incorporated into protein and could be recovered by precipitation with 5% (w/v) trichloroacetic acid (Salari and Ward, 1981). This chlamydial threonine incorporation was abolished by the addition of the antichlamydial agent rifampicin (Sigma) at 1 μ g per ml to infected cultures immediately after challenge.

2.6 Preparation of Purified Inocula for Large Scale Chlamydial Culture

HeLa 229 cells were grown as monolayers in DMEM in 260 ml disposable tissue culture flasks. The tissue culture medium was decanted and replaced with DMEM containing 1 μ g per ml emetine hydrochloride. After 2 h incubation at 36°C the emetine-containing medium was discarded and the cells were washed in HBSS. After a further wash in HBSS the cells were inoculated with 8 ml of DMEM containing sufficient chlamydiae to infect >90% of the cells. The flasks were centrifuged in swing-out microtitre tray centrifuge carriers at 1000 g for 1 h using an MSE GF8 centrifuge. After 2 h incubation to permit chlamydial adsorption, the cells were washed with HBSS then incubated in 20 ml DMEM containing 1 μ g per ml emetine

hydrochloride for 48 h. Infected cells were washed in PBS, detached by brief incubation at 36°C with 0.125% (w/v) trypsin (Flow Laboratories) in PBS, collected in DMEM containing 10% (v/v) foetal calf serum and pelleted for 5 minutes at 200 g. The cell pellet was resuspended in 5 ml PBS and homogenized with a Dounce homogenizer (Jencons, London) in order to rupture the inclusions but leave the cell nuclei intact. The progress of the homogenization was confirmed by observing small samples of the homogenate using interference contrast microscopy. Nuclei and cell debris were sedimented at 500 g for 5 minutes at 4°C. Two portions of the resulting partially purified chlamydial suspension were overlaid on a discontinuous gradient of 19 to 60% (w/v) Triosil 440 (Nyegaad + Co; Oslo, Norway). Triosil-440 is a dense, non-ionic solution of balanced salts of a tri-iodinated organic acid, metrizoic acid. Triosil-440 is relatively cheap and readily available because of its widespread use as an X-ray contrast medium. It gives excellent separation of chlamydiae from host cell material (Salari and Ward, 1981) and unlike density gradient materials based on colloidal silica (Percoll), is easily removed from purified chlamydiae by washing using an Eppendorf microcentrifuge.

The Triosil gradient (16 ml centrifuge tubes) consisted of a base layer of 2 ml 60% (w/v) Triosil 440 overlaid successively with 3 ml, 3 ml, and 2 ml of 37.5% 25% and 19% (w/v) Triosil 440, respectively. After centrifugation at 100,000 g for 45 min at 2°C in an M.S.E. 6x16 ml titanium swing-out rotor, chlamydial elementary bodies were harvested as a sharp band at the boundary between the 25% and 37.5% (w/v) Triosil. The purified chlamydiae were washed in PBS at 12,000g in a microcentrifuge and suspended in cryoprotective sucrose phosphate buffer (2SP) consisting of 68.46 g sucrose, 2.088 g K₂HPO₄, 1.088 g KH₂PO₄ in 100 ml distilled water, adjusted to pH 7.2. The number of chlamydial particles in suspension was calculated as described in Section 2.4, the inoculum standardised to 4-8 x 10¹⁰ particles per ml and stored frozen at -196°C. Infectivity was determined by titration on HeLa 229 cells (Section 2.5).

2.7 Preparation of Intrinsically Radiolabelled Chlamydiae

HeLa 229 cells were grown to lightly confluent monolayers in DMEM in 260 ml disposable tissue culture flasks. The inoculation procedure was essentially the same as described in Section 2.6 with minor modifications. Following emetine treatment of the cell monolayers, 8 ml of an appropriate dilution of chlamydiae was inoculated onto the cells in TD-DMEM. This medium is not readily available commercially and was therefore prepared in 6 L batches from its individual components (Table 3). After 22 h incubation in TD-DMEM, 160 µl DL-[³H] threonine containing 160 µCi (5.92MBq) was added to each flask and incubated for a further 24-48 h. The cells were washed in PBS, detached from their substratum by brief treatment with 0.125% (w/v) trypsin in PBS at 36°C, collected in ordinary DMEM containing 10% (v/v) foetal calf serum and sedimented at 200 g for 5 min. After homogenization, the radiolabelled chlamydiae were separated from the host cell debris by triosil density gradient centrifugation (Section 2.6) and stored frozen at -196°C in 2SP.

2.8 Liquid Scintillation Counting

Chlamydial infected cells labelled with DL-[³H] threonine (Section 2.5.2) and intrinsically radiolabelled chlamydiae used for attachment (Section 2.11) and uptake (Section 2.12) experiments were solubilised in 1% (w/v) SDS in 0.1M NaOH as described. The radioactivity of the solubilised cells was determined using disposable scintillation vial inserts containing 5 ml of liquid scintillation fluid 'Pico-Fluor TM15' (Packard Instrument Company Inc. 2200 Warrenville Road, Downers Grove, III 60515 U.S.A.) This scintillation medium is based on 1, 2, 4 trimethyl benzol and has a high capacity for aqueous samples with good counting efficiency. Samples were counted in an LKB (Wallac) 1211 Minibeta Liquid Scintillation counter with a typical counting efficiency (for tritium) of 35%. In the absence of significant quenching radioactive counts were expressed as counts per minute.

TABLE 3

Composition of threonine deficient Dulbeccos Minimal
Essential Medium (TD-DMEM)

<u>Compound</u>	<u>mg per L</u>	<u>Source</u>
L - arginine - HCl	84.0	BDH
L - cystine - disodium salt	56.8	BDH
L - glutamine	584.0	BDH
glycine	30.0	BDH
L - histidine HCl	42.0	BDH
L - isoleucine	104.8	BDH
L - leucine	104.8	BDH
L - lysine HCl	146.2	BDH
L - methionine	30.0	BDH
L - phenylalanine	66.0	Sigma
L - serine	42.0	BDH
L - tryptophan	16.0	BDH
L - tyrosine	72.0	Sigma
L - valine	93.6	BDH
D - pantothenate Na salt	4.0	Koch-Light
choline chloride	4.0	BDH
folic acid	4.0	BDH
l - inositol	7.0	Sigma
nicotinamide	4.0	BDH
pyridoxal HCl	4.0	Sigma
riboflavin	0.40	Sigma
thiamin-HCl	4.0	Sigma
sodium pyruvate	110.0	BDH
glucose	450.0	BDH
Fe(No ₃) ₂ · 9H ₂ O	0.10	Hopkin & Williams
KCl	400.0	BDH
NaCl	6400.0	BDH
NaHCO ₃	3700.0	BDH
NaH ₂ PO ₄ · 2H ₂ O	141.3	BDH
CaCl ₂ · 2H ₂ O	265.0	BDH
MgSO ₄ · 7H ₂ O	200.0	BDH
Sodium phenol red	110.0	Sigma

2.9 Protein Quantitation

Proteins were determined by the method of Lowry et al (1951). The first stage of the process is the reaction of peptide bonds with copper to form a copper-protein complex. The intensity of colour is increased greatly in alkaline conditions and is greatest at pH 10. The second stage is the reduction of the yellow Folin-Ciocalteau phenol reagent to give a blue-coloured product. In the absence of copper the reagent oxidises only the phenolic side chain of tyrosine and, to a lesser extent, the indole and imidazole rings of tryptophan and histidine. Although the colour intensity varies with different proteins, and is not exactly proportional to protein concentration, this method is more sensitive than measuring absorbance at 280 nm and more sensitive than a ninhydrin reaction.

Alkaline copper reagent was prepared by mixing 1 ml of 1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ with 1 ml of 2% (w/v) potassium tartrate and adding 1 ml of this mixture to 50 ml of 2% (w/v) Na_2CO_3 in 0.1 M NaOH. 100 μl aliquots of each protein sample were compared with bovine serum albumin (BSA) at 5, 10, 20, 30, 40, 60, 80 μg used as a standard. After 15 minutes incubation at room temperature, 100 μl aliquots of 1M Folin-Ciocalteu's phenol reagent (BDH) were rapidly added to the protein samples and mixed quickly. The tubes were left for 30 minutes at room temperature and read against a water blank at 750 nm in a Unicam SP 1800 spectrophotometer (Pye Unicam Ltd., York Street, Cambridge, England).

2.10 Determination of HeLa Cell Viability and Numbers

Experiments involving the use of potentially cytotoxic drugs or large inocula of chlamydiae required the number and viability of the HeLa cells to be monitored throughout the experiment to ensure that data obtained, was not just a reflection of cell loss or death. Cell monolayers in these experiments were qualitatively assessed by phase contrast microscopy, for evidence of cell loss or morphological change. If there was any evidence of cell loss, treated and untreated (control) cells were fixed for 10 minutes at room temperature with

absolute methanol, washed briefly with PBS then stained for 30 minutes at room temperature with a filtered solution of 0.18% (w/v) Safranin O (BDH colour index 50240) in distilled water. Unbound dye was removed by four successive washes over 15 minutes with distilled water and the stained cells were solubilised with 1% (w/v) SDS in 0.1M NaOH for 30 minutes at 36°C. After careful mixing, the solutions were transferred to 96 well microlitre trays and the O.D. 492nm determined using an automated ELISA spectrophotometer (Titertek ^R Multiskan Flow Laboratories). There was a linear relationship between cell numbers and O.D. 492 within the range 0.1-0.8 with a standard deviation for 8 replicate samples less than 5% of the mean.

Cell viability was monitored using the trypan Blue exclusion test, which depends upon the fact that viable cells do not take up the stain whereas non-viable cells do. Cell monolayers were briefly washed in PBS to remove foetal serum proteins then 500 μL of 0.1% (w/v) trypan Blue (BDH colour index 23850) in PBS was added, and the cells incubated at room temperature for 10 minutes. The number of stained and nonstained cells amongst a total of 500 cells was noted, and the percentage of viable cells calculated.

2.11 Measurement of Chlamydial Attachment to HeLa 229 Cells

Adhesion of [^3H]-labelled chlamydiae to drug treated (test) or untreated (control) cells was compared. HeLa 229 cells were grown in DMEM overnight in 24 well polystyrene tissue culture trays to lightly confluent monolayers. Precise details of cell treatments in individual experiments are given in the results. Generally 3 to 8 replicate wells were used for each experimental variable and each well was inoculated with a standard inoculum of [^3H]-labelled organism contained in 500 μL DMEM, with or without test agent. After incubation for 4 to 6 h at 36°C in moist 5% (v/v) CO_2 in air to permit attachment, each well was washed 4 times with 5 ml volumes of HBSS to remove unbound chlamydiae. The cells were solubilised with 700 μl of 1% (w/v) SDS in 0.1M NaOH for 30 min at 36°C and the radioactivity representing chlamydiae both attached to and ingested by the host cell determined by liquid scintillation counting (Section 2.8). Standard

deviation of replicate counts was less than 3% of the mean counts; attachment to the polystyrene of the tissue culture wells was negligible.

2.12 Measurement of Chlamydial Uptake into HeLa 229 Cells

The method used as a modification of that of Byrne (1978) who showed that chlamydial uptake but not adhesion is prevented at low temperature and that adherent chlamydiae, unlike intracellular chlamydiae, can be removed from the host cell surface by trypsinisation.

HeLa 229 cells were grown overnight to confluence in 24-well tissue culture trays. After washings in HBSS, the monolayers were incubated for the times indicated at 36°C in DMEM, either with or without (control) the agent under test. Precise details of individual treatments are given in the results. The trays were then placed on ice, the supernatant tissue culture medium removed, and 500 µl of ice cold DMEM buffered at pH 7.2 with 30 mM-HEPES was added containing a standardised inoculum of [³H]-labelled organism. The radiolabelled chlamydiae were centrifuged onto the host cells for 1 h at 1000g at 8°C to promote attachment. The supernatant medium was gently replaced with 500 µl of HEPES-buffered ice cold DMEM, the trays were rapidly warmed to 36°C by flotation in a water bath, then incubated for 30 min to permit chlamydial uptake. After washing three times with 5 ml of ice cold PBS, 140 µl of 0.125% (w/v) trypsin (Flow Laboratories) containing 0.01% EDTA in PBS was added to each well and the trays were incubated at 36°C for 15 minutes to detach the cells. The trypsin was inactivated by adding 85 µl of ice cold HBSS containing 20% (v/v) foetal calf serum, the cells were transferred to an ice cooled 96 well U-bottom microtitration tray and sedimented for 5 min at 100 g at 4°C. The supernatant medium was aspirated and the cell pellet solubilised in 150 µl of 1% (w/v) SDS in 0.1M NaOH. The radioactivity of each sample due to intracellular chlamydiae was determined by liquid scintillation counting (Section 2.8).

2.13 Electron Microscopy

Cell monolayers adherent to 24-well tissue culture trays were fixed at 0°C in 1% (v/v) glutaraldehyde in 0.1M cacodylate buffer pH 7.2, post fixed in 1% (w/v) osmium tetroxide in Palades buffer for 1 h at room temperature, washed in cacodylate buffer then postfixed for 30 min at room temperature in 1.5% (w/v) aqueous uranyl acetate. The fixed cells were dehydrated in graded ethanols and the monolayer was removed intact from the substratum by brief treatment with 2-epoxy propane (BDH) and flat-embedded in Spurr low viscosity epoxy resin (Taab Laboratories, Reading, Berks). Trypsinised cell suspensions were processed in 1.5 ml microcentrifuge tubes and embedded in 1.5% (w/v) Ionagar No 2 (Oxoid) in distilled water after fixation prior to dehydration and embedding. Some cell monolayers were stained with 1% (w/v) colloidal thorium hydroxide (Polysciences, Moulton Park, Northampton) in 3% (v/v) acetic acid pH 2.6 for 24 h at room temperature after glutaraldehyde fixation, to delineate the host cell glycocalyx (Ward et al 1975). At pH 2.6, colloidal thorium binds to ionised sialic acid and carboxyl groups in the glycocalyx. This permitted a distinction to be made between chlamydiae which were genuinely intracellular and chlamydiae which were sitting in transversely sectioned invaginated cups in the host cell surface. In the former case, no colloidal thorium particles would be present on the endosomal membrane because colloidal thorium does not penetrate intact cell membranes. In the latter case particles of colloidal thorium adherent to the glycocalyx would be apparent on host cell membrane surrounding the chlamydiae.

Other monolayers were fixed with tannic acid to accentuate clathrin (Aggeler and Werb 1982). In some experiments purified horse ferritin (Miles Labs. Stoke Poges, Bucks) or horse raddish peroxidase (Sigma grade VI) at 1 mg per ml were added to DMEM as a tracer during chlamydial uptake. Peroxidase was demonstrated histochemically with diaminobenzidine (Graham and Karnovsky 1966) as substrate after minimal fixation of the cells for 15 min at room temperature with 0.1% (v/v) glutaraldehyde in 0.1M phosphate pH 7.2. Preparations were sectioned with a diamond knife, stained with Reynold's lead citrate as

necessary and examined with a Philips EM 300 transmission electron microscope at 40 to 100 Kv.

2.14 Data Processing

Statistical analysis of experimental data was performed using a programme of 14K bytes written in this laboratory for a Commodore 4032 microcomputer (Commodore Computers). This programme subtracts appropriate background from replicate radioactive count data and prints the mean standard deviation and 95% confidence interval for the data. A menu then permits disc filing of the data, t testing and corresponding P values for each column of replicates compared against every other column, 2 way analysis of variance within replicates and between columns, and the option of fitting the data to linear or polynomial models. Much of the programming was based on algorithms published by Cooke, Craven and Clarke 1982 from which test data to verify the accuracy of the programme was also obtained.

2.15 Assay of Cyclic Nucleotides Using Tritiated Label

2.15.1 Assay for Adenosine 3',5'-Cyclic Monophosphate (cAMP)

2.15.1.1 Principle of the Method

The assay is based on the competition between unlabelled cAMP (standard or unknown) and a fixed quantity of the tritium labelled compound for binding to a bovine adrenal protein which has a high specificity and affinity for cAMP (Brown et al 1971). The amount of labelled cAMP-protein complex formed is inversely related to the amount of unlabelled cAMP present. Separation and quantitation of the bound from free cAMP is achieved by adsorption of the free nucleotide onto coated charcoal, followed by liquid scintillation counting of the supernatant, protein-bound [3 H]-cAMP. The concentration of unlabelled cAMP is then calculated from a standard curve.

2.15.1.2 Preparation of cAMP Binding Protein

Bovine adrenals, freshly collected from a Slaughter house 20 miles away, were transported on ice to the laboratory. The adrenal cortices were separated, chopped and homogenized in a tissue emulsifier (Silverson Machines Ltd., London SE1 U.K.) with 1.5 volumes of an ice cold medium comprising 0.25M sucrose, 50 mM Tris-HCl buffer, pH 7.4, 25mM KCl and 5 mM MgCl₂. After centrifugation of the homogenate at 1000 g for 5 minutes, the supernatant was respun at 7000 g for 15 minutes. The supernatant containing the crude binding protein was stored at -20°C. Each batch of binding protein was standardised by preparing a series of dilutions (1/5 to 1/25) in 50 mM Tris buffer pH 7.5 containing 4 mM EDTA (Fig. 2). That dilution of binding protein which bound 20-30% of a standard amount of [³H]-cAMP (10,000 cpm) was used for subsequent assays.

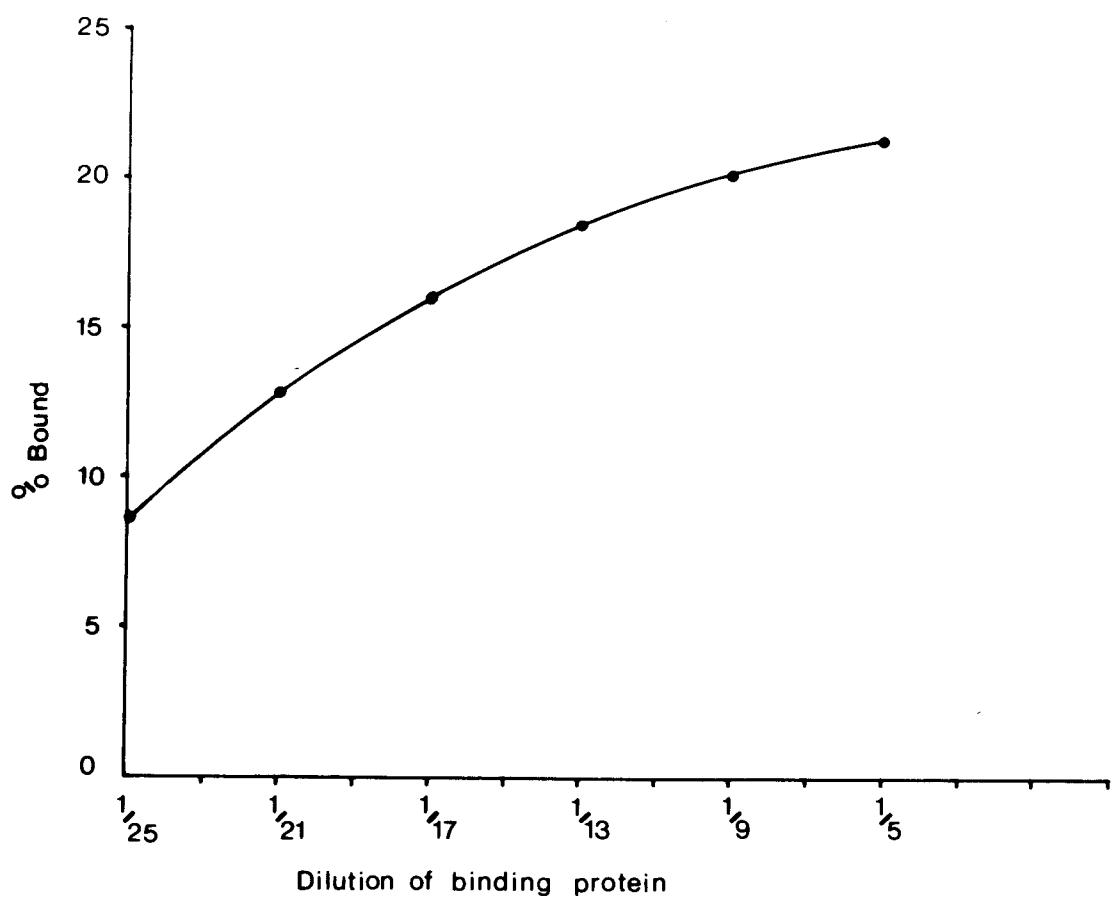
2.15.1.3 Reagents and Sample Preparation

[2, 8 ³H] Adenosine 3'5'-cyclic phosphate Ammonium salt, (Amersham International, 37 Ci per mmol, 250 μ Ci in 250 μ l) was diluted in 50 mM Tris buffer pH 7.5 containing 4 mM EDTA (Tris-EDTA buffer) so that 10 μ l contained a standard 10⁴ c.p.m. The charcoal solution for separating free from bound cyclic nucleotide was prepared by adding 500 mg Norit GSX charcoal (Hopkins and Williams) to 50 mg Bovine serum albumin fraction 4 (Sigma) in 25 ml Tris-EDTA buffer. The charcoal was kept on ice and maintained in suspension by gentle stirring.

A stock solution of 1 mg per ml cAMP (sodium salt)(Sigma) was prepared in distilled water, aliquoted in 100 μ l volumes and kept frozen at -20°C. Prior to each assay, an aliquot was thawed and diluted 1 in 10,000 in Tris-EDTA buffer, to contain 15.2 pmoles per 50 μ l. Doubling dilutions were prepared to contain 7.6, 3.8, 1.9 and 0.95 pmol per 50 μ l.

To minimise changes in cyclic nucleotide levels during sampling, the cells were rapidly washed in PBS at 36°C, and 70% ethanol at 80°C was added to the monolayer to extract cyclic nucleotide and to

Figure 2 Standardisation of bovine adrenal binding protein concentration. A series of dilutions of binding protein were prepared in 50 mM Tris buffer pH 7.5 containing 4 mM EDTA. Duplicate 100 μ l dilutions (1/5 to 1/25) were incubated on ice for 2 h with 10 μ l of [3 H]-cAMP (10^4 cpm) and 200 μ l Tris-EDTA buffer pH 7.5. Following separation of bound from free [3 H]-cAMP using dextran coated charcoal the percentage of radiolabelled cyclic nucleotide bound to the protein was plotted against the dilution of protein on linear graph paper. That dilution which bound 20-30% of the [3 H]-cAMP was used for subsequent assays. Thus a 1 in 5 dilution was used of this particular batch of binding protein.



inactivate any degradative phosphodiesterase present. After overnight incubation at 4°C the ethanolic sample was removed and evaporated to dryness at 36°C in a conical container. The samples were either assayed immediately or sealed and stored dry at -20°C.

To assay, the dried extracts were shaken in Tris-EDTA buffer, at 36°C for 1 h then centrifuged to remove any insoluble debris. The supernatant was diluted in Tris-EDTA buffer and assayed directly.

2.15.1.4 Assay Procedure

Tubes and reagents were kept on ice throughout the assay and their contents are shown in Table (4). After addition of the binding protein, the tubes were mixed thoroughly and incubated for 2 h. The charcoal suspension was then added, the tubes were mixed and centrifuged in a refrigerated centrifuge for 5 minutes at 700 g to sediment the charcoal. Without disturbing the sediment, a known volume of supernatant was removed and its radioactivity determined by liquid scintillation counting (Section 2.8).

2.15.1.5 Calculations

The mean c.p.m. in tubes 1 and 2 (Table 4) was determined to obtain the non specific counts bound to the assay tubes. This value was subtracted from all other tubes.

The mean c.p.m. in tubes 3 and 4 was determined so as to give the c.p.m. bound in the absence of unlabelled cAMP (Co).

The remaining mean c.p.m. for each pair of duplicates was determined to give the c.p.m. bound in the presence of standard or unknown cAMP (Cx).

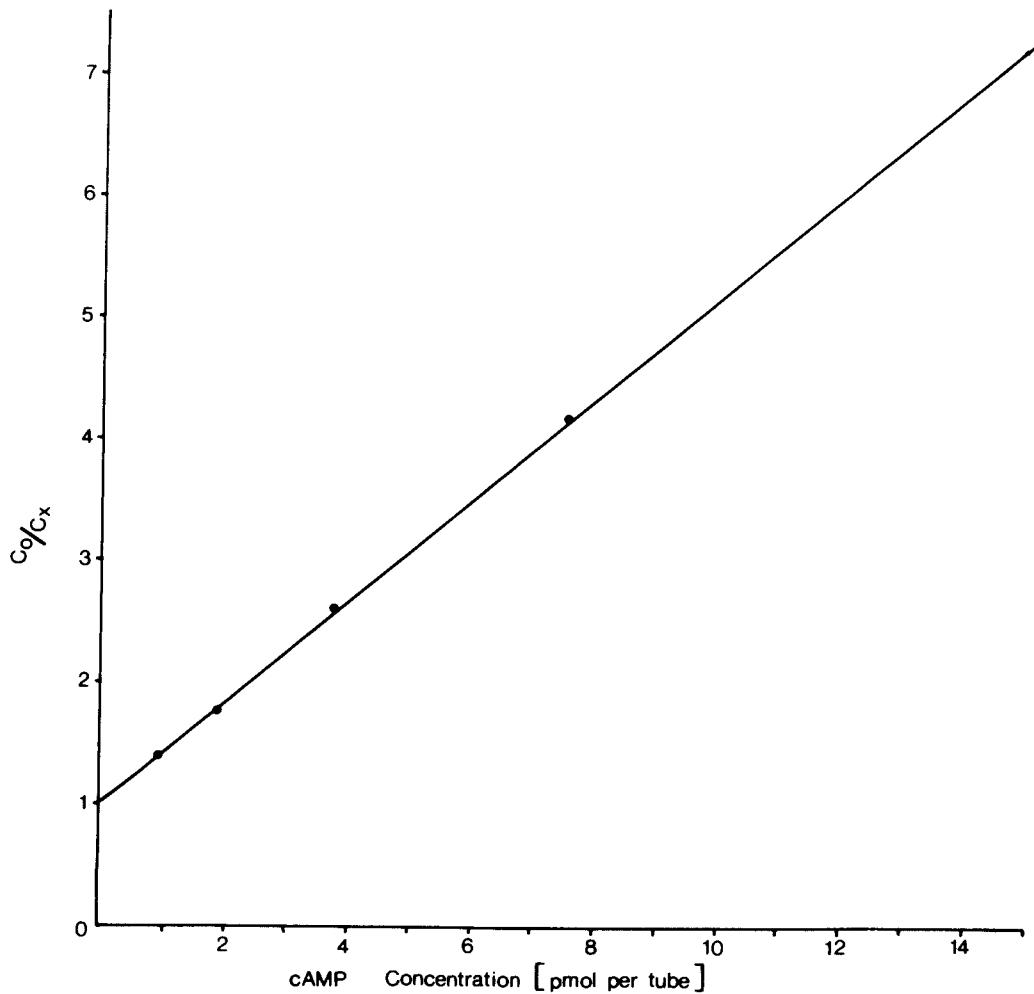
The Co/Cx was calculated for the standard cAMP samples and plotted against their concentration per tube on linear graph paper.

TABLE 4 cAMP assay protocol (adrenal binding protein assay)

Tube No.	Tris-EDTA buffer pH 7.5	Standards	Unknowns	[³ H]-cAMP	Binding protein
1,2	300	-	-	10	- Charcoal blank
3,4	200	-	-	10	100 Zero dose
5,6	150	50	-	10	100 15.2 pmol standard
7,8	150	50	-	10	100 7.6 pmol standard
9,10	150	50	-	10	100 3.8 pmol standard
11,12	150	50	-	10	100 1.9 pmol standard
13,14	150	50	-	10	100 0.95pmol standard
15,etc.	150	-	50	10	100 unknowns

All volumes are in microlitres.

Figure 3 A typical cAMP standard curve (binding protein assay). The mean cpm of [^3H]-cAMP bound in the absence of unlabelled cAMP is calculated to give (C_0). The binding of [^3H]-cAMP in the presence of increasing amounts of unlabelled cAMP is then calculated (C_x), all counts being corrected for background. C_0/C_x (ordinate) is plotted on linear graph paper against the concentration of cAMP per tube (abscissa) giving a straight line with an intercept of 1.0 on the ordinate.



The Co/Cx value for the unknowns was calculated on the corresponding number of pmoles of cAMP per tube from the standard curve (fig 3).

2.15.2 Assay for Guanosine 3'5'-cyclic Monophosphate (cGMP)

2.15.2.1 Principle of the Method

The assay is commercially available as a Kit from Amersham International and is based on the competition between unlabelled cGMP (standard or unknown) and a fixed quantity of the tritium labelled compound for binding to an antiserum which has high specificity and affinity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Separation of the antibody-bound cGMP from the unbound nucleotide is achieved by ammonium sulphate precipitation, followed by centrifugation. The precipitate containing the antibody-bound complex is dissolved in water and its activity determined by liquid scintillation counting. The concentration of unlabelled cGMP is then calculated from a linear standard curve.

2.15.2.2 Reagent and Sample Preparation

Reconstitution of freeze dried reagents.

1. Tris-EDTA buffer - 25 ml of distilled water was added to give a 0.05M Tris solution at pH 7.5 containing 4 mM EDTA.
2. Antiserum - the reagent was reconstituted with 7.5 ml of distilled water.
3. [8-³H] Guanosine 3'5' cyclic phosphate - the reagent was reconstituted with 10 ml of distilled water.
4. Guanosine 3'5' cyclic phosphate standard - 5 ml of distilled water was added to give a solution containing 8 pmol cGMP per 100 μ l. Doubling dilutions were made of this to give 4, 2, 1 and 0.5 pmol per 100 μ l.
5. Reagent blank - 2 ml of distilled water was added. This solution completely displaces tritiated cGMP from its antibody binding sites.

6. Ammonium sulphate - 150 ml of distilled water was added to yield a 60% saturated solution.
7. Preparation of cells for assay - samples were prepared and extracted for assay as described in Section 2.15.1.3.

2.15.2.3 Assay Procedure

All tubes and reagents were maintained on ice throughout the assay. Contents of the tubes are shown in Table 5. After the addition of the antiserum, the tubes were mixed thoroughly and incubated for 90 minutes. $(\text{NH}_4)_2\text{SO}_4$ was then added, followed by mixing and incubation for 5 minutes to permit precipitate formation. After centrifugation at 700 g for 10 min at 4°C the supernatant fluid was discarded, the pellet was dissolved in distilled water and the radioactivity was determined by liquid scintillation counting (Section 2.8).

2.15.2.4 Calculations

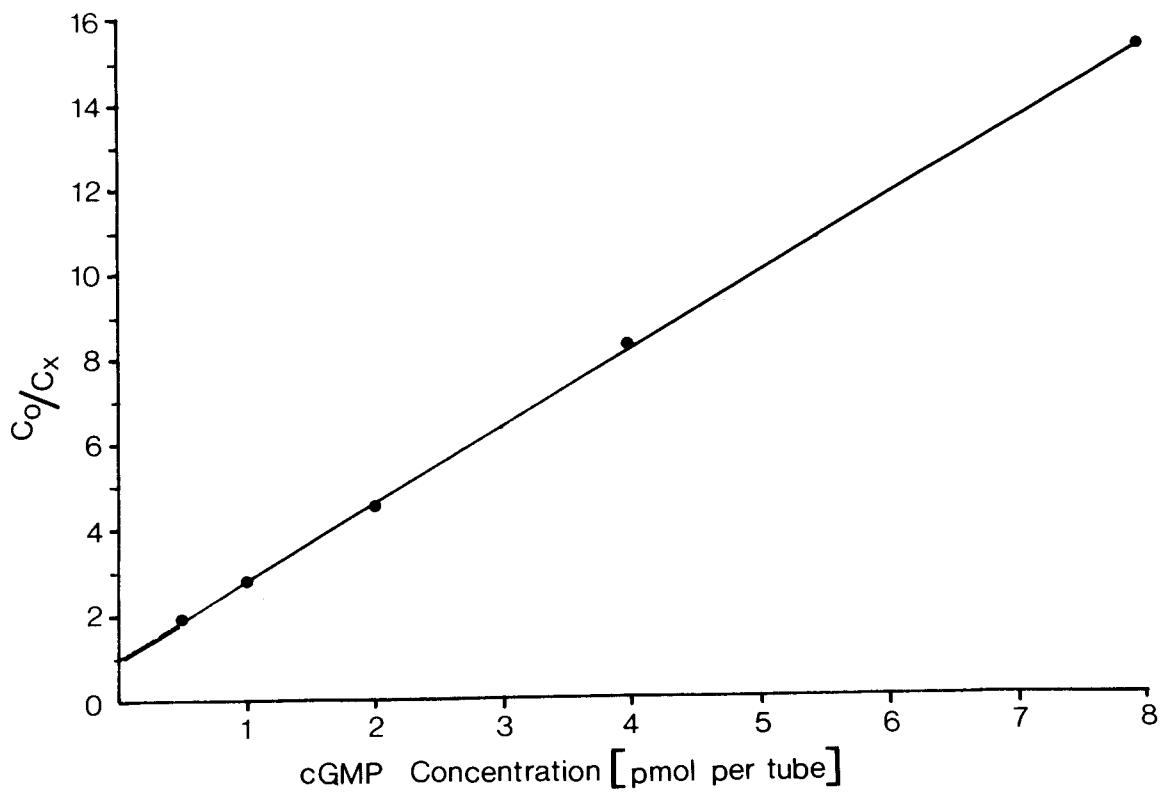
The mean c.p.m. in tubes 13 and 14 was determined to obtain the background c.p.m. due to non specific counts bound to the assay tubes. This value was subtracted from all other counts. The mean c.p.m. in tubes 1 and 2 was determined to give the counts bound in the absence of unlabelled cGMP (Co). The c.p.m. for each pair of duplicates was determined to give the c.p.m. in the presence of standard or unlabelled unknown cGMP (Cx). The Co/Cx was calculated for each of the assay samples of known (reference) or unknown cGMP content. The Co/Cx of reference samples was plotted as before against the concentration of cGMP per tube on linear graph paper. The Co/Cx value for the unknown samples was calculated and the number of picomoles of cGMP per tube determined by reference to the standard curve (see fig 4).

TABLE 5 cGMP assay protocol ($[^3\text{H}]$ -based R.I.A. method)

Tube No.	[^3H]-cGMP	Tris-EDTA buffer pH 7.5	Standards	Unknowns	Reagent blank	Antiserum
1,2	50	100	-	-	-	50 Zero dose
3,4	50	-	100	-	-	50 0.5 pmol standard
5,6	50	-	100	-	-	50 1.0 pmol standard
7,8	50	-	100	-	-	50 2.0 pmol standard
9,10	50	-	100	-	-	50 4.0 pmol standard
11,12	50	-	100	-	-	50 8.0 pmol standard
13,14	50	-	-	-	100	50 Blank
15,etc.	50	-	-	100	-	50 Unknowns

All volumes are in microlitres.

Figure 4 A typical cGMP standard curve. ($[^3\text{H}]$ -based R.I.A. method). The derivation of the cGMP standard curve is identical to that described for cAMP in Fig. 3.



2.16 Radioimmunassay of Cyclic Nucleotides Using Iodinated Label

2.16.1 Assay for Adenosine 3',5'-cyclic Monophosphate (cAMP)

2.16.1.1 Principle of the method

Greatly increased sensitivity in cyclic nucleotide radioimmunassay (RIA) can be achieved using ^{125}I and formed the basis of most of the assay results described in Chapter 3.

The basic principle of the method is again the competition between unlabelled cAMP (standard or unknown) and a fixed quantity of an iodinated labelled derivative (tyrosyl methyl ester) of cAMP for binding to an antiserum with a high specificity and affinity for cAMP. The amount of labelled cAMP bound to the antiserum is inversely related to the amount of cAMP present in the assay sample. Antibody-bound cAMP is separated from the free nucleotide by Polyethylene glycol precipitation in the presence of an excess of carrier protein (bovine gamma globulin). The activity of the precipitate, which contains the antibody-bound complex, is determined by counting in a gamma counter. Sensitivity of the assay can be further increased by acetylation of the known and unknown cyclic nucleotide with acetic anhydride prior to assay. The structure of the iodinated derivative of cAMP and the reaction scheme for the acetylation of cAMP is shown in figures 5 and 6 respectively.

2.16.1.2 Preparation of Specific cAMP Antisera

Antiserum was purchased from Miles-Yeda (Kiryat Weizmann, Rehovot Israel), and was a selected single bleed from a rabbit immunized with repeated injections of 2'0 succinyl-cAMP conjugated to bovine serum albumin. Each ampoule contained freeze dried antiserum sufficient for 100 tubes and an inert carrier, polyvinylpyrrolidone.

2.16.1.3 Preparation of Iodinated Derivative of cAMP

The method for iodination was adapted from Hunter and Greenwood 1962. A 1 mg per ml solution of chloramine T (Fisons Scientific

Figure 5 Structure of Adenosine 3'5' cyclic phosphoric acid
[¹²⁵I]-2'-O succinyl iodotyrosine methyl ester.

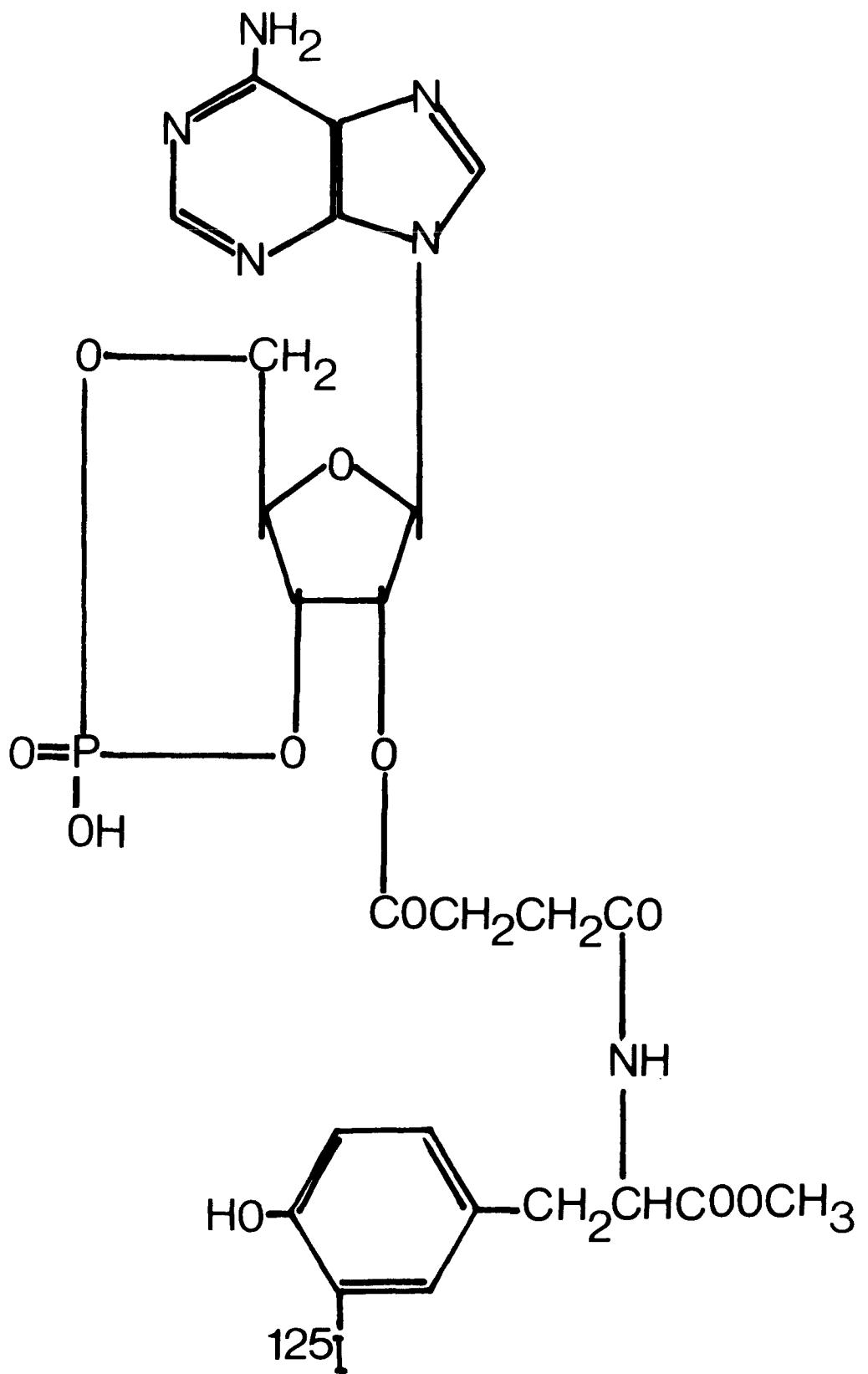
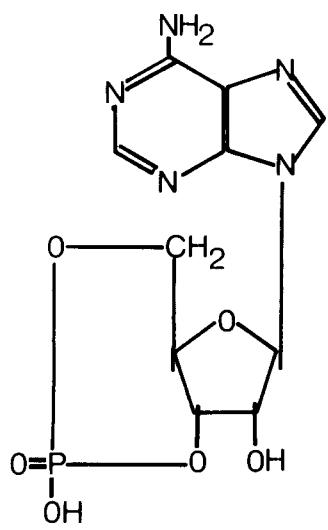
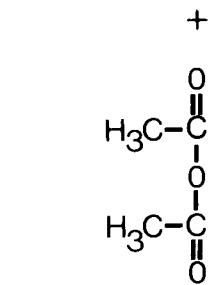
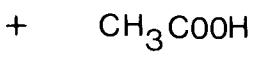
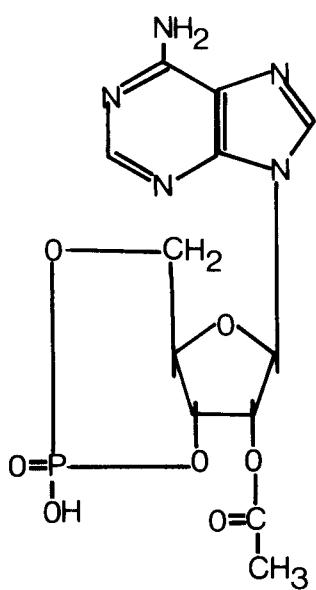


Figure 6 Acetylation of Adenosine 3'5' cyclic monophosphate. cAMP (A) in 0.05M sodium acetate buffer pH 6.2 was acetylated at the 2'0-position using acetic anhydride to yield 2'-0-acetylated cAMP (B). Sufficient triethylamine was provided to make the solution basic in order to catalyze the acetylation, and to neutralize excess acetic acid resulting from hydrolysis of acetic anhydride. Thus, the final solution is acidic, a condition in which the acetyl cyclic nucleotide is relatively stable.

A



B



Apparatus, Loughborough, Leics Eng.) and a 0.6 mg per ml solution of sodium metabisulphite (B.D.H.) were prepared in 0.1 M sodium phosphate buffer pH 7.5. 2.5 μ l of sodium metabisulphite solution was added to 50 μ l 1M sodium phosphate buffer pH 7.5 containing 2.5 nanomoles of 0^{2'} monosuccinyl adenosine 3'5' cyclic monophosphate tyrosyl methyl ester (Sigma). 0.5mCi Na¹²⁵I (Amersham International) was then added followed by 10 x 1 μ l volumes of Chloramine T at 3 second intervals. The reaction was terminated by the addition of 25 μ l of metabisulphite solution followed by 250 μ l of 0.1 M Citrate buffer pH 6.2 containing 0.5 g per L sodium azide.

Separation of the unlabelled ester and its labelled derivatives was achieved using a 0.9 x 60 cm G25 Sephadex Column with 0.1 M citrate buffer pH 6.2 containing 0.5 g per L sodium azide as the eluting buffer. 10 μ l samples were taken from each 4 ml fraction and counted in an LKB 1280 Ultragamma gamma counter. The elution profile is shown in Fig 7.

The monoiodinated derivative was pooled, 2 g per litre of human serum albumin added as stabiliser, and the material aliquoted such that each tube contained 2 x 10⁶ c.p.m. Aliquots were stored frozen at -20°C for a maximum of 2 months.

2.16.1.4 Reagents and Sample Preparation

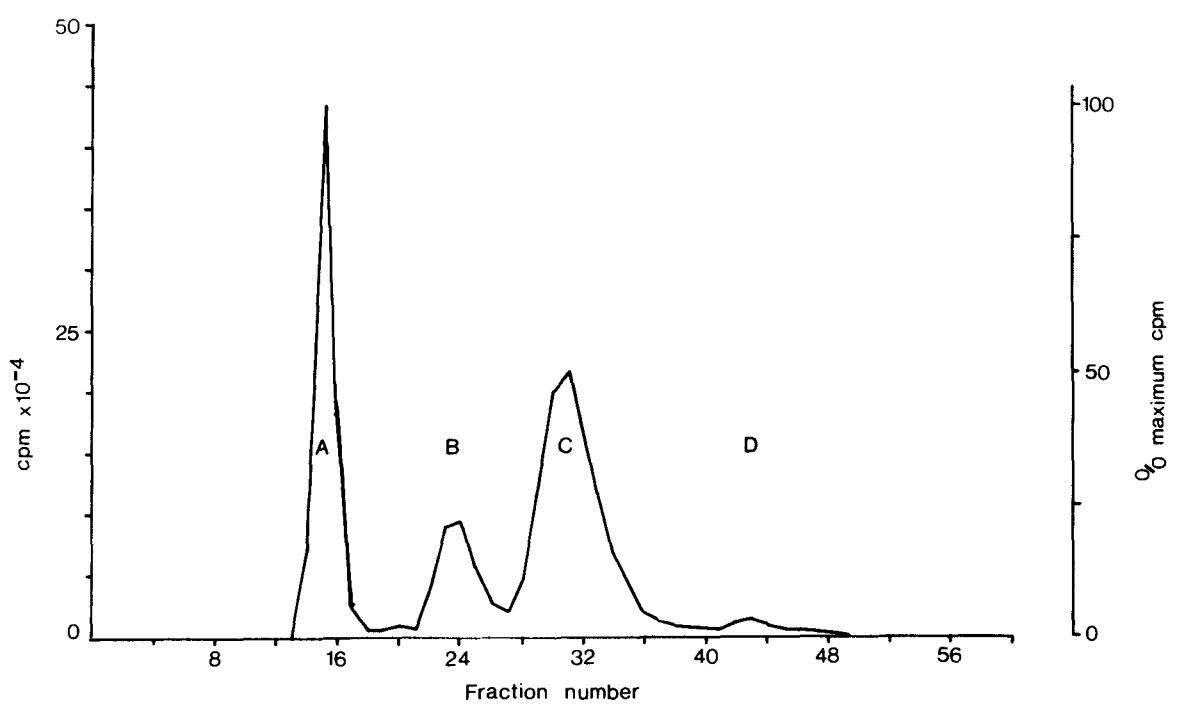
1. 0.05 M Sodium Acetate Buffer pH 6.2

4.1 g sodium acetate was dissolved in 1 L distilled water and the pH adjusted to 6.2 with acetic acid.

2. cAMP Antiserum

A freeze dried ampoule of antisera (section 2.16.1.2) was reconstituted in distilled water containing 0.1% (w/v) B.S.A. as required. Unused antiserum was snap frozen in liquid N₂ and stored at -20°C.

Figure 7 Elution profile from a G25 sephadex column of the radiolabelled derivatives following iodination of succinyl cAMP tyrosine methyl ester (Suc. cAMPTME). 2.5 nmoles of suc.cAMPTME was iodinated with Na^{125}I by the chloramine T method of Hunter and Greenwood 1962. The iodination reaction was terminated with sodium metabisulphite. The reaction mixture was applied to a 0.9 x 60 cm G25 Sephadex column and eluted with 0.1 M citrate buffer pH 6.2. Peaks A, B, C and D represent $^{125}\text{I}^-$, iodosuccinyl tyrosine methyl ester, mono [^{125}I]-succinyl cAMPTME and di[^{125}I]-succinyl cAMPTME respectively.



3. [I¹²⁵] - Succinyl cAMP Tyrosine Methyl Ester

Aliquots sufficient for 100 tests (section 2.16.1.3) were rapidly thawed and made up to 10 ml in 0.05M sodium acetate buffer, pH 6.2.

4. Standard cAMP

A 0.2 μ M stock solution of cAMP (sodium salt) (Sigma) was prepared in distilled water, and frozen at -20°C. Working solutions containing between 200 and 3.125 femtomoles of cAMP per 100 μ l were prepared by diluting the stock solution in 0.05 M sodium acetate buffer, pH 6.2.

5. Acetylation Reagent

A solution consisting of 1 volume acetic anhydride(BDH Analar) plus 2 volumes triethylamine (BDH Analar) was made immediately before use.

6. Polyethylene Glycol (P.E.G.)

A 16% (w/v) solution of P.E.G. 6000 (Hopkins and Williams) was prepared in 0.05 M sodium acetate buffer, pH 6.2.

7. Gamma Globulin Carrier Protein

A 20 mg per ml of bovine gamma globulin Cohn fraction II(Sigma) was prepared in 0.05 M sodium acetate buffer pH 6.2.

8. Preparation of Samples for Assay

Dried extracts were prepared as described in Section 2.15.1.3. Prior to assay the extracts were resuspended in 0.05 M sodium acetate buffer pH 6.2 and shaken at 36°C for 1 h. Insoluble material was removed by centrifugation, and the resultant supernatant was assayed following acetylation.

2.16.1.5 Assay Procedure

Prior to assay, the standards and unknowns contained in 100 μ l of 0.05 M sodium acetate buffer were acetylated by introducing 5 μ l of acetylating reagent under the surface of each sample and immediately mixing for 1-2 seconds. The tubes were then kept on ice throughout the assay. Following the addition of the antiserum, the tubes were mixed well and incubated for 18 h (Table 6).

Separation of the antibody-bound cAMP from the free nucleotide was achieved by adding 1 ml of 16% (w/v) P.E.G. 6000 and 50 μ l bovine gamma globulin carrier protein to each tube. The tubes were mixed and allowed to stand for 20 minutes, centrifuged for 2 min at 12,000 g in an eppendorf centrifuge, and the radioactivity in the pellets determined by gamma counting. Counting efficiency for I^{125} was 70%.

2.16.1.6 Calculations

The mean cpm in tubes 3 and 4 was determined. This represents the radioactivity which cannot be aspirated from the tubes and also includes the instrument background count. This figure was deducted from the gross mean counts of all tubes to give the net mean cpm. The net mean counts of each standard and unknown (B) was expressed as a percentage of the net mean counts of tubes 5 and 6, due to radioactivity bound to antibody in the absence of added cAMP. (B^o):

$$\% \frac{B}{B^o} = \frac{\text{mean net counts (standards or unknown)}}{\text{mean net counts of tubes 5 \& 6}} \times 100$$

The $\% \frac{B}{B^o}$ was plotted for each standard against the corresponding concentration of cAMP per tube on log logit graph paper (fig.8). The concentration of cAMP in each unknown was determined by interpolation from the standard curve.

2.16.2 Assay for Guanosine 3'5'-Cyclic Monophosphate (cGMP)

2.16.2.1 Principle of the Method

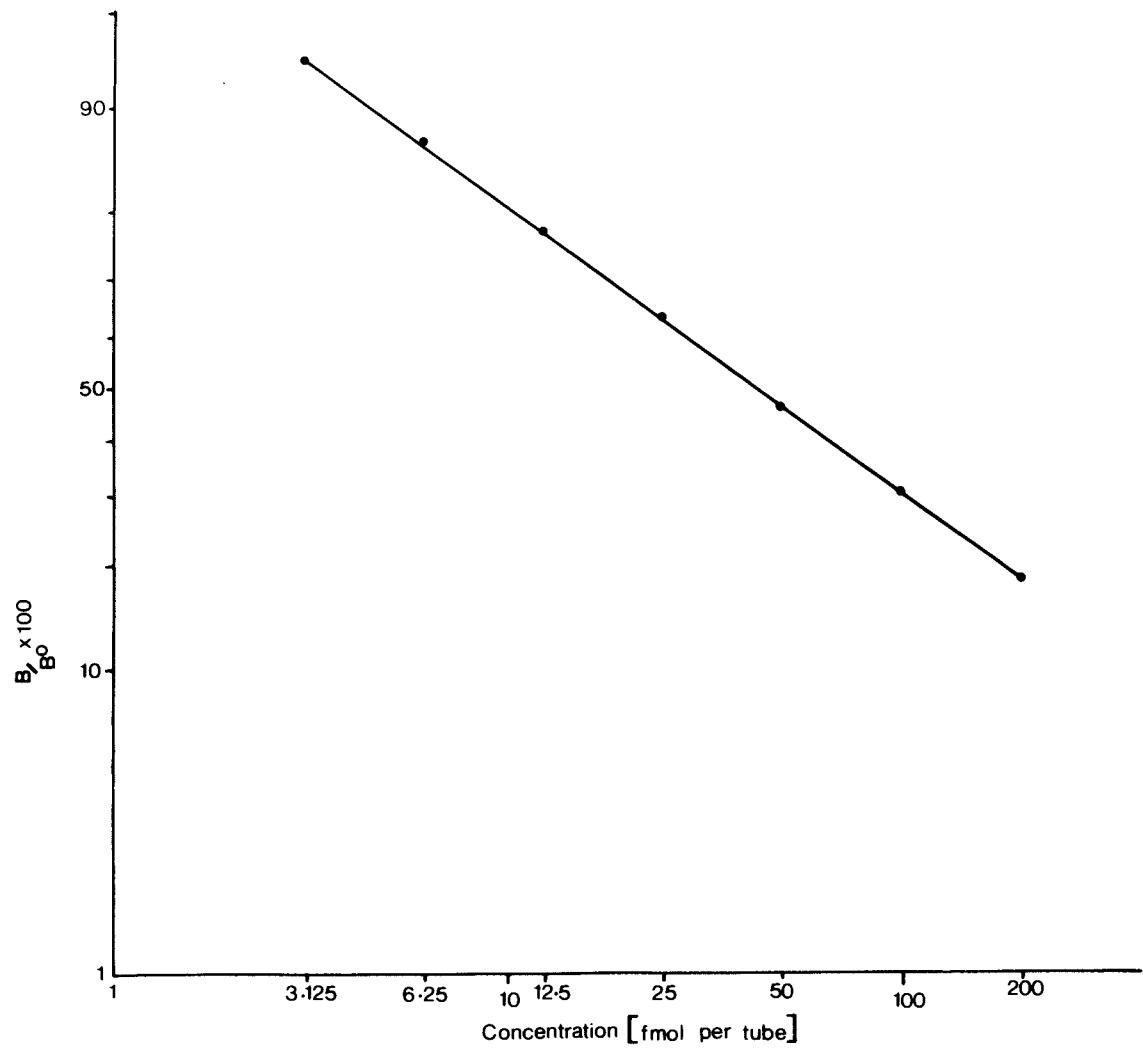
The principle of this assay is similar to that for cAMP (see

TABLE 6 [I¹²⁵] Radioimmunoassay protocol

Tube No.	sodium acetate buffer pH 6.2	acetylated sample or standard	[I ¹²⁵]-labelled derivative	antibody	
1,2	-	-	100	-	Total counts
3,4	200	-	100	-	non specific binding
5,6	100	-	100	100	zero dose
7,8	-	100	100	100	200 fmole standard
9,10	-	100	100	100	100 fmole standard
11,12	-	100	100	100	50 fmole standard
13,14	-	100	100	100	25 fmole standard
15,16	-	100	100	100	12.5 fmole standard
17,18	-	100	100	100	6.25 fmole standard
19,20	-	100	100	100	3.125 fmole standard
21, etc.	-	100	100	100	unknowns

All volumes are in microlitres.

Figure 8 A typical standard curve for cAMP and cGMP radioimmunoassay using iodinated label. The binding of [I^{125}]-cAMPTME or [I^{125}]-cGMPTME in the absence of unlabelled cyclic nucleotide is taken as 100% (B_0). Binding of iodinated derivative in the presence of increasing amounts of unlabelled cyclic nucleotide (B) is expressed as a percentage of B_0 , all values being previously corrected for background. $B/B_0 \times 100$ (ordinate) is plotted on a LOGIT scale against the concentration of cyclic nucleotide (abscissa) on a log scale, each concentration being assayed in duplicate.



section 2.16.1.1) but sensitivity is even more crucial because of the much lower levels of cGMP present in cells.

2.16.2.2 Preparation of Specific Antisera

Antisera raised against bovine serum albumin - 2'-O-Succinyl cGMP was a kind gift from Dr. P.J. Wood, Department of Chemical Pathology, Southampton General Hospital. The method for its production was briefly as follows:

2'-O-Succinyl cGMP was conjugated to Bovine Serum using the carbodiimide 'EDC' (1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl) (Steiner et al 1972). The conjugate consisted of six molecules of cGMP per molecule of bovine serum albumin from spectrophotometric data and was stored freeze dried in aliquots each containing 1 mg protein. Four half-lop rabbits (Ranch Rabbits Ltd) each received a suspension of 0.25 mg conjugate in complete Fruend adjuvant injected at multiple sites subcutaneously.

Booster injections were performed at monthly intervals and bleeds were tested by incubating a 60 fold dilution of the antiserum with a standard amount of [I^{125}]-cGMP. When the ratio of bound to total radioactivity exceeded 70% the antiserum was deemed satisfactory and the rabbit bled three times a week.

The antiserum chosen for [I^{125}]-cGMP RIA, (G3(X111)24/12) was aliquoted and freeze dried, each aliquot containing 12.5 μ l antiserum, 11.3 μ l non immune rabbit serum, 1.13 mg Lactose and 1.25 ml 50 mM Tris buffer pH 7.5 containing 5 mM EDTA.

2.16.2.3 Preparation of Mono-iodinated Derivative of cGMP

The method of preparation was identical to the preparation of iodinated derivative of cAMP (Section 2.16.1.3). The derivative used was $0^{2'}$ monosuccinyl guanosine $3'5'$ cyclic monophosphate tyrosyl methyl ester (Sigma). The elution profile of the iodinated derivatives from the G25 sephadex column is shown in Fig. 9. The

Figure 9 Separation of iodinated mono [^{125}I]-suc.cGMPTME from reaction products on sephadex G25. The iodination was performed as described for Fig. 6. Peaks A, B, C and D are: $^{125}\text{I}^-$, iodosuccinyl tyrosine methyl ester, mono [^{125}I]-succinyl cGMPTME and di[^{125}I]-succinyl cGMPTME respectively.

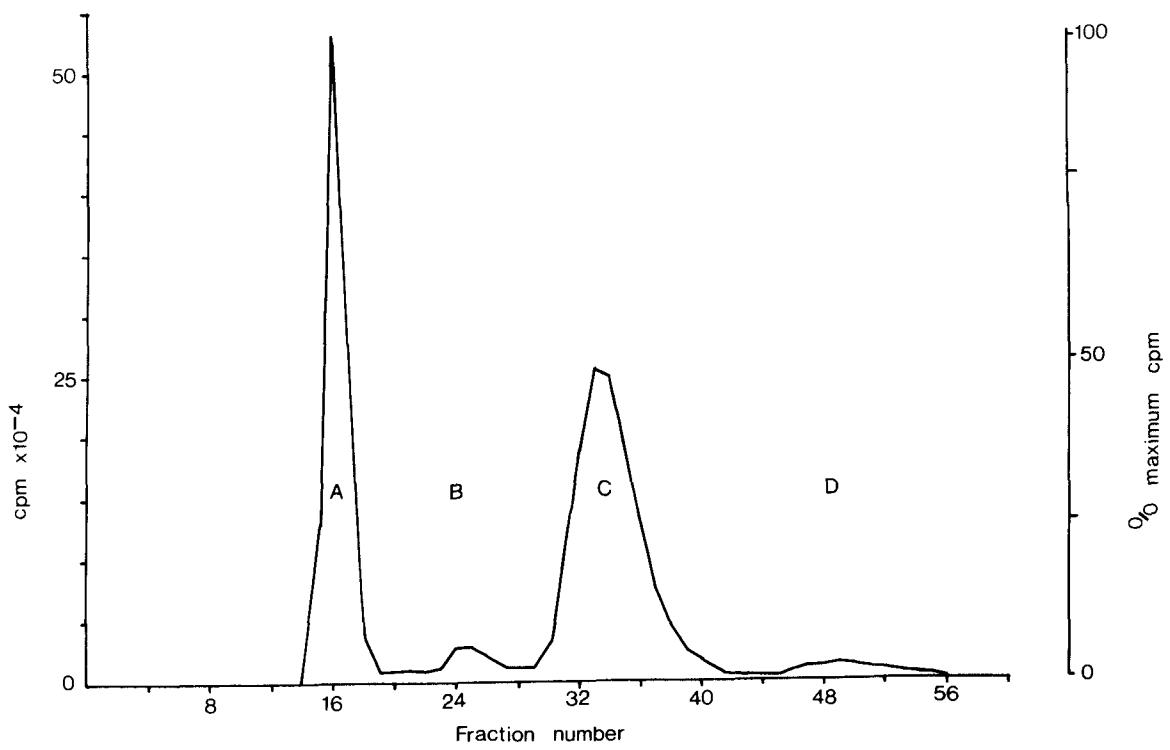


Figure 10 Structure of Guanosine 3'5' cyclic phosphoric acid
[¹²⁵I]-2'-O succinyl iodotyrosine methyl ester.

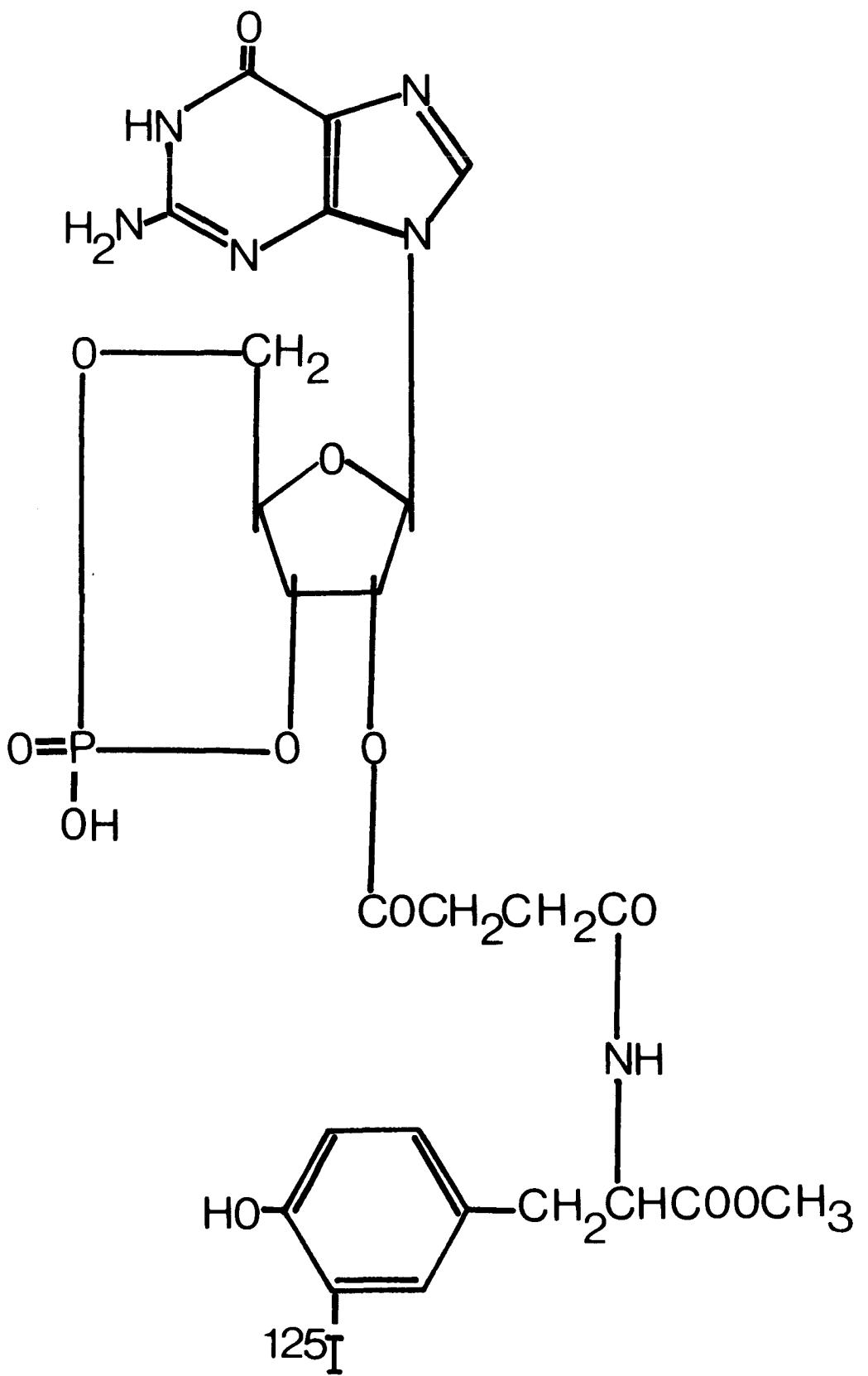
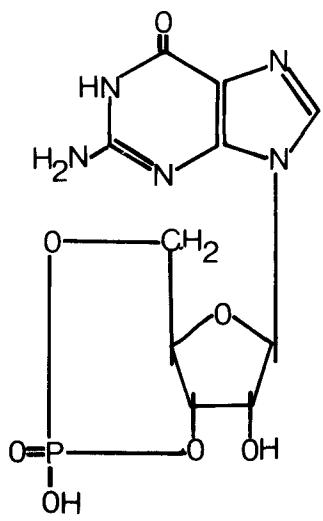
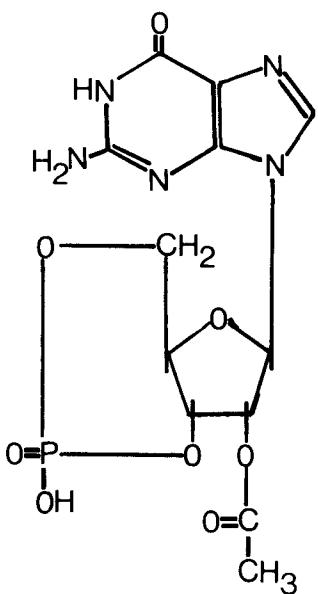


Figure 11 Acetylation of Guanosine 3'5' cyclic monophosphate. The reaction scheme for the acetylation of cGMP(A) is the same as for cAMP, described in Fig. 6. The product of the reaction, 2'-O-acetylated cGMP(B) has a higher affinity for the antibody than the unsubstituted cyclic nucleotide and therefore displaces the iodinated derivative more effectively; resulting in increased sensitivity of the assay.

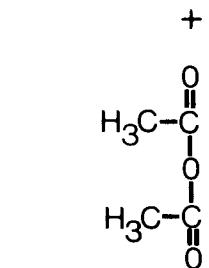
A



B



+ CH₃COOH



structure of the iodinated derivative of cGMP and the reaction scheme for the acetylation of cGMP are given in figures 10 and 11 respectively.

2.16.2.4 Reagents and Sample Preparation

1. 0.05 M sodium acetate buffer pH 6.2. As section 2.16.1.4

2. cGMP antiserum

A freeze dried ampoule sufficient for a 1000 assay tubes (Section 2.16.2.2) was dissolved in distilled water containing 0.1% (w/v) B.S.A. as required. Unused antiserum was aliquoted, snap frozen, and stored at -20°C.

3. $[I^{125}]$ - Succinyl cGMP Tyrosine Methyl Ester

Aliquots sufficient for 100 tests (section 2.16.2.3) were rapidly thawed and made up to 10 ml in sodium acetate buffer pH 6.2.

4. Standard cGMP

A 0.2 μ M stock solution of cGMP (sodium salt) (Sigma) was prepared in distilled water and frozen at -20°C. Working solutions, containing between 200 and 3.125 femtmoles cGMP per 100 μ l were prepared by diluting the stock solution in 0.05 M sodium acetate buffer pH 6.2.

5. Preparation of the acetylation reagent, polyethylene glycol, gamma globulin carrier protein and the methods of sample preparation were as described in Section 2.16.1.4 for cAMP assay. Assay procedure and calculations were as described in Sections 2.16.1.5 and 2.16.1.6 respectively.

A typical standard curve for $[I]^{125}$ -cGMP R.I.A. is shown in Fig.8.

2.17 Radioimmunoassay of Prostaglandin E and F₂ α

The facility for assaying prostaglandin E and F₂ α was kindly provided by Dr. K. Hillier of the Clinical Pharmacology Group, Faculty of Medicine of this University. The assays were performed by Mr. R. Jewell.

2.17.1 Principle of the Method

The principle of the method was essentially the same as described previously for the RIA of cAMP and cGMP (Section 2.16.1.1). Briefly, unlabelled prostaglandin competed with a tritium labelled derivative for binding to a highly specific antibody. Separation of antibody-bound prostaglandin from free prostaglandin was achieved using coated charcoal followed by centrifugation. The radioactivity of the supernatant containing protein-bound [³H]-prostaglandin was measured by liquid scintillation counting. The concentration of the unlabelled prostaglandin was calculated from a standard curve.

2.17.2 Reagents and Sample Preparation

The radioactive derivatives [5,6,8,9,11,12,14,15,(n)-³H] prostaglandin F₂ α (³H-PGF₂ α) (Amersham International, 180 Ci per mmol, 495 mCi per mg) and [5,6,8,11,12,14,15,(n)-³H] prostaglandin E₂ (³H-PGE₂) (Amersham International, 160 Ci per mmol 438 mCi per mg) were diluted in 0.1 M potassium phosphate buffer pH 7.5 so that 100 μ l contained 5500 and 7000 cpm for PGF₂ α and PGE₂ respectively.

The charcoal suspension for separating free from bound prostaglandin was prepared by adding 625 mg Norit GSX charcoal (BDH) to 125 mg dextran (BDH) in 50 ml 0.1 M potassium phosphate buffer pH 7.5. The charcoal was kept on ice and maintained in suspension by gentle stirring with a magnetic follower.

Standard prostaglandin solutions of PGF₂ α and PGE₂ were prepared in 0.1 M potassium phosphate buffer pH 7.5 so as to give 5, 10, 20, 50, 100, 200 and 500 pg prostaglandin per 100 μ l.

Tissue culture medium from infected (test) or uninfected (control) HeLa cell monolayers, was rapidly frozen in the presence of 10^{-6} M indomethacin and stored at -70°C until assayed. Indomethacin prevents prostaglandin degradation by cyclooxygenase. The cell monolayers were rapidly washed in ice cold PBS containing 10^{-6} M indomethacin and the cells detached into a known volume of PBS/ 10^{-6} M indomethacin using a 'rubber policeman'. The resulting cell suspension was immediately snap frozen at -196°C in liquid nitrogen until assayed.

2.17.3 Assay Procedure

Samples were acidified to pH 3-4 with 1.0 M citric acid and the prostaglandins extracted into an equal volume of distilled ether by vigorous shaking for 10 minutes. Following centrifugation at 400 g the ether layer was aspirated and retained. The aqueous phase was re-extracted in ether and the two ether extracts (approximate volume 2 ml) were pooled before evaporating to dryness under a stream of nitrogen.

The dried residue was redissolved in sodium phosphate buffer pH 7.5 and 100 or 200 μl aliquots were used for assay. The prostaglandin standard solutions in 100 μl of sodium phosphate buffer together with the unknowns were then mixed with either 100 μl of a 1 in 2,500 dilution of rabbit anti PG $\text{F}_2\alpha$ antisera (K.H₁₀(20.4.77) or a 1 in 200 dilution of rabbit anti PGE antisera (KH.Eq(20.4.77) (both antisera were raised by Dr. K. Hillier). 100 μl of the appropriate labelled derivative ($^3\text{H-PGF}_2\alpha$ or $^3\text{H-PGE}_2$) was added and the tubes were incubated for 18 h at 4°C .

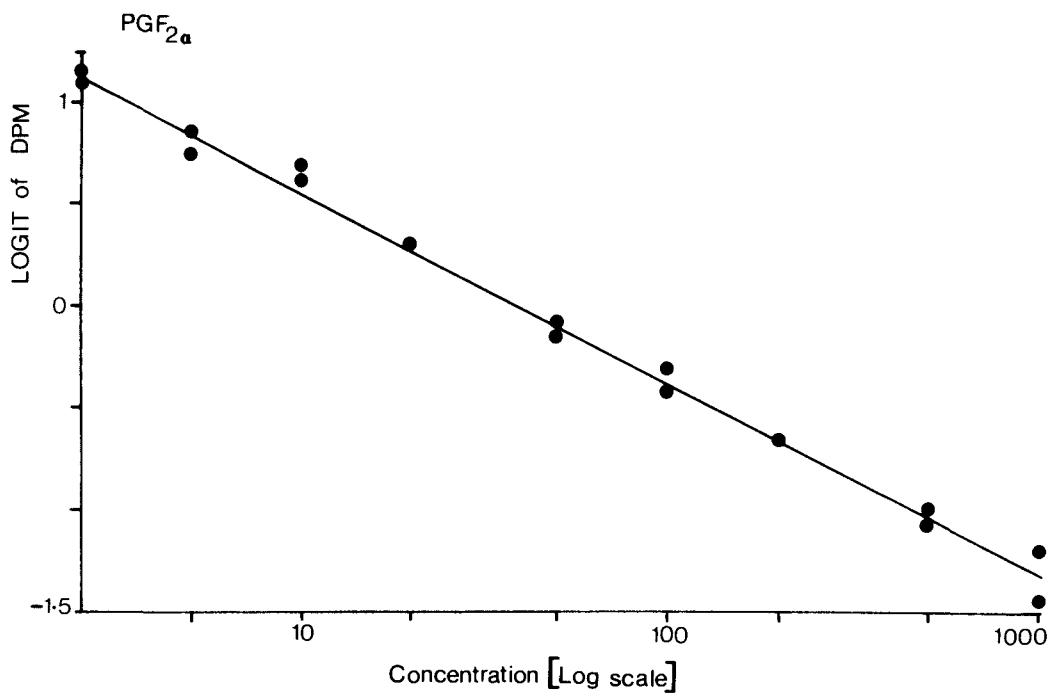
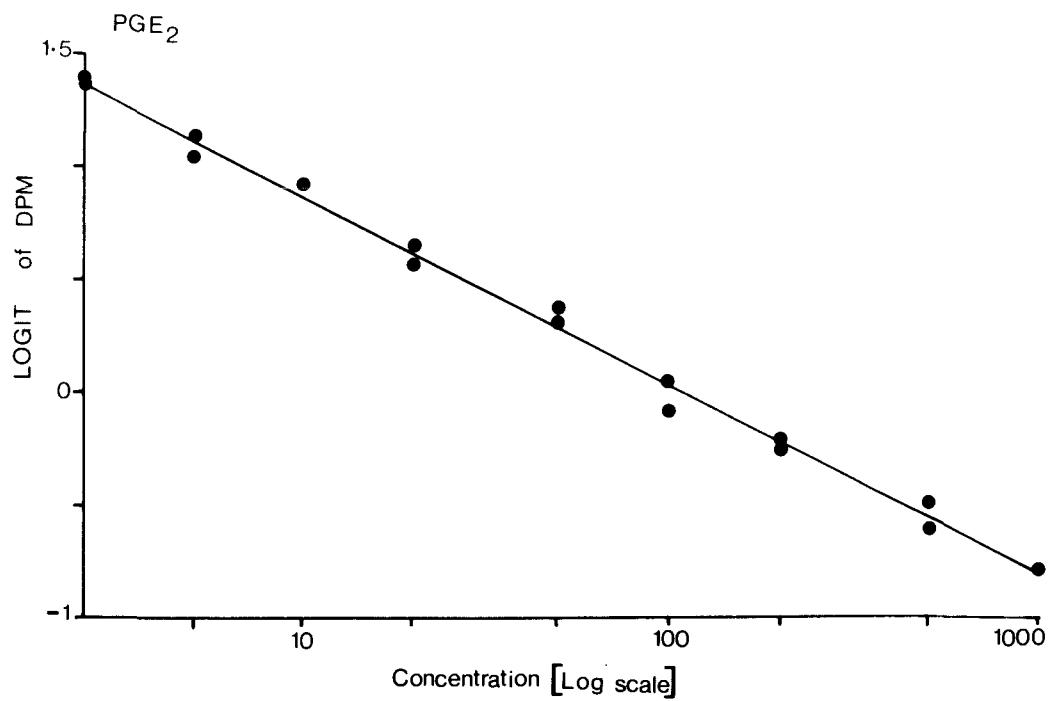
Free and antibody-bound prostaglandin were separated by adding 250 μl of the 1.25% (w/v) stirred charcoal suspension (Section 2.17.2). The tubes were well mixed, centrifuged at 400 g for 10 mins at 4°C and the supernatant was decanted into 5 ml of scintillation fluid and each tube counted for 5 minutes (Section 2.8).

A typical standard curve for PGE₂ and PGF₂ α is shown in Fig. 12.

Figure 12 Standard curves for PGE₂ and PGF₂α radioimmunoassay. The binding of [³H]-PGE₂ or [³H]-PGF₂α in the absence of unlabelled prostaglandin is taken as 100%. Binding of [³H]-prostaglandin (ordinate) in the presence of increasing amounts of unlabelled prostaglandin (abscissa) is expressed as:

$$\text{logit} = \log_{10} \left[\frac{\text{dpm}}{\text{maximum recovery} - \text{dpm}} \right]$$

all values being previously corrected for background. The curves shown are typical standard curves, each concentration being assayed in duplicate.



CHAPTER 3

RESULTS

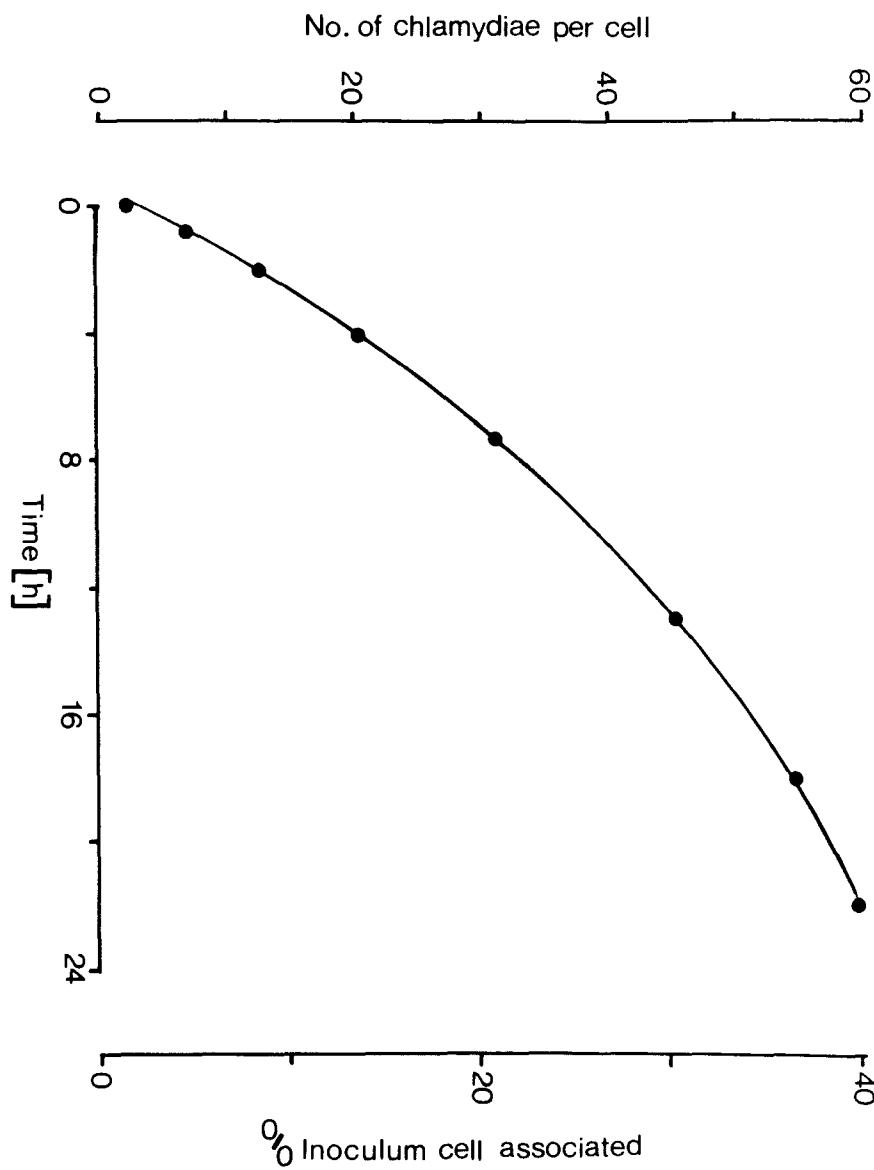
3.1 The Kinetics of Chlamydial Attachment to HeLa 229 Cells

Attachment of chlamydiae to the host cell surface is an essential prerequisite to ingestion. The kinetics of chlamydia - host cell association was determined in duplicate monolayers of HeLa 229 cells in 24-well tissue culture trays at 36°C over 24 h. fig. 13 500 µl DMEM containing [³H]-labelled LGV 434 (6000 cpm) at a chlamydiae: cell ratio of 160:1 was added and at the times indicated, the monolayers were washed and the radioactivity due to cell-associated chlamydiae determined. Chlamydial association with the host cells was slow, the maximum association rate was 8 chlamydiae per cell h⁻¹ which occurred during the first hour of incubation. After 18 h only 35% of the inoculum was cell-associated showing that the association constant of LGV 434 for HeLa cells was low.

3.2 The Kinetics of Chlamydial Ingestion by HeLa 229 Cells

Clearly the slow rate of chlamydial (LGV 434) attachment in the HeLa cell model must be a major limit on uptake. Accordingly the kinetics of uptake were measured at 36°C following centrifugation of the organism onto the HeLa cells at 8°C to ensure attachment had occurred. Lightly confluent monolayers of the HeLa 229 cells on coverslips in 24-well tissue culture trays were cooled on ice and inoculated with purified tritiated LGV 434 (40 chlamydiae per cell) in 1 ml HEPES-buffered DMEM. Following centrifugation at 8°C for 1 h at 1000 g to promote attachment the supernatant medium was aspirated and replaced with 1 ml HEPES-DMEM per tube at 0°C. Ingestion of the organism was initiated by rapid warming to 36°C (zero time). At the times indicated adherent non-ingested chlamydiae were eluted with trypsin-EDTA from 3 replicate monolayers and the cells were centrifuged at 100 g for 5 min. at 0°C. The radioactivity in the cell pellet, attributed to ingested chlamydiae was measured by liquid scintillation counting (section 2.8).

Figure 13 Kinetics of adhesion of C.trachomatis LGV 434 to HeLa 229 cells. Replicate monolayers of HeLa 229 cells in 24-well tissue culture trays were incubated at 36°C with 500 μ l DMEM containing [3 H]-labelled LGV 434 at a chlamydia: cell ratio of 160:1. At the times indicated, duplicate monolayers were washed and the radioactivity due to cell-associated chlamydiae determined. Points plotted are the means of the duplicate samples and the experiment was performed twice. Maximum rate of chlamydiae-HeLa cell association was 8 chlamydiae per cell h^{-1} .



The results (Fig. 14) indicated that ingestion was virtually complete after only 30 min incubation, with some 24% of the inoculated chlamydiae being intracellular. In this figure the small number (6% of the inoculum) which resisted trypsin elution at time zero have been subtracted from the data. Electron microscopic observations suggested that the remaining cell associated organisms were not ingested because they failed, despite centrifugation, to achieve sufficiently intimate contact with the HeLa cell membrane (Dr. M.E. Ward. Personal Communication). Control experiments performed to validate the uptake assay included:

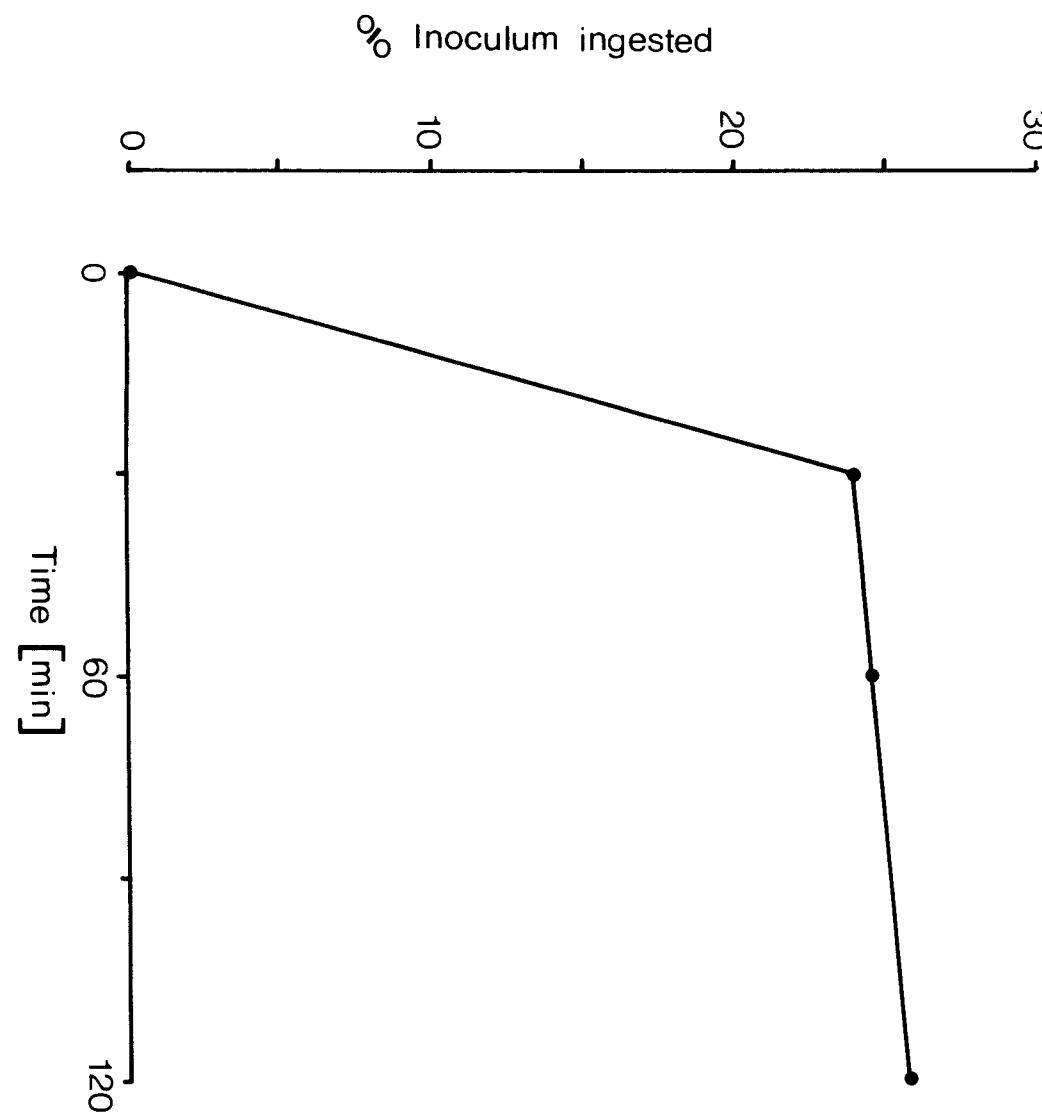
1. The demonstration that the rate of chlamydial ingestion was saturable with respect to particle concentration (see section 3.5.5).
2. The demonstration that at time zero only low levels of LGV 434 resisted trypsin elution. It was calculated that approximately 94% of the inoculum was removed from the cell surface after centrifugation of the organism onto the host at 8°C. The residual 6% was attributed to chlamydiae inevitably internalized as the cells were warmed to 36°C for enzyme digestion.
3. Electron microscopic observations of monolayers following centrifugation of the organism onto the host at 8°C confirmed that, uptake of chlamydiae did not occur at low temperature but was rapid on warming to 36°C (see Section 3.4.3).

These data satisfied the criteria suggested by Stossel 1975 for the valid measurement of ingestion.

3.3 The Role of Calmodulin in Chlamydial Infectivity

The infectivity of C. trachomatis for HeLa 229 cells was enhanced by pretreatment of the cell monolayer with A23187 (Ward and Salari 1982), an ionophore which increases the permeability of eukaryotic cell membranes to Ca^{2+} (Reed and Lardy 1972). Moreover, movement of Ca^{2+} across the host cell membrane has been demonstrated when purified C. trachomatis EBs were centrifuged onto HeLa 229 cell monolayers (Ward

Figure 14 Kinetics of ingestion of C.trachomatis LGV 434 by HeLa 229 cells. [3 H]-labelled LGV 434 in HEPES-buffered DMEM (40 chlamydiae per cell) were centrifuged at 8°C onto replicate coverslip cultures of HeLa 229 cells. The supernatant medium was aspirated and replaced with HEPES-DMEM at 0°C. Uptake was initiated by rapid warming to 36°C (zero time). At the times indicated, 3 replicate monolayers were digested with trypsin-EDTA to elute adherent, non-ingested chlamydiae, the cells were centrifuged at 100 g, 0°C for 5 min and radioactivity in the cell pellets due to ingested chlamydiae was determined. Points plotted are the means of 3 replicate samples; the experiment was performed 3 times with different levels of inocula with essentially similar results. The data show that chlamydial ingestion was rapid and virtually maximum after 30 min incubation, with 10 chlamydiae ingested per cell.



and Salari 1982) suggesting that chlamydial attachment is associated with rapid movement of Ca^{2+} and perhaps other ions across the HeLa cell membrane. Such movement of Ca^{2+} during the initial stages of infection might facilitate chlamydial uptake given the role of calcium in endocytosis (Salisbury et al 1980; Horowitz et al 1981). In these systems Ca^{2+} regulates endocytosis by interaction with calmodulin a calcium binding protein, ubiquitously distributed in eukaryotic cells. The role of calmodulin in chlamydial infectivity was therefore investigated by measuring chlamydial attachment, uptake and development in the presence and absence of calmodulin inhibitors. The feasibility of using chlamydial dependent uptake of threonine as a parameter of chlamydial growth was first assessed.

3.3.1 The relationship between chlamydial threonine uptake and infectivity

The radiolabelling of C.Psittaci to high specific activity with [^{14}C]-L-threonine, a non-essential amino acid for chlamydial growth, was demonstrated by Allan and Pearce in 1982. It was therefore decided to investigate if tritiated threonine uptake by chlamydial infected cells could be used in a quantitative assay of chlamydial development. Preliminary experiments established that the efficiency of tritiated threonine incorporation was dependent on the amount of unlabelled threonine present in the medium (Fig. 15). Threonine-deficient Dulbeccos minimum essential medium (TD-DMEM) was prepared by reconstituting DMEM from its individual components, but omitting L-Threonine. (Table 3).

Replicate (12) monolayers of HeLa 229 cells were challenged with different numbers of chlamydiae and the incorporation of DL-[3H]-threonine by the infected cells measured 42 h after challenge. The results are shown in fig 16. The standard deviation of threonine incorporation by the replicate samples over a range of chlamydial inocula (10-160 chlamydiae per cell) varied between 3-7% of the sample mean, permitting regression analysis by the least squares method. The regression equation for the relationship plotted in fig 16 can be

Figure 15 Relationship between chlamydial dependent DL-[³H]-threonine incorporation by HeLa 229 cells and the concentration of L-threonine in the tissue culture medium. HeLa 229 cells in 24-well tissue culture trays (approx 5×10^5 cells per well) were inoculated with LGV 434 (200 chlamydiae per cell) in 1 ml TD-DMEM containing 1 μ g per ml emetine hydrochloride. Following 4 h adsorption at 36°C, the medium was removed and the cells incubated for a further 20 h in 1 ml TD-DMEM containing emetine and 0-100 μ g per ml L-threonine as appropriate. At 24 h post infection, 2 μ Ci per ml DL-[³H]-threonine was added to each well and the cells incubated for a further 24 h at 36°C. After 4 washes in HBSS the cell associated radioactivity was measured by liquid scintillation counting. The points plotted are the mean of 4 replicate samples (●) infected cells, (○) uninfected cells.

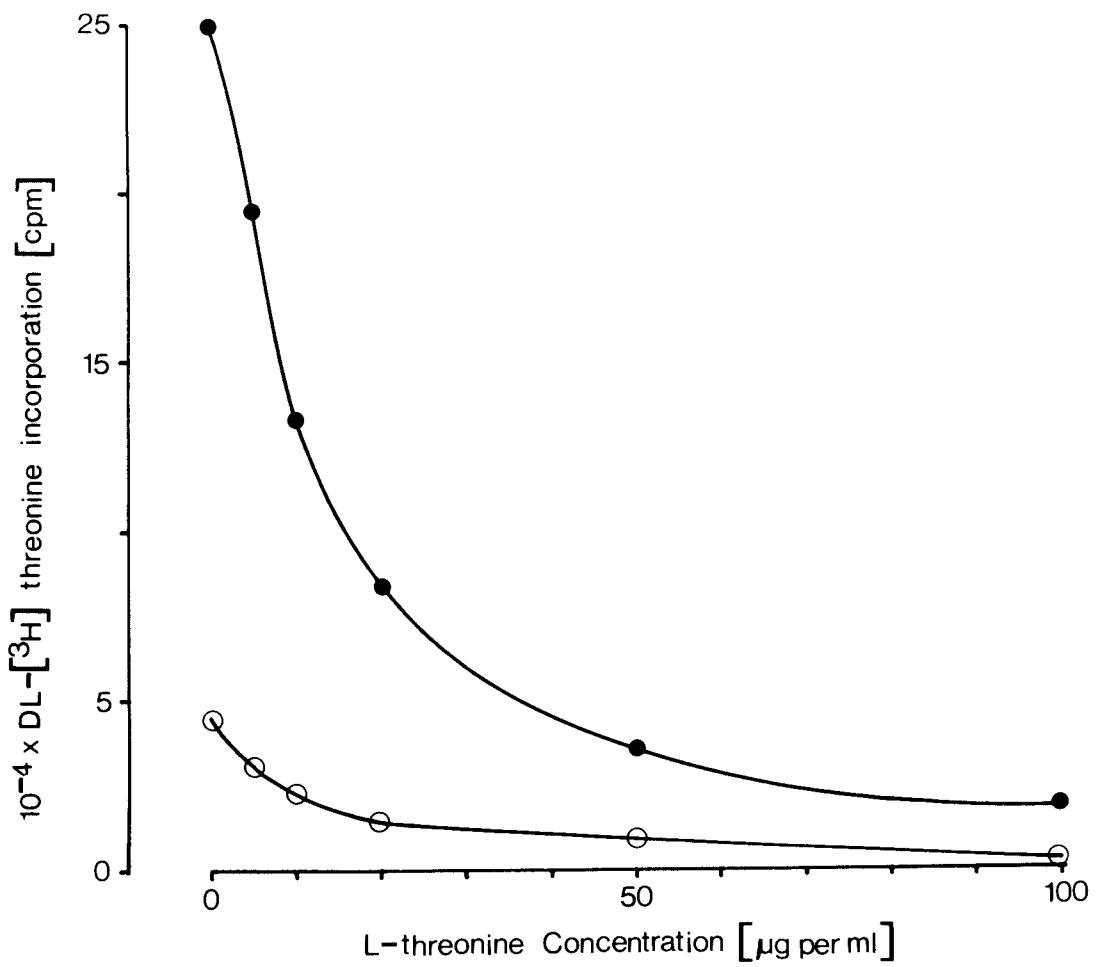
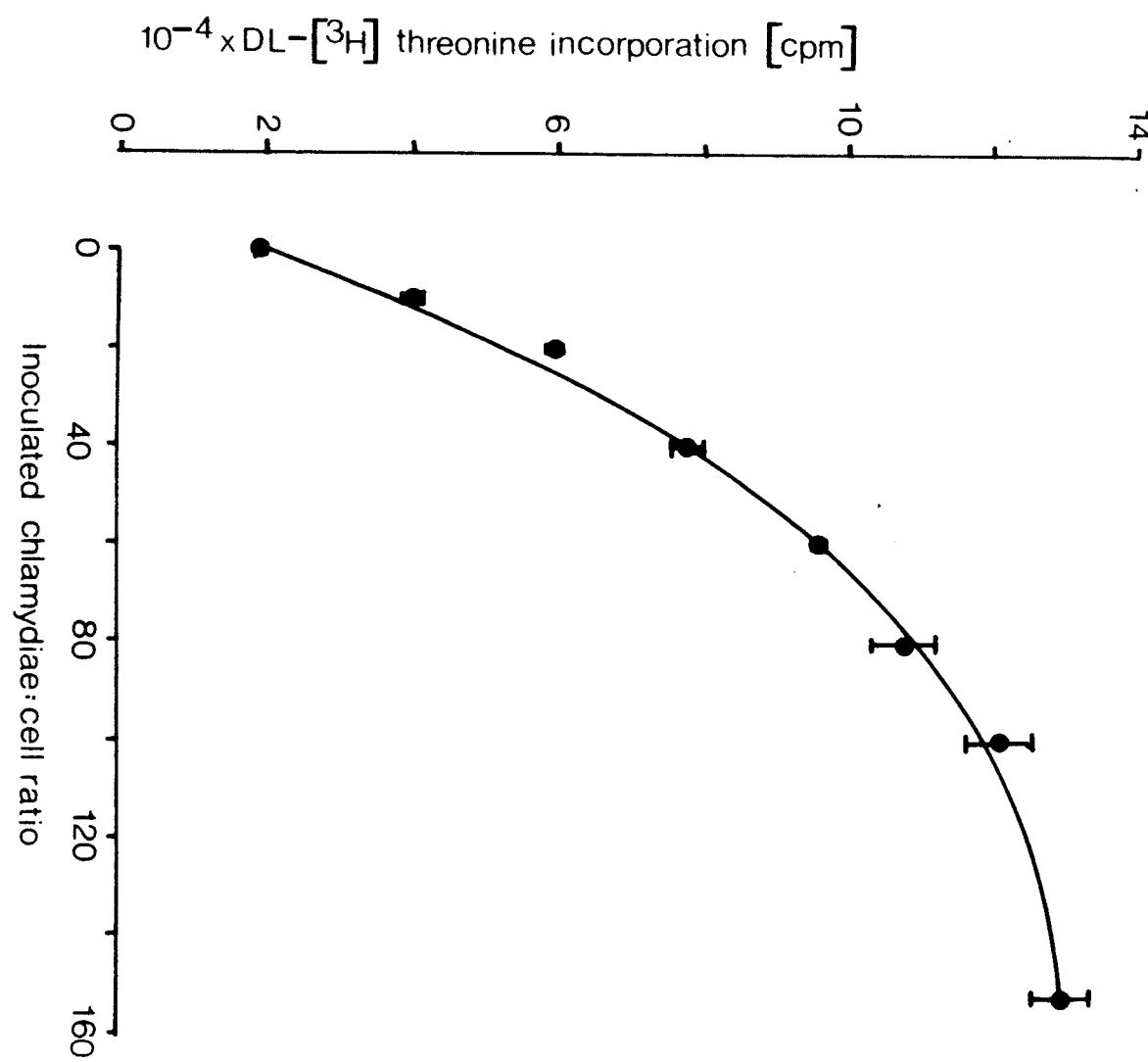


Figure 16 Dependence of [^3H]-threonine uptake by HeLa 229 cells infected with LGV 440 on the number of organisms inoculated. Threonine incorporation was measured at 42 h post infection in the presence of emetine. The points plotted are the mean and 95% confidence interval of 12 replicate samples. The line plotted is the computed curvilinear (quadratic) regression for the data with a coefficient of determination of 0.991 and a correlation coefficient of 0.995. All points were significantly different to each other on a 1-tail t test with $p<0.01$.



expressed as a polynomial of general form:

$$y = ax + bx^2 + cx^3 + \dots + nx^n$$

i.e. an nth order polynomial. For the data plotted the correlation coefficients were .996 and .997 for quadratic and cubic polynomials with standard errors for the estimated curves of 439 and 401 respectively. Thus the relationship between chlamydial infectivity and threonine uptake was clearly curvilinear, implying that as the inoculum increased the proportion of cells infected also increased but the probability that a given elementary body would infect a hitherto uninfected cell decreased, particularly at a chlamydiae : cell ratio in excess of 100. Between the limits of 20 - 100 chlamydial particles inoculated per cell, small changes in chlamydial numbers produced significant changes in [³H]-threonine uptake with $p < 0.01$ for the sample means tested against each other using a 1-tail t test (Cooke et al 1982).

3.3.2 The effect of calmodulin inhibitors on chlamydial infectivity

The following calmodulin inhibitors were investigated for their ability to modulate chlamydial infectivity in HeLa 229 cells:

10-[3-(4-methylpiperazin-1-yl) propyl]-2-trifluoro-methylphenothiazine (trifluoperazine; TFP) (a gift from Smith, Kline and French Laboratories Ltd., Welwyn Garden City, Herts, UK,)

1-[1-(4,4-Bis(p-fluorophenyl)butyl] piperid

4-yl benzimidazolin-2-one (pimozide) and

(4-p-chlorophenyl-4-hydroxypiperidine)-p fluorobutyrophenone (haloperidol)(from Janssen Pharmaceutical Ltd., Marlow, Bucks, UK)

10-(2-dimethylaminopropyl) phenothiazine (promethazine) (from May and Baker Ltd., Dagenham, Essex, UK)

2-Chloro-10-(3-dimethylaminopropyl) phenothiazine (chlorpromazine)

(Sigma). Concentrated solutions of haloperidol and pimozide were dissolved in ethanol at $10^{-2}M$, chlorpromazine hydrochloride, TFP and promethazine were dissolved at $10^{-2}M$ in de-aerated saline buffered at pH 7.2 with 30 mM-HEPES. All solutions were freshly prepared before each experiment and protected from light to prevent possible deterioration.

HeLa 229 cells were grown overnight to confluent monolayers in 1.0 ml volumes of DMEM in 24-well tissue culture trays. After washing in HBSS, the monolayers were incubated in DMEM containing 1 µg per ml emetine hydrochloride and a range of concentrations of each of the five different calmodulin inhibitors for 1 h before chlamydial challenge. The inhibitors were maintained on the cells throughout the subsequent chlamydial challenge and incubation. After a further wash in HBSS the cells were inoculated with C.trachomatis LGV 440 as described in section 2.5.2. Following chlamydial adsorption, the cells were exposed to 1.0 ml of TD-DMEM containing 2 µCi (74KBq) DL-[³H]-threonine, emetine and test agents. Chlamydial infectivity was determined after 42 h by washing the cells thoroughly in HBSS and solubilising in SDS (section 2.5.2) followed by liquid scintillation counting (section 2.8). Each experiment routinely included as controls, uninfected cells plus and minus emetine to ensure the emetine block was functional as well as emetine treated uninfected cells plus and minus test agent to ensure that the agent was not cytotoxic.

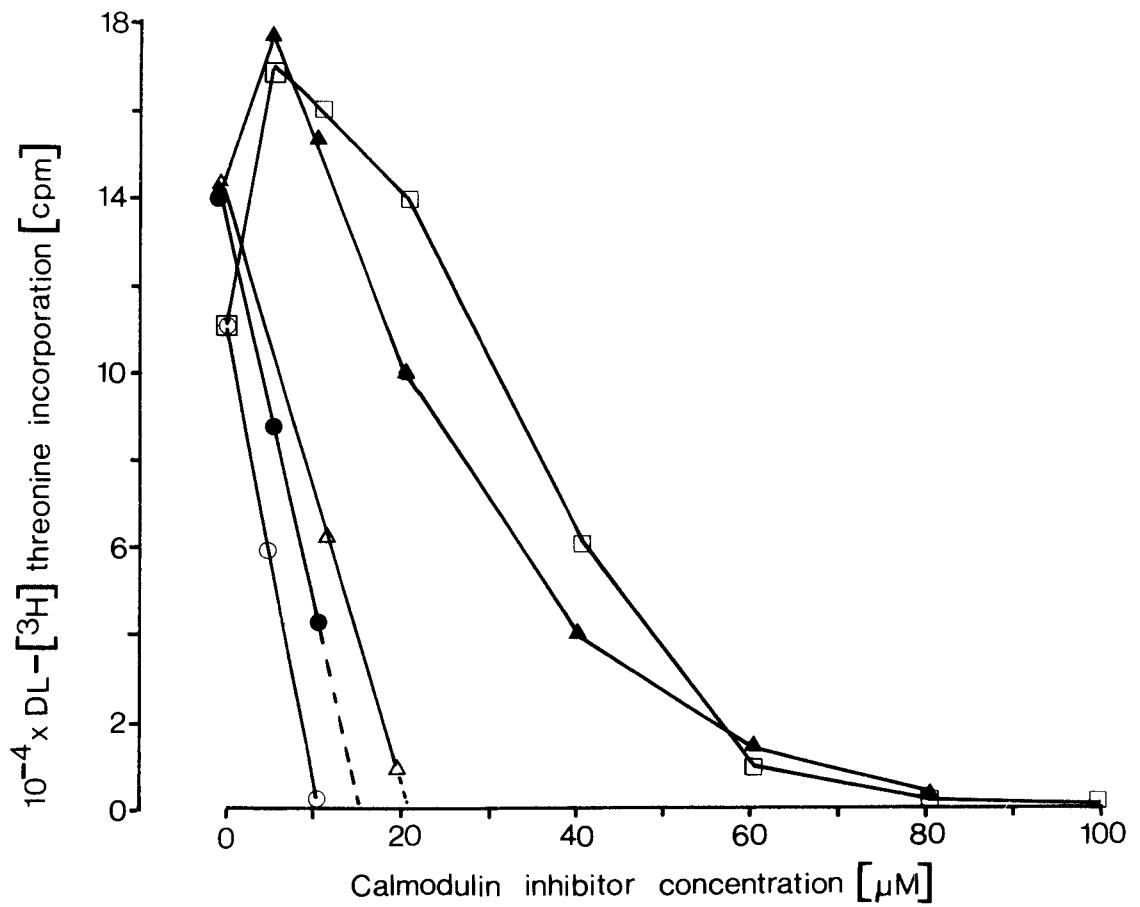
The results of this experiment (Fig. 17) showed that pimozide, TFP and chlorpromazine were potent inhibitors of chlamydial growth in HeLa cells; the concentration of drug required to inhibit chlamydial threonine uptake by 50% (ID₅₀) being 5, 7 and 11 µM respectively. Haloperidol and promethazine were less active, the corresponding ID₅₀ being 45 and 47 µM. Low concentrations of haloperidol and promethazine caused a consistent small increase in threonine uptake by both infected and uninfected cells, the reasons for which are unknown.

3.3.3 The action of continuous TFP treatment at different stages of the chlamydial growth cycle

Having established that TFP was a potent inhibitor of C.trachomatis growth in HeLa 229 cells, a further experiment was performed to examine the effects of continuous treatment with the drug at different stages of the chlamydial growth cycle.

HeLa 229 cells were grown overnight to a confluent monolayer in

Figure 17 Effect of varying concentrations of calmodulin inhibitors on chlamydial [^3H]-threonine uptake in HeLa cells. The inhibitors were added 1 h before chlamydial challenge and were present throughout the experiment. The inhibitors tested were pimozide (o), TFP (●), chlorpromazine (Δ), haloperidol (\blacktriangle) and promethazine(\square). The organism was LGV 440 and the threonine incorporation was determined 42 h after challenge in the presence of emetine. All the drugs tested, inhibited chlamydial dependent [^3H]-threonine uptake with pimozide, TFP and chlorpromazine the most potent.



DMEM in 24-well tissue culture trays. After washing in HBSS the cells were inoculated (time zero) with C.trachomatis LGV 434 in DMEM (section 2.5.2).

Non adherent chlamydiae were washed off the cells in HBSS and 1.0 ml volumes of TD-DMEM containing emetine and 2 μ Ci per ml DL-[3 H]-threonine was added to each well and the cells incubated to permit chlamydial maturation (section 2.5.2). At 2h before inoculation (pre-treatment), at inoculation or at 2, 22 and 34 h after inoculation, a 10 μ l concentrate of TFP (2×10^{-3} μ M in TD-DMEM) was added to the tissue culture medium so as to achieve a final concentration of 20 μ M in each well. The TFP was maintained on the cells until the chlamydial tritiated threonine incorporation was determined 42 h after challenge.

The results (Table 7) indicated that the inhibitory effect of TPF on chlamydial development increased with the duration of the treatment. TFP added 2 h before inoculation resulted in the chlamydial [3 H]-threonine incorporation being reduced by 95.1% whilst the addition of TFP 34 h post inoculation caused a 38.9% reduction. Taken together these results suggested that TFP has an inhibitory effect on chlamydial infection throughout the chlamydial growth cycle.

3.3.4 The action of short term treatments with TFP on chlamydial multiplication at different stages of the growth cycle

To establish if the inhibitory effect of TFP on chlamydial development was reversible, the experiment in section 3.3.3 was repeated, except that the inhibitor was added for only 2 h periods. Chlamydial growth was determined both by measuring chlamydial dependent tritiated threonine uptake and, in a parallel experiment, by the number of inclusions developing (section 2.5.1).

Replicate overnight confluent monolayers of HeLa 229 cells grown on 13 mm diameter coverslips were inoculated with C.trachomatis LGV 434 in DMEM (Section 2.5.2). After washing off any non adherent chlamydiae, the infected cells were incubated in DMEM for 40 h to

TABLE 7

Replicate confluent monolayers of HeLa 229 cells in 24 well tissue culture trays were washed in HBSS and inoculated with DMEM containing LGV 434 at a chlamydiae:cell ratio of 160:1. Following centrifugation at 1000 g and 2 h incubation at 36°C, non adherent chlamydiae were removed by washing in HBSS and the cells incubated for 42 h in 1.0 ml TD-DMEM containing emetine and 2 μ Ci per ml DL-[³H]-threonine. At the appropriate times TFP was added to the tissue culture medium so as to achieve a final concentration of 20 μ M in each well. The inhibitor was maintained on the cells until the chlamydial [³H]-threonine incorporation was determined 42 h after challenge.

TABLE 7

Effect of continuous TFP treatment on
Chlamydial [³H]-threonine uptake in HeLa 229 cells
infected with LGV 434

Time of TFP addition	$10^{-1} \times$ chlamydial [³ H]-threonine incorporation (c.p.m)*
2 h before inoculation	627 (± 136)
At inoculation (0h)	1116 (± 127)
2 h post inoculation	2519 (± 279)
22 h post inoculation	4572 (± 366)
34 h post inoculation	7761 (± 783)
No TFP added	12695 (± 2943)

* Mean ($\pm 95\%$ confidence interval for mean) of six replicates
42 h after chlamydial challenge.

permit inclusion development. TFP was added for 2 h either 2 h before inoculation, at inoculation (0h), or 2, 20, or 30 h after challenge. The inclusions were observed by interference microscopy as described in Section 2.5.1. The effect of short term TFP treatment on chlamydial threonine uptake and the number of chlamydial inclusions is shown in Table (8).

In seven different experiments TFP treatment was always maximally effective when added at the time of chlamydial challenge. Statistical analysis of the data showed that the number of inclusions that developed following TFP treatment at time 0 h, was significantly reduced compared with the untreated controls ($p<0.01$, 1-tail t test) whilst threonine uptake was reduced by 30-50%. This suggested that TFP interfered with an early event in chlamydial infection such as attachment and/or the uptake of the organism. Alternatively TFP might have a directly toxic action on chlamydiae.

3.3.5 Toxicity of TFP for chlamydiae

To determine if TFP was having a direct toxic action on chlamydiae a concentrated inoculum of C.trachomatis LGV 440 (section 2.6) was incubated at 36°C for 2 h in the presence of 0, 20, 40, 60, 80 or 100 μ M TFP in TD-DMEM. These concentrations of TFP tested were greatly in excess of the ID₅₀ of 7 μ M (Section 3.3.2). The chlamydiae were then diluted 20 fold in TD-DMEM and inoculated onto replicate monolayers of emetine-treated HeLa 229 cells grown in 24-well tissue culture trays. Chlamydial growth was quantitated, as before, by measurement of DL-[³H]-threonine incorporation into the HeLa 229 cells (Section 2.5.2). The results of this experiment are shown in Table (9).

Incubation of chlamydiae directly with TFP at concentrations up to 80 μ M caused no statistically significant difference in chlamydial tritiated threonine uptake. A significant decrease in infectivity was observed when 100 μ M TFP was incubated with chlamydiae ($p<0.05$), but this could be entirely attributed to the residual 5 μ M TFP incubated with the host cells after dilution of the treated inoculum.

TABLE 8

The experimental procedure was similar to that described for table 7 except the inhibitor was added for only 2 h periods. HeLa cells grown on coverslips were washed in PBS and examined by interference microscopy for inclusion development 40 h after challenge.

TABLE 8

Effect of 2 h treatments with TFP on chlamydial
development in HeLa 229 cells

Time of TFP addition	Chlamydial [³ H]-threonine uptake (c.p.m.)*	Number of inclusions per high power field †
2 h before inoculation	14365 (± 201)	16.3 (± 3.34)
At inoculation (0h)	14217 (± 575)	10.8 (± 2.15)
2 h post inoculation	15466 (± 922)	17.4 (± 5.37)
20 h post inoculation	21900 (± 2101)	17.2 (± 3.81)
30 h post inoculation	21449 (± 1526)	16.6 (± 2.63)
No TFP added	20328 (± 1478)	20.9 (± 2.58)

* Mean ($\pm 95\%$ confidence interval for the mean) of four replicate counts, 40 h after challenge.

† Mean ($\pm 95\%$ confidence interval for the mean) of the number of inclusions in 10 fields.

Table 9

A concentrated purified LGV 440 inoculum was incubated for 2 h at 36°C in the presence 0-100 μ M TFP in TD-DMEM. The chlamydiae were then diluted 20 fold in TD-DMEM, and inoculated onto replicate monolayers of emetine treated HeLa 229 cells. Following centrifugation at 1000 g for 1 h, non adherent chlamydiae were removed by washing in HBSS and the cells incubated at 36°C in 1 ml TD-DMEM containing emetine and 2 μ Ci(74k Bq) DL-[³H] threonine. Incorporation of [³H]-threonine into the HeLa cells was determined after 43 h incubation.

TABLE 9

Effect of incubating chlamydiae with high concentrations of TFP

Concentration of TFP in incubation with chlamydiae (μ M)	Final concentration of TFP on HeLa 229 cells (μ M)	Mean chlamydial threonine uptake (cpm)*	Probability†
0	0	95745	-
20	1	99035	0.17
40	2	95140	0.45
60	3	91385	0.16
80	4	90045	0.18
100	5	76735	<0.01

* Mean of four replicate samples determined 43 h after chlamydial challenge.

† Probability on a 1-tail t test that threonine incorporation by TFP-treated chlamydiae is less than in the untreated control.

It was concluded therefore, that the inhibitory effects of TFP on chlamydial development were due to an effect of the inhibitor on host cells, rather than due to a direct toxic effect of the drug on the chlamydiae.

3.3.6 The effect of TFP on adhesion of chlamydiae to HeLa 229 cells

One possibility was that calmodulin might be important for chlamydial adhesion to host cells. This was investigated in the HeLa cell model by comparing the adhesion of [³H]-C.trachomatis LGV 440 to TFP treated (test) or untreated (control) cells.

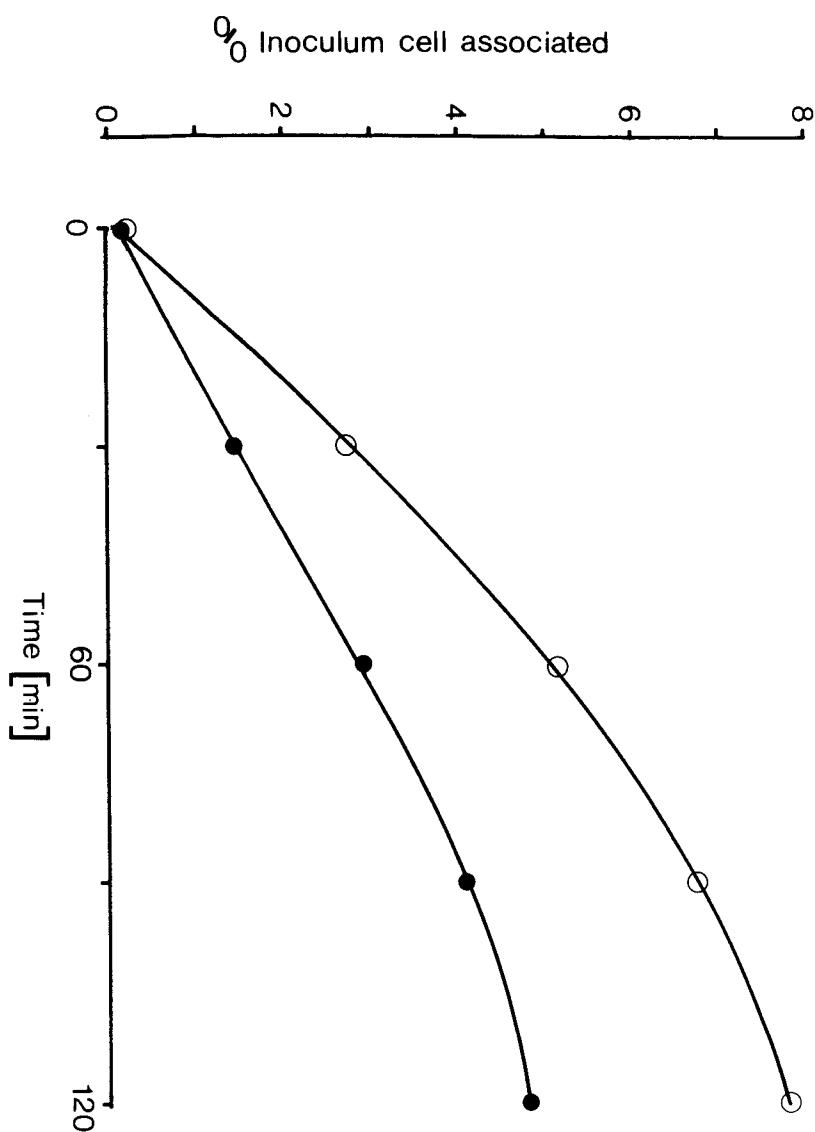
HeLa 229 cells were grown overnight in DMEM to lightly confluent monolayers on glass coverslips in 24 well polystyrene tissue culture trays. After washing in HBSS the test cells were incubated for 2.5 h at 36°C with 30 μ M TFP in DMEM. Control cells were incubated for the same period in DMEM. The supernatant tissue culture medium was removed and 200 μ l DMEM buffered with 30 mM-HEPES pH 7.2 containing 5000 cpm radiolabelled chlamydiae and 30 μ M-TFP as appropriate was added to each well. After gentle rocking at 36°C to promote attachment, triplicate monolayers at 0, 0.5, 1, 1.5 and 2 h after chlamydial challenge were washed 4 times with 5 ml volumes of HBSS to remove non adherent chlamydiae. The coverslips were removed to a fresh 24-well tissue culture tray, the host cells solubilised in 1% (w/v) SDS in 0.1M NaOH (Section 2.11) and the radioactivity due to cell associated chlamydiae determined by liquid scintillation counting (section 2.8). The results are shown in (fig 18).

In the two experiments performed it was found that the proportion of cell associated chlamydiae was reduced in cells treated with 20 or 30 μ M TFP compared to the untreated controls. This suggested that calmodulin was involved in the process of chlamydial attachment to HeLa cells.

3.3.7 Effect of TFP on chlamydial ingestion by HeLa cells

Clearly calmodulin might be involved in chlamydial uptake as well

Figure 18 Comparison of the kinetics of adhesion of radiolabelled LGV 440 to untreated HeLa 229 cells (o) and to cells treated with 30 μ M TFP (•). Cells were treated with 30 μ M-TFP for 2.5 h at 36°C before chlamydial challenge and during subsequent incubation periods. The cell associated radioactivity due to chlamydiae both attached and ingested by the host cells was expressed as a percentage of the original inoculum added. The points plotted represent the mean of 3 replicates and the experiment was repeated twice.



as chlamydial attachment. This possibility was investigated by measuring the penetration of C.trachomatis LGV 434 into host cells in the presence of increasing concentrations of TFP. The object was to establish whether there was a dose-dependent relationship between TFP concentration and chlamydial uptake.

HeLa 229 cells were grown overnight to light confluence in 24-well tissue culture trays. After washing in HBSS, replicate wells (8) were incubated with either 10, 15, 20, 25 or 30 μ M TFP in DMEM for 1 h at 36°C. Control cells were incubated with DMEM alone. The medium in each well was replaced with 500 μ l of a suspension of purified tritium labelled C.trachomatis LGV 434 (6000 cpm) in ice cold DMEM buffered at pH 7.2 with 30 mM-HEPES containing the appropriate concentration of TFP. Chlamydiae were centrifuged onto the host cells at 1000 g for 1 h at 8°C to facilitate attachment and the medium was replaced with 1 ml of HEPES buffered ice cold DMEM in the presence (test) or absence (control) of TFP. The trays were rapidly warmed to 36°C by flotation on a water bath and incubated for 30 minutes to permit chlamydial uptake to occur. Following a wash in ice cold PBS chlamydiae adherent to the host cell surface were eluted with trypsin-EDTA and separated by differential centrifugation (section 2.12). The cell associated radioactivity due to internalised chlamydiae was solubilised in 1% (w/v) SDS in 0.1M NaOH (Section 2.12) and measured by liquid scintillation counting (section 2.8). The experiment was repeated three times and Fig (19) shows a typical result.

Uptake of tritiated LGV 434 into the HeLa 229 cell was reduced by TFP and this effect was linearly dependent on the concentration of the inhibitor over the range 5-30 μ M. This supported the concept that the uptake of chlamydiae into host cells was dependent on the functional integrity of host cell calmodulin.

3.3.8 Electron microscopic observations of chlamydial infected cells treated with calmodulin inhibitors

Phase contrast light microscopy on TFP and pimozide treated monolayers suggested that the calmodulin inhibitors were impairing

Figure 19 Dose-dependent inhibition of uptake of LGV 43⁴ by TFP. HeLa 229 cells were treated with appropriate concentrations of TFP in DMEM for 1 h at 36°C prior to chlamydial challenge. Control cells received DMEM alone. Uptake was measured after 30 mins incubation at 36°C following centrifugation of the organism onto the cells at 8°C. The graph shows the mean and 95% confidence interval for the population mean determined from 8 replicate samples. The line plotted is the linear regression for the data with a correlation coefficient of 0.992. The open symbol represents a control experiment in which no inhibitor was added.

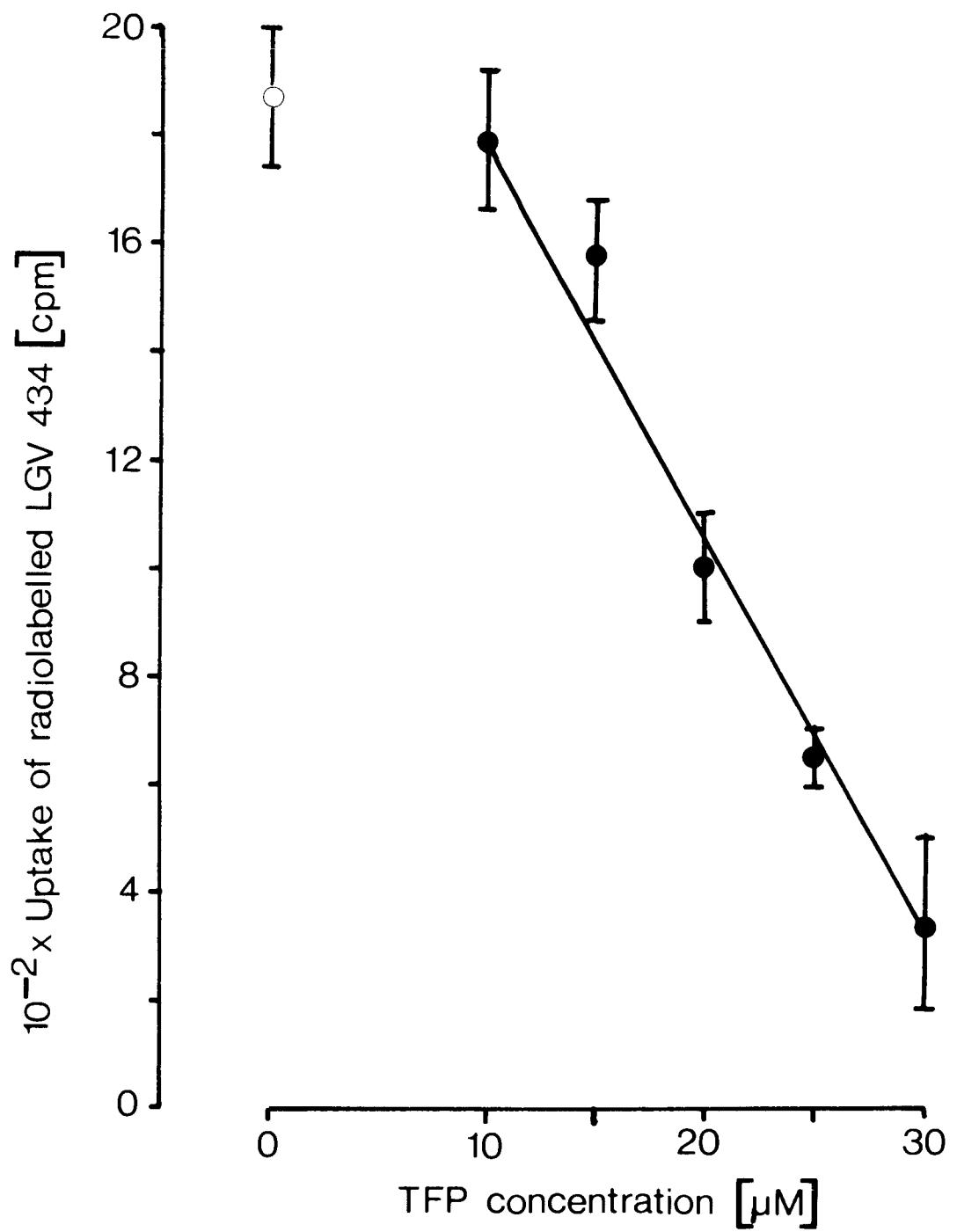
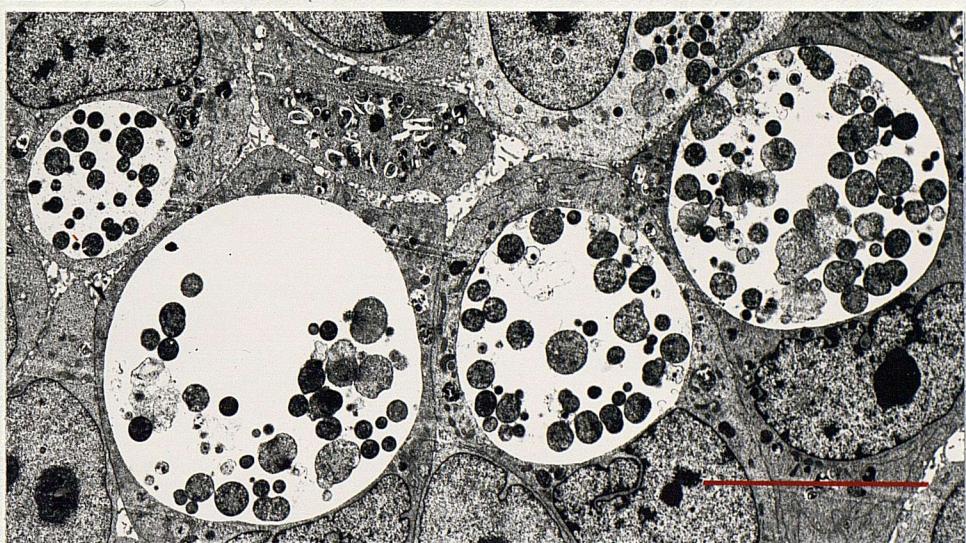


Figure 20 Transmission electron micrographs of HeLa 229 cells infected with C.trachomatis LGV 440. (A) Control cells 42 h after infection showing large inclusions containing numerous infectious elementary bodies. (B) Cells treated with 20 μ M TFP. The inclusions are smaller and less mature than in the control. (C) Typical small immature inclusion of cells treated with 10 μ M-pimozide. No infectious elementary bodies are present. The bars represent: (A) and (B) 10 μ m and (C) 1.0 μ m.

A



B



C



inclusion development. Chlamydial inclusions in cells treated with these inhibitors were noticeably smaller. Accordingly the cells were examined by electron microscopy to see if the calmodulin inhibitors altered the chlamydial growth cycle. HeLa 229 cell monolayers in polystyrene tissue culture trays were challenged with C. trachomatis LGV 440 in the presence (test) or absence (control) of calmodulin inhibitor. The infected cells were incubated with the inhibitor for 42 h at 36°C then fixed in situ with 1% v/v glutaraldehyde in 0.1 M cacodylate buffer, post fixed in 1% w/v osmium tetroxide in Palades buffer, stained with 1% w/v aqueous uranyl acetate and dehydrated in graded ethanols. The dehydrated cell sheet was then washed from the polystyrene substratum with propylene oxide and flat embedded in Spurr low viscosity epoxy resin. Ultra thin sections were stained with the conventional Reynolds lead citrate and examined in a Philips EM 300 transmission electron microscope by Dr. Ward. The results are described in the legend to Fig 20. TFP (20 μ M), pimozide (10 μ M) and chlorpromazine (20 μ M, data not shown) retarded chlamydial inclusion development. Pimozide had the most dramatic effect, virtually abolishing the development of infectious EBs within inclusions.

3.4 The Importance of the Receptor-Mediated Pathway in Chlamydial Endocytosis

The internalization of a diverse group of molecules including polypeptide hormones, serum proteins such as α_2 macroglobulin, certain toxins, and some viruses eg vesicular stomatitis virus and Semliki Forest virus, into eukaryotic cells can be attributed to a single functional pathway, receptor-mediated endocytosis (RME) (Marsh and Helenius 1980; King and Cautrecasas 1981; Matlin et al 1982; Paston and Willingham 1981 and Morris et al 1983). Biochemical studies have shown that receptor-mediated endocytosis can be inhibited by primary amines such as methylamine, amantadine or monodansyl cadaverine (Paston and Willingham 1981). Therefore, if receptor-mediated endocytosis is an important route for chlamydial endocytosis these compounds would be expected to inhibit uptake. The hypothesis that chlamydial uptake involves receptor-mediated endocytosis was tested experimentally both by determining the effect of mono-

dansylcadaverine on chlamydial - host cell interactions and by direct electron microscopic observation of the endocytic process.

3.4.1 The effect of monodansylcadaverine on chlamydial attachment to host cells

Monodansylcadaverine an inhibitor of vesicular stomatitis virus uptake and receptor-mediated endocytosis of α_2 macroglobulin (Schlegel et al 1982) was tested for its ability to affect chlamydial adhesion to HeLa 229 cells. A concentrated solution of monodansylcadaverine at 5 mM was freshly prepared in PBS pH 7.2 before each experiment. HeLa 229 cells were grown to lightly confluent monolayers in DMEM overnight in 24-well polystyrene tissue culture trays. After washing in HBSS replicate monolayers were incubated for 1 h at 36°C in 0-500 μ M monodansylcadaverine in DMEM buffered with 30 mM-HEPES to pH 7.2. The supernatant tissue culture medium was removed and replaced with 500 μ l DMEM buffered with 30 mM-HEPES pH 7.2 containing tritiated *C.trachomatis* LGV 434 plus the appropriate concentration of drug. After $4\frac{1}{2}$ h incubation at 36°C the monolayers were washed 4 times with 5 ml volumes of HBSS to remove non adherent chlamydiae and the cells solubilised with 800 μ l of 1% (w/v) SDS in 0.1 M NaOH for 30 minutes (section 2.11). The radioactivity representing chlamydiae both attached and ingested by the host cell was measured by liquid scintillation counting (section 2.8). Cell loss from the monolayers due to drug treatment was monitored using Safranin O (section 2.10). The results are shown in table (10).

Monodansylcadaverine did not affect chlamydial attachment to HeLa 229 cells. However, the drug at concentrations above 300 μ M caused cell loss from the monolayer. Safranin O dye uptake experiments enabled cell numbers to be determined for each concentration of drug, allowing an estimate of the mean number of chlamydiae attached per cell to be calculated (Table 10). The results showed that attachment of chlamydiae to host cells was not primary amine-inhibitable. This suggested that the clustering of receptors in clathrin coated pits at the host cell surface was not essential for chlamydial adhesion.

Table 10

Replicate monolayers of HeLa 229 cells were incubated for 1 h at 36°C in DMEM containing 0-500 μ M monodansylcadaverine.

After washing in HBSS the cells were incubated at 36° in DMEM containing [3 H]-LGV 434 (280 chlamydiae per cell; 1.21×10^4 cpm) and the appropriate concentration of drug. Cell associated radiolabelled chlamydiae were determined after 4.5 h incubation.

TABLE 10

Effect of monodansylcadaverine on the random association of
[³H]-labelled C. trachomatis LGV 434 with HeLa 229 cells

Concentration of inhibitor added (μM)*	Mean cell-associated [³ H]-labelled LGV 434 (cpm)†	Mean no. of chlamydiae attached per cell ‡
0	1493	35
50	1358	32
100	1639	41
200	1498	39
300	1537	47
500	1124	39

* cells in 24-well trays were treated with inhibitor at the indicated concentrations throughout the experiment.

† Mean of three replicates.

‡ Safranin O dye uptake showed there were 5×10^5 cells in untreated wells and a minimum 3.4×10^5 cells after incubation in 500 μM inhibitor. Number of chlamydiae adherent per cell has been corrected for cell loss.

3.4.2 The effect of monodansylcadaverine on the uptake of chlamyiae into host cells

The ability of monodansylcadaverine to effect the internalisation of chlamyiae into host cells was investigated. Purified tritium-labelled *C.trachomatis* LGV 434 were centrifuged at 1000 g for 1 h at 8°C onto replicate monolayers of HeLa 229 cells in 24-well tissue culture trays. The monolayers had been pretreated for 1 h at 36°C with 0-500 µM monodansylcadaverine in DMEM buffered with 30 mM HEPES to pH 7.2. The supernatant medium was gently replaced with 500 µl of HEPES buffered ice cold DMEM containing the appropriate concentration of drug. The trays were rapidly warmed to 36°C by flotation in a water bath, then incubated for 30 min to permit chlamydial uptake. After washing in ice cold PBS, chlamyiae adherent to the host cell surface were eluted with trypsin-EDTA and separated by differential centrifugation (section 2.12). The internalised tritium labelled chlamyiae in the cell pellet were measured by liquid scintillation counting (section 2.8). The results are shown in table (11).

At concentrations up to 500 µM the drug had no effect on chlamydial ingestion. These findings together with the failure of the drug to inhibit chlamydial adhesion to host cells suggested that receptor-mediated endocytosis was not a significant route for chlamydial endocytosis.

3.4.3 Ultrastructural features of chlamydial endocytosis

Electron microscopy was used to determine the morphological features of chlamydial endocytosis. Purified chlamydial elementary bodies (LGV 434) were centrifuged at 8°C for 1 h onto the surface of HeLa 229 monolayers in 24 well tissue culture trays to promote attachment. Chlamyiae were inoculated at a ratio of 2000 organisms per cell in order to provide a reasonable chance of visualising the early stages of endocytosis. This number of organisms did not prove cytotoxic when judged by microscopy or cell viability (trypan blue dye exclusion) but unusually large inclusions developed in the cells at only 24 h after challenge, presumably due to infection of the cells by

Table 11

Replicate monolayers of HeLa 229 cells were pre-treated for 1 h at 36°C in DMEM containing 0-500µM monodansylcadaverine. Following a wash in ice cold HBSS the monolayers were inoculated with [³H]-LGV 434 (145 chlamydiae per cell; 1.45x10⁴ cpm) and centrifuged at 1000 g for 1 h at 8°C in the presence of the appropriate concentration of drug. After gentle washing in ice cold PBS, uptake was determined after 30 minutes incubation at 36°C.

TABLE 11

Effect of monodansylcadaverine on uptake of
[³H]-labelled C.trachomatis LGV 434 by HeLa 229 cells

Concentration of inhibitor added (μ M)	Mean uptake of [³ H]-labelled LGV 434 (c.p.m)*	Mean no. of chlamydiae ingested per cell	Probability †
0	4452 (\pm 418)	45	-
50	4717 (\pm 172)	47	0.12
100	4475 (\pm 353)	45	0.90
200	4283 (\pm 315)	43	0.36
300	4381 (\pm 679)	44	0.80
500	4044 (\pm 393)	40	0.08

* Mean of four replicates (\pm 95% confidence interval for population mean).
The experiment was performed twice.

† Probability on a 2-tail t test that uptake was significantly greater or less than in the control. Two-way analysis of variance showed that variance between and within replicates was not significant ($p>0.05$).

multiple elementary bodies. After challenge the cells were rapidly warmed to 36°C to permit uptake and the cell monolayers were fixed either 5 min or 30 min later. The fixative used was the glutaraldehyde-tannic acid - osmium tetroxide formulation of Aggeler and Werb, 1982 which was chosen to accentuate any clathrin coats which might be associated with the process of chlamydial endocytosis. The in situ arrangement of the cell monolayer was preserved using the method described in section 3.3.8 and the ultra thin sections were examined in a Philips EM300 transmission electron microscope by Dr. Ward. The results are shown in Figs. 21, 22, 23 and 24 and are described in detail in the legends to the figures. Chlamydial entry into the host cells occurred by the formation of a phagocytic cup at the cell surface (Fig 21 A & B). Clathrin coated pits (Fig 21 C) and vesicles (Fig 21 D) were readily visualised in tannic acid fixed material but were never seen associated with chlamydial endocytosis. This suggested that the clathrin-dependent mechanism of receptor mediated endocytosis was not involved. At 5 min after initiating uptake the chlamydiae in tight endocytic vesicles were lying between bundles of microfilaments close to the cell surface (Fig 22A). These chlamydiae were genuinely intracellular and not lying in invaginated cups at the cell surface because colloidal thorium marking the cell surface membrane (Fig 22B) was not present on the membrane of the chlamydial endosome. At 30 min after initiating uptake, very large numbers of organisms were present deep within the cell (Fig 23A). When peroxidase (molecular weight 40,000) or ferritin (molecular weight 450,000) was present during chlamydial uptake in the extracellular tissue culture medium, only peroxidase became incorporated into the endosomes in around 40% of chlamydial-containing endocytic vesicles (Fig 23B). The much larger ferritin was totally excluded indicating the close, tight interaction between the host cell and chlamydial surfaces during formation of the endocytic vesicles. These tight, endocytic vesicles are shown at greater resolution in Fig 24. The involvement of cell surface processes in the initial endocytosis (Fig 21 A & B) and the absence of clathrin-cloated chlamydial endosomes, taken together suggests that chlamydiae enter cells by a microfilament dependent process and not by micropinocytosis.

Figure 21 Early stages in the endocytosis of C.trachomatis LGV 434 by HeLa 229 cells. Chlamydiae were centrifuged onto the host cells for 1 h at 8°C to bring about attachment. The cell monolayer was then rapidly warmed to 36°C to initiate uptake and fixed after 5 min. (A) Engulfment of adherent chlamydiae by microvillous processes at the host cell surface. (B) Chlamydial uptake was associated with the formation of a tight endocytic cup. In glutaraldehyde-tannic acid-osmium tetroxide fixed specimens, clathrin coated pits (C) and vesicles (D) were readily visualised but a clathrin coat was never observed to be associated with chlamydial endocytosis. The bar markers represent: (A) and (B) 0.5 μ m (C) and (D) 0.1 μ m.

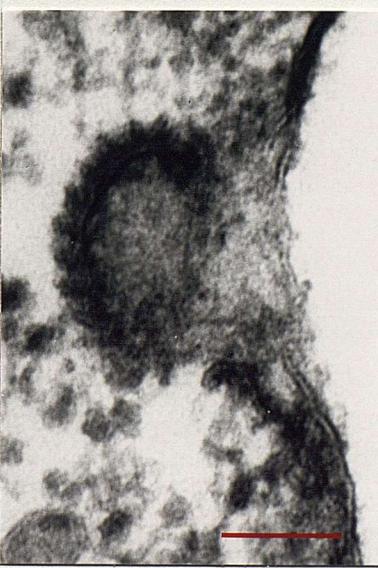
A



B



C



D

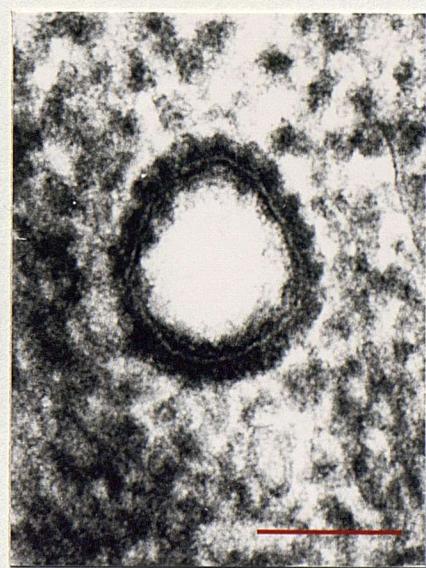
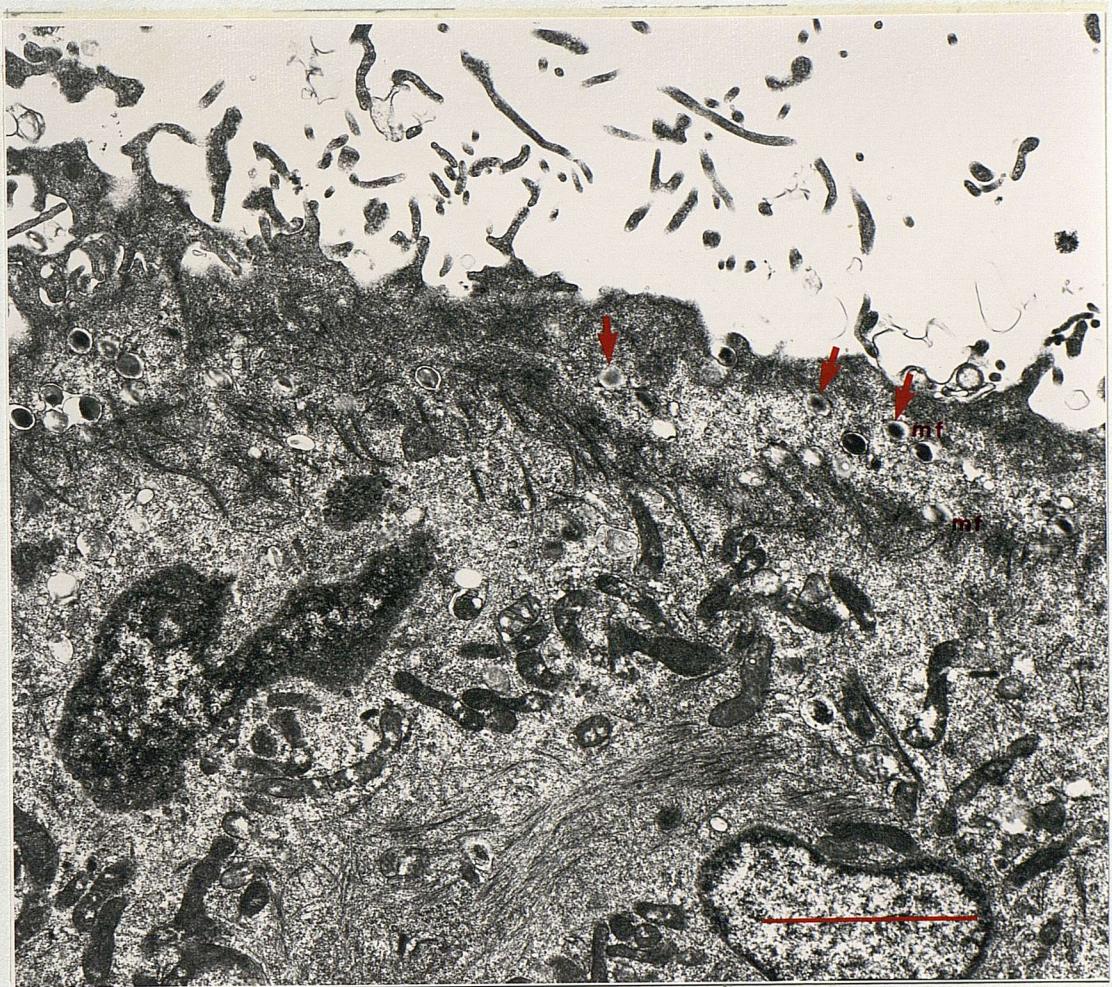


Figure 22 (A) Five minutes after initiating ingestion chlamydiae could be seen (arrowed) near the cell surface lying within endocytic vacuoles. Two parallel regions close to the cell surface rich in microfilaments (MF) were frequently seen; chlamydial endosomes at this stage often lay between these two regions. (B) To confirm that these superficially located chlamydiae were in fact intracellular rather than laying in transversely sectioned invaginated cups on the host cell surface, the host cell surface (glycocalyx) was stained en bloc with 1% colloidal thorium hydroxide. The membranes surrounding the intracellular chlamydiae remained unstained showing that the surrounding membrane was not connected with the host cell surface. Thus it was demonstrated that chlamydiae are rapidly interiorised within host cells only 5 min after initiating uptake. The bars represent (A) 2.5 μ m and (B) 1.0 μ m.

A



B

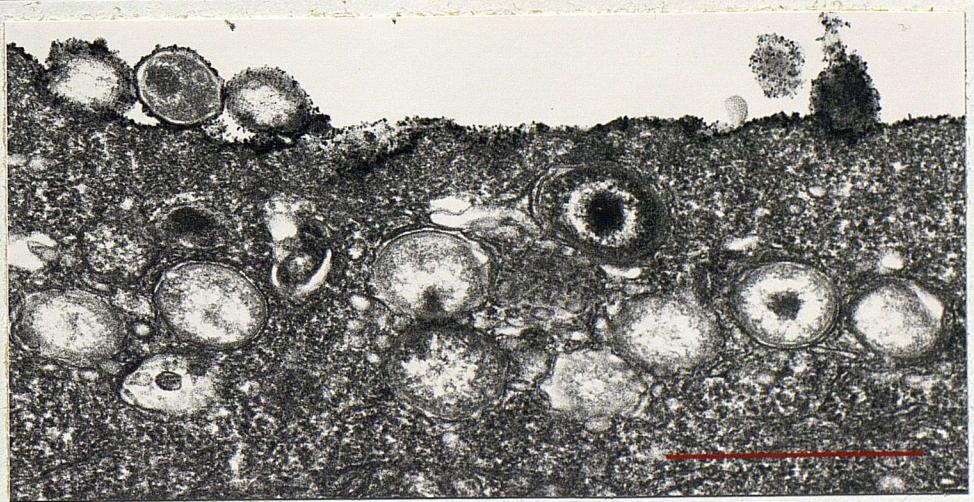
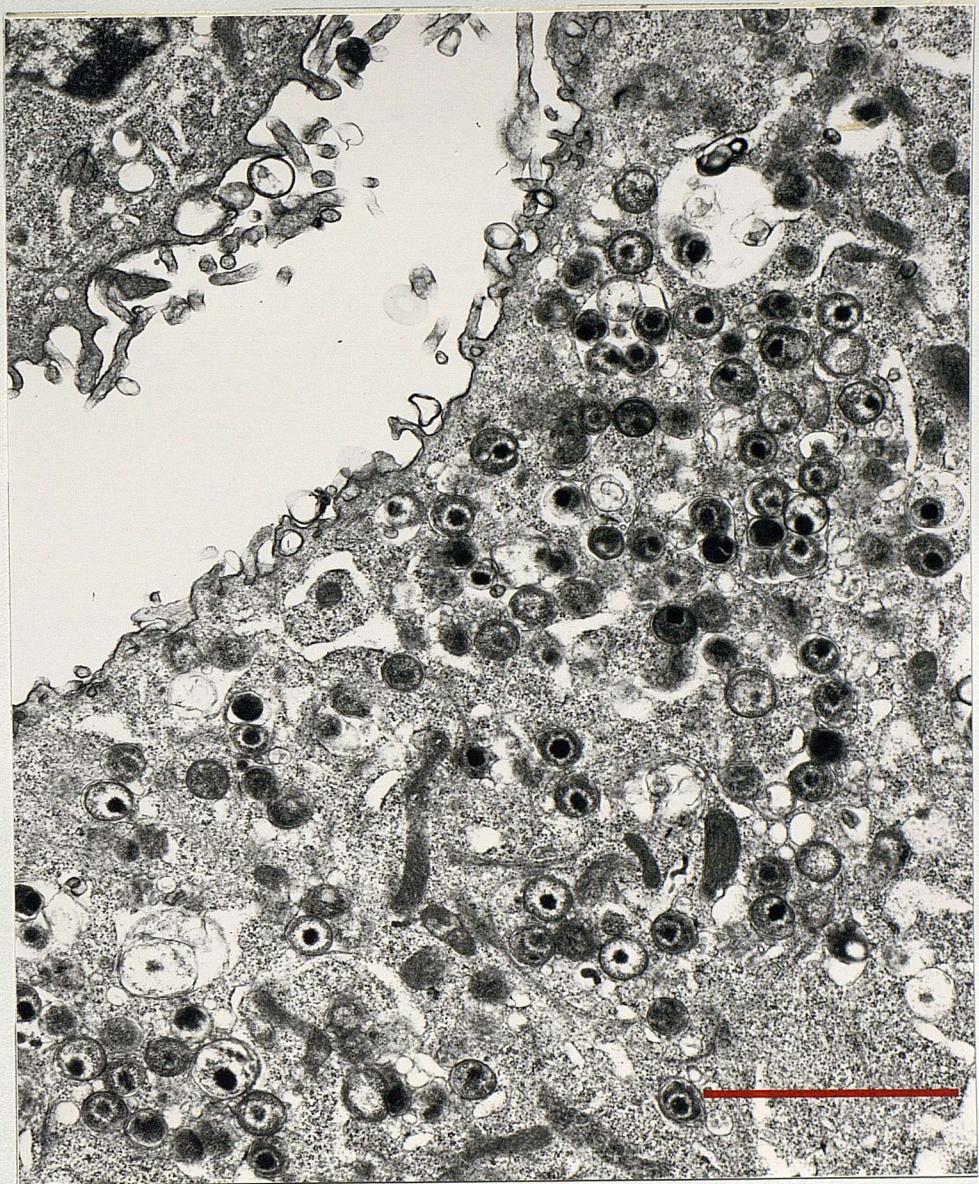


Figure 23 (A) Thirty minutes after initiating uptake large numbers of chlamydiae were observed deep within the host cell cytoplasm. (B) When peroxidase was present in the extracellular tissue culture medium during endocytosis the reaction product of the enzyme with diaminobenzidine and osmium tetroxide could be detected (P) between the chlamydial and endocytic membranes in approximately one half of the endocytic vacuoles. A small clathrin coated vesicle (CL) was also visible. Under the same circumstances ferritin, with its much greater size, was completely excluded from the endocytic vesicles. The bars represent (A) 2.0 μm , (B) 0.1 μm .

A



B





Figure 24 Some 30 min after initiating ingestion the majority of the chlamydial elementary bodies were present within the cell surrounded by tight-fitting endocytic membranes. In this picture some of the chlamydiae are already undergoing expansion and differentiation towards reticulate bodies (arrowed) although it is not clear whether this occurred before or after uptake. Experiments using the radiolabelled sucrose as a marker of the extracellular fluid phase confirmed the low free fluid volume within the endocytic vesicles which is suggested by this electron micrograph. The bar in this figure represents 0.25 μ m.

3.5 The Participation of Surface Receptors in Chlamydial Attachment and Uptake

If host cell surface receptors are required for either chlamydial attachment or interiorisation in the HeLa cell model, it should be possible to demonstrate competition between chlamydiae for these receptors. The ability of purified C. trachomatis LGV 434 to modify attachment or uptake of the homologous organism was therefore determined.

3.5.1 Effect of increasing numbers of unlabelled chlamydiae on the random attachment at 36°C of the [³H]-labelled homologous organism

HeLa 229 cells were grown overnight in DMEM to lightly confluent monolayers in 24-well polystyrene tissue culture trays (5×10^5 cells per well). Following a wash in HBSS, replicate monolayers were inoculated with 500 μ l DMEM containing a constant 287 [³H]-LGV 434 organisms per cell (1.23×10^4 cpm) and 0-10,000 unlabelled LGV 434 per cell. After 5 h incubation at 36°C, the monolayers were washed 4 times with 5 ml volumes of HBSS to remove non adherent chlamydiae, and the cells solubilised with 800 μ l of 1% (w/v) SDS in 0.1M NaOH for 30 minutes (section 2.11). The radioactivity associated with the solubilised cells was measured by liquid scintillation counting (section 2.8). The results are shown in table (12).

After 5 h random association at 36°C in the absence of unlabelled chlamydiae 8% of the inoculum became cell associated equivalent to 23 chlamydiae bound per cell. In the presence of increasing concentrations of unlabelled chlamydiae the proportion of radioactive chlamydiae binding to the cells increased significantly in a dose-dependent manner. This result suggested that chlamydiae might enhance their own attachment to host cells, a phenomenon which may be due to agglutination or a metabolic effect on the host cell surface induced by large numbers of chlamydiae.

Table 12

Replicate monolayers of HeLa 229 cells were washed in HBSS and inoculated with 500 μ l DMEM containing a constant 287 [3 H]-LGV 434 organisms per cell (1.23×10^4 cpm) and 0-10,000 unlabelled LGV 434 per cell. After 5 h incubation at 36°C the monolayers were washed in HBSS and the cell associated radiolabelled chlamydiae determined.

TABLE 12 Effect of increasing numbers of unlabelled LGV 434 on the random attachment at 36°C of the [3H]-labelled homologous organism

Ratio of chlamydiae inoculated per cell	Mean association of [3H]-labelled LGV 434 with cells (cpm)*	Mean number of chlamydiae attached per cell	Increase over control (%)	Probability +
0	981 (± 110)	23	-	-
200	1055 (± 78)	25	7.5	0.07
500	1136 (± 190)	27	15.8	0.04
1000	1336 (± 100)	31	36.2	<0.01
5000	2120 (± 156)	50	116.1	<0.01
10000	2535 (± 143)	59	158.4	<0.01

* Mean of four replicates ($\pm 95\%$ confidence interval for population mean). Three experiments were performed using different batches of inoculum.

+ Probability on a 1-tail t test that cell associated [3H]-labelled LGV 434 was increased compared to the control. Two-way analysis of variance showed variance between treatments was highly significant ($p<0.001$) whilst variance within replicates was not significant ($p>0.05$).

3.5.2 Effect of increasing numbers of unlabelled chlamydiae on the random attachment at 0°C of the [³H]-Labelled homologous organism

Any chlamydial-induced metabolic effect at the host cell surface would probably be temperature dependent. The experiment described in section 3.5.1 was repeated with attachment measured at 0°C instead of 36°C. Replicate monolayers of HeLa cells were inoculated with ice cold DMEM (500 µl) buffered with HEPES to pH 7.2 containing a constant 287 [³H]-LGV 434 particles per cell (1.01x10⁴ c.p.m.) and 0-5000 unlabelled LGV 434 per cell. After 20 h incubation at 0°C the monolayers were thoroughly washed in ice cold HBSS, the cells solubilised as before and the cell associated radioactivity determined. The results are shown in table (13).

Again there was no evidence that unlabelled chlamydiae competitively inhibited the random attachment of the homologous radiolabelled organisms to the host cell. The increase in binding observed with the higher doses of unlabelled chlamydiae was unlikely to be due to a metabolic effect at 0°C. The observed increased association was probably due to aggregation of the large numbers of chlamydial particles in suspension, an effect which would be dose-dependent at high concentrations.

3.5.3 The effect of centrifuging increasing numbers of unlabelled chlamydiae onto host cells on the random attachment at 36°C of the [³H]-labelled homologous organism

Given that chlamydial attachment to HeLa 229 cells by random association was inefficient, it could be argued that insufficient non radiolabelled chlamydiae were attached to each cell to saturate or compete for putative receptor sites. This possibility was examined more thoroughly using centrifugation to increase the total number of unlabelled chlamydiae attached per cell.

Confluent monolayers of HeLa 229 cells (5x10⁵ cells per well) were cooled on ice, washed in ice cold HBSS and inoculated with 500 µl

Table 13

The experimental procedure was similar to that described for Table 12. A constant 287 [^3H]-LGV particles per cell (1.01×10^4 cpm) and 0-5000 unlabelled LGV 434 were inoculated in 500 μl DMEM buffered to pH 7.2 with HEPES. Cell associated radiolabelled chlamydiae were determined after 20 h incubation at 0°C.

TABLE 13 Effect of increasing numbers of unlabelled LGV 434 on the random attachment at 0°C of the [³H]-labelled homologous organism

Ratio of chlamydiae inoculated per cell	Mean association of [³ H]-labelled LGV 434 with cells (cpm)*	Mean number of chlamydiae attached per cell	Probability †
0	754 (± 129)	18	-
250	689 (± 45)	16	0.2
500	746 (± 107)	17	0.90
1000	758 (± 99)	18	0.96
2500	868 (± 56)	20	0.06
5000	1012 (± 119)	24	0.02

* Mean of four replicates ($\pm 95\%$ confidence interval for population mean) determined 20 h after chlamydial challenge.

† Probability on a 2-tail t test that cell associated [³H]-labelled LGV 434 was significantly greater or less than in the control.

of ice cold DMEM buffered to pH 7.2 with HEPES containing 0-5000 LGV 434 particles per cell. Following centrifugation for 1 h at 1000 g in a precooled centrifuge at 8°C, the supernatant medium was carefully removed, 500 µl DMEM containing a standard inoculum of [³H]-LGV 434 (287 particles per cell) was added and the cells incubated at 36°C for 5 h. After thoroughly washing the monolayers in HBSS, the cell associated chlamydiae were then solubilised in SDS and the radioactivity measured by liquid scintillation counting (section 2.8). Cell numbers were determined by Safranin O dye uptake (Section 2.10). The results are shown in Table (14).

Up to 5000 unlabelled LGV 434 deposited on each cell made no significant difference to the proportion of [³H]-LGV 434 inoculum subsequently attaching to the cell by random association during 5 h incubation. One explanation might be that at 36°C attachment receptors were regenerated as adherent chlamydiae were ingested by the cell.

3.5.4 The effect of centrifuging increasing numbers of unlabelled chlamydiae onto host cells on the random attachment at 0°C of the [³H]-labelled homologous organism

To eliminate the possibility of receptor regeneration following ingestion of chlamydiae by the host cell, the previous experiment (section 3.5.3) was repeated at 0°C, to prevent uptake but permit attachment. HeLa 229 cells were grown in 24-well tissue culture trays and between 0-5000 LGV 434 particles per cell were centrifuged onto the monolayer at 8°C as previously described (section 3.5.3). Following the removal of the supernatant medium 500 µl ice cold DMEM containing [³H]-LGV 434 (287 particles per cell) was added and the cells incubated for 20 h at 0°C. The supernatant medium was removed, the cells were thoroughly washed, solubilised (section 2.11) and the radioactivity determined (section 2.8). The results are shown in table (15).

In the presence of increasing concentrations of unlabelled chlamydiae the proportion of radioactive chlamydiae binding to the

Table 14

Confluent monolayers of HeLa 229 cells were cooled on ice and inoculated with DMEM containing 0-5000 LGV 434 particles per cell. Following centrifugation at 1000 g for 1 h at 8°C the supernatant medium was removed and replaced with 500 µl DMEM containing a constant 287 [³H] LGV particles per cell (1.49 x 10⁴ cpm). After 5 h incubation at 36°C the cell associated radiolabelled chlamydiae was determined.

TABLE 14 The effect of centrifuging increasing numbers of unlabelled LGV 434 onto host cells on the random attachment at 36°C of the [³H]-labelled homologous organism

Ratio of chlamydiae inoculated per cell	Mean association of [³ H]-labelled LGV 434 with cells (cpm)*	Mean number of chlamydiae attached per cell	Probability †
0	1255 (± 213)	29	-
250	1287 (± 52)	30	0.68
500	1294 (± 88)	30	0.62
1000	1266 (± 78)	30	0.90
2500	1256 (± 94)	29	1.0
5000	1062 (± 57)	25	0.04

* Mean of four replicates ($\pm 95\%$ confidence interval for population mean) measured 5 h after chlamydial challenge.

† Probability on a 2-tail t test that cell associated [³H]-labelled LGV 434 was significantly greater or less than in the control.

Table 15

The experimental procedure was similar to that described for Table 14. Following the centrifugation of the unlabelled organisms onto the monolayer, the supernatant medium was removed and replaced with 500 μ l DMEM containing [3 H]-LGV 434 (287 chlamydiae per cell; 1.49×10^4 cpm). Cell associated radiolabelled chlamydiae were determined after 20 h incubation at 0°C.

TABLE 15 The effect of centrifuging increasing numbers of unlabelled LGV 434 onto host cells on the random attachment at 0°C of the [³H]-labelled homologous organism

Ratio of chlamydiae inoculated per cell	Mean association of [³ H]-labelled LGV 434 with cells (cpm)*	Mean number of chlamydiae attached per cell	Probability †
0	1013 (\pm 38)	24	-
250	1065 (\pm 60)	25	0.08
500	1104 (\pm 49)	26	0.02
1000	1245 (\pm 170)	29	0.02
2500	1333 (\pm 162)	31	0.02
5000	1257 (\pm 231)	29	0.02

* Mean of four replicates (\pm 95% confidence interval for population mean) determined 20 h after chlamydial challenge.

† Probability on a 2-tail t test that cell associated [³H]-labelled LGV 434 was significantly greater or less than in the control.

cells again increased. As before, there was no evidence of competition between [³H]-labelled and non-labelled LGV 434 for specific attachment sites on the host cell surface. This suggested that LGV 434 attachment in the HeLa cell model probably involves low avidity non specific mechanisms rather than high avidity specific receptors.

3.5.5 The effect of increasing numbers of unlabelled chlamydiae on the uptake of the [³H]-labelled homologous organism

Specific receptors might still be essential for chlamydial uptake as opposed to attachment. Thus, the ability of increasing numbers of *C. trachomatis* LGV 434 to inhibit the uptake of a standard [³H]-labelled inoculum of the homologous organism into HeLa 229 cells was investigated. HeLa 229 cells were grown overnight to confluence in 24-well tissue culture trays (5×10^5 cells per well). After washing in ice cold HBSS the cells were cooled on ice, inoculated with 500 μ l DMEM containing 0-10,000 purified non-radioactive LGV 434 and a constant 115 [³H]-LGV 434 particles per cell (1.5×10^4 c.p.m.). The trays were centrifuged at 1000 g for 1 h at 8°C. The supernatant medium was gently replaced with 500 μ l of HEPES-buffered ice cold DMEM, the trays were rapidly warmed by flotation on a 36°C water bath, then incubated for 30 min to permit uptake. After 4 washes in ice cold PBS, the chlamydiae adherent to the host cell surface were eluted with trypsin-EDTA and separated by differential centrifugation as before (section 2.12). Cell associated radioactivity due to internalised tritium labelled LGV 434 was determined by solubilisation of the cells in SDS (section 2.12) and liquid scintillation counting (section 2.8). The results are shown in table (16).

In the presence of 500 or more unlabelled chlamydiae per cell a significant and dose related reduction in the uptake of the [³H]-labelled organisms was observed. This was in direct contrast with the failure to demonstrate competitive inhibition of attachment and suggests that the mechanisms involved in attachment and uptake were distinct.

Table 16

Confluent monolayers of HeLa 229 cells were washed in ice cold HBSS and inoculated with 500 μ l DMEM containing 0-10,000 unlabelled LGV 434 and a constant 115 [3 H]-LGV 434 particles per cell (1.5×10^4 cpm). Following centrifugation at 1000 g for 1 h at 8°C, the supernatant medium was replaced with ice cold HEPES-buffered DMEM and uptake of the radiolabelled organism determined after 30 minutes incubation at 36°C.

TABLE 16 The effect of increasing numbers of unlabelled LGV 434 on the uptake of the [³H]-labelled homologous organism

No of unlabelled chlamydiae centrifuged onto each cell	Mean uptake of [³ H]-labelled LGV 434 (c.p.m.)*	Mean number of chlamydiae ingested per cell	Reduction in uptake (%)
0	5985 (± 210)	60	-
500	5267 (± 399)	53	12.0
1000	4789 (± 259)	48	20.0
2500	4170 (± 248)	42	30.3
5000	2555 (± 181)	26	67.3
10000	1365 (± 113)	14	77.2

* Mean of seven replicate samples ($\pm 95\%$ confidence interval for population mean).

The presence of 500 or more unlabelled chlamydiae per cell significantly reduced the uptake of the [³H]-labelled organism compared with the control (1 tail t test, $p < 0.01$).

3.6 Fluid Phase Ingestion During Chlamydial Endocytosis

One possibility might be that chlamydiae are ingested following the stimulation of wide-spread endocytic activity by the host cell. In that event, a concomitant increase in fluid phase ingestion by the host cells should be demonstrable. Conversely, passive entry of chlamydiae into pinocytotic vesicles during the normal process of receptor mediated endocytosis, would be reflected in a reduction in fluid uptake due to the volume of fluid displaced by the chlamydiae. This concept was investigated by measuring fluid phase uptake during chlamydial endocytosis using [³H]-sucrose, a small molecular weight, non permeable marker of the extracellular fluid space (Marsh and Helenius 1980).

3.6.1 Ingestion of tritiated sucrose during chlamydial endocytosis

HeLa 229 cells were grown in DMEM to lightly confluent monolayers in 24-well tissue culture trays. The trays were washed in ice cold HBSS and 0-5000 LGV 434 particles per cell contained in 500 µl of DMEM were centrifuged onto the monolayers at 1000 g for 1 h at 8°C. The supernatant medium was replaced at 0°C with 250 µl per well of DMEM containing 2% (v/v) foetal calf serum, 100 nM unlabelled sucrose and 1x10⁶ c.p.m. [³H]-sucrose. The unlabelled sucrose was present to saturate any surface receptors for sucrose. Chlamydial uptake was stimulated synchronously by warming to 36°C. After 30 minutes incubation the cells were washed three times in ice cold HBSS containing 10 mM non-radioactive sucrose then solubilised in 700 µl SDS for 30 min at 36°C and the cell associated radioactivity due to fluid phase ingestion determined (Section 2.8).

The results (Table 17) showed that as the chlamydial inoculum was increased there was a very small but significant, ($p < .001$) linear increase in sucrose uptake above that due to normal, constitutive level of incorporation. In 8 separate experiments using different batches of [³H]-LGV 434 inoculum the mean proportion of the challenge chlamydiae endocytosed after 30 min was 35%. On this basis, the mean volume of fluid endocytosed as each chlamydial particle was ingested

Table 17

Confluent monolayers of HeLa 229 cells were washed in ice cold HBSS and inoculated with 500 μ l DMEM containing 0-5000 LGV 434 particles per cell. Following centrifugation at 1000 g for 1 h at 8°C the supernatant medium was removed and replaced with 250 μ l DMEM containing 1×10^6 c.p.m. [3 H]-sucrose. After 30 minutes incubation at 36°C the cells were washed in ice cold HBSS and the cell associated radioactivity determined.

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 TABLE 17 The effect of chlamydial uptake on the endocytosis by HeLa 229 cells of extracellular fluid containing [³H]-sucrose

No. of chlamydiae centrifuged onto each cell	Mean [³ H]-sucrose uptake (c.p.m.)	
	Uptake observed after 30 min at 36°C*	Uptake calculated from linear fit †
0	516 (± 20)	518
250	525 (± 29)	525
500	535 (± 20)	532
1000	546 (± 39)	547
5000	661 (± 60)	661

* Mean of eight replicate samples ($\pm 95\%$ confidence interval for population mean). The experiment was repeated twice.

† Correlation coefficient for the linear fit was 0.99. Two-way analysis of variance showed variance between treatments to be highly significant ($p < 0.001$) whilst variance within replicates was not significant ($p > 0.05$).

could be calculated as 64 al (6.4×10^{-17} L). This figure was in broad agreement with the electron microscope observations showing the presence of tight endocytic vacuoles, (Fig. 24). However, it was not possible from electron microscopy to calculate the fluid volume with sufficient accuracy due to lens hysteresis and the likelihood of dimensional changes during processing.

3.7 Role of the Host Cytoskeleton in Chlamydial Endocytosis

Numerous workers have reported that chlamydial infectivity is unaffected by the microfilament inhibitor cytochalasin B. However, the lack of evidence for uptake of chlamydiae via receptor mediated endocytosis, prompted a reinvestigation of the importance of the host microfilament-microtubule system during chlamydial endocytosis. The microfilament inhibitors cytochalasin B and D and the microtubule inhibitors vinblastine, vincristine and colchicine were selected to investigate the importance of the functional integrity of the HeLa cytoskeleton during chlamydial endocytosis.

3.7.1 Effect of microfilament and microtubule inhibitors on attachment of chlamydiae to host cells

Concentrated solutions of the microfilament inhibitors cytochalasin B and D (both Sigma) were prepared in analytical grade dimethylsulphoxide at 1 mg per ml and stored frozen at -70°C. Microtubule inhibitors colchicine (Sigma), vincristine sulphate (Lilly Ind. U.K., Basingstoke Hants) and vinblastine sulphate (Lederle UK, Gosport Hants) were dissolved immediately before use in HBSS. Replicate monolayers of HeLa 229 cells grown overnight in DMEM in 24-well tissue culture trays were washed in HBSS and incubated for 1 h at 36°C with (test) or without inhibitor (control) in threonine-deficient DMEM. The medium was removed and 500 µl volumes of [³H]-LGV 434 in inhibitor containing DMEM equivalent to 6000 c.p.m. (140 chlamydiae per cell) was added to each well. After 6 h incubation at 36°C, the cells were thoroughly washed in HBSS, solubilised in SDS (Section 2.11) and the radioactivity due to cell associated chlamydiae

determined. Any effect of inhibitors on cell numbers or viability was determined at the end of the experiment by Safranin O staining or trypan blue dye exclusion (section 2.10). Light and scanning electron microscopy performed by Dr. M. Ward (data not shown) demonstrated that cytochalasin D but not B had a marked morphological effect on HeLa cells, causing them to round up but not detach from the substratum. Cell numbers (Safranin O staining) and cell viability (trypan blue exclusion) were not affected by any of the inhibitors in these relatively short term experiments.

The results (Table 18) showed that both cytochalasin B and D caused a significant reduction in chlamydial adherence compared with the untreated controls (1-tail t test, $p<0.01$). The microtubule inhibitors vincristine, vinblastine and colchicine were less effective, reducing attachment by only 7, 12, and 11% respectively compared with the untreated control. This suggested that microfilaments may be important for chlamydial attachment to host cells whereas microtubules were relatively unimportant.

3.7.2 Effect of microfilament and microtubule inhibitors on the ingestion of chlamydiae by host cells

The effect of microfilament and microtubule inhibitors on chlamydial ingestion was next determined. Replicate monolayers of HeLa 229 cells grown in DMEM in 24-well tissue culture trays were incubated for 1 h at 36°C in DMEM buffered with 30 mM HEPES to pH 7.2 containing either microfilament or microtubule inhibitor, or DMEM alone (control). The medium was removed, the trays cooled on ice and the cells were challenged by centrifugation of 140 [3 H]-LGV 434 organisms per cell at 8°C for 1 h at 1000 g in inhibitor-containing HEPES-DMEM. The supernatant fluid was carefully removed and fresh inhibitor containing medium was added. The cells were then rapidly warmed to 36°C and incubated for 30 minutes to permit uptake to occur. After washing in ice cold PBS chlamydiae adherent to the host cell surface were eluted with trypsin-EDTA and separated by differential

Table 18

Replicate monolayers of HeLa 229 cells were washed in HBSS and incubated for 1 h at 36°C in TD-DMEM containing the appropriate concentration of inhibitor. The medium was removed and 500 µl volumes of [³H]-labelled LGV 434 in inhibitor containing medium equivalent to 6000 cpm (140 chlamydiae per cell) was added to each well. After 6 h incubation at 36°C and washing in HBSS, radioactivity due to cell associated chlamydiae was determined.

TABLE 18

Effect of microfilament and microtubule inhibitors on the attachment of [³H]-labelled LGV 434 to HeLa 229 cells

Inhibitor (con ^c , µg per ml)	Mean * attachment (c.p.m. ± S.D.)	Mean No. of chlamydiae attached per cell	Reduction (%)†
None	1253 (±418)	29	-
Cytochalasin B (1.0)	1061 (±14)	25	15
(2.5)	776 (±59)	18	38
Cytochalasin D (1.0)	905 (±69)	21	28
(2.5)	865 (±71)	20	31
Vincristine	1165 (±14)	27	7
Vinblastine	1100 (±54)	26	12
Colchicine	1117 (±45)	26	11

* Mean (±S.D) for three or four replicates. The experiment was performed twice.

† Percentage reduction compared with untreated control.

centrifugation (section 2.12). The cell associated radioactivity due to internalised chlamydiae was solubilised in SDS (Section 2.12) and measured by liquid scintillation counting (Section 2.8). The effect of the various drug treatments is shown in Table (19).

Cytochalasin D at 1 μ g per ml reduced chlamydial ingestion by 51% but cytochalasin B was less effective. Vinblastine and vincristine reduced chlamydial ingestion by 47 and 44% respectively compared with the untreated control but colchicine was relatively ineffective. The reduction in chlamydial ingestion due to drug treatments was not a result of elution of the chlamydiae from the host cell following centrifugation (data not shown). These results suggested that microfilaments and microtubules were both involved in the uptake of chlamydiae into host cells.

3.7.3 Effect of microfilament inhibitors on chlamydial infectivity for HeLa cells

The long term effects of cytochalasin B and D on chlamydial infectivity for HeLa 229 cells was examined by measuring chlamydial dependent [3 H]-threonine uptake (section 2.5.2) as a direct measure of chlamydial growth.

Replicate monolayers of HeLa 229 cells in 24-well tissue culture trays were washed in HBSS and incubated at 36°C for 1 h prior to chlamydial challenge in TD-DMEM containing 1 μ g per ml emetine hydrochloride (control) or the same medium with 1 μ g per ml of either cytochalasin B or D (test). Following incubation, the tissue culture medium was removed and the cells were inoculated with 1 ml volumes of TD-DMEM containing LGV 434 (140 chlamydiae per cell) and 1 μ g per ml emetine hydrochloride in the presence (test) or absence (control) of microfilament inhibitor. After 48 h incubation at 36°C the monolayers were washed 4 times in 5 ml HBSS then solubilised as before in SDS at 36°C for 30 minutes. Cell associated radioactivity was measured by liquid scintillation counting (section 2.8) and cell numbers were monitored using Safranin O staining (section 2.10).

Table 19

Replicate monolayers of HeLa 229 cells were washed in HBSS and incubated for 1 h at 36°C with inhibitor in DMEM buffered with 20 mM-HEPES at pH 7.2. [3H]-labelled LGV 434 (140 chlamydiae per cell) were centrifuged onto the cells at 8°C for 1 h in inhibitor-containing HEPES-DMEM. Following centrifugation, fresh inhibitor-containing medium was added, the cells incubated at 36°C for 30 min to promote uptake and the radioactivity due to intracellular chlamydiae determined.

TABLE 19

Effect of microfilament and microtubule inhibitors on the
ingestion of [³H]-labelled LGV 434 to HeLa 229 cells

Inhibitor (con ^c , µg per ml)	Mean uptake (c.p.m.)*	Mean No. of chlamydiae ingested per cell	Reduction † in uptake %
None	2335 (± 202)	55	-
Cytochalasin B (1.0)	1698 (± 215)	40	27
(2.5)	1674 (± 147)	39	28
Cytochalasin D (1.0)	1152 (± 168)	27	51
(2.5)	1104 (± 88)	26	53
Vincristine (2.5)	1309 (± 129)	31	44
(5.0)	1342 (± 76)	31	43
Vinblastine (2.5)	1246 (± 174)	29	47
(5.0)	1249 (± 91)	29	47
Colchicine (1.0)	1888 (± 218)	44	19
(2.0)	1980 (± 184)	46	15

* Mean of six replicates ($\pm 95\%$ confidence interval for population mean).
The experiment was performed twice.

† Percentage reduction compared with untreated control. All inhibitors significantly reduced chlamydial ingestion compared with the untreated control. (1-tail t test, $p < 0.01$).

Long term (48 h) cytochalasin B or D treatment inevitably caused cell loss from the monolayers, with losses of 13 and 31% respectively (Table 20). The mean chlamydial dependent [³H]-threonine incorporation \pm standard deviation for 4 replicates in infected, untreated cells was 311,910 \pm 23,240 c.p.m. This was reduced 33 and 79% respectively by cytochalasin B or D treatment to 208,100 \pm 22,190 and 65,380 \pm 2590 c.p.m. Thus, allowing for cell loss, cytochalasin B and D reduced chlamydial threonine incorporation and hence chlamydial infectivity for HeLa 229 cells by 23% and 70% respectively.

3.8 Requirement for Circumferential Engulfment of Chlamydiae by Host Cell prior to Ingestion

The close interaction between the chlamydial and host cell surfaces during ingestion and the participation of host cell microfilaments suggested that chlamydial ingestion might occur following sequential binding of the host cell membrane to the chlamydial surface. To investigate this possibility, the ability of rabbit hyper-immune chlamydial antibody, to prevent the ingestion of radiolabelled chlamydiae was determined.

3.8.1 Effect of rabbit hyper-immune LGV 434 antibody on the ingestion of chlamydiae into host cells

Replicate HeLa 229 cell monolayers in 24-well tissue culture trays were washed in ice cold HBSS. [³H]-LGV 434 (145 chlamydiae per cell) in 500 μ l DMEM buffered to pH 7.2 with HEPES were centrifuged for 1 h at 1000 g, 8°C, onto the cells. Non adherent chlamydiae were aspirated and the medium was replaced with ice cold DMEM containing dilutions of hyperimmune rabbit anti LGV 434 serum (test) or the same concentration of pre-immune serum (control). Antiserum was obtained from a rabbit repeatedly immunized subcutaneously and intramuscularly with 500 μ g LGV 434 in Freunds adjuvant over 2 months. The resulting hyperimmune serum had an ELISA titre of 1:1,638,400 using 3,5',5,5' tetramethybenzidine (TMB; Miles) as substrate. After incubation on ice for 1 h to permit antibody binding, the cells were rapidly warmed to 36°C and incubated for 30 minutes to permit synchronous chlamydial

Table 20

Replicate monolayers of HeLa 229 cells in 24-well tissue culture trays were washed in HBSS and incubated at 36°C for 1 h in TD-DMEM containing inhibitor and 1 μ g per ml emetine hydrochloride. Following incubation, the medium was removed and replaced with 1 ml volumes of TD-DMEM containing LGV 434 (140 chlamydiae per cell), emetine hydrochloride and inhibitor as appropriate. Cell associated radioactivity was determined after 48 h incubation at 36°C. Cell numbers were monitored using Safranin O staining.

TABLE 20

Effect of microfilament inhibitors on
chlamydial development in HeLa 229 cells

Inhibitor (con ^c , µg per ml)	Chlamydial dependent [³ H]-threonine uptake* (c.p.m.)	% reduction in [³ H]-threonine uptake †	% loss of cells from monolayer
0	311,910 (± 23240)	-	none
cytochalasin B (1.0)	208,100 (± 22190)	33	13
cytochalasin D (1.0)	65,380 (± 2590)	79	31

* Mean (\pm S.D) for four replicates.

† Percentage reduction compared with untreated control.

ingestion. Cell monolayers were then washed in PBS, treated with trypsin-EDTA (section 2.12) to remove surface adherent chlamydiae and the radioactivity in the cell pellet attributable to interiorised chlamydiae was determined as before (section 2.8).

It was assumed that antibody would be unable to bind to points where chlamydiae were tightly adherent to the host cell, but would be free to bind elsewhere to the chlamydial surface. This concept was supported by the fact that antibody failed to elute bound chlamydiae from the host cell surface (data not shown). The results (Table 21) showed that pre-immune serum at high concentrations had only a slight effect on chlamydial uptake presumably due to non-specific interactions. By contrast, hyperimmune serum from the same animal at comparable concentrations blocked chlamydial uptake to a significant extent ($p<0.01$) and in a dose dependent manner. This experiment showed that high levels of polyclonal antibody were capable of blocking the ingestion of chlamydiae already adherent to the cell surface. The most plausible explanation was that antibodies bound to the chlamydial surface at points distal to the point of attachment, prevented circumferential binding between ligands on the chlamydial and host cell surfaces.

3.9 Host Cell Cyclic Nucleotide and Prostaglandins - their role during chlamydial infection

The cyclic nucleotides cAMP and cGMP together with calcium are major components of an internal signalling system regulating many fundamental cell activities. Ward and Salari (1982) reported that alterations in the level of HeLa cell cyclic nucleotides (cAMP and cGMP) and prostaglandins (PGE and PGF₂ α) affected the susceptibility of HeLa cells to chlamydial infection. It was suggested that chlamydial infection of HeLa cells might be under bidirectional cyclic nucleotide control with cGMP or Ca²⁺ promoting chlamydial infectivity and cAMP antagonising it. The possibility that chlamydial infection of HeLa cells might itself affect host cell cyclic nucleotide or prostaglandin synthesis was investigated.

Table 21

[³H]-LGV 434 were centrifuged for 1 h at 1000 g and 8°C onto monolayers of HeLa 229 cells. The supernatant medium was replaced with ice-cold HBSS containing the indicated concentration of rabbit LGV 434 antiserum or pre-immune serum and incubated for 1 h at 0°C to permit antibody binding. Uptake of the organism was determined following incubation of the monolayers for 30 min at 36°C.

TABLE 21 Effect of rabbit hyper-immune LGV 434 antibody bound asymmetrically to the chlamydial surface at points distal to the site of chlamydial attachment to the host cell on the ability of HeLa cells to ingest chlamydiae

Dilution of antibody or pre-immune serum	Mean uptake of [³ H]-labelled LGV 434 in presence of pre-immune serum (cpm)*	Mean uptake of [³ H]-labelled LGV in presence of antibody (cpm)*	Reduction in uptake due to antibody (%)†	Probability *
1/10	3256 (\pm 579)	1760 (\pm 312)	46.0	<0.01
1/25	3220 (\pm 477)	2084 (\pm 299)	35.3	<0.01
1/50	3024 (\pm 165)	2436 (\pm 229)	19.5	<0.01
1/100	3008 (\pm 674)	2696 (\pm 127)	10.4	0.11

* Mean of four replicates (\pm 95% confidence interval for population mean). The experiment was repeated three times.

† Percentage reduction in uptake due to antiserum compared with the corresponding pre-immune serum.

* Probability on a 1-tail t test that [³H]-labelled LGV 434 uptake was significantly reduced compared with corresponding pre-immune control. Two-way analysis of variance showed that variance between antibody treatments was highly significant ($p<0.01$) whilst variance within replicates was not significant ($p>0.05$). Variance between pre-immune serum treatments or within replicates was not significant ($p>0.05$).

3.9.1 Assay of endogenous host cell cAMP and cGMP using tritiated cyclic nucleotides

The first essential was to determine the endogenous levels of host cell cyclic nucleotides in order to calculate the number of cells required for the assays. HeLa 229 cells were grown overnight in DMEM to light confluence in 80 sq. cm tissue culture flasks (approx 2×10^7 cells per flask). Following a brief wash in PBS, 5 ml of 70% (v/v) ethanol at 80°C was added to each flask to simultaneously extract the cyclic nucleotides and to inactivate any degradative phosphodiesterases. After overnight equilibration at 4°C the ethanolic extracts (5 ml) were transferred to a conical container and evaporated to dryness at 36°C. The dried residue was redissolved in 200 µl of 50 mM Tris buffer pH 7.5 containing 4 mM EDTA. Any insoluble material was removed by centrifugation at 1000 g for 5 min and assays were performed on the supernatant material as described in section 2.15. Figs. 3 & 4 show typical standard curves for the cyclic nucleotide assays. The protein content of the HeLa cell monolayer was also determined after solubilisation of the cells in 1.0 M NaOH using the method of Lowry et al 1951 (section 2.9). The results of the cyclic nucleotide assay are tabulated in Table 22.

The mean endogenous levels of cAMP and cGMP in HeLa 229 cells were 0.78 and 0.033 picomoles per mg of cell protein respectively. These levels were equivalent to 6.3 and 0.26 picomoles cAMP and cGMP respectively per 70 sq cm. flask of HeLa 229 cells. These low levels of cyclic nucleotides particularly cGMP, necessitated the development of very sensitive assay systems.

3.9.2 Radioimmunoassay of cAMP and cGMP using iodinated cyclic nucleotides

The sensitivity of a radioimmunoassay (RIA) is principally determined by the specific activity of the radiolabelled antigen and the affinity of antigen for the antisera. cAMP and cGMP can be labelled to a higher specific radioactivity with I^{125} , using the

Table 22

Confluent monolayers of HeLa 229 cells grown in DMEM in tissue culture flasks were washed in PBS and the cyclic nucleotides extracted into 5 ml 70% (v/v) ethanol at 80°C. Following overnight equilibration at 4°C the ethanolic extracts were evaporated to dryness at 36°C. The dried residues were redesolved in Tris-EDTA buffer pH 7.4 and assayed for cAMP and cGMP.

TABLE 22

Assay of endogenous cAMP and cGMP levels in
HeLa 229 cells using tritiated label

monolayer*	cAMP picomoles † per mg protein	cGMP picomoles † per mg protein
1	0.90	0.045
2	0.65	0.035
3	0.80	0.020

* Approximately 2×10^7 cells per monolayer.

† Mean of two replicates. The experiment was performed three times.

succinyl tyrosine methyl esters, than is possible using ^3H . Moreover, cyclic nucleotides substituted at the 2'-O-position have a higher affinity for antibody and thus displace the labelled derivative more effectively than the unsubstituted cyclic nucleotide (Steiner et al 1972). Frandsen and Krishna 1976 demonstrated that acetylation of samples with acetic anhydride at the 2'-O-position increased assay sensitivity down to the femtomole range (10^{-15}M). This use of acetylation to increase assay sensitivity and its applicability to tissue culture extracts was investigated using ^{125}I -labelled cyclic nucleotide derivatives.

3.9.3 Validation of cyclic nucleotide assays

3.9.3.1 Specificity of the antisera

The specificity of the antisera to cAMP and cGMP was tested by determining the quantity of structurally related nucleotides required to inhibit the binding of the antisera to its target cyclic nucleotide by 50%. The following compounds cAMP, cGMP, cIMP, cCMP, AMP, ADP, ATP and the succinyl derivates of cAMP and cGMP were tested at 500, 50, 5, 0.5, 0.05 0.005 and 0.0005 picomoles per tube. All these compounds were selected because of their structural relationship to either cAMP or cGMP and the likelihood that they might be present in biological material. The compounds, dissolved in 100 μl of sodium acetate buffer pH 6.2, were acetylated with 5 μl acetylating reagent (section 2.16.1.4) and incubated for 18 h with either [^{125}I]-succinyl cAMP-TME and anticAMP antisera at 0°C (cAMP assay) or with [^{125}I]-succinyl cGMP-TME and anticGMP antisera (cGMP assay). Following the separation of antibody-bound compound from unbound nucleotide (section 2.16.1.5) the radioactivity bound to the antibody was determined by gamma counting with a counting efficiency for ^{125}I of approximately 70%.

The results shown in table 23 indicate that cIMP showed the most cross-reactivity in both assays, with 0.1% and 1.3% for cAMP and cGMP respectively. Other structurally related compounds tested did not cross-react by more than 0.01%. Given that cIMP levels in biological

Table 23

The percentage cross-reactivity of the antibodies to other nucleotides at the 50% level of inhibition was calculated as:

100X concentration of cAMP or cGMP required for <u>inhibition of $[I^{125}]$ binding to 50%</u>	
concentration of test substance required for inhibition of $[I^{125}]$ binding to 50%	

TABLE 23

Specificities of cyclic nucleotide antisera

cAMP

<u>Compound</u>	<u>at I₅₀</u>	<u>Cross-reactivity</u>
cAMP		100
suc cAMP		100
cIMP		0.01
cGMP		<0.01
AMP		<0.01
ADP		<0.01
ATP		<0.01

cGMP

<u>Compound</u>	<u>at I₅₀</u>	<u>Cross-reactivity</u>
cGMP		100
suc cGMP		100
cIMP		1.3
cAMP		0.01
cCMP		0.01
AMP		<0.01
ADP		<0.01
ATP		<0.01

material are some 100 fold lower than cGMP levels both assays were considered to have an acceptable degree of specificity.

3.9.3.2 Precision of the assays

Precision refers to the magnitude of the random errors and the reproducibility of the assay measurements and was estimated by calculating the mean and standard deviation of replicate assays of cell extract. Confluent monolayers of HeLa 229 cells grown in DMEM in 6 well trays were washed in PBS, the cyclic nucleotides were extracted in 70% ethanol at 80°C and evaporated to dryness at 36°C. (Section 2.15.1.3). The dried extracts were redissolved in 300 µl sodium acetate buffer pH 6.8 and aliquots taken for cyclic nucleotide assay (section 2.16.1.5). Twenty replicate samples gave a mean and standard deviation of 63.61 ± 8.38 and 26.60 ± 3.31 femtomoles per tube for cAMP and cGMP respectively. This was considered an acceptable degree of intra-assay reproducibility.

3.9.3.3 Recovery

The recovery of cyclic nucleotides from the cell extracts was monitored by adding [I^{125}]-labelled cAMP and cGMP to 10 replicate ethanolic extracts prior to evaporating to dryness. The number of counts recovered in the sodium acetate buffer was expressed as a percentage of the counts added, to give the percent recovery. Confluent monolayers of HeLa 229 cells grown in DMEM in 6 well trays were washed in PBS and 2 ml of 70% ethanol at 80°C containing either 4603 c.p.m. [I^{125}]-cAMP or 3877 c.p.m. [I^{125}]-cGMP was added. The ethanolic extracts were evaporated to dryness at 36°C followed by reconstitution in 300 µl sodium acetate buffer pH 6.8. The radioactivity in the sodium acetate buffer was then determined by gamma counting.

It was found that the mean recovery of [I^{125}]-cAMP and [I^{125}]-cGMP was 90% (coefficient of variation 2.37%) and 91% (coefficient of variation 4.8%) respectively. The coefficient of variation was calculated as the ratio of the standard deviation to the population mean expressed as a percentage.

3.9.4 Host cell cyclic nucleotide levels during the initial stages of chlamydial infection

The levels of cAMP and cGMP in HeLa 229 cells during the first 2 h of chlamydial infection were compared with similarly treated uninfected control cells to determine if there were early changes in cyclic nucleotides following chlamydial challenge. Confluent monolayers of HeLa cells grown overnight in DMEM in 6 well polystyrene trays were washed in HBSS and cooled to 0°C on ice. Three wells of each tray were inoculated with purified LGV 434 at a chlamydial : cell ratio of 320:1 in 5 ml of ice cold DMEM. The remaining 3 wells of each tray were inoculated with 5 ml of buffered DMEM alone (controls). Following centrifugation at 1000 g for 1 h at 8°C the cells were warmed to 36°C to permit uptake to occur. At 0, 15, 30, 60 and 120 mins after centrifugation the medium was removed, the cells were quickly washed in PBS at 36°C and the cyclic nucleotides extracted into 2 ml of 70% (v/v) ethanol at 80°C. After overnight equilibration in ethanol at 4°C the extracts were pooled and evaporated to dryness at 36°C. Following resuspension of the dried extracts in 300 µl sodium acetate buffer pH 6.2, 2 x 100 µl aliquots of neat extract were assayed directly for cGMP and 2 x 100 µl aliquots of a 1/50 dilution of extract were assayed for cAMP (section 2.16). The protein content of the cell monolayers was determined after solubilisation of the cells in 1.0 M NaOH using the method of Lowry et al 1951 (section 2.9).

The results (table 24) showed no evidence of any reproducible chlamydial dependent alteration in total HeLa cell cAMP or cGMP during the first 2 h of infection. Repeated experiments showed, however, that perturbation of cyclic nucleotide levels was occurring following centrifugation at the initial sampling time. This led to shifts in the baseline levels of both cAMP and cGMP, was observed in both infected and control cells, and probably would mask any transient alterations in cyclic nucleotide levels during the initial chlamydial-host cell interaction.

Table 24

Confluent monolayers of HeLa 229 cells in 6 well tissue culture trays were washed in HBSS and cooled to 0°C on ice. Purified LGV 434 (320 chlamydiae per cell) contained in 5 ml DMEM were centrifuged onto the cells at 1000 g for 1 h at 8°C, uninfected cells received 5 ml DMEM alone. Following centrifugation, the cells were warmed to 36°C to promote uptake and at the times indicated, the monolayers washed in PBS and the cyclic nucleotides extracted into 1ml 70% (v/v) ethanol at 80°C. After overnight equilibration at 4°C the ethanolic extracts were evaporated to dryness at 36°C, redissolved in sodium acetate buffer pH 6.2 and assayed for cAMP and cGMP.

TABLE 24 Host cell cyclic nucleotide levels during the initial stages of LGV 434 infection of HeLa 229 cells

		Time (mins)				
		0	15	30	60	120
* cAMP (femtomoles per assay tube)	uninfected (control)	52	47	47	37	41
	infected †	50	44	41	37	39
* cGMP (femtomoles per assay tube)	uninfected (control)	21	21	23	23	24
	infected †	17	18	21	19	21

* mean of 2 replicates. The experiment was repeated 6 times with different batches of inocula with essentially similar results.

† chlamydiae: cell ratio 320:1

3.9.5 Host cell cyclic nucleotide levels during the chlamydial growth cycle

To overcome the perturbing effects of centrifugation on host cell cyclic nucleotide levels a further experiment was performed to monitor the cyclic nucleotide responses during unassisted infection. In this system because attachment and endocytosis of the organism occurs over an extended period of time, the sample times were selected to cover the entire growth cycle. Replicate (3) confluent monolayers of HeLa 229 cells grown overnight in DMEM in 6 well trays were inoculated with purified LGV 434 (320 chlamydiae per cell) in 5 ml of DMEM. The remaining three wells containing uninfected (control) cells received 5 ml DMEM alone. The cells were incubated at 36°C in an atmosphere of 5% (v/v) CO_2 in air. At the sampling times, the DMEM was removed, the cells were rapidly washed with PBS at 36°C and the cyclic nucleotides extracted as before into 70% ethanol at 80°C (section 3.9.4) and assayed for cAMP and cGMP. Total protein content of the infected and uninfected cell monolayers after ethanol extraction was measured by the method of Lowry et al 1951 (section 2.9).

The results (table 25) showed that a chlamydial dependent alteration in cAMP and cGMP was not detected in the early stages of chlamydial infection. However, towards the end of the growth cycle the mean cAMP and cGMP levels of infected cells increased significantly compared to the uninfected controls, with increases of 47 and 51% respectively at 48 h for cAMP and cGMP.

3.9.6 The effect of Rifampicin on the chlamydial dependent modulation of host cell cyclic nucleotides

The growth of chlamydiae in tissue culture can be inhibited by Rifampicin. (Lepetit Pharmaceuticals Ltd). This drug blocks chlmaydial replication by inhibiting the synthesis of DNA-dependent RNA polymerase (Nabli 1971). The effect of Rifampicin on the observed increase in cAMP and cGMP during the later stages of the chlamydial growth cycle was therefore investigated.

Table 25

Confluent monolayers of HeLa 229 cells were washed in HBSS, inoculated with 5 ml DMEM containing purified LGV 434 (320 chlamydiae per cell) and incubated at 36°C. Uninfected control cells received 5 ml DMEM alone. At the times indicated, the cells were washed in PBS and the cyclic nucleotides extracted into hot (80°C) 70% ethanol. After overnight equilibration at 4°C, the samples were evaporated to dryness at 36°C, the extracts redissolved in sodium acetate buffer pH 6.2 and the cyclic nucleotide levels determined. Protein content of the monolayers were measured following solubilisation of the cells in 1.0M NaOH.

TABLE 25 Cyclic nucleotide levels during the growth of LGV 434 in HeLa 229 cells

		Time after inoculation											
		minutes			hours								
		0	5	15	30	60	2	5	10	24	36	48	
*cAMP													
(femtomoles per assay tube)	uninfected (control)	17	20.5	26	20	20.5	15	28	29	30	31	39.5	
	infected	18	23.5	23.5	25.5	21.5	17.5	28	29.5	34	45	58	
*cGMP													
(femtomoles per assay tube)	uninfected (control)	24.5	23.5	26.5	18	21	29	22.5	27	44.5	38	47	
	infected	29.5	28.5	27	28	29	29.5	30.5	34	46	45.5	71	
+ Protein													
(mg)	uninfected (control)	2.6	2.7	2.9	2.9	2.5	2.5	3.0	3.1	3.5	4.2	5.1	8
	infected	2.6	2.9	2.9	2.9	2.5	2.6	3.0	3.2	4.5	4.7	6.1	

* Mean of 2 replicates. The experiment was repeated 3 times

† Total protein in 3 replicate wells

§ i.e. 1 mg of chlamydial protein per 3 infected wells 48 h after challenge

HeLa 229 cells grown overnight to light confluent monolayers in DMEM in 6 well trays were inoculated with LGV 434 (320 chlamydiae per cell) in the absence or presence of 1 µg per ml Rifampicin. Control cells received either DMEM alone or DMEM containing 1 µg per ml Rifampicin. After inoculation the cells were incubated at 36°C in an atmosphere of 5% (v/v) CO_2 in air. At selected time intervals the cells were rapidly washed in PBS at 36°C and the cyclic nucleotides extracted into 2 ml 70% ethanol at 80°C. Protein estimations and cyclic nucleotide assays were performed as before (section 3.9.4) The results are shown in table 26.

Rifampicin treatment of chlamydial infected cells abolished the chlamydial induced increase in cAMP and cGMP previously described (section 3.9.5) showing that this increase was not a delayed consequence of chlamydial infection and that chlamydial replication in the cells was essential.

3.9.7 Prostaglandin E and $\text{F}_2\alpha$ levels in host cells during chlamydial infection

Ward and Salari (1982) demonstrated that prostaglandins as well as cyclic nucleotides were capable of modulating the susceptibility of HeLa 229 cells to chlamydial infection. Moreover, there was evidence to suggest that stimulation of HeLa cell susceptibility to chlamydial infection by cGMP was inhibited by four different prostaglandin inhibitors, implying that prostaglandins or related compounds may be involved. Accordingly the levels of PGE and $\text{PGF}_2\alpha$ in HeLa 229 cells and tissue culture supernatants during infection with *C. trachomatis* LGV 434 were assayed.

Confluent monolayers of HeLa 229 cells grown in DMEM in 6 well trays were washed in HBSS and inoculated with 4 ml DMEM containing 320 purified LGV 434 particles per cell. Uninfected controls received 4 ml DMEM alone. The cells were incubated at 36°C in an atmosphere of 5% (v/v) CO_2 in air. At appropriate sample times, the tissue culture medium from 3 chlamydial infected wells (12 ml) and the corresponding control medium from 3 uninfected wells were each pooled in glass

Table 26

Replicate monolayers of HeLa 229 cells were washed in HBSS and inoculated with 4 ml DMEM containing LGV 434 (320 chlamydiae per cell) in the absence or presence of 1 μ g per ml Rifampicin. Control cells received either DMEM alone or DMEM containing Rifampicin. Following incubation at 36°C, the cyclic nucleotides were extracted at the times indicated into 70% ethanol at 80°C. After evaporating to dryness the ethanolic extracts were redissolved in sodium acetate buffer pH 6.2 and the cyclic nucleotide levels determined. Protein content of the monolayers was measured following solubilisation of the cells in 1.0M NaOH.

TABLE 26

The effect of Rifampicin on the chlamydial dependent modulation of HeLa 229 cell cyclic nucleotide levels

		Time after inoculation										
		minutes				hours						
		0	15	30	60	2	5	10	24	36	48	
cAMP												
	(femtomoles per assay tube)*	uninfected	40	45	38	42	50	46	46	55	75	70
		uninfected + Rifampicin †	40	45	45	45	50	55	46	50	75	76
		LGV 434 infected	40	46	42	42	55	46	46	52	83	90
		LGV 434 infected + Rifampicin †	35	31	45	46	42	52	46	52	75	75
cGMP												
	(femtomoles per assay tube)*	uninfected	33	26	30	27	33	27	41	50	54	60
		uninfected + Rifampicin †	27	29	26	26	36	35	43	45	49	64
		LGV 434 infected	30	30	24	26	33	41	41	50	50	72
		LGV 434 infected + Rifampicin †	30	32	29	36	43	41	48	50	50	55
Protein † (mg)												
		uninfected	3.2	3.3	3.4	3.4	3.5	3.6	3.2	4.0	5.2	5.1
		uninfected + Rifampicin †	3.2	3.4	3.3	3.4	3.6	3.6	3.2	4.1	5.2	5.1
		LGV 434 infected	3.2	3.2	3.2	3.3	3.2	3.6	3.1	3.9	5.4	6.0
		LGV 434 infected + Rifampicin †	3.2	3.3	3.2	3.3	3.4	3.6	3.1	4.0	5.2	5.0

* Mean of 2 replicates

† total protein in 3 replicate wells

‡ Rifampicin added at time of inoculation (1 μ g per ml)

§ i.e. 1 mg of chlamydial protein per 3 infected wells 48 h after challenge

tubes, 12 μ l 10^{-3} M indomethacin was added, to minimise prostaglandin degradation and the samples were frozen at -70°C . The remaining cell monolayers from these wells were rapidly washed twice in ice cold PBS containing 10^{-6} M indomethacin, scraped from the substratum using a 'rubber policeman' and pooled into 2 ml PBS containing 10^{-6} M indomethacin and snap frozen to -196°C in liquid nitrogen.

For assay, the samples were acidified with citric acid, the prostaglandins were extracted into ether, evaporated to dryness under nitrogen and the extracts dissolved in sodium phosphate buffer pH 7.5 (section 2.17.3). The reactivity of other prostaglandins in the PGE and $\text{PGF}_2\alpha$ radioimmunoassays is shown in table 27. The results of prostaglandin assays in infected and uninfected cells are shown in Table 28.

Cell levels of $\text{PGF}_2\alpha$ were below the detection limit of the assay at all time points. PGE was at the detection limit for all time points except 36 and 48 h which showed increases of 16.6 and 206.6% respectively in infected monolayers compared with uninfected controls. The overlying tissue culture medium had detectable levels of $\text{PGF}_2\alpha$ but no difference was observed between infected and control cells. However, PGE in the infected cell supernatants was raised at 48 h by 406.25% compared with the uninfected control. Clearly, chlamydial infection of HeLa 229 cells caused a marked increase in host cell PGE levels but these could be detected only late in the growth cycle. The question therefore arose as to whether this raised prostaglandin level was due to endogenous chlamydial prostaglandin or was a result of chlamydial induced synthesis of host cell prostaglandin by the HeLa cells. Accordingly the level of PGE and $\text{PGF}_2\alpha$ in purified chlamydiae was determined.

Four tissue culture flasks of HeLa 229 cells (approx 2×10^7 cells per flask) were inoculated with LGV 434 (320 chlamydial particles per cell), producing infection in $>90\%$ of the cells. At 46 h the infected cells were harvested and the chlamydiae purified on a triosil gradient (section 2.6). The endogenous prostaglandin levels of the purified chlamydiae were equivalent to only 0.050 ng PGE and

Table 27

Confluent monolayers of HeLa 229 cells in 6 well tissue culture trays were inoculated with 4 ml DMEM containing 320 purified LGV 434 particles per cell and incubated at 36°C. Uninfected control cells received 4 ml DMEM alone. At the times indicated the supernatant tissue culture medium from infected and control cells were each pooled, indomethacin added to give a final concentration of 10^{-6} M and the samples frozen at -70°C. The cell monolayers from these wells were scraped from the substratum and pooled into 2 ml PBS containing 10^{-6} M indomethacin and snap frozen at -196°C in liquid nitrogen. For assay, the samples were acidified, extracted into ether and evaporated to dryness under nitrogen. The dried extracts were redissolved in sodium phosphate buffer pH 7.5 and assayed for PGE and PGF₂^α.

TABLE 27 Prostaglandin E and F₂^α levels in HeLa 229 cells and supernatant tissue culture medium during infection with LGV 434

Cells*	PGE (ng)	uninfected	Time after inoculation											
			minutes				hours							
			0	15	30	60	2	5	10	24	36	48		
- 128	PGF ₂ ^α (ng)	uninfected	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	0.3	0.45		
		infected	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	0.35	1.38		
	Supernatant tissue culture medium †	uninfected	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3		
		infected	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3		
	PGE (ng)	uninfected	0.92	ND	ND	ND	ND	ND	0.9	0.89	0.61	0.96		
		infected	1.0	ND	ND	ND	ND	ND	1.0	0.9	1.01	4.86		
	PGF ₂ ^α (ng)	uninfected	1.25	ND	ND	ND	ND	ND	1.51	2.02	2.0	2.54		
		infected	1.57	ND	ND	ND	ND	ND	2.10	2.10	2.51	2.43		

* Total prostaglandin E and F₂^α in 3 replicate wells. Figures are the mean of duplicate samples.

† Total prostaglandin E and F₂^α in supernatant tissue culture medium from 3 replicate wells (12 ml).

ND Not done

Table 28

Cross-reactivity was calculated by incubating prostaglandins with antibody to PGE and $[^3\text{H}]$ - PGE₂ or antibody to PGF_{2 α} and $[^3\text{H}]$ - PGF_{2 α} under the same conditions used in the radioimmunoassay. The inhibition of binding of radioactive PG's produced by the substance under test was then compared with the inhibition produced by standard concentrations of PGE₂ or PGF_{2 α} . The percentage cross-reactivity of the antibodies to other PG's or metabolites at 50% level of inhibition was calculated as

$$\frac{100 \times \text{Concentration of PGE}_2 \text{ (or PGF}_2\alpha\text{) required for inhibition of } [^3\text{H}] \text{ binding to 50\%}}{\text{concentration of test substance required for inhibition of } [^3\text{H}] \text{ binding to 50\%}}$$

TABLE 28 - Specificity of antibodies to prostaglandins

PGE₂

Compound	% Cross-reactivity at I ₅₀
PGE ₂	100.00
PGE ₁	46.00
PGB ₂	2.50
15-keto-PGE ₁	2.10
PGF ₂ α	0.30
PGA ₁	0.28
PGD ₂	0.17
13,14-dihydro-15-keto-PGE ₂	0.04
6-keto-PGF ₁ α	0.04
TxB ₂	< 0.04
Arachidonic acid	< 0.04

PGF₂ α

Compound	% Cross-reactivity at I ₅₀
PGF ₂ α	100.00
PGD ₂	6.70
PGF ₁ α	3.80
15-keto-PGF ₂ α	0.06
PGE ₁	0.05
13,14-dihydro-PGE ₁	0.04
15-keto-PGE ₁	0.02
13,14-dihydro-PGE ₂	0.01
PGE ₂	< 0.01
PGA ₁	< 0.01
15-keto-PGE ₁	< 0.01
13,14-dihydro-15-keto-PGE ₂	< 0.01
13,14-dihydro-15-keto-PGE ₁	< 0.01
13,14-dihydro-15-keto-PGF ₂ α	< 0.01
13,14-dihydro-15-PGF ₂ α	< 0.01

(Data supplied by Mr. R. Jewell)

0.02 ng PGF₂^α per mg of chlamydial protein. On the basis of these figures it was calculated that 18.6 mg of chlamydiae would be required to account for the 0.93 ng difference in PGE observed at 48 h between infected and uninfected control cells, if it were entirely attributable to endogenous chlamydial prostaglandins. As only some 1 mg of chlamydial protein is produced under these experimental conditions, (see Tables 25 and 26) the raised prostaglandin level seen in infected cells at 48 h must largely be a result of chlamydial induced prostaglandin synthesis by the host cell. Nevertheless the fact that prostaglandin like products were detected by radioimmunoassay in purified chlamydiae is of interest as prostaglandins have rarely been reported in prokaryotic cells. Further work would be needed to verify this point.

3.9.8 The effect of Rifampicin on the chlamydial dependent modulation of host cell prostaglandin

The chlamydial dependence of the net PGE increase in infected cells was investigated further by measuring prostaglandin levels in tissue culture supernatants at 48 h following the addition of Rifampicin at different stages of the chlamydial growth cycle.

Replicate (3) overnight confluent monolayers of HeLa 229 cells grown in DMEM in 6 well trays were washed in HBSS and inoculated with either 5 ml DMEM containing LGV 434 (320 particles per cell) or 5 ml DMEM alone (uninfected controls). The cells were incubated at 36°C, and 5 µl Rifampicin (1 mg per ml) in distilled water was added (final concentration 1 µg per ml) either at the time of inoculation or at 20, 30 or 40 h post inoculation. Control infected and uninfected monolayers received 5 µl of distilled water. At 48 h, samples of the tissue culture medium from the infected or control cells were pooled (15 ml) then frozen at -70°C in the presence of 10⁻⁶M indomethacin. Prostaglandin assays were performed as previously described (section 2.17).

An increase in PGE in the supernatant tissue culture medium at 48 h post infection was again observed (table 29). The mean control level was 0.603 ng per 15 ml compared with 1.54 ng per ml for the infected supernatant tissue culture fluid, an increase for infected medium of 155%. The earlier the addition of Rifampicin, the more effective was its action, suggesting that the presence of mature chlamydial particles were necessary for the increased PGE synthesis.

Table 29

Confluent monolayers of HeLa 229 cells in 6 well tissue culture trays were inoculated with either 5 ml DMEM containing 320 LGV 434 particles per cell or 5 ml DMEM alone (uninfected controls). The cells were incubated at 36°C and at the times indicated Rifampicin was added to give a final concentration of 1 µg per ml. Following 48 h incubation at 36°C the supernatant tissue culture medium from infected and control cells were each pooled then frozen at -70°C in the presence of 10⁻⁶M indomethacin. For assay, the samples were acidified, extracted into ether and evaporated to dryness under nitrogen. The dried extracts were redissolved in sodium phosphate buffer pH 7.5 and assayed for PGE and PGF₂ α.

TABLE 29

The effect of Rifampicin on prostaglandin E and $F_2\alpha$ levels in supernatant tissue culture medium from HeLa 229 cells infected with LGV 434

		Time of addition of Rifampicin (1 μ g per ml) after chlamydial challenge				No Rifampicin added
		At inoculation (0h)	20h	30h	40h	
PGE*(ng)	uninfected (control)	0.65	0.55	0.49	0.58	0.60
	infected	0.51	0.43	0.70	1.01	1.55
PGF $_2\alpha$ (ng)	uninfected (control)	2.36	2.69	2.65	2.43	2.53
	infected	2.87	2.74	2.67	3.01	2.86

* Total prostaglandin E and $F_2\alpha$ in supernatant tissue culture medium from 3 replicate wells (15 ml) measured 48 h after chlamydial challenge. The experiment was repeated twice.

CHAPTER 4

DISCUSSION

4.1 The effect of chlamydial infection on host cell cyclic nucleotides and prostaglandins

The results (section 3.9) showed that chlamydial development within HeLa cells resulted in an elevation of cAMP, cGMP and PGE during the later stages of the growth cycle. In this section the function of cyclic nucleotides and prostaglandins is discussed first followed by its relevance to the pathogenesis of chlamydial infection.

The primary function of cAMP is to mediate the cell's response to changes in the extracellular environment. The plasma membrane acts as a transducer with ligand-receptor interactions modulating the synthesis of the nucleotide, which is ultimately responsible for adjusting and regulating cellular activity. (Rodbell 1980; Dunwiddie and Hoffer 1982; Johnson et al 1971; Sheppard 1972). cAMP is synthesized by adenyl cyclase, an enzyme structured in the lipid framework of the cell membrane. The substrate for the reaction is adenosine triphosphate (ATP) with Mg^{2+} required as a co-factor. The enzyme catalyzing the degradation of cAMP, phosphodiesterase, does so by hydrolytic cleavage of the 3' phosphodiester bond to yield 5' adenosine monophosphate.

The role of cGMP in biological systems is less well understood. Certain hormone-receptor interactions in intact tissues in the presence of extracellular calcium alter cGMP levels. (Kuehl et al 1974; Flandroy and Garland 1975; Hardman et al 1969). However, none of these hormones has had convincing, reproducible, or physiologically relevant effects on guanylate cyclase activity in cell free systems. This suggests that hormone-receptor interactions are apparently less tightly coupled to guanylate cyclase activation and cGMP synthesis than the adenylate cyclase cAMP system.

The intracellular levels of cGMP are regulated by guanylate cyclase and phosphodiesterase, which are present in both particulate and soluble fractions of tissue homogenates (Murad et al 1978) implying that these enzymes are both membrane bound and free in the cytoplasm.

The role of cyclic nucleotides in chlamydial infectivity has been investigated by Ward and Salari 1982. Their working hypothesis was that an adherent elementary body somehow has to induce its own endocytosis by the host cell and that modulation of host cell secondary messengers like cAMP might provide the means. They demonstrated that exogenous dibutyryl cGMP or compounds such as carbamylcholine and prostaglandin F₂ α known to increase endogenous eukaryotic cell cGMP levels, increased the susceptibility of HeLa cells to chlamydial infection. In contrast to this stimulatory effect, dibutyryl cAMP or prostaglandin E₂ which promotes endogenous eukaryotic cell cAMP synthesis (Samuelsson et al 1978) depressed chlamydial infectivity.

A corollary to the regulatory actions of exogenous cAMP and cGMP on chlamydial infectivity in HeLa cells could be that modulation of endogenous cyclic nucleotide levels by the invading organism facilitates successful parasitization of the host. This hypothesis was investigated by measuring cAMP and cGMP levels during both the initial stages of chlamydial infection and throughout the developmental cycle. Alteration of cellular cyclic nucleotide levels as a result of hormone-receptor interactions are rapid, for example, glucagon stimulated rat hepatocytes give a maximal cAMP response within 2 minutes of adding the hormone (Cherrington and Exton 1976). Therefore, to overcome the slow rate of chlamydial attachment in the HeLa cell model, chlamydiae were centrifuged onto the host cell surface in the cold and the cyclic nucleotides measured after rapid warming to 36°C. Unfortunately, this process was found to perturb the basal cyclic nucleotide levels in both the infected and uninfected control cells. Repeated experiments failed to show any consistent chlamydial-dependent modulation of total cAMP and cGMP levels during the initial stages of infection. However, this observation did not necessarily exclude a role for cyclic nucleotides in chlamydial

attachment and uptake. One possibility could be that assay sensitivity was not sufficient to detect the changes or perhaps overall cellular cyclic nucleotide levels remained constant during compartmentalized synthesis and degradation. Such a localized activation of cellular adenyl cyclase activity has been described by Atkinson et al 1975. These workers used cyclic nucleotide immunohistochemistry to investigate phagocytic stimuli in human lymphocyte preparations. They showed that when low numbers of latex particles were incubated with lymphocytes, immunofluorescence immediately adjacent to the site of particle attachment was observed. This indicated that the particles were activating adenyl cyclase only in the region of the plasma membrane that had bound the particle.

Changes in cGMP compartmentalization have also been demonstrated during liver cell regeneration using fluorescent antibodies to cGMP (Steiner et al 1978). These changes were observed during regeneration whilst measurements of cGMP levels showed no significant fluctuations.

The finding of a chlamydial-dependent increase in both cAMP and cGMP, detectable some 36-48 h after challenge is worthy of further investigation although its significance is unknown.

cAMP is involved in the regulation of HeLa and other eukaryotic cell cycles (Zeilig et al 1976; Costa et al 1976). High levels of cAMP in the premitotic stage (G_2) of the HeLa cell cycle inhibits progression through G_2 to the mitotic phase (M). This inhibition of G_2 traverse by cAMP, together with the demonstration that endogenous cAMP levels normally fall during G_2 (Zeilig et al 1976) suggests the existence of a cAMP-mediated G_2 control point in HeLa cells. In contrast to the premitotic inhibitory effects of cAMP on G_2 , once the cells had reached the metaphase stage of mitosis, addition of analogs or inducers of cAMP enhanced the rate at which cells completed mitosis and entered the postmitotic stage G_1 . (Zeilig et al 1976; Friedman et al 1976). This cAMP-induced stimulation of cell division was mimicked by increased extracellular calcium or by the addition of Ca^{2+} ionophore A23187, suggesting that the action of cAMP was mediated

through increased cytosolic Ca^{2+} (Friedman et al 1976). It has been reported that once free Ca^{2+} concentration in the cytoplasm is in the region of 100 μM , several cell proteases, nucleases and phospholipase may be significantly activated (Chien et al 1978).

Therefore, chlamydial induced increases in cAMP may have a modulatory role both in the control of the host cell cycle and in the activation of hydrolytic enzymes. Thus, elevated cAMP levels at the end of the growth cycle may be important for the release of chlamydiae from the host cell.

Attempts to associate and correlate cGMP with various cellular processes has proved difficult to demonstrate (Mittal and Murad 1982). An unusual feature of guanylate cyclase is that it can be activated by sodium azide, (Kimura et al 1975; Levilliers et al 1976; DeRobertis and Craven 1976) with oxidising and reducing agents modifying the activation. This finding has led to the suggestion that guanylate cyclase may be a monitor of redox states or free radical formation within cells, with the product (cGMP) formed to permit feedback control of these events. (Mittal and Murad 1982). Redox states are likely to be important in chlamydial development as cysteine-rich disulphide-cross-linked protein complexes found in C.psittaci EB's are severely deficient in the replicating form of the organism (Hatch et al 1984). The RB outer membrane of C.trachomatis (LGV 434) is deficient in cysteine-rich proteins and is more readily derivatised in SDS than the outer membrane of the EB (Hatch et al 1984). This suggests that formation and reduction of disulphide bonds occurs during the developmental cycle of both chlamydial species, a process that is likely to involve altered redox states within the cell.

Chlamydial MOMP also contains cysteine and has properties analogous to the porins of *E.coli* (Nikaido & Rosenberg 1983) or the protein 1 of gonococci (Heckels 1981). In EB's the MOMP forms disulphide-linked oligomers (Newhall and Jones, 1983) which are partially reduced in reticulate bodies, increasing the porosity of the cell wall (Bavoil, et al 1984). Reduction of the disulphide linked MOMP after exposure to reducing conditions (e.g. reduced glutathione)

within the host cell might facilitate ingress of critical nutrients into the EB, stimulating morphogenetic transformation. In a cell-free system Hackstadt and Caldwell, 1984 obtained preliminary evidence that reducing agents could stimulate the initial transformation of purified EB into RB. The concept that cGMP might reflect altered redox states within the cell is therefore of particular interest in relation to chlamydial development.

The ability of cGMP to stimulate HeLa cell susceptibility to chlamydial infection was abolished by pretreatment of the host cell with non-steriod anti-inflammatory drugs such as aspirin, indomethacin, froben and mefenamate. (Ward and Salari 1982). The inhibitory action of these agents was mediated through their ability to inhibit prostaglandin biosynthesis. The prostaglandins E (PGE₁ and PGE₂) and F₂ α (PGF₂ α) which were assayed in this study are metabolites of arachidonic acid, a constituent of the structural phospholipids of cell membranes (Irvine 1982). The synthesis of PGE and PGF₂ α initially involves the hydrolytic cleavage of arachidonic acid from esterified stores in complex lipids by the enzyme phospholipase A₂ (Lands and Samuelsson 1968). The resulting free acid can then be oxidised via the cyclo-oxygenase pathway to yield prostaglandin and thromboxane derivatives (Bakhle 1983).

The finding of a chlamydial-dependent increase in PGE released into the supernatant tissue culture medium some 36-48 h after challenge is of particular interest in view of the modulatory role prostaglandins exhibit in both humoral and cell mediated immunity. Exogenously added PGE has been shown to inhibit in-vitro functions of lymphocytes, (Smith et al 1971; Gordon et al 1976) invivo responses mediated by lymphocytes, (Quagliata et al 1973) and to stimulate the non-specific B-cell responses induced by pokeweed mitogen (Staite and Panayi 1982; Ceuppens and Goodwin 1982). It has been suggested that T-cell proliferative responses are inhibited by PGE₂ (Goodwin et al 1977).

Thus, a chlamydial infected cell may be capable of mediating

immunosuppression and possibly thereby escape immunological surveillance. Chlamydial modulation of host immune defences is consistent with the fact that many chlamydial infections (e.g. trachoma) are chronic, latent infections characterised by acute exacerbation of infection leading to the release of infectious chlamydiae. Further work is needed to investigate these hypothesis - in this study assay of cyclic nucleotides and prostaglandin was greatly limited by resources, as each experiment including the preparation of cells, their infection and assay, took three weeks to complete.

4.2 The Role of Calmodulin in Chlamydial Infectivity

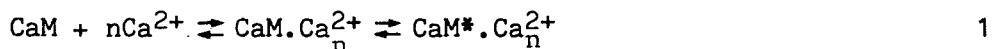
Centrifugation of chlamydial elementary bodies onto the host cell surface is accompanied by the movement of Ca^{2+} across the host cell membrane (Ward and Salari 1982). The possibility that a Ca^{2+} flux may facilitate chlamydial uptake by activating some cellular process dependent on calmodulin was investigated.

Calmodulin is now well established as the major calcium-binding protein in non muscle cells (Means et al 1982) and was first described by Cheung in 1970 as a heat-stable protein activator of brain cyclic nucleotide phosphodiesterase. Calmodulin with calcium have been found to mediate many vital cell activities including, protein phosphorylation (Schulman and Greengard 1978); myosin light chain kinase activity (Yerna et al 1979; Kuo and Coffee 1976); microtubule assembly/disassembly (Welsh et al 1978); Ca^{2+} - Mg^{2+} ATPase activity (Gopinath and Vincenzi 1977) and phospholipase A₂ activity (Wong and Cheung 1979; Moskowitz et al 1983).

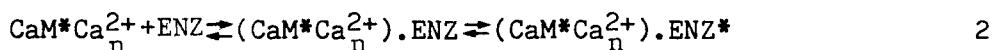
Calmodulin exists as a monomer of molecular weight 16,700 (Watterson et al 1980) and in the presence of Ca^{2+} resists denaturation (boiling 8M urea, 1% SDS) (Dedman et al 1977). The primary structure appears to have been well conserved throughout evolution with calmodulin from phylogenetically distinct species showing almost identical primary sequences (Watterson et al 1980; Dedman et al 1978; Grand et al 1981). The molecule contains four

metal binding sites (Teo and Wang 1973) which when occupied by calcium, result in an increase in the α -helicity of the molecule, exposing a highly lipophilic region (Laporte et al 1980; Tanaka and Hidaka 1980). This region interacts with enzymes under its control; the process occurring in at least two steps (Klee, et al 1980).

First calmodulin (CaM) binds calcium according to equation 1



The active conformer, indicated with an asterisk, then interacts with the enzyme (ENZ) which is inactive or partially active. The enzyme undergoes a conformational change (Liu & Cheung 1976) which is accompanied by activation (ENZ*) according to equation 2



The Stoichiometry of the active $\text{CaM} \cdot \text{Ca}^{2+}$ complex has not yet been determined (n can vary between one and four) and could vary with different calmodulin-binding proteins.

A variety of pharmacological agents bind in a Ca^{2+} dependent manner to the lipophilic region of calmodulin, resulting in inhibition of the molecule's function (Weiss and Levin 1978). Three classes of calmodulin inhibitor were examined for their effect on chlamydial infectivity for HeLa 229 cells - the phenothiazines: trifluoperazine (TFP), chlorpromazine and promethazine, the diphenylbutylpiperidine: pimozide and the butyrophenone: haloperidol. All these structurally diverse compounds inhibited HeLa cell susceptibility to chlamydial infection. The most active compound was pimozide followed in order by TFP, chlorpromazine, haloperidol and promethazine. Interestingly, the ability of these compounds to inhibit chlamydial infection paralleled their ability to inhibit calmodulin-dependent, Ca^{2+} mediated activation of cyclic nucleotide phosphodiesterase activity in a cell free system which was determined by the relative affinity of the inhibitors for the hydrophobic binding site on calmodulin. (Weiss et al 1980). This suggested that the inhibitors were exhibiting their

effect through calmodulin. In addition, the lack of evidence for direct toxicity of TFP on chlamydiae, and the absence of calmodulin-like compounds in prokaryotic organisms also implied that the inhibitor effect was mediated via host cell calmodulin.

There were two clearly distinguishable effects of calmodulin inhibitors on chlamydial-host cell interactions. Firstly, short term (2 h) treatments with TFP at or immediately prior to chlamydial challenge reduced chlamydial infectivity as shown by a reduction in the number but not the size of the inclusions, with an associated reduction in chlamydial growth as measured by [³H]-threonine incorporation. This short term effect was due to both a reduction in chlamydial attachment and subsequent uptake by the host cell.

Continuous treatment of infected cells with calmodulin inhibitor caused a reduction in both the size and the maturity of the inclusions. This effect was related to the length of time of treatment and was most marked for pimozide.

The impairment of chlamydial maturation by continuous treatment with calmodulin inhibitors was expected. Chlamydiae are energy parasites (Moulder 1962, 1970; Weiss and Wilson 1969; Hatch 1975) and depend on the host cell for their supply of high energy compounds. Calmodulin inhibitors are known to impair mitochondrial energy production and the adenylate charge of cells, possibly due to the inhibitory effect on calmodulin-regulated enzymes such as Ca^{2+} dependent ATPase (Corps et al 1982) and NAD kinase (Epel et al 1981).

The mechanism for the effect of calmodulin inhibitor on chlamydial attachment and uptake is open to speculation at this point. The most likely explanation is that the calmodulin inhibitors are blocking chlamydial uptake by some critical, calmodulin-dependent process. This might be mediated through the inhibition of phospholipase A₂ whose activity destabilises membrane integrity. Calmodulin has been shown to modulate phospholipase A₂ activity in human platelets. (Samuelsson et al 1978) and in cell free systems (Moskowitz et al 1983).

Calmodulin inhibitors might also interfere with endocytosis by blocking the activation of myosin light chain kinase which is necessary for microfilament contraction and movement of the cell surface. This enzyme is absolutely dependent on calmodulin, and catalyzes the phosphorylation of a 20,000 dalton myosin light chain. Phosphorylation of the light chain activates the actomyosin ATPase, which leads to the hydrolysis of ATP, contraction of the myosin and membrane movement. Alternatively, TFP might block chlamydial endocytosis by its effect on membrane fluidity. TFP is a tricyclic compound and is highly hydrophobic. Such compounds can bind to host cell surfaces reducing membrane fluidity (Norman 1980; Kanoho et al 1982) and might therefore indirectly reduce chlamydial endocytosis. This is difficult to distinguish from their action on calmodulin because the hydrophobicity of the inhibitors is itself important in their ability to bind to calmodulin.

Finally, calmodulin is also a major component of clathrin coated vesicles (Aggeler and Werb 1982) which form part of the receptor mediated endocytic pathway. Thus, TFP might block chlamydial endocytosis by reducing the recruitment of clathrin to the cell surface essential for the recycling of membrane during receptor mediated endocytosis (Aggeler and Werb 1982; Salisbury et al 1980). The role of receptor mediated endocytosis and clathrin in chlamydial endocytosis is discussed in the next section.

4.3 Mechanisms of Chlamydial Endocytosis

The attachment and uptake of chlamydiae into mucosal cells in the course of the natural disease probably involves a high affinity mechanism as chlamydial infections are ubiquitous within the animal kingdom. (Schachter and Caldwell 1980). The first stage of chlamydial endocytosis is attachment of the organism to the host cell which is likely to be mediated through macromolecules on the chlamydial outer-membrane, although any adhesins involved in such a process have so far not been identified. The fact that attachment of C.psittaci and C.trachomatis LGV 440 required a heat labile and trypsin-sensitive

site on the EB (Byrne 1976; Byrne and Moulder 1978) suggested that the adhesin is a protein. However, attachment of a trachoma isolate of C. trachomatis to McCoy cells was not affected by heating at 60°C for 3 mins. or by digesting the host cell with trypsin (Lee 1981). It is likely that the chlamydial biovars utilize different attachment mechanisms as they exhibit differences in the pathogenicity and immunology. Kuo et al 1973 demonstrated the inhibition of the non LGV biovars of C. trachomatis infection of HeLa cells by treatment of the host cell with neuraminidase. This implied that sialic acid residues were involved in the receptor for trachoma agents. Levy 1979 implicated N-acetylglucosamine as a component of the host cell receptor as wheat-germ agglutinin blocked infection with C. psittaci 6BC and C. trachomatis LGV 440. More recently, Söderlund and Kihlström 1983a demonstrated that oligomers of B1-4 linked N-acetyl-D-glucosamine inhibited attachment and uptake of C. trachomatis LGV 440 in McCoy cells. The N-acetylamino group appeared to be crucial as the B1-4 linked disaccharide of glucose (cellobiose) failed to have an effect. However, caution must be exercised when interpreting lectin binding data. Chlamydial adhesion may be inhibited due to steric hindrance caused by the bulky lectin molecule bound close to the chlamydial receptor.

Chlamydial attachment to host cells is also influenced by non-specific factors. The glycolipids and glycoproteins on the epithelial cell membrane comprise negatively charged residues due to an excess of ionised carboxyl groups including those of sialic acid. The chlamydial surface also, carries a net negative electric charge at physiological pH. (Kraaijpoel and Van Duin 1979). Therefore, at some stage in the approach of chlamydiae towards the host cell surface, the surrounding potential fields must overlap, producing forces of electrostatic repulsion between the two surfaces. The practical importance of this electrostatic barrier to chlamydial attachment is shown by the fact that the polycations DEAE-dextran or poly-L-lysine potentiate the susceptibility of HeLa 229 cells to infection with non LGV biovars of C. trachomatis (Kuo et al 1972).

Unassisted attachment of chlamydiae to HeLa 229 cells is a slow

process. In this study only some 35% of the inoculum became cell-associated after 18 h incubation under conditions designed to maximize chlamydial-host cell interaction. One possible explanation for this observation is that in the HeLa cell model, attachment of chlamydiae to the cell surface is relatively weak, time being required to firmly bind the organism to the host at multiple attachment sites. This multipoint attachment would be achieved by local movement of the cell membrane in areas adjacent to the chlamydial particle. Electron microscopic evidence of this process is shown (Fig. 21).

In mammalian cells active cell membrane movement is known to be mediated through microfilaments. The membrane movement around chlamydial particles probably involved microfilaments as the fungal metabolite cytochalasin B inhibited attachment of the organism to the host, however, thermodynamic forces may also be utilized. Theoretical considerations suggest that invagination of the cell membrane following attachment of influenza virus (Patterson et al 1979) or other polyvalent ligand minimizes the change in free energy and is thus the most stable configuration for the host cell membrane to adopt. Extrapolation of this concept to particles the size of chlamydiae is speculation, however, in this study it was not possible to totally inhibit chlamydial attachment to the host cell with microfilament inhibitors.

Evidence for the importance of non-specific factors in chlamydial attachment stems from the fact that up to 10,000 chlamydial particles per cell failed to inhibit the homologous radiolabelled organism from attaching to the cell surface. The ability of chlamydiae to utilize non-specific mechanisms of attachment in vivo may be an evolutionary adaptation, serving to increase the range of cells susceptible to chlamydial infection.

To further characterize the endocytic pathway of chlamydiae in HeLa cells it was necessary to overcome the slow rate of attachment which must inevitably limit the rate of uptake. The approach in this study was to first promote chlamydial attachment to the cell surface by centrifugation in the cold, and then to synchronize uptake by rapid

warming to 36°C. Centrifugation aids chlamydial attachment to host-cells mechanically, possibly by overcoming electrostatic forces; and may even partially offset the neutralizing effect of antibody (Ainsworth et al 1979). This approach assumes that ingestion of the organism following centrifuge assisted attachment at low temperature, is identical to the normal unassisted random association of chlamydiae with host cells. In support of this concept, electron microscopic observations of the morphological features of chlamydial ingestion following either centrifugation or unassisted attachment were indistinguishable (Ward, M.E. personal communication). The uptake of radiolabelled chlamydiae into HeLa cells was found to be competitively inhibited by the homologous organism in a dose dependent manner (Table 16). This data could be interpreted as providing evidence for a specific uptake mechanism. One could speculate, in the HeLa cell model, that following the initial non-specific attachment to the host cell surface, binding to a specific saturable cell receptor occurs, the binding to this receptor being mandatory for uptake to proceed. However, the synchronous uptake of particles the size of chlamydiae must involve the endocytosis of considerable amounts of host cell surface over a short period of time. Indeed, under these conditions large numbers of chlamydiae were found to be ingested after the cell monolayer was warmed to 36°C for 5 minutes. Thus, competitive inhibition of chlamydial ingestion might simply reflect a limited ability of the host cell to recycle endocytosed membrane back to the cell surface.

.. Söderlund and Kihlström (1983b) have suggested that chlamydiae might enter cells as part of the normal process of micropinocytosis. This form of uptake, generally referred to as receptor-mediated endocytosis is the process utilised by eukaryotic cells for the internalization of a diverse group of molecules including polypeptide hormones, proteins such as α_2 macroglobulin, low-density lipoprotein (LDL) and maternal immunoglobulins (Salisbury et al 1983) within clathrin coated vesicles. The intrinsic features of receptor mediated endocytosis are that it is saturable, receptor dependent, fast and highly efficient, (Fries and Helenius 1979) and it is insensitive to agents that disrupt cytoskeletal elements. (Davies et

al 1971). The pathway is initiated by the binding of a given ligand to its specific cell surface receptor which are typically found in a diffuse distribution at the cell surface. Ligand binding induces receptor complexes to cluster or patch in the plane of the membrane (Salisbury et al 1983). Subsequently, ligand-receptor complexes come to lie over specialized regions of the plasma membrane called coated pits (Salisbury et al 1980). These are largely composed of the molecule clathrin, (Molecular Wt. 180,000) (Pearse 1975) which forms a basket-like network on the inside surface of cell membranes.

Transglutaminase may be involved in the covalent cross-linking of receptor-ligand complexes prior to internalization of coated vesicles. (Maxfield et al 1979; Levitzki et al 1980; Cheng et al 1980). Internalization of the clustered ligand-receptor complexes occurs by membrane vesiculation which involves the assembly and molecular rearrangement of the clathrin coat material (Kanaseki and Kadota 1969; Heuser and Evans 1980) which results in a "geodesic dome"-like structure containing the ligand-laden membrane. As coat assembly nears completion, the membrane-containing clathrin coat closes and the newly formed vesicles are drawn into the cytoplasm. (Salisbury et al 1980).

The evidence that chlamydiae utilize such a route comes from the observation that monodansylcadaverine and methylamine, inhibitors of receptor mediated endocytosis, reduced the internalization of chlamydiae into loosely confluent McCoy cells (Söderland and Kihlstrom 1983b). Certainly, these and related compounds block the entry of vesicular stomatitis, Semliki Forest and other viruses into host cells. (Marsh and Helenius 1980; Schlegel et al 1982; Talbot and Vance, 1982). The mechanism of action of these primary amines is thought to be mediated through the inhibition of transglutaminase activity. However biochemical studies of receptor mediated endocytosis indicate that ligand binding, receptor clustering and internalization proceeds normally in the presence of inhibitors of transglutaminase. (McKenna et al 1979; Haigler et al 1979; Dorland et al 1981; Schneider et al 1981;) So, the mechanism of action of these drugs is at present unclear. The data presented in this study

suggests that chlamydial uptake is not mediated through the receptor mediated endocytic pathway. Electron micrographs of chlamydial endocytosis failed to show any chlamydia containing clathrin coated endocytic vesicles which were readily demonstrable in the same tannic acid-fixed preparations. In vitro studies also indicate that chlamydiae associate with random McCoy cell membrane vesicles but not to similarly prepared clathrin coated vesicles (Greogry 1983). Also, chlamydiae are approximately 300 nm in diameter, which is much larger than the coated vesicles normally seen in cells (See Fig. 23). Experiments using [³H] sucrose as the non-permeant fluid-phase marker, demonstrated that unlike Semliki Forest virus, chlamydiae failed to reduce the basal rate of fluid uptake by the host cell. Finally, monodansylcadaverine an inhibitor of receptor mediated endocytosis did not block chlamydial ingestion in confluent monolayers of HeLa 229 cells.

Byrne and Moulder 1978 hypothesized that the selection pressure on chlamydiae to constantly gain access to the inside of a host cell induced the evolution of a mechanism for enhancing their own phagocytosis. This hypothesis predicts the presence of structures on the surface of both host cell and parasite that facilitate chlamydial attachment and ingestion. Uptake by this route would involve microfilament mediated movement of the host's cell membrane, in order to bring about sequential interaction between ligands on the chlamydial and host cell surface. The process repeats until plasma membranes of the advancing pseudopods meet and fuse with one another to form a tight endocytic vesicle. This 'zipper' mechanism has been described for a model system involving the ingestion of opsonized erythrocytes by mouse macrophages. This ingestion required tight, circumferential, multipoint binding of the host cell membranes to ligands on the particle surface. (Griffin et al 1975, 1976). This study shows that chlamydiae have similar requirements for ingestion. The close interaction of chlamydiae with the host cell membrane was demonstrated by electron microscopy (Fig. 21) and by the minute volume of the external fluid phase ($6.4 \times 10^{-17} \text{ L}$ for each vesicle) ingested with chlamydiae, calculated from [³H]-sucrose uptake data. The fact that large amounts of antibody bound to the chlamydial

surface at sites distal to the points of chlamydial attachment to the host cell inhibited ingestion, suggests that chlamydial uptake requires the sequential binding of the HeLa cell membrane to chlamydial surface ligands.

The role of microfilaments in chlamydial endocytosis has been obscured by the observation that pretreatment of host cells with cytochalasin B has little effect on chlamydial development. (Sompolinsky and Richmond 1974; Stirling and Richmond 1977; Kuo, 1978; Gregory et al 1979; Lee 1981). In this study, cytochalasin B pretreatment of HeLa 229 cells had little effect on chlamydial dependent [3 H]-threonine incorporation 42 h after challenge. However, such long term measurements of overall chlamydial infectivity do not give any insight into the rate of attachment or ingestion of the organism into the host cell. Recent studies on the effects of cytochalasin B on actin-myosin association at particle binding sites show that the effect of this inhibitor is to reduce the bulk polymer strength of the network affecting the propagation of long-range forces at the membrane. Nevertheless, these forces are still sufficient to permit the uptake of particles less than 600 nm in diameter, including chlamydiae. (Painter et al 1981). This notion is further emphasized by the fact that cytochalasin D, a more potent inhibitor than cytochalasin B which acts by causing a rearrangement or disruption of the microfilaments (Tannenbaum et al 1980; Casella et al 1981) significantly reduced chlamydial attachment and uptake.

Within the cell cytoplasm, microtubules serve as anchorage points for microfilaments. Microtubules are 30 nm diameter structures of a non-contractile globular protein, tubulin, consisting of two subunits with a molecular weight of approximately 120,000 (Olmsted and Borisy 1973). Tubulin molecules are arranged in helical fashion to form the wall of a tube, the diameter of which is determined by the number of tubulin molecules in its circumference. The formation of microtubules in cells is characteristically inhibited by certain agents long known to be inhibitors of cell division in eukaryotes, namely the alkaloid colchicine and the mould products vinblastine and vincristine. The mechanism of action of these two groups of compounds is quite

different and this was reflected in their ability to inhibit chlamydial uptake. Vincristine and vinblastine interfere with the assembly of microtubules by causing precipitation of tubulin, inducing the formation of highly birefringent paracrystalline arrays which were observed by electron microscopy. These alkaloids inhibited chlamydial uptake but not attachment. Colchicine binds to tubulin, disturbs the dynamic equilibrium between monomeric and polymeric tubulin, does not form paracrystalline arrays (Olmsted and Borisy 1973; Margolis and Wilson 1977) and was a much less effective inhibitor of chlamydial uptake. Interestingly, the ability of cytochalasin B and D to block chlamydial attachment was independent of microtubules and may have been due to drug induced alterations in the surface area of the cells available for attachment.

Confirmation of the involvement of the host cytoskeleton may offer an explanation as to the modulatory role of cyclic nucleotides and calmodulin in chlamydial infectivity. Cyclic nucleotides affect the assembly and function of both microfilaments and microtubules (Dedman et al 1979) and calmodulin inhibitors such as TFP inhibit microfilament activity by blocking the activity of myosin light chain kinase. (Salisbury et al 1981). The important accessory role of microfilaments in chlamydial ingestion suggests a mechanism by which movement or displacement of Ca^{2+} at the cell membrane induced by chlamydial binding (Ward and Salari 1982) might lead to membrane movement. Actin activation of heavy chain ATPase occurs only after phosphorylation of the light chains by myosin light chain kinase, and a direct relationship exists between calcium concentration, the degree of light chain phosphorylation and ATPase activity (Sobieszek 1977). It is the degree of light-chain phosphorylation which determines the tension development (Lebowitz and Cooke 1978).

Despite the observation that chlamydiae are endocytosed 10-100 times more efficiently than Escherichia coli or polystyrene latex beads (Byrne and Moulder 1978) it is unlikely they contribute metabolically to the process. Ultra-violet-irradiated chlamydiae or chlamydial outer envelope preparations are ingested with reasonable efficiency (Lee 1981; Eissenberg et al 1983).

In conclusion, this study suggests that in the HeLa cell model, chlamydiae bind to the host by multiple weak and probably non specific interactions. Attachment of the organism does not induce a generalized endocytic response in the host. Ingestion occurs following sequential circumferential binding of the host cell membrane to the chlamydial surface, with membrane mobility limited to the area adjacent to the particle. Membrane movement around the organism is probably a calmodulin dependent process mediated through microfilaments perhaps aided by thermodynamic forces. Thus chlamydial uptake most closely resembles microfilament-dependent rickettsial uptake (Wisseman 1981) rather than the recently described mechanisms of receptor mediated endocytosis for virus uptake.

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