STUDIES OF THE PROPAGATION AND ANTIBIOTIC SENSITIVITY OF C.TRACHOMATIS.

A Thesis submitted to the University of London

for the degree of Master of Philosophy

1989

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ABSTRACT

CHLAMYDIAE, BEING INTRACELLULAR PARASITES WITH A UNIQUE LIFE CYCLE, EVADED CLOSE LABORATORY SCRUTINY UNTIL 1932 WHEN BEDSON AND BLAND FOLLOWED THE LIFE CYCLE OF CHLAMYDIA PSITTACI IN THE TISSUES OF DEAD ANIMALS. IN 1957, SUCCESSFUL ISOLATION OF CHLAMYDIA TRACHOMATIS IN EMBRYONATED HENS' EGGS OPENED A WHOLE NEW CHAPTER IN THE ELUCIDATION OF THE LIFE CYCLE OF THIS ORGANISM. A PART OF THE WORK PRESENTED IN THIS THESIS FOLLOWS THE LIFE CYCLE OF C.TRACHOMATIS USING TRANSMISSION ELECTRON MICROSCOPY, TIME LAPSE PHOTOGRAPHY AND A NUMBER OF STAINING TECHNIQUES SUITABLE FOR LIGHT MICROSCOPY.

THE ISOLATION OF C.TRACHOMATIS IN EGGS BY T'ANG ET AL (1957) AND LATER IN TISSUE CULTURE BY GORDON AND QUAN (1965b) WAS A MAJOR TECHNICAL ADVANCE IN PRODUCING CHLAMYDIAL ANTIGEN IN BULK. AN ALTERNATIVE, EASIER TECHNIQUE WHICH GIVES BOTH A LARGE YIELD OF C.TRACHOMATIS AND SAVES THE BURDEN OF INOCULATING EGGS OR SEVERAL BOTTLES OF TISSUE CULTURE, AS IS CURRENT PRACTICE, IS PRESENTED IN THIS THESIS. THIS WORK INVOLVES THE USE OF MICROCARRIERS WHICH HAVE ELSEWHERE BEEN USED FOR GROWING VIRUSES,
CELLS AND THEIR PRODUCTS IN BULK.

DURING THE LAST DECADE, C.TRACHOMATIS HAS BEEN RECOGNISED AS ONE OF THE MOST COMMON SEXUALLY TRANSMITTED PATHOGENS AS WELL AS THE COMMONEST CAUSE OF PREVENTABLE BLINDNESS. IN ADDITION TO UNCOMPLICATED INFECTIONS, NAMELY URETHRITIS IN BOTH SEXES, CERVICITIS IN WOMEN AND NEONATAL CONJUNCTIVITIS, SERIOUS SEQUELAE SUCH AS EPIDIDYMITIS, PERIHEPATITIS, PELVIC IMFLAMMATORY DISEASE (WITH THE ATTENDANT RISK OF

INFERTILITY) AND IN INFANTS A CHARACTERISTIC PNEUMONITIS SYNDROME MAY RESULT. EFFECTIVE CHEMOTHERAPY AGAINST CHLAMYDIAL INFECTION HAS BEEN POSSIBLE SINCE THE ADVENT OF SULPHONAMIDES, TETRACYCLINES AND ERYTHROMYCIN. PART OF THE WORK PRESENTED IN THIS THESIS EXAMINES THE EFFECT OF OVER 40 DIFFERENT ANTIMICROBIAL AGENTS AGAINST C.TRACHOMATIS USING A STANDARD TISSUE CULTURE TECHNIQUE.

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CHAPTER 1

INTRODUCTION

1.1. AN OVERVIEW.

Chlamydia has been recognised as one of the major human pathogens from the earliest records. Trachoma, as a chronic inflammation of the conjunctiva and cornea, was the predominant cause of blindness in ancient Chinese and Egyptians and was widespread in ancient Greece and Rome. The name "trachoma" was first used by a Sicilian physician, Pedanius Diascarides, in 60 AD, and a century later the 4 stages of the disease were delineated by Galen. In Europe, the disease was often referred to as "military ophthalmia" since it was contracted by English and French soldiers in Egypt who brought the disease back home with them. Psittacosis, a chlamydial infection of birds, has been recognised as a disease of man for many centuries. Though not a major public health problem in terms of the total number of reported humans affected by this disease, most countries have import regulations to prevent psittacosis.

The role of *Chlamydia* as a sexually transmitted disease pathogen was first shown by Heymann in 1910, and since that time accumulating evidence points towards a high prevalence of this infection amongst sexually active young people with possible serious sequelae.

1.2. HISTORICAL BACKGROUND.

In 1907, Halberstaedter and Von Prowazek made the first aetiological observation on trachoma whilst working in Java. They described the cytoplasmic inclusion bodies in the epithelial scrapings from the conjunctiva of humans and apes suffering from the disease. They called the organisms "Chlamydozoa" or mantle bodies, because when stained with Giemsa the small reddish elementary bodies, which they believed to have been the agent of trachoma, seemed to be embedded in a blue matrix or mantle. In 1909, these workers found similar inclusions in cases of non-gonoccocal ophthalmia neonatorum. They declared that these organisms were independant of gonoccocal infection. This finding agreed with the work of Lindner who, in 1909, showed inclusions in most cases of non-gonoccocal ophthalmia neonatorum. He defined this condition as "inclusion blennorrhoea". Lindner fulfilled Koch's postulates by producing the same disease in the eyes of baboons using genital secretions from mothers of babies with inclusion blennorrhoea. In 1910, Fritsch and Hoffstatter also demonstrated the same phenomenon with material obtained from men with non-gonococcal urethritis (NGU) and, in the same year, Halberstaedter and Von Prowazek reported findings of such inclusions in female genital epithelial cells. Lindner, using a wet fixation technique, demonstrated that the blue material which Halberstaedter and Von Prowazek had called "Plastin" was a collection of large coccoid bodies. These he termed "initial bodies" as they were seen first in developing inclusions.

In 1913, Nicolle et al passed the clinical material from a case of trachoma through Berkefeld filters. The filtrate was inoculated into

the conjunctiva of a chimpanzee and produced an experimental disease for which a trachomatous nature was established by transmission of the disease to the human conjunctiva. This experiment was repeated and the results confirmed by other workers (Julianelle & Harrison 1935 and Thygeson & Proctor 1935).

There was not much further progress with regard to C.trachomatis infection until the isolation of this agent first by T'ang et al (1957) from the eye in hyperendemic trachoma, and later by Jones et al (1959) from the genital tract and eyes in cases of paratrachoma.

C.trachomatis is now recognised worldwide as a major sexually transmitted disease pathogen. During the early 1970's C.trachomatis was established not only as the most important causative agent of non-gonococcal urethritis in men, but was also recognised to be responsible for cervicitis in women (Oriel et al 1972 and 1974). The aetiological role of C.trachomatis in complicated genital infections in females, namely salpingitis (Mardh et al 1977), endometritis (Mardh et al 1981), perihepatitis and peritonitis (Muller-Schoop et al 1978 and Wolner-Hanssen et al 1980) and recently in peri-appendicitis (Mardh & Wolner-Hanssen 1985) has renewed attention. The role of C.trachomatis as an aetiological agent causing symptomatic and asymptomatic genital infection in men (Schachter & Dawson 1978), epididymitis (Berger et al 1978) and proctitis (McCormack 1986) has gathered interest. C.trachomatis remains an important cause of neonatal and infant morbidity (Harrison 1986). From amongst several symptoms associated with this agent, pneumonia in newborns (Beem & Saxon 1977 and Harrison et al 1978) and inclusion blennorrhoea (Mordhorst & Dawson 1971 and Beem & Saxon 1982) are the best studied.

1.3. MICROBIOLOGY OF CHLAMYDIA SP.

1.3.1. CLASSIFICATION.

1.3.2. MORPHOLOGY.

i) ELEMENTARY BODY (EB).

ii) RETICULATE BODY (RB).

1.3.3. ANTIGENIC CHARACTERISTICS.

i) SPECIES SPECIFIC ANTIGEN.

ii) SEROVAR SPECIFIC ANTIGEN.

1.3.4. DEVELOPMENTAL CYCLE.

1.3.5. PROPAGATION METHODS.

i) ANIMAL INOCULATION.

ii) YOLK SAC INOCULATION.

ii) TISSUE CULTURE INOCULATION.

1.3.1. CLASSIFICATION.

Because of their unique life cycle, these agents have been placed in a single order, the Chlamydiales (Storz & Page 1971). There is one genus, Chlamydia, and two species, C.psittaci and C.trachomatis (Bergey's 8th Manual of Determinative Bacteriology). The species are differentiated on the basis of their inclusion types and sulphonamide sensitivity. C.trachomatis produces inclusions which contain glycogen and thus can be stained with iodine and it is sensitive to sulphonamides. C.psittaci produces inclusions which do not contain glycogen and it is resistant to sulphonamides.

1.3.2. MORPHOLOGY.

The chlamydiae are obligate intracellular procaryotic organisms characterised as small Gram negative cocci. They exist in two morphological forms.

i) ELEMENTARY BODY (EB). A small spherical (200-300 nm in diameter) particle, slightly basophilic which stains bluish red with Giemsa stain as first described by Bedson and Bland (1934). It is infectious, metabolically inactive and has an electron dense nucleoid (Schachter & Dawson 1978). The elementary body acts as the extracellular transport form of the agent and is adapted for survival in transit between the cells. It has a DNA:RNA ratio of 1:1, and is resistant to trypsin digestion.

ii) RETICULATE BODY (RB) or Initial Body (IB). A larger (800-1200 nm in diameter) pleomorphic, more basophilic, less infectious, metabolically active particle (Schachter & Dawson 1978) which divides inside the intracytoplasmic vesicle by a process of binary fission to form a para nuclear inclusion. It has a DNA:RNA ratio of 1:3, and is sensitive to trypsin digestion (Ward 1983).

Following Bedson and Bland's (1934) observations on the growth cycle of C.psittaci several workers (Meyer & Eddie 1942, Lwoff 1957 and Stanier 1964) produced evidence, based on morphology, staining reaction and biochemical tests, that these organisms were bacteria. The most definitive argument for classifying these organisms as bacteria came from Moulder who, in 1964, also rejected the idea of chlamydiae as being the "missing link" between viruses and bacteria.

The unique life cycleof the agents of ornithosis and trachoma was used by Storz and Page (1971) to place these organisms in a separate order, Chlamydiales.

1.3.3. ANTIGENIC CHARACTERISTICS.

Chlamydiae have a major antigenic component which exists throughout their growth cycle. This component is a cell wall associated, heat stable, group specific, complement fixing antigen (Reeves & Tavern 1962). This antigen was shown by Dhir et al (1972) to be a lipid moiety with an acidic carbohydrate component which was later identified by Caldwell and Hitchcock (1984) as 2-keto-deoxyoctonic acid (KDO). In addition to this heat stable, genus specific antigen, Caldwell et al (1975a) have described another antigen found in both C.trachomatis and C.psittaci which is heat labile.

As techniques for the isolation, purification and specific staining of this organism improved, the heat stable, genus specific antigen was extracted from infected cells (Wahlstrom et al 1984) in the overlaying supernatant fluid (Stuart & McDonald 1984) and was observed on the cytoplasmic membrane of these infected cells (Richmond & Sterling 1981). It also became possible to determine directly the composition of isolated chlamydial components both immunologically and biochemically (Schachter & Caldwell 1980). As a result of these advances, several antigenic components of the organism became apparent and the genus, species and serovar specific epitopes were revealed.

i) SPECIES SPECIFIC ANTIGEN.

Using two dimensional immunoelectrophoresis, Caldwell et al (1975a) demonstrated nineteen different antigenic components in C.trachomatis, one of which was cross reacting with C.psittaci. Using cross immunophoresis (Caldwell et al 1975b) these workers also identified a 155 KD heat labile protein which is present in all 15 serotypes of C.trachomatis species but is absent in C.psittaci and the mouse pneumonitis agent. Salari and Ward (1981) have described a similar antigen, equivalent to a polypeptide mass, and have suggested that it is located in the chlamydial outer membrane.

Caldwell and Perry (1982) also demonstrated the presence of an antigen in the outer membrane protein of C.trachomatis. This antigen represents 60% of the outer membrane protein and is designated the major outer membrane protein (MOMP), having sub-species and serovar specific epitopes (Stephens et al 1982). Using an immunoblotting technique, a 60-62 KD species polypeptide has been found which is assumed to be equivalent to the cysteine rich polypeptide of outer membrane described by Hatch et al (1984).

ii) SEROVAR SPECIFIC ANTIGEN.

Although many workers have shown antigenic differences between isolates of C.psittaci (Banks et al 1970 and Schachter et al 1975b), there is still no useful technique for differentiating C.psittaci on the basis of antigenic structure. In contrast C.trachomatis may be immunotyped with much greater ease using the micro immunofluorescence technique developed by Wang and Grayston (1970) and Wang et al (1975). On the basis of their study, members of the species C.trachomatis are divided into 15 serotypes. Serotypes A, B, Ba, C (the trachoma group), serotype D, E, F, G, H, I, J and K (the urogenital group) and LGV 1, 2 and 3 (lymphogranuloma venereum group). Several different serovar specific antigens have been demonstrated by a number of workers to be present in the lipid phase extract. Sacks et al (1978), Sacks and McDonald (1979) and Hourinhan et al (1980) have described a pronase sensitive, heat labile, antigenically active molecule of 300 KD.

1.3.4. DEVELOPMENTAL CYCLE.

The developmental cycle of chlamydiae is complex and not fully understood. Schachter and Dawson (1978) have described a simplified chain of events. Briefly, attachment of EBs to the surface of the susceptible cell is followed by active phagocytosis of the EB into a phagocytic vesicle in the host cell cytoplasm. Transformation into the metabolically active reticulate body occurs 6-8 hrs post infection with the loss of the electron dense nucleoid and changes in the cell wall allowing the exchange of metabolites. The RBs synthesize macro-molecules, multiply by binary fission and yield several daughter cells. This process stops at about 18 hrs post infection. At this stage another reorganization occurs and reticulate bodies are transformed into EBs. If infected cells are stained at 18 hrs post infection, inclusions are visible as microcolonies inside the susceptible cells. The EBs are released from the inclusion between 48-72 hrs post infection (fig 1.1). This chain of events has been observed by several workers, including Bedson & Bland (1932) and Thygeson (1934) who used different staining techniques and light microscopy and by Pollard et al (1960) who used cytochemical tests and fluorescence microscopy. Matsumoto and Manire (1970a and 1970b), Doughri et al (1972), de la Maza et al (1984) and Evans (1982) used transmission electron microscopy and de la Maza et al (1982) used scanning electron microscopy.

Giemsa stain was first used by Von Prowazek to detect inclusions produced by trachoma in 1907. C.trachomatis mature inclusions may be detected by dark-field microscopy when they exhibit a distinct "autofluorescing" apple green colour. By bright field microscopy, the

inclusions are well defined, reticulate in structure and are contained within a limiting membrane. Evans and Woodland (1983) observed that the particles within the inclusion stained blue (RBs) or purple (EBs). This staining reaction suggests that the RBs contain RNA and that EBs contain DNA. This observation is confirmed by acridine orange stain with which these particles stain orange and yellow green respectively (Pollard et al 1960).

C.psittaci does not autofluoresce when stained by Giemsa . The inclusions appear to be larger and are less well defined when examined by bright field microscopy. Methylene blue stain combined with dark field microscopy has been used to demonstrate C.psittaci and C.trachomatis inclusions (Johnson et al 1978).

The glycogen matrix found in C.trachomatis inclusions may be stained brown by iodine (Gordon & Quan 1965a), red by carmine (Johnson 1975) or magenta by periodic acid Schiff (Mallinson et al 1981). These stains detect inclusions from about 24 hrs post infection (Evans & Woodland 1983) and, as such, the amount of glycogen produced in the inclusions varies during the growth cycle.

C.psittaci lack glycogen matrix and therefore cannot be detected by any of the above stains.

Fluorescein isothiocynate (FITC) and peroxidase labelled antibody are used for the detection of Chlamydia. They have been shown to have greater sensitivity than Giemsa bright field for the detection of C.psittaci (Woodland et al 1978 and Munday et al 1980), but have the same order of sensitivity for C.trachomatis observed by dark field microscopy. In an immunoperoxidase technique used by Woodland et al

(1978), the inclusions were less prominent in comparison with immunofluorescence but had the advantage that permanent preparations were produced which could be observed by normal bright field microscopy.

Richmond and Ashley (1977), using immunoferritin to detect extracellular Chlamydia from urethral and cervical samples report a sensitivity comparable to tissue culture isolation of these agents. The use of this technique, however, requires the availability of an electron microscope.

Many of the staining properties of Chlamydia are affected when certain antimicrobial drugs are present during the life cycle. The presence of erythromycin interferes with glycogen synthesis (Clark et al 1982) and sulphonamides inhibit the autofluorescing properties of C.trachomatis. It is therfore possible to choose certain staining techniques to follow the life cyclė of Chlamydia and, by introducing antimicrobial drugs during the developmental cycle, study the effect of the drug on the appearance of the inclusions produced. Furthermore, the morphology of such affected inclusions can be more closely studied by electron microscopy.

A part of the work presented in this thesis describes the findings on the developmental cycle of C.trachomatis serotype L2 by light microscopy, using different staining techniques, and by electron microscopy in the presence and absence of penicillin.

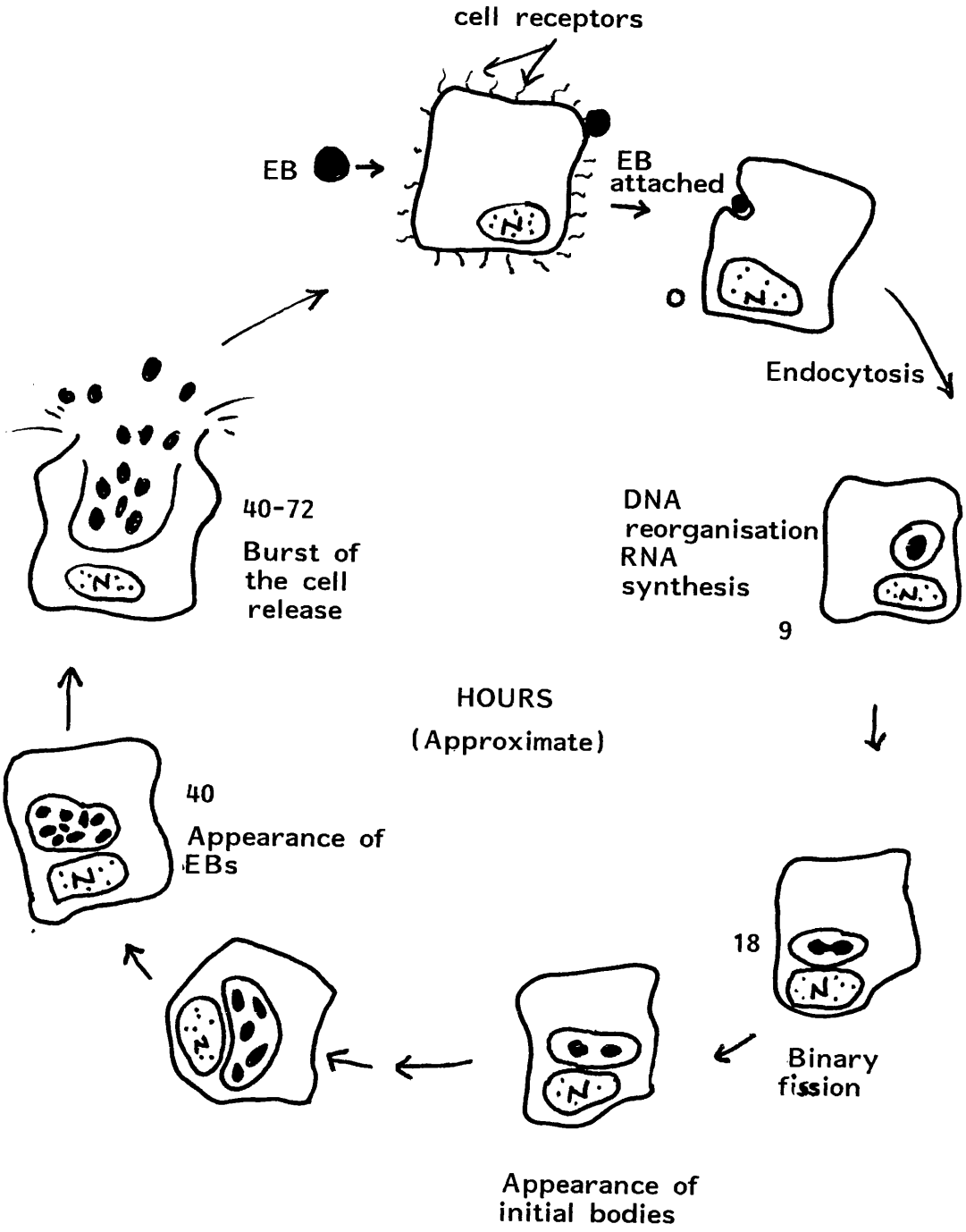

fig 1.1. Life cycle of CHLAMYDIA

1.3.5. PROPAGATION METHODS.

The growth of chlamydiae in the laboratory demands the use of a sensitive and reliable technique. In the past, three in-vitro systems have been used.

i) ANIMAL INOCULATION.

ii) YOLK SAC INOCULATION OF EMBRYONATED HENS' EGGS.

iii) CELL CULTURE.

i) ANIMAL INOCULATION. The orang-outang was first used in 1907 as an animal model by Halberstadter and Prowazek. Since then several studies have been performed on human volunteers (Jones & Collier 1962, Tarizzo et al 1967). Because such practices are ethically restricted, several species of laboratory animals such as mice have been used for the purposes of research in chlamydial disease (Darougar et al 1977, Kuo & Chen 1980 and Barron 1982). However, for the purpose of routine laboratory isolation of Chlamydia, the use of animals is not practical.

ii) YOLK SAC INOCULATION. The earliest technique for the isolation of chlamydiae was described by T'ang et al (1957) who inoculated the embryo of a fertilised hen's egg with a sample from trachoma and grew the organisms. Although this technique has become well established and detects all chlamydiae, it is a laborious procedure, prone to contamination (Harper et al 1967 and Dunlop et al 1967) and several blind passages may be needed for the isolation of certain strains. Furthermore, seasonal variation may affect the susceptibility of eggs (Jawetz 1962). The technique is not as sensitive as cell culture but it is an efficient method for the production of high titres of antigen.

iii) TISSUE CULTURE INOCULATION. Previously grown chlamydiae in yolk sac were adapted to grow on suitable cell lines (Furness et al 1960). Although many cell lines may be used for the isolation of these organisms, McCoy and Hela 229 cells are the most widely used.

McCoy cells: These cells were originally human synovial tissue from a patient suffering from degenerative arthritis (Pomerat et al 1957), but some time in their history they became contaminated with mouse karyotype similar to L-929 cells. Gordon and Quan (1965b) successfully used McCoy cells for the isolation of C.trachomatis and since then they have been widely used.

Hela 229: This cell line, established by Gey et al (1952), was originally endocervical cells from a patient, Henrietta Lacks, who died of cervical cancer in 1951 (Jones et al 1971). These cells were first used by Jenkins (1966) and later by Kuo et al (1972a) for the isolation of C.trachomatis.

Several other cell lines such as MK2, Hep2, Hela M (Croy et al 1975) and Buffalo green monkey (Hobson et al 1982) have been used and are reported to have different degrees of sensitivity for the isolation of chlamydiae.

The isolation procedure of Chlamydia in tissue culture entails:

a) Inoculation of the sample onto a suitable cell line.

b) A centrifugation step.

c) Incubation of infected cells in a suitable environment for 2-3 days at 35-37°C.

d) Staining.

a) SUITABLE CELL LINES.

Gordon et al (1972) observed that a higher proportion of the multinucleated giant McCoy cells (present in a small number) than of the normal McCoy cells contained inclusions. They therefore undertook an experiment to produce giant cells by the process of gamma irradiation used by Pomerat et al in 1957. Using this process, they observed 4-8 times greater number of inclusions than in non-irradiated cells. Similarly, pre-treatment of the cells with cytotoxic agents rendering them non replicating prior to inoculation has been studied by several workers. Wentworth and Alexander (1974) used 5-iodo-2-deoxy-uridine (IUDR), a thymine analogue which inhibits DNA synthesis and eventually prevents cell division. This inhibitor enhances the isolation of chlamydiae in McCoy cells but does not increase the sensitivity of Hela 229 cells. Cytochalasin B, used by Sompolinsky and Richmond (1974), is a microfilament inhibitor and blocks the uptake of particles which are larger than 600 nm in diameter (Painter et al 1981). Cycloheximide, an inhibitor of protein and DNA synthesis in eucaryotic cells, was used by Ripa and Mardh (1977) at a concentration to depress but not completely inhibit the metabolism of McCoy cells for the cultivation of C.trachomatis. These workers observed a greater number of inclusions in cycloheximide treated cells than the cells pretreated with IUDR. Other inhibitors such as cortisol (Bushnell & Hobson 1978) and emetine (Paul 1982) have also been used.

Harrison (1970) and Rota and Nichols (1971) reported on the use of DEAE-Dextran to enhance the growth of chlamydiae in cells. Kuo et al (1972a) found Hela 229 cells treated with DEAE-dextran enhanced the

primary isolation of trachoma inclusion conjunctivitis agent but had no advantage for the isolation of LGV serotypes. Croy et al (1975) compared the susceptibility of eleven mammalian cell lines to infection with trachoma organisms and observed that this compound was most effective when used with Hela 229 cells.

DEAE-dextran is a positively charged macromolecule presumably changing the surface charge of the cell so as to permit the attachment of a greater number of the EBs to the cell membrane. This process is aided by a centrifugation step which according to the studies by Rota and Nichols (1971) and Kuo et al (1972a & b) plays a major part for increasing infectivity.

The use of other cell lines such as untreated BHK21 (Blyth & Taverne 1974), DEAE treated Hela 229 (Kuo et al 1972a), MK2, McCoy, Hela M and Hela 229 (Croy et al 1975) and Buffalo green monkey cells (Hobson et al 1982) has been reported, showing different degrees of activity for the isolation of chlamydiae. However, there seems to be no significant difference in the isolation rate between Buffalo green monkey cells and McCoy cells (Willis et al 1984) and McCoy cells and Hela 229 cells for the isolation of C.trachomatis. Culture of C.psittaci has been carried out using L.929 cells (Morgan & Bader 1957) as well as McCoy cells.

b) CENTRIFUGATION.

Centrifugation of clinical material onto the cell line enhances the entry of the elementary body into the susceptible cells. Gordon and Quan (1965b) showed a better isolation rate by centrifugation of the samples onto irradiated McCoy cells. The reason for the necessity of this step in the isolation procedure is not clear. One possible explanation is that the centrifugal forces overcome the electrostatic charges that exist between the cell and the EBs. Becker et al (1969a) showed that EBs already absorbed onto the cell membrane can be eluted when the cell surface is treated with heparin. An enhancing effect of DEAE-dextran, a positively charged macromolecule, on the isolation of Chlamydia in cell culture has been demonstrated by Harrison (1970), Rota and Nichols (1971). Kuo et al (1973), believing that ionized surface structures are involved in the adhesion of chlamydiae to the host cells, investigated the effect of polycations, polyanions and neuraminidase on the infectivity of TRIC agents and LGV serovars. They found that DEAE-dextran and neuraminidase did not affect the infectivity of LGV serotypes while that of TRIC agents was enhanced by DEAE-Dextran treatment and inhibited by neuraminidase. It was therefore concluded that the attachment points or receptor sites for the LGV and TRIC agents are different. Kraaipoel and Van Duin (1979) and Vance and Hatch (1980) showed that chlamydiae carry a net negative charge on their surface. Schiefer et al (1982) applied cytochemical techniques using polycationic ferritin and methanolic HCL as ultrastructural markers and found that the major negative ionogenic species on the outer membranes of chlamydiae are free hydroxyl groups.

The temperature during centrifugation affects the sensitivity of

the isolation system. Darougar et al (1974) showed a four times increase in the inclusion count when the temperature of centrifugation was increased from 18°C to 35°C at 1800 G for 1 hr.

c) INCUBATION OF INFECTED CELLS IN A SUITABLE ENVIRONMENT.

The isolation and the number of inclusions produced in the cell system are affected by such factors as media constituents, ionic concentration, and the incubation temperature.

Media: Eagle's Minimum Essential Medium (MEM) supplemented with serum is used for the growth of Chlamydia. Allan and Pearce (1977) found that the type and concentration of serum will influence the isolation of these organisms. 10% foetal calf serum added to the MEM gives a larger number of inclusions (Johnson & Hobson 1976). Factor V of bovine serum albumin, sodium oleate and fetuin together give the same results as 10% foetal calf serum (Karayiannis & Hobson 1981). Evans (1980) showed that batch quality control should be performed regardless of the type and concentration of serum used in cell culture system.

pH: Harrison (1970) found that there is a drop in the number of inclusions of a C.psittaci strain when the pH of the cell culture media is below 7.0 and suggested that certain enzymes necessary for the growth of Chlamydia are inactivated at acid pH values. Rota and Nichols (1973) and Johnson and Hobson (1976) found that a pH of 7.0 was optimal for the growth of C.trachomatis and that a variation of the order ±0.4 in the pH value may reduce the number of inclusions developed.

Temperature: Ward and Murray (1984) suggested that the engulfment of EBs is achieved by a microfilament-dependent movement of the host cell surface accompanied by sequential binding of Chlamydia-host cell surface dependant legands. The chlamydial entry has been shown to become blocked at a temperature of 4-8°C (Ward 1986) hence making it distinct from the process of attachment. An incubation temperature of 35°C for 48 hrs or of 37°C for 72 hrs increases the number of inclusions produced (Rota & Nichols 1973 and 1977).

Glucose concentration: Verdos and Gordon (1963) studied the effects of a high concentration of glucose, 10-40 mM/ml of media, on the growth of C.trachomatis grown in tissue culture as well as in eggs. They observed a 10-fold increase in the number of iodine inclusions produced compared with cultures grown on unsupplemented media.

d) STAINING.

Chlamydia grown in tissue culture may be observed microscopically using several staining methods. The most commonly used techniques for routine diagnosis are:

Iodine: This is a simple and rapid technique for the detection of C.trachomatis inclusions in tissue culture. It is, however, the least sensitive for this purpose as the glycogen matrix of C.trachomatis is only present for a relatively short period during the developmental cycle (Schachter & Dawson 1978). This lack of sensitivity also applies to other staining techniques, namely periodic acid Schiff (Mallinson et al 1981) and carmine (Johnson

1975) which also stain the glycogen matrix. C.psittaci does not produce the polysaccharide glycogen and hence cannot be stained by these methods.

Giemsa: This stain is more sensitive than iodine (Darougar et al 1971) for the detection of C.trachomatis. However, the sensitivity of this method is reduced if incorrect buffering and inferior quality Giemsa stain are used. Artefacts such as microcolonies of contaminating bacteria may look like chlamydial inclusions to an inexperienced observer. Inclusions of C.trachomatis stained with Giemsa may be observed by bright field as well as dark field microscopy. Inclusions of C.psittaci have to be examined by bright field microscopy. Methylene blue stain used by Johnson et al (1978) for enzootic ovine abortion strain of C.psittaci is claimed to be useful in dark field microscopy.

Immunofluorescent stain: The use of immunofluorescent stain for the detection of chlamydial antigen grown in tissue culture has been demonstrated by several workers (Thomas et al 1977, Woodland et al 1978 and How et al 1985) and found to be superior to conventional histological stains. Monoclonal antibodies conjugated with a fluorescein label are now commercially available and have been shown to be the method of choice in detecting chlamydial inclusions in tissue culture (Stephens et al 1982, Stamm et al 1983 and Tjiam et al 1985).

1.4. CHLAMYDIAL DISEASES OF MAN AND ANIMALS.

1.4.1. CHLAMYDIA PSITTACI.

i) PSITTACOSIS.

ii) TWAR AGENTS (TW/183,IOL/207).

1.4.2. CHLAMYDIA TRACHOMATIS.

i) OCULAR CHLAMYDIAL INFECTION.

ii) GENITAL CHLAMYDIAL INFECTION IN WOMEN.

iii) CHLAMYDIAL INFECTION IN MEN.

iv) LYMPHOGRANULOMA VENEREUM.

v) CHLAMYDIAL INFECTION OF CHILDREN AND NEONATES.

1.4.1. CHLAMYDIA PSITTACI.

i) PSITTACOSIS.

Psittacosis is a symptomatic chlamydial infection caused by C.psittaci which may be contracted by humans through exposure to infected birds. The term psittacosis was applied to the chlamydial infection of psittacine species, namely parrots and parakeets and related birds. The name ornithosis was used to describe chlamydial infection of other avian species such as pigeons, turkeys etc.

Ritter reported an outbreak of pneumotyphus in Switzerland in 1879-1880. This disease was a kind of severe pneumonia with stupor and scattered rose spots. This outbreak was associated with exposure to exotic pet birds and was reconfirmed in outbreaks in France in the 1890's. In 1895, Morange gave the name Psittacosis, derived from the Greek word for parrot, to this condition. Several other outbreaks were noted, namely in France in 1892-3, in Italy in 1897, in the USA in 1904 and 1925. In 1930, Barros observed an unusual form of pneumonia in Argentina which was associated with exposure to parrots. The disease soon spread to Europe and North America and reached England, Germany and the USA later that year. This condition had a mortality rate of 40% in certain outbreaks. Many laboratory workers investigating the causative organisms became infected. Reports from the scientists working with the agent indicated a characteristic coccoid organism and inclusions were found in the tissues of infected birds and humans.

In 1930, Bedson and Western isolated the agent by inoculating human and avian material into parakeets and demonstrated a complement

fixing antibody response to these organisms. In the USA, Krumwiede et al (1930) and in England, Gordon (1930) successfully transmitted the agent into mice. This success in the isolation and demonstration of antibody response provided a useful screening technique and led to the development of serological tests for diagnosis (Bedson 1935).

In 1938, Haagen and Mauer traced an atypical pneumonia in infected fulmers in the Faroe Islands. This disease had a fatality rate of 24.1%. In 1950, Vaag observed that amongst humans this disease was much more severe in pregnant women, with a mortality rate of 78%. The term ornithitis was used to describe this condition, making a distinction between the causative organisms originating from psittacine birds and those originating from others. This was shown by Pinkerton and Swank (1940) by isolating the agent from pigeons. Karrer et al (1950) showed that chickens could also be a source of infection. Chlamydial infection associated with turkeys was proved by Iron et al (1985) and Meyer and Eddie (1953). Several other outbreaks followed in other parts of the USA. Ducks were found to be a significant reservoir for human infection (Anon 1953 and Andrews et al 1981). It was clear that the so called exotic birds were not the only ones capable of harbouring the infection.

Non-avian forms of C.psittaci infection are very common in lower mammals. Members of C.psittaci have been found in mammals such as ewes and koalas. Enzootic abortion of ewes and infertility in female koalas are well recognised conditions. The infection of ewes may be transmitted to humans. Beer et al (1982) and Mckinley et al (1985) suggested that human pregnancy may be terminated as a result of infection with C.psittaci. Johnson et al (1985) and Wong et al (1985) reported convincing case histories of pregnant women who had been

exposed to aborting ewes. The women aborted between 25-28 weeks of pregnancy and C.psittaci was isolated from the fetal tissues and, hence, abortion in humans due to C.psittaci from a mammalian source was established.

While studying the potential for arthopod transmission of the bovine abortion agents, Schachter (1986) found that the organism could remain viable on dried litter or on surfaces of ectoparasites for months and, therefore, suggested that it is likely that potential exposure of humans is quite high and as such a dose effect may be important in establishing the disease.

Since the recognition of human psittacosis there have been isolated cases where no animal exposure could be identified and the possibility of person to person transmission has been suggested (Pether et al 1984). Serological evidence showing the prevalence of psittacosis infection amongst humans has led to the suggestion that psittacosis is a more common disease than can be explained by avian exposure alone (Darougar et al 1980).

Repeated isolation of C.psittaci from diseased koalas has been reported since early 1970 (Cockram & Jackson 1974 and Brown & Grice 1984). The chlamydial infection which is currently responsible for mortality in koalas may cause a variety of symptoms including conjunctivitis, urogenital infection and infertility in female koalas.

ii) TWAR AGENTS (TW183, IOL/207).

The human C.psittaci strains "TWAR" have been focused on recently. Isolate TW183 was grown in 1965 at the University of

Washington (UW) from a conjunctival sample taken from a school child in Taiwan. The isolate GB/IOL/207 was grown from a conjunctival sample collected in Iran. Both isolates were initially grown in eggs and were difficult to grow in tissue culture. The name "TWAR" was coined by the UW workers when a second agent similar to TW183 was isolated from a patient in an acute respiratory disease study. These isolates resemble C.psittaci. They are glycogen negative and do not react with C.trachomatis specific monoclonal antibodies. They are not sexually transmitted and a serological survey suggests that the infections are acquired in childhood between the ages of 5-10 and peak at 30 years (Forsey et al 1986).

As more of these agents are isolated and studied, their taxonomy can be elucidated. They are identified as antigenically related to C.psittaci but it is not clear that they are in fact the same species*.

* Footnote added in proof. Recently this organism has been designated as a separate species, C.pneumoniae(J.T. Grayston, C. Kuo, L.A. Campbell and S. Wang. Chlamydia pneumoniae sp. nov. for Chlamydia sp. strain TWAR. International Journal of Systemic Bacteriology, Jan 1989 vol 39 p 88-90).

1.4.2. CHLAMYDIA TRACHOMATIS.

i) OCULAR CHLAMYDIAL INFECTION.

C.trachomatis infection of the eye occurs in two distinctly different epidemiological situations, each having a different significance for the affected communities (Dawson 1982).

a) TRACHOMA (rough swelling - Greek).

This is an inflammation of the mucous membrane lining the eyelids and eyeballs (Schachter & Dawson 1978). It is caused by C.trachomatis serotypes A, B, Ba and C. Trachoma is a major cause of preventable blindness in the world and is estimated to affect approximately 500 million people. It is a major public health problem in Africa, the Middle East, the Indian subcontinent and South East Asia. Several pockets of trachoma also exist in Australia, the Pacific Islands and Latin America. It is more prevalent in people from low socio-economic backgrounds and in such communities there are annual or biannual epidemics, associated with the increased number of flies (Dawson 1982).

In the early stages of the disease, trachoma appears as a chronic follicular conjunctivitis with particular involvement of the upper tarsal conjunctiva. Conjunctival follicles appear as elevated avascular lesions 0.2-2 mm in diameter. They may be yellowish or translucent. Trachomatous inflammation may undergo spontaneous resolution or progress, with repeated infections, to conjunctival scarring. After a period of years, this leads to inturning of the upper eyelid (entropion), resulting in corneal ulceration from abrading

eyelashes. Ultimately blindness will result.

The hypersensitivity to, and an understanding of the mechanisms which result in blinding chlamydial infection are not fully understood. Animal studies have shown that pannus develops in a small proportion of animals infected with C.trachomatis. The essential factor for the development of pannus has been shown to be the history of previous exposure to Chlamydia, be it ocular infection or immunization. Therefore, it is hypothesised that the response to infection is most important in pannus formation and that a hypersensitivity reaction, which is not strain specific, plays a major role in this regard (Wang & Grayston 1967). Taylor et al (1981), after repeatedly infecting the eyes of cynomolgus monkeys with serotype E, produced conjunctival scarring. Monnickendam and Pearce (1983) have attempted to give reasons as to why the severity of trachoma varies so much. They suggest that:

(a) There is a difference in the rate of infection in blinding trachoma and para-trachoma.

(b) there may be major biological differences between serotypes A-C and D-K, or differences in the degree of protection offered by immune responses evoked by these serotypes.

Transmission of the disease is by direct or indirect contact with infected material. Factors like the crowding together of children, lack of adequate hygiene and clean water, plus prevalence of flies, all contribute towards the spread of the disease.

b) INCLUSION CONJUNCTIVITIS.

This disease is caused by the sexually transmitted chlamydiae, serotypes D-K. It presents itself as inclusion blennorrhoeà in newborns or paratrachoma in adults.

Inclusion blennorrhoea (chlamydial ophthalmia neonatorum): Inclusion conjunctivitis in the newborn usually appears 4-12 days post partum. Earlier onset can result if there is premature rupture of the placental membrane. The disease in the newborn is characterised by swelling of the eyelids, with hyperemia and inflammation of the conjunctiva accompanied by a purulent discharge (Dawson 1986). Untreated cases of neonatal inclusion conjunctivitis will run a course of 3-12 months. Infants who are treated late or not at all may develop conjunctival scarring and superficial corneal vascularization (Beem & Saxon 1982).

Paratrachoma in adults: In 1899, an epidemic of eye infection amongst clients of a swimming pool in Berlin was recorded by Schultz. In 1900, Fehr deduced that this syndrome was different and distinct from trachoma. The first evidence of adult chlamydial eye infection was shown by Huntenmuller and Padderstein in 1913, when they successfully inoculated the eyes of a monkey with the infected material. After this, several workers demonstrated the presence of chlamydial infection in extra genital sites. It was not until the early 1960s that it was pointed out by Jones (1964) that genital infection by Chlamydia can be manifested as adult inclusion conjunctivitis without corneal involvment. Further, this condition, when acquired during the neonatal period can continue into adolescence, resembling classic trachoma with pannus.

Using serological techniques, it became apparent that isolates from patients with trachoma from the endemic areas are of serotypes A, B, Ba and C, whereas isolates from the genital tract are mostly serotypes D-K with a few C, B and Ba types. Dawson et al (1962) and Grayston and Wang (1975) demonstrated that serotypes A-C had a lesser pathogenicity for primates' eyes than serotypes D-K.

Adult inclusion conjunctivitis occurs most often in sexually active adults aged between 18-30 years (Dawson 1986). In 1981, Tullo et al estimated that 1 in 300 patients with genital chlamydial infection will develop adult inclusion conjunctivitis. The disease in volunteers inoculated with genital Chlamydia types D and F had an incubation period of 2-19 days, depending on the concentration of the inoculum (Dawson et al 1966). Both in volunteers and in naturally occurring infection, corneal involvment is a regular feature, and diffuse punctate epithelial keratitis and swelling of limbus are present (Schachter & Dawson 1978). The infection of the eye usually presents itself as an acute follicular conjunctivitis with hyperemia, mucoid discharge and, after two weeks, leads to epithelial keratitis (Jones & Collier 1962). In many inoculated volunteers, the disease subsided gradually and in some instances healed without any chemotherapy (Schachter & Dawson 1978).

Dawson and Schachter (1967) observed that middle ear inflammation is a common complication of ocular infection and occurred in 14% of the infected volunteers. This observation was confirmed by isolation of the organism from fluid drained from the middle ear (Schachter & Dawson 1978).

ii) GENITAL CHLAMYDIAL INFECTION IN WOMEN.

The first suggestion that chlamydiae may cause genital infection in females was made by Heymann who, in 1910, saw the inclusions in the cervical cells of infected women. In the same year, Fritsch and co-workers, infected rhesus monkeys' conjunctiva with scrapings from the eyes of babies with inclusion blennorrhoea, with secretions from their mothers' cervix and their fathers' urethra. They observed that in each case the monkeys developed inclusion conjunctivitis with identical pathology, irrespective of the source of infection. The first isolation of the agent from the female genital tract was described by Jones et al (1959). They inoculated embryonated hens' eggs with cervical secretions to demonstrate the growth of Chlamydia from the cervix of mothers with babies suffering from inclusion conjunctivitis.

Since the introduction of tissue culture, many studies on different populations have been done which have helped towards an understanding of the prevalence of this organism. Hare and Thin (1983), after studying several reports from the USA from the years 1975-1982, state that C.trachomatis is a highly infectious agent with a high prevalence amongst women. The incidence varies in different groups, being related to age and patterns of sexual behaviour. Oriel et al (1972), Holmes et al (1975) and Alani et al (1977) have reported that as many as 45-68% of sexual partners of men with proven C.trachomatis urethritis contracted the infection. Concomitant gonorrhoea and C.trachomatis cervical infections have also been described by several authors (Hilton et al 1974 and Davies et al 1978) and it has been shown that 50% of women with gonorrhoea are also infected with C.trachomatis.

a) INFECTION OF THE LOWER GENITAL TRACT OF FEMALES.

The clinical symptoms of infection of the female lower genital tract by C.trachomatis include discharge and vaginal soreness. Although it is generally accepted that the cervix is the main site of infection, the urethra can also be infected (Dunlop et al 1972a & 1972b and Woolfitt & Watt 1977).

The pathological changes observed in the cervix have been the subject of some controversy. Terms such as "cervical erosion", "chronic cervicitis" or "acute cervicitis" have been used to indicate the presence of C.trachomatis infection and have been interpreted differently by different authors. Hare and Thin (1983) state that some degree of inflammation is induced by C.trachomatis but is not specific to chlamydial infection and that "it is unwise to imply that chlamydial infection can be diagnosed from these appearances".

b) INFECTION OF THE UPPER GENITAL TRACT OF FEMALES.

Since the mid 1970s the role of C.trachomatis in pelvic inflammatory disease (PID) has been recognised. This complex includes salpingitis (Mardh et al 1977), endometritis (Mardh et al 1981a), peritonitis and perihepatitis (Muller-Schoop 1978 and Wolner-Hanssen et al 1980 & 1982a & b) and periappendicitis (Mardh & Wolner-Hanssen 1985).

PID has been defined as "the clinical syndrome attributed to the ascending spread of microorganisms (unrelated to pregnancy or surgery) from the vagina and cervix to the endometrium, fallopian tube and/or contiguous structures" (Centre for Disease Control 1982). The aetiological agent for such conditions may be endogenous (i.e. normal flora of the lower genital tract), mainly anaerobic organisms (Mardh 1980), or exogenous, most of which are sexually transmitted pathogens eg. Neisseria gonorrhoeae, C.trachomatis and Mycoplasma hominis.

Salpingitis: C.trachomatis was first isolated from fallopian tubes by Eilard et al (1976) and Mardh et al (1977). Since then other groups of workers have confirmed these findings (Moller et al 1979 and Wolner-Hanssen et al 1982a). Infection by C.trachomatis in the fallopian tubes may cause infertility and an increased risk of ectopic pregnancy, but further studies are needed to define this risk.

The spread of the infection by C.trachomatis from the lower genital tract to the uterus and fallopian tubes is thought to be canalicular. Evidence for this is presented by Moller and Mardh (1980). Considering the fact that salpingitis does not occur in women after sterilisation and in pregnant women, they used grivet monkeys as animal models and showed that the spread of the infection to the fallopian tubes was from the cervix via the endometrium.

Both mechanical and physiological factors for the canalicular spread of chlamydial infection have been considered. Changes during the menstrual cycle and sexual stimulation leading to uterine muscle contraction, and mechanical factors such as the use of IUD (Westrom et al 1981), elective and therapeutic abortion (Moller et al 1982 and Quivastad et al 1982) have been shown to increase the risk of tubal infection in carrier women.

Perihepatitis: The first description of perihepatitis was made in 1920 by Stajono. He described the relationship between upper abdominal peritonitis, perihepatitis and salpingitis. In 1930, Curtis described the perihepatic adhesions ("violin strings") and associated them with evidence of N.gonorrhoeae salpingitis. In 1934, Fitz-Hugh described the acute clinical features of perihepatitis. These findings attracted little attention until the late 1970s and early 1980s when there was an increased interest in chlamydial infection as well as in its aetiological role in the female genital tract. Muller-Schoop et al (1978), Wolner-Hanssen et al (1980) and Dalakar et al (1981) presented laparoscopic findings consistent with the observation made by Curtis. Several other workers found a correlation between the presence of N.gonorrhoeae and typical clinical features of perihepatitis (Curtis Fitz-Hugh syndrome). In recent years, patients with clinical evidence of perihepatitis have been studied for the presence of N.gonorrhoeae and C.trachomatis and of anti-chlamydial antibodies.
Muller-Schoop et al (1978) found laparoscopic evidence of Curtis Fitz-Hugh syndrome in 7 women, 4 of whom had high serum levels of chlamydial antibodies and negative culture for N.gonorrhoeae. Two had elevated C.trachomatis antibodies and positive N.gonorrhoeae culture and one had neither. Wolner-Hanssen et al (1980) isolated C.trachomatis from the cervix of two of their three patients suffering from perihepatitis. The third patient had high levels of C.trachomatis antibodies. These workers also succeeded in isolating C.trachomatis from the liver capsule and cervix of a patient with perihepatitis (Wolner-Hanssen et al 1982b).

c) INFECTIONS OF OTHER SITES.

Though chlamydiae have been isolated from Bartholin ducts (Davies et al 1978), the contamination of the sample by C.trachomatis from other genital sites is not ruled out. Dunlop et al (1972b) and Paavonen (1979) have reported the isolation of C.trachomatis from the urethra of women with urinary symptoms. The role of this organism as an aetiological agent in otherwise unexplained urinary symptoms requires further investigation.

Chlamydia have been isolated from the rectum of females (Dunlop et al 1971). The significance of this finding in carrier women is not clear.

iii) GENITAL CHLAMYDIAL INFECTION IN MEN.

C.trachomatis causes a variety of diseases in sexually active men. These include:

a) Symptomatic or asymptomatic urethritis.

b) Epididymitis.

c) Proctitis.

a) URETHRITIS.

In 1879, Neisser identified N.gonorrhoeae and, in 1911, Lindner first described infection of the male and female genital tract by C.trachomatis. With the introduction of a tissue culture technique by Gordon and Quan (1965b) the study of chlamydial disease took a new turn. However, it was not until the mid 1960s that the first isolates from the male urethra were reported by Jones (1964) and Dunlop et al (1966). These isolates were from the urethra of the fathers of babies with inclusion blennorrhoea, men with inclusion conjunctivitis and male contacts of women with inclusion conjunctivitis. Later, Dunlop et al (1972b) studied men with non-gonococcal urethritis (NGU) and found that about 40% of these patients had chlamydial infection verified by culture. Schachter and Dawson (1978) state that this agent is the cause of 30-50% of cases of NGU.

NGU is a sexually transmitted disease of complex aetiology (Oriel and Ridgway 1983). Organisms other than Chlamydia may be responsible for this condition. Ureaplasma urealyticum is identified in such

patients and is thought to be responsible for 25% of the cases of NGU (Taylor-Robinson & McCormack 1980), but the evidence for its causal role is not fully understood. Trichomonas vaginalis, Herpes simplex virus and Candida are also found in a few cases of NGU.

A diagnosis of NGU cannot reliably be made on clinical grounds alone. NGU has a longer incubation period, a gradual onset, generally milder dysuria and a less purulent discharge. However, 20% of all men who have gonococcal urethritis are also infected with C.trachomatis and may develop post gonococcal urethritis (PGU) following treatment with a beta-lactam antibiotic (Stamm et al 1984). Many NGU infections are subclinical.

b) EPIDIDYMITIS.

The epithelium of the male genital tract is largely composed of columnar cells which are particularly susceptible to infection by C.trachomatis. As there is no animal model with which to study the pathogenesis of infection at this site, there is difficulty in understanding the spread of the infection further from the urethra into the coiled tubules of the epididymis.

The first indication of possible chlamydial aetiology in epididymitis came from studies made by Heap (1975). She observed a significant rise in chlamydial antibody in two patients with acute epididymitis. Later studies by Harnisch et al (1977) demonstrated that epididimitis caused by N.gonorrhoeae and C.trachomatis occurred more frequently in men under the age of 32. Berger et al (1978) confirmed this finding by isolating C.trachomatis from the epididymal aspiration of 5 of 6 men under the age of 35. The aspirates from the other 10 men in this study who were over the age of 35 grew coliforms. No N.gonorrhoeae or C.trachomatis were isolated from these latter men. Hawkins et al (1986) found C.trachomatis in 48% of patients under 35 years old as opposed to 15% in older patients. There is some speculation as to the role of C.trachomatis alone for this condition. Oriel and Ridgway (1983) express uncertainty about whether an initial chlamydial infection is maintained by other microbes, or immunological reactions are involved in the pathogenesis of the disease. They also draw attention to the significance of this condition for its effect on fertility and the need for further research in this area.

c) PROCTITIS.

Men who practise anorectal intercourse may suffer chlamydial proctitis. The proctitis caused by LGV serovars of C.trachomatis is more severe than that caused by non LGV serovars. Other organisms for this condition in this group of people include N.gonorrhoeae, Herpes simplex virus and trauma (Quinn et al 1981). Chlamydial proctitis can be diagnosed by isolation of the organism from the rectum or by serological evidence using paired sera in the presence of symptoms. Goldmeier and Darougar (1977) isolated C.trachomatis from the rectum of 2 homosexuals, in one of whom severe congestion and numerous follicles were seen with an operating microscope. C.trachomatis has also been isolated from the rectum of 6/150 homosexuals, two of whom also had rectal gonorrhoea. The other four patients were asymptomatic (McMillan et al 1981). Munday and Taylor-Robinson (1983), considering the available evidence, concluded that although C.trachomatis has repeatedly been isolated from the rectum of this group of people, it is not necessarily a common cause of proctitis in homosexuals nor that C.trachomatis isolated from the rectum of such men is the cause of symptoms or abnormal physical signs.

iv) LYMPHOGRANULOMA VENEREUM (LGV).

This condition was recognised in 1913 by Durand, Nicholas and Favre as a sexually transmitted disease. It is caused by C.trachomatis serotypes L1, L2 and L3. These agents have a greater virulence both in-vivo and in-vitro, and the organisms often infect mononuclear cells, specially macrophages, rather than the columnar epithelial cells which are the target for serotypes A-K. In-vitro, they are capable of growing in tissue culture without the need for centrifugation.

The characteristic clinical feature of LGV is inguinal lymphadenopathy with enlarging buboes. The clinical course of the disease may be divided into three stages: the primary stage involves initial infection and early lesions, the secondary stage involves the regional lymph nodes, and the tertiary stage involves the late sequelae which covers a variety of conditions resulting in necrotic lesions. Anorectal syndromes such as proctitis, rectal stricture, perirectal abscesses and formation of fistula occur in women, and elephantiasis of the genitalia in both sexes.

LGV is worldwide in its distribution, but it is believed to be more common in tropical and developing countries.

v) CHLAMYDIAL INFECTION OF CHILDREN AND NEONATES.

In communities with blinding trachoma, infection of the eye due to C.trachomatis serovars A-C (trachoma group) occurs most in children between the ages of 1-2 years and during childhood and adolescence [see section on trachoma p.37].

In infants, infection with C.trachomatis serovars D-K (urogenital group) occurs during vaginal delivery through an infected cervix. True congenital infection has not been reported. Neonatal chlamydial infection of the eye has been noted since the beginning of this century. Chlamydial infection of this site (ophthalmia neonatorum) is at least 5 times as common as gonococcal infection. The symptoms appear most in infants between the 3rd and 13th days of their life (Rees et al 1977), and represent a wide range of clinical diseases varying from mild conjunctivitis to severe inflammation (Oriel & Ridgway 1982) [see section on neonatal conjunctivitis p.44].

However, during the last few years, the number of neonatal syndromes in which Chlamydia has been implicated have increased. These include respiratory tract infection and otitis media.

Colonization of the upper respiratory tract by Chlamydia is not necessarily associated with disease (Beem & Saxon 1977). Dawson and Schachter (1967) and Tipple et al (1979) have found C.trachomatis in the ear of infants with otitis media. Infection of the middle ear may follow pharyngeal infection by respiratory pathogens, and Oriel and Ridgway (1982) suggest that the same may apply to infection by C.trachomatis.

Chlamydial pneumonitis in infants may present itself as an afebrile and relatively non-toxic illness appearing between the 4th and 12th weeks of life. Several authors (Schachter at al 1975a, Beem & Saxon 1977 and Tipple et al 1979) have described this condition. Serologically, the infants show raised IgG, IgM amd IgA titres. The disease runs a protracted course with eventual recovery.

Infants infected with C.trachomatis, particularly those with pneumonia, shed a large amount of antigen in their faeces. Whether C.trachomatis causes gastroenteritis is not clear (Harrison 1986).

In the absence of detailed information about the long term effects of untreated chlamydial infection of the respiratory and gastrointestinal tracts in infancy, there is a clear need for both an animal model and case control studies.

A number of serological studies of children from infancy to puberty show an increase in titres of specific antibody through childhood (Black et al 1981, San Joaquin et al 1982 and Grayston et al 1982). This antibody response may be due to the serovars called "TWAR" [see section on TWAR agents p.40].

1.5. THERAPY.

The Ebers Papyrus (1500 BC) mentions the exudate and cicatrical features of trachoma and its treatment with copper salts.

The first effective specific chemotherapy for trachoma was described in 1938. This was in the form of orally administered sulphonamides. Later, topically administered tetracyclines were used.

Untreated chlamydial infections may persist for long periods. Man infected by C.psittaci, LGV and trachoma may harbour the organisms for years. Infection with genital chlamydiae may persist for months in the cervix of untreated women (McCormack et al 1979) and in the urethra of men for weeks or longer (Prentice et al 1976 and Johannison et al 1979). Inclusion conjunctivitis of adults and newborns, if not treated, may lead to corneal scarring and vascularization.

Although tetracyclines and erythromycin have for many years been the antibiotics of choice for the treatment of chlamydial infection, they are not ideal. Disadvantages include:

(a) 80% cure rate when used in the treatment of lower genital tract infections (Ridgway 1986).

(b) Gastrointestinal upsets caused by both agents.

(c) Poor bioavailability of erythromycin and chelating of tetracycline in the gut, affecting bio-availability.

(d) The unsuitability of tetracycline in the treatment of pregnant and lactating women.

As a genital infection in which the aetiological agent is solely chlamydial is unusual, and as erythromycin and tetracycline are not reliably active against all other concurrent genital organisms

(eg. N.gonorrhoeae, M.hominis and anaerobic micro-organisms), other antimicrobial agents have been investigated. Moreover, as even a mild or asymptomatic chlamydial infection may lead to serious complications and particularly as the prevalence of these organisms in different communities around the world may be alarmingly high, the need for research to find an effective and safe cure is justified. A major part of the work presented in this thesis deals with the in-vitro testing of antibiotics for the treatment of chlamydial infection.

Since the early 1950s, the antimicrobial activities of chemotherapeutic agents against Chlamydia have been studied in mice, embryonated hens' eggs (Weiss 1950, Gogelack & Weiss 1950, Weiss & Gordon 1958 and Gordon & Quan 1962) and tissue culture (Alexander et al 1977, Kuo et al 1977, Treharne et al 1977, Lee et al 1978, Bowie et al 1978, Ridgway et al 1978 and Bailey et al 1984).

Ridgway (1986) has divided antimicrobial agents according to their activity against C.trachomatis in vitro into three classes:

1. High activity - those with minimum inhibitory concentration (MIC) of < 0.06 mg/L.
2. Intermediate activity - those with MIC of 0.125-32 mg/L.
3. Low activity - those with MIC of >64 mg/L.

These values were obtained in the laboratory using cell culture and usually a laboratory adapted strain of Chlamydia. Several antimicrobials investigated by this method have been tested at different centres not only against a fast growing laboratory strain (eg. SA2f) but also against recent clinical isolates.

Multiple passage studies (Ridgway et al 1978) have been performed to establish the minimum bactericidal concentration (MBC) of the drugs

involving up to ten blind passages in cell culture after the withdrawal of the drug.

Using the above techniques the work presented in this thesis includes the:

1) Determination of MICs of antimicrobial drugs against SA2f.
2) Determination of MBCs of antimicrobial drugs against SA2f.
3) Determination of MICs of 13 antimicrobials against recent clinical isolates of C.trachomatis.

Different classes of antimicrobial agents studied in this thesis are as follows:

1) Tetracyclines.
2) Macrolides.
3) 4-Quinolones.
4) Beta-lactams (penicillin, cephalosporin, clavulanic acid and Augmentin).
5) Aminoglycosides / Aminocyclitols.
6) Pseudomonic acids.
7) Imidazoles.
8) Courmermycin and Novobiocin.
9) Thiamphenicol.
10) Rifamycins.
11) Folic acid pathway inhibitors (Cotrimoxazole / Fansidar).

1.5.1. TETRACYCLINES.

Tetracyclines are a family of antibiotics the first of which, aureomycin, was discovered in 1948 (Garrod et al 1981). Since then, several additions have been made, namely oxytetracycline, chlortetracycline, doxycycline, minocycline, clomicycline, lymecycline and rolitetracycline.

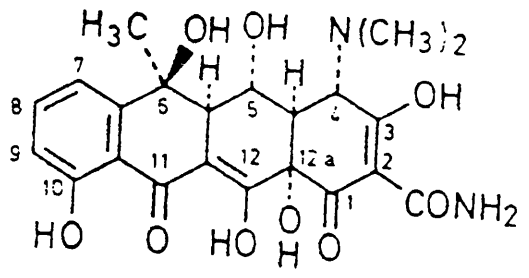

fig 1.2. structure of oxytetracycline.

Tetracyclines have broad spectrum activity and are active against Gram positive organisms which are sensitive to penicillin.They are also active against many Gram negative species which are not sensitive to penicillin. In addition, tetracyclines are active against mycoplasmas, rickettsias, chlamydiae and Treponema pallidum. They are bacteriostatic and act by interfering with protein synthesis at the 30S ribosomal level.

Tetracyclines are usually administered by mouth but a number of preparations are available for intramuscular use. They behave much like penicillins in their diffusion into serous cavities, the foetal

circulation, glandular secretions and cerebro-spinal fluid. They are deposited in areas of the body where bone is being laid down. This can happen *in utero* and early childhood which may result in the pigmentation of teeth.

The role of tetracyclines against chlamydial infection has been well studied. They have been the drug of choice for the treatment of this infection for many years. Some early treatment failures which have been observed are probably due to poor compliance. Although there are no isolates of chlamydiae which show resistance, or relative resistance, to tetracyclines so far, the possibility of this occurrence cannot be ruled out.

The study of different tetracyclines against genital pathogens may result in the discovery of an agent having not only higher activity against *Chlamydia* and possibily *N.gonorrhoeae* and *Ureaplasma*, but also one having less adverse effects on the gastrointestinal system, kidneys and other contra indications associated with this group of antimicrobials.

The work presented in this thesis includes the *in-vitro* activity of 3 different tetracyclines, namely oxytetracycline, doxycycline and minocycline, against *C.trachomatis*.

1.5.2. MACROLIDES.

The macrolides comprise a large group of related antibiotics, the first of which was discovered by Brockmann and Hankel in 1950. The term macrolide was suggested by Woodward in 1954 and is a combination of two words "macro", meaning large, and "olide", a suffix pertaining to the lactone ring.

Since the discovery of erythromycin by McGuire in 1952, a large number of compounds have been described.

The chemical structure of macrolides is characterised by a large lactonic cycle with 12, 14 or 16 atoms. This cycle is substituted

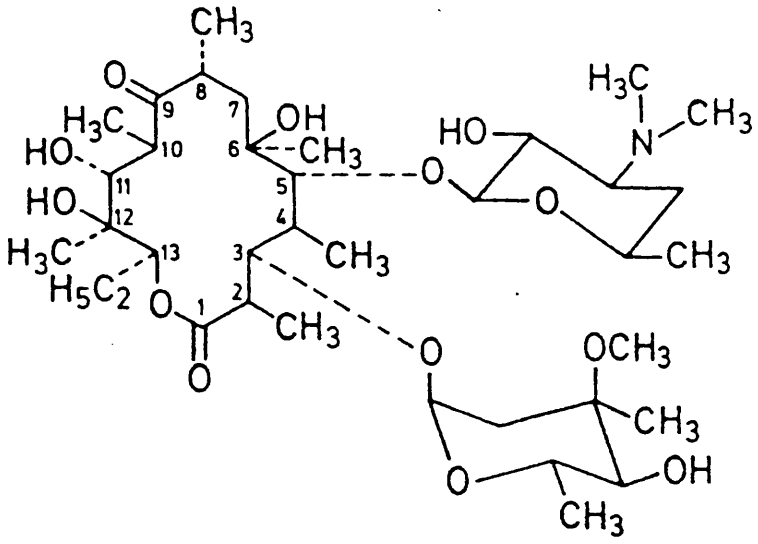

fig 1.3. Structure of erythromycin.

by hydroxyl and alkyl groups, one ketone in the 12 and 14 membered macrolides, and one aldehyde group in 16 membered ones. One, two and sometimes three hydroxyl groups are substituted by sugars. There are 2 species of sugars:

1) Neutral (mainly deoxy) with an a-glycoside linkage.

2) Basic (three amino sugar) with a b-glycoside linkage.

In the 12 and 16 membered groups there is at least one double bond.

The most widely used and documented of this class of antibiotics is erythromycin which is nearly always the most active of this group against sensitive micro-organisms in-vitro. Its activity against C.trachomatis is well documented. However, an increasing appreciation of the role of C.trachomatis in genital infections has led to the activity of the other macrolides being investigated in experimental systems. The activity of rosaramicin in a group of patients with NGU has been investigated by Darne et al (1982), and trials with roxithromycin and CP62993 are underway in several centres in the UK and in other parts of Europe.

Of the several new macrolides which have been investigated eg. josamycin, miocamycin and rosaramicin, none have been found to have in-vitro advantages over erythromycin. However, the Oxime-ether macrolide, roxithromycin, has an advantage over these compounds by virtue of its superior oral absorption and maintenance of a comparable antimicrobial spectrum (Jones 1985). The results of pharmokinetic studies in animals (Chantot and Bryskier 1985) have shown good bioavailability and tissue penetration. Moreover, relative resistance to erythromycin in-vitro has been reported in two clinical isolates of Chlamydia (Mourad et al 1980). Survival of 2 isolates in the presence

of 1mg/l of erythromycin was seen in single cycle studies. However, this relative resistance is regarded as clinically irrelevant since erythromycin levels above 1 mg/l were sustained for long periods during therapy.

The work presented in this thesis investigates the activities of 8 different macrolides in-vitro using McCoy cell culture, and looks at the possibility of inducing roxithromycin resistance Chlamydia in-vitro.

1.5.3. 4-QUINOLONES.

In early 1976, Crumplin and Smith discovered that the specific action of nalidixic acid on chromosome replication in Escherichia coli was to cause the abnormal accumulation of single strand DNA precursor of M.Wt 18.8 x 10^6 D. They calculated that, given that a single strand of a chromosome of E.coli is 1250 x 10^6 D, there must have been 66 chromosomal precursors per chromosome. Since this calculation corresponded well with the number of domains of supercoiling, they concluded that the accumulation of these precursors was the result of nalidixic acid not allowing the precursor to be made into chromosomal DNA. Crumplin and Smith (1976) suspected the presence of an enzyme which is inhibited specifically by nalidixic acid. Later that year, Gellert et al identified this enzyme and called it DNA-gyrase.

DNA gyrase is composed of 4 subunits, 2A monomers and 2B monomers. Their sequence of action is as follows:

1. The A subunit makes a nick into each strand of double stranded DNA.
2. The B subunit supercoils the DNA using 1 mole of ATP for each negative supertwist produced.
3. The A subunit locks the supercoil back into the chromosome and closes the nick.

Recently, several compounds have been synthesised based on the quinolone ring system with one fluorine atom subsituted. These compounds are norfloxacin, ofloxacin, pefloxacin, ciprofloxacin, A-56619 and A-56620.

Gellert and co-workers proposed that the 4-quinolones prevented

the a-subunits of DNA gyrase from finally sealing the nick originally introduced to the chromosomal DNA.

The new quinolones, like their predecessors (eg. nalidixic acid), have good antimicrobial activity (Inque et al 1985) against a range of aerobic and anaerobic bacteria. The action of quinolones against N.gonorrhoeae, including beta lactamase producing strains, has been studied by Felmingham et al (1983) and, at the same time, the action of these drugs on other genital pathogens, particularly C.trachomatis and genital mycoplasma, has also been studied (Meir-Ewert et al 1984, Heppleston 1985, Van Roosbraeck et al 1984, Bowie 1986 and Retting 1986). Some of the earlier work done on these agents (Ridgway et al 1984) comprises part of the work presented in this thesis. A total of 16 different quinolones have been studied, and the results are presented here.

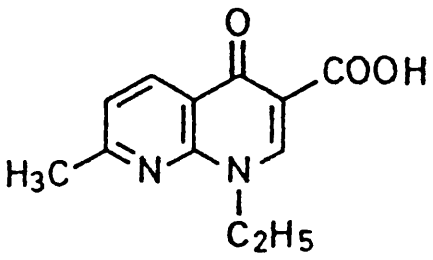

fig 1.4. Structure of nalidixic acid.

1.5.4. BETA-LACTAM ANTIBIOTICS (penicillin, cephalosporins, and clavulanic acid).

i) PENICILLIN.

Penicillin was the first antibiotic found to be suitable for systemic use. It was obtained from Penicillium chrysogenum.

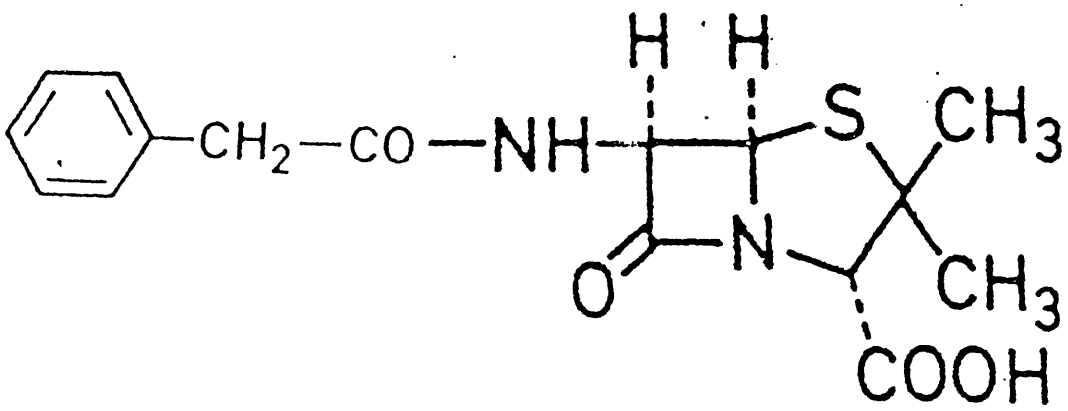

fig 1.5. Structure of benzyl penicillin.

Acylation of the penicillin nucleus with different carboxylic acid chlorides or mixed anhydrides has permitted the modification of the penicillin side chain, resulting in the production of several semisynthetic penicillins. Of these, amoxycillin is of particular interest because of its rapid bactericidal effect against Gram negative organisms. This agent has a more rapid cidal effect than ampicillin and is of value in the treatment of gonorrhoea.

ii) CEPHALOSPORINS.

The first of this group of antimicrobials was produced from a fungus Cephalosporium acremonium which was dicovered in Italy by G. Brotzu in 1945. Florey, in 1955, and Abraham, in 1962, established that several other antibiotics were derived from the compound cephalosporin C. This antimicrobial drug has a high degree of resistance to staphylococcal penicillinase.

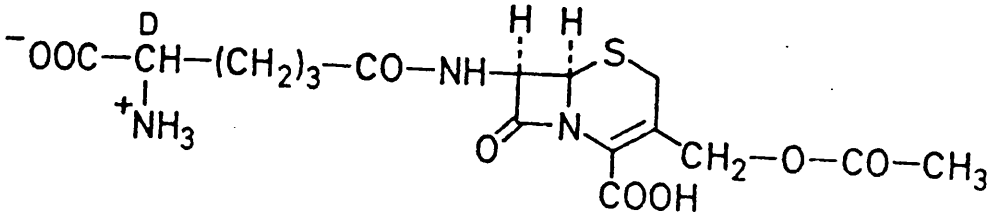

fig 1.6. 7-Amino cephalosporanic acid.

The compounds derived from cephalosporin C are bactericidal and inhibit cell wall formation. They can safely be given to patients with penicillin sensitization.

The efficacy of many of these compounds has been established against N.gonorrhoeae by showing a >95% cure rate in females after a single dose of a cephalosporin via the intramuscular route. It is, therefore,

justified to examine the antichlamydial activity of the newer products in this group.

iii) CLAVULANIC ACID.

This compound has little antibacterial activity but is a potent inhibitor of beta-lactamase production. However, many bacteria are resistant to penicillins and cephalosporins because of their ability to produce beta-lactamase. Therefore there is some scope for combining the activity of such compounds with clavulanic acid which inhibits the production of this enzyme.

iv) AUGMENTIN.

Augmentin is a combination of amoxycillin and clavulanate. The two agents together act against a wide range of beta-lactamase producing bacteria. This activity is achieved by the binding of clavulanate to the beta-lactamase, rendering it inactive, and leaving the organisms exposed to the action of amoxycillin. The combination drug is rapidly absorbed into the blood stream, penetrating the soft tissues. Its activity is not hindered by either the concentration of the organisms or by the pH of the environment and, as such, it is a good candidate for the treatment of genital infections. The action of Augmentin against Chlamydia has been examined in this work.

1.5.5. AMINOGLYCOSIDES/AMINOCYCLITOLS.

This group of antibiotics is typified by the presence of amino sugars glycosidically linked to aminocyclitols (Garrod et al 1981). Interest in this group of antibiotics emerged when it was found that

1) Gentamicin, a member of this group, is active against Pseudomonas.

2) Clinically acquired resistance to agents of this group is due to the ability of resistant bacteria to degrade these drugs enzymatically.

3) Modifications and chemical manipulations to overcome the acquired resistance is possible.

Four members of this group, namely neomycin, tobramycin, spectinomycin and trospectinomycin have been looked at in this work.

Trospectinomycin: Trospectinomycin (6'-n-propylspectinomycin) is a novel aminocyclitol, with greater activity against N. gonorrhoeae than spectinomycin. Because of its potential use for the treatment of gonorrhoea, its action against C.trachomatis is of interest.

The activity of this antimicrobial is studied in this thesis.

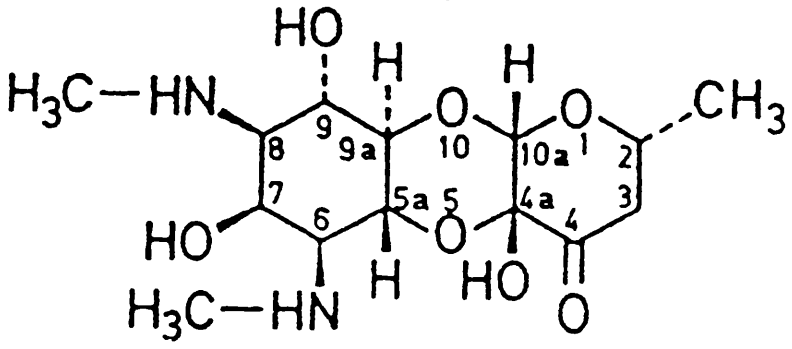

fig 1.7. Structure of spectinomycin.

1.5.6. PSEUDOMONIC ACIDS.

Produced by Pseudomonas fluorescens, pseudomonic acids are broad spectrum antibiotics which act by inhibiting protein synthesis of the bacterial cell wall. They are active against both Gram-positive and Gram-negative organisms particularly those associated with skin infections. Two compounds belonging to this group have been studied against C.trachomatis.

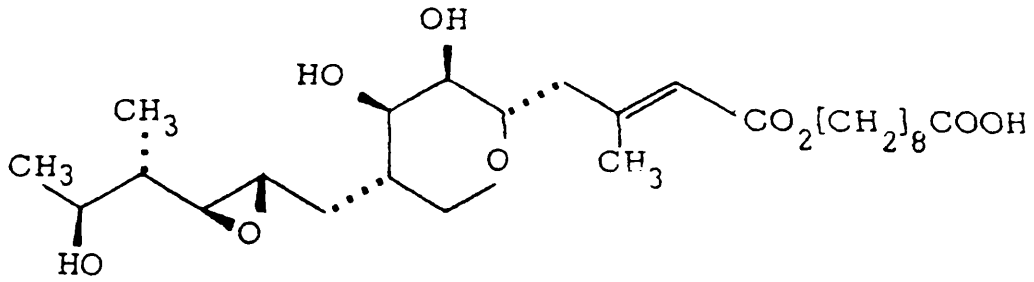

fig 1.8. Structure of pseudomonic acid.

1.5.7. IMIDAZOLES.

Imidazoles are antifungal agents used for topical treatment of vaginal candidosis or infection by dermatophytes. Some, like miconazole, have considerable activity against Gram-positive bacteria as well as being free from serious toxicity.

Concomitant infection of genital candida and Chlamydia is possible and an antifungal drug with activity against C.trachomatis is desirable.

The six imidazoles examined in this work were looked at with this aspect of chlamydial treatment in mind.

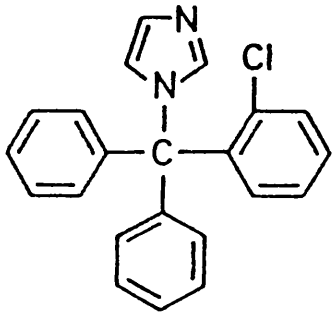

fig 1.9. Structure of clotrimazole.

1.5.8. COUMERMYCIN AND NOVOBIOCIN.

Novobiocin and Courmermycin are structurally related antibiotics containing carbohydrate and coumerin moieties respectively. Both drugs inhibit bacteria, with Gram positive cells being more susceptible than the Gram negatives. They primarily inhibit DNA synthesis, though RNA is also inhibited to a lesser extent.

It has been suggested that these drugs inhibit the DNA gyrase. In this study, the action of these drugs against Chlamydia has been investigated with this in mind.

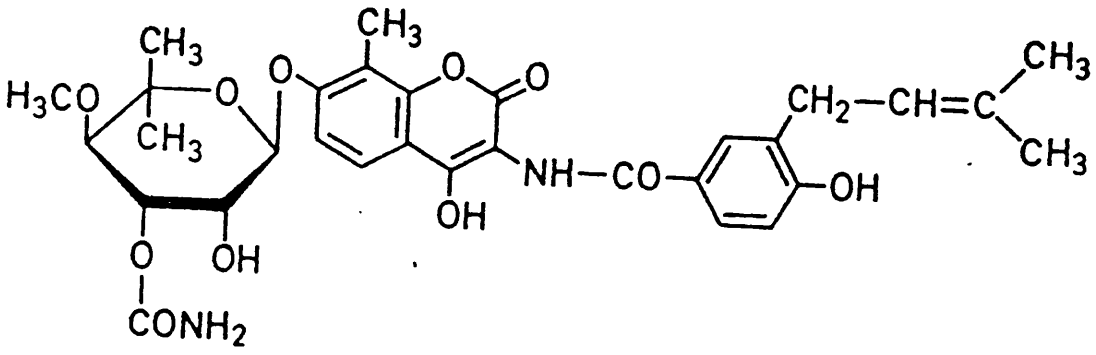

fig 1.10. Structure of novobiocin.

1.5.9. THIAMPHENICOL.

Substitution of the nitro group of chloramphenicol by a sulfomethyl group resulted in the production of thiamphenicol. This compound shows several differences from chloramphenicol, namely enhancement of bactericidal action against species of Haemophilus and Neisseria (Garrod et al 1981). This fact, as well as evidence of reduced risk from irreversible bone-marrow toxicity makes thiamphenicol a better candidate for study against genital tract pathogens.

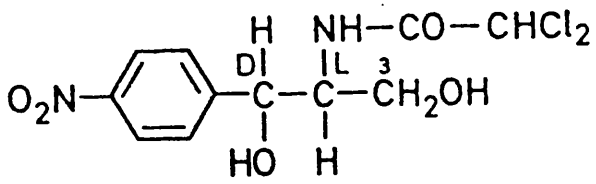

fig 1.11. Structure of chloramphenicol.

1.5.10. RIFAMYCINS.

Rifamycins act by inhibiting RNA synthesis and are active against Gram positive bacteria as well as mycobacteria. Rifamycin was rescovered from Streptomyces mediterranei in 1957.

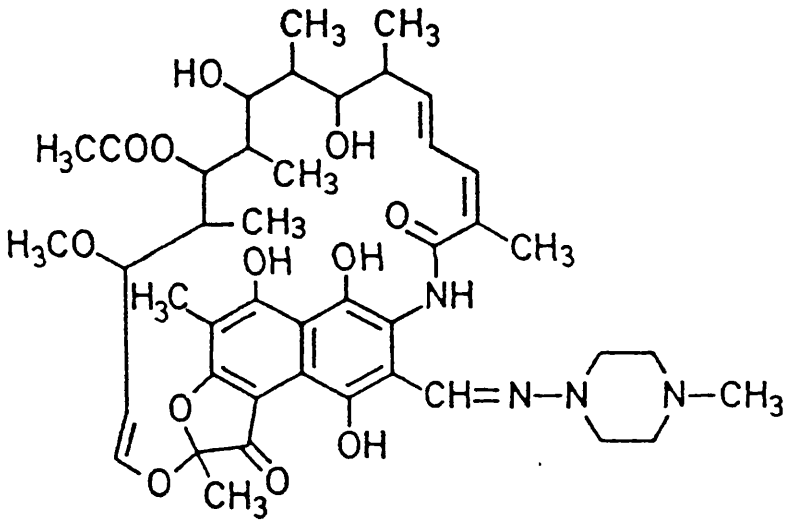

fig 1.12. Structure of rifampicin

The action of rifampicin against C.trachomatis has been studied by Becker and Zachay-Rones (1969). These workers reported that the trachoma agent is sensitive to rifampicin early in its growth cycle both in-vitro and in embryonated eggs in-vivo. Menke et al (1979) showed that rifampicin is clinically active in the therapy of LGV. Keshishyan et al (1973) noted the development of resistance to rifampicin by other bacteria and, using eggs, demonstrated the same phenomenon for C.trachomatis. Ridgway et al (1980) induced resistance in this organism in-vitro with relative ease. These workers, using rifampicin, rifapentin and rifamide, found that the final inhibitory concentrations of resistant SA2f which had emerged after treatment with these drugs were: >200 µg/ml, 0.5 µg/ml and >200 µg/ml respectively. The resistance of the organism beyond the 200µg/ml mark was not ascertained, because some toxicity was exhibited by the cells. As rifampicin has been used in the topical treatment of trachoma with no apparent adverse effect (Darougar et al 1977), a study was devised as part of this work to establish the possible toxic effect of these drugs on the McCoy cells.

1.5.11. FOLIC ACID PATHWAY INHIBITORS (Cotrimoxazole and Fansidar).

Trimethoprim is an inhibitor of folic acid synthesis inhibiting the dihydrofolate reductase. Sulphonamide is a competitive inhibitor of para amino benzoic acid (PABA) and inhibits the growth of C.trachomatis, indicating that it synthesises its own folic acid. In combination, the two drugs (Cotrimoxazole) have strong potentiating action which results as a consequence of sequential blockade of biochemical pathway that leads to the synthesis of coenzyme f. and, therefore, the combination is active against a number of common pathogenic bacteria.

Several workers have reported the results of the combination of sulphamethoxazole/trimethoprim in fixed ratios against C.trachomatis (Johannison et al 1979 and Hammerschlag 1982) and demonstrated an additive response only. At no time were combinations used where the concentration of trimethoprim exceeded that of sulphamethoxazole. How et al (1985) used a chequerboard titration, allowing the sulphamethoxazole/trimethoprim ratios to be reversed. After a single cycle of growth, they concluded that the combination was synergistic with the trimethoprim/sulphamethoxazole ratios of 64:1 or 4:1. However, folic acid pathway inhibitors are essentially bacteristatic against Chlamydia and in order to reach a stable end point there is a necessity for multiple passages. A part of the work undertaken in this thesis was to confirm the findings of How et al, using a chequerboard titration of the two drugs and examining the results after one cycle of growth and, again, following multiple passages over four weeks (passage 10).

Pyrimethamine is an antimalarial drug with a structure resembling that of trimethoprim. It acts on the malaria parasite by inhibiting the utilization of folic acid. Sulfadoxine inhibits the synthesis of folic acid. Reeves and Tavern (1968) demonstrated the presence of dihydrofolate reductase in trachoma by testing the activity of trimethoprim and pyrimethamine separately and in conjunction with sulphonamide. A part of the work presented in this thesis is the study of the effects of combinations of pyrimethamine and sulfadoxine (Fansidar) in a chequerboard titration after one passage and after ten passages.

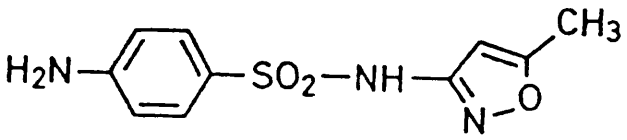

fig. 1.13. Structure of sulphamethoxazole.

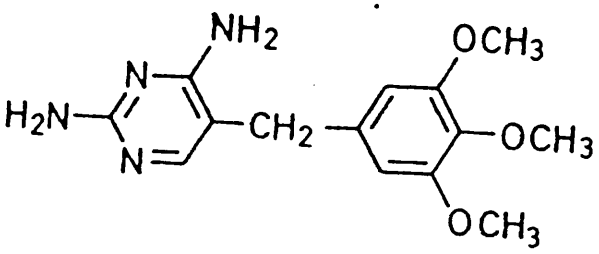

fig. 1.14. Structure of trimethoprim.

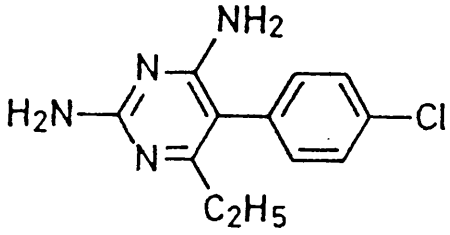

fig. 1.15. Structure of pyrimethamine.

CHAPTER 2

OBJECTIVES OF THE PRESENT INVESTIGATIONS

2.1. OBSERVATIONS ON THE LIFE CYCLE OF C.TRACHOMATIS.

It is proposed to follow the developmental cycle of SA2f in McCoy cells grown on coverslips. The developmental cycle will be aborted at different intervals post infection and examined by:

2.1.1. THE USE OF DIFFERENT STAINING TECHNIQUES SUITABLE FOR BRIGHT FIELD (BFM), DARK GROUND (DGM) AND FLUORESCENT MICROSCOPY.

It is proposed that Giemsa, iodine, fluorescent and acridine orange stains are used to study the morphology of the inclusions.

2.1.2. THE USE OF ELECTRON MICROSCOPY (EM).

It is proposed that thin sections of McCoy cells infected with SA2f are examined at intervals during it s life cycle so as to observe the morphological changes of the particles inside the inclusions.

2.1.3. THE USE OF BFM AND EM FOR THE OBSERVARION ON THE EFFECT OF PENICILLIN AT DIFFERENT STAGES OF THE DEVELOPMENTAL CYCLE.

It is proposed that penicillin is introduced at different intervals during the life cycle of SA2f growing in McCoy cells. The inclusions in McCoy cells grown on coverslips are examined by bright field microscopy and thin section preparations by electron microscopy using the staining methods mentioned in Chapter 3. Penicillin was chosen because of its therapeutic use in the treatment of gonoccocal infections, its apparent lack of activity against C.trachomatis in-vivo and for its peculiar effect in inhibiting C.trachomatis growth when present in cell culture.

2.1.4. THE USE OF TIME LAPSE PHOTOGRAPHY.

Nomarski's system of differential interference contrast (DIC) is used to observe the continuous appearance, enlargement and subsequent disruption of SA2f inclusions without interrupting the life cycle.

2.2. INVESTIGATION INTO THE DEVELOPMENT OF A NOVEL CELL CULTURE TECHNIQUE TO OBTAIN LARGE AMOUNTS OF CHLAMYDIAL ANTIGEN.

The isolation of SA2f and two clinical isolates in McCoy cells grown in a microcarrier system (cytodex B2) has been undertaken. The results obtained are discussed and the advantages of this technique for the production of bulk antigen are considered.

2.3. INVESTIGATION OF THE SENSITIVITY OF SA2f TO ANTIMICROBIAL AGENTS.

2.3.1. MINIMUM INHIBITORY CONCENTRATION (MIC).

It is proposed that the sensitivities, ie. minimum inhibitory concentrations (MICs) of antimicrobial drugs for SA2f are investigated using IUDR treated McCoy cells grown on coverslips. These compounds include newer drugs as well as those which have been available previous to this study.

2.3.2. MINIMUM BACTERICIDAL CONCENTRATION (MBC).

Further, it is proposed that if, and when, an antimicrobial is shown to have reasonable activity against SA2f, the minimum bactericidal concentration (MBC) of the drug is also measured using multiple cell culture passages.

2.3.3. STUDY OF CLINICAL ISOLATES.

For some of these drugs, the sensitivity of several recent clinical isolates is measured.

2.3.4. RESISTANCE STUDY.

It is further proposed that attempts are made to induce in-vitro resistance of SA2f to roxithromycin. Roxythromycin was chosen for this study because of its possible advantage in clinical use over other macrolides including erythromycin.

2.3.5. TOXICITY STUDY.

Possible toxicities of rifapentin and rifampicin for McCoy cells are investigated. This study is undertaken to confirm or refute the belief that high concentrations of rifapentin or rifampicin in tissue culture cannot be investigated because of their possible toxic effect on the cells. The results of this study are discussed.

2.3.6. SYNERGY AND ANTAGONISM STUDY.

The investigation of synergistic or antagonistic activity of combinations of trimethoprim/sulphamethoxazole and pyrimethamine/sulfadoxine against SA2f was undertaken. The purpose of this study was to establish the synergy or antagonism of the combination of these drugs in vitro.

CHAPTER 3

MATERIALS AND METHODS

3.1. OBSERVATIONS ON THE LIFE CYCLE OF CHLAMYDIA.

3.1.1. THE CONTROL ORGANISM (SA2f).

The control organism used in the studies in this thesis was "SA2f", a laboratory adapted strain of C.trachomatis. This organism is an LGV2 serotype of C.trachomatis which is widely cross reactive with the other serotypes.

3.1.2. PREPARATION OF McCOY CELL CULTURES.

McCoy cells were grown in McCoy growth media (MGM) containing Eagles minimum essential medium, supplemented with foetal calf serum (10% V/V), glutamine (1% stock solution containing 30 mg/l), vitamins (1% V/V 100 X concentrated) and 1.4% of bicarbonate (7.5% stock solution) in tissue culture bottles (200 ml, Flow Laboratories). When confluent, the cells were treated with trypsin/versene. The cells were then diluted in MGM supplemented with 5-iodo-2 deoxyuridine (IUDR) 25 mg/l to obtain 10^5 cells/ml. One ml of this suspension was added to a flat bottomed 5 mls plastic vial containing a 12 mm glass coverslip. The tubes were incubated at 36°C for at least 3 days to obtain a confluent growth of cells on the coverslips and then infected with SA2f on the 3rd to 7th day after seeding.

Small tissue culture bottles (50 mls, Sterilin) were seeded at $1X10^6$ cells/bottle in the presence of MGM supplemented with IUDR (25 mg/l), incubated at 35°C for 3 days to obtain a monolayer and infected with SA2f on the 3rd to 7th day after seeding.

3.1.3. INOCULATION OF CELLS WITH SA2f.

i) INOCULATION OF TUBES.

Stock suspension of SA2f was diluted in Chlamydia Transport Media (CTM), or McCoy growth medium supplemented with 1% of 3 Molar glucose, to obtain 1,000 inclusion forming units/ml (IFU/ml). This was used to infect IUDR treated McCoy cells (1 ml/tube). The inoculated tubes were centrifuged at 3,000g and incubated at 36°C. The growth cycle was interrupted at 16, 24, 30, and 42 hrs post inoculation when the cells were fixed and stained with iodine, Giemsa, immunofluorescent or acridine orange stain.

ii) INOCULATION OF BOTTLES.

Stock suspension of SA2f was diluted in CTM to obtain 3,000 IFU/ml and used to infect small bottles containing monolayers of IUDR treated McCoy cells. The bottles were incubated at 36°C and the growth cycle was stopped at 16, 24, 30, and 42 hrs post inoculation when the cells were fixed and prepared for EM.

iii) ADDITION OF PENICILLIN TO INOCULATED TUBES.

Monolayers of IUDR treated McCoy cells were infected as in section i). Penicillin at a final concentration of 0.125 µg/ml was added to each tube at inoculation times 0 hr (PT0), 16 hrs post infection (PT16), 24 hrs post infection (PT24), 30 hrs post infection (PT30) and 40 hrs post infection (PT40). After incubation at 36°C, the cells were fixed and stained with iodine, Giemsa, immunofluorescent and acridine orange stain 42 hrs post infection.

iv) ADDITION OF PENICILLIN TO INOCULATED BOTTLES.

Small bottles of IUDR treated McCoy cells were infected with SA2f, 3,000 IFU/ml. Penicillin at a final concentration of 0.125 µg/ml was added to each bottle at PT0, PT16, PT24, PT30 and PT40. After incubation at 36°C, the cells were fixed and prepared for electron microscopy.

v) INOCULATION OF THE CHAMBER.

McCoy cells ($5x10^5$/ml) were grown on a glass coverslip loaded over a perfusion chamber (fig 3.1.). The chamber was air sealed by candle wax and incubated at 36°C for 3 days to obtain confluent growth of the cells. Using a surgical needle, the overlaying fluid was then carefully drawn away from the cell surface without disturbing the coverslip. An inoculum of SA2f containing 1,000 IFU/ml was prepared in CTM containing 2 µg/ml of cycloheximide. The inoculum was injected into the chamber making sure that it had covered the cell monolayer. Excess fluid was carefully withdrawn, the chamber was resealed with wax and incubated at 36°C before being positioned and focused under a Nomarski phase contrast microscope equipped with a cine-camera (x40 objective).

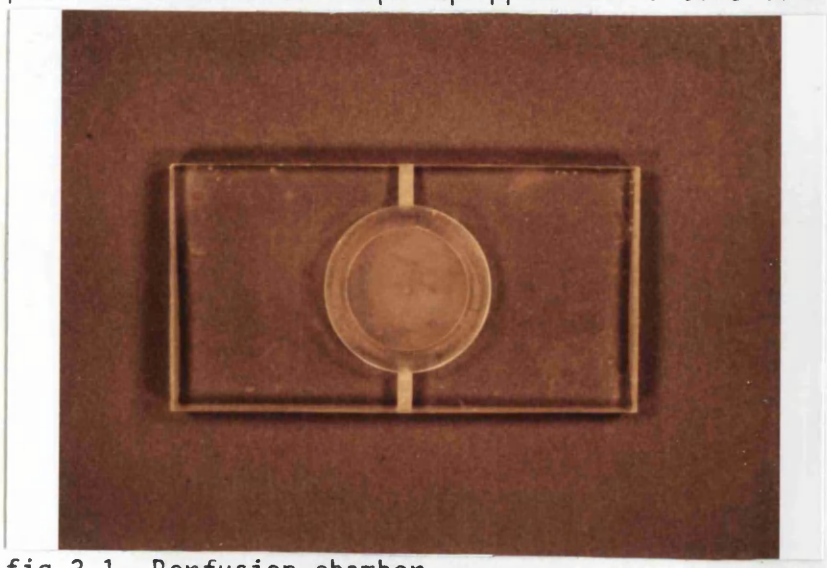

fig 3.1. Perfusion chamber.

The timer on the camera was set to take one photograph every 3 minutes (20 frames/hr).

Nomarski's system of differential interference contrast (DIC) microscopy consists of a uniaxial birefringent crystal. The optic axis of one of these prisms is inclined at a certain angle to the upper bounding face. An advantage of using this system is that the resultant image, which is an optical expression of the microscopic object, shows much greater detail in three dimensions without the problems of the "halo effect" associated with conventional phase contrast microscopy.

3.1.4. STAIN AND STAINING METHODS.

i) IODINE STAIN.

a) STAIN. The stain used was Lugol's iodine made up to 10% with distilled water.

b) STAINING METHOD. The cells were fixed in methanol by replacing the supernatant medium with 1ml of methanol. After 10 minutes, the methanol was replaced with iodine stain and left for 5 minutes. Iodine stain was then removed and the coverslips knocked out on a piece of blotting paper, blotted on both sides, air dried, mounted on glass slides in DEPEX mounting fluid and examined by BFM X 40 objective. The normal inclusions stained by iodine are brown, intracytoplasmic and reticulate in structure.

ii) GIEMSA STAIN.

a) STAIN. Giemsa stain (Raymond A. Lamb) was diluted 1:10 in freshly made Sorensen's buffer just before use.

b) BUFFER. Sorensen's buffer (Mercia-Brocades Ltd) was diluted 1:200 in distilled water just before use.

c) STAINING METHOD. The cells were fixed in methanol as above for 30 minutes. The methanol was replaced with diluted Giemsa stain and left for a further 30 minutes. The stain was discarded and the cells washed 3 times in diluted buffer. The coverslips were knocked out of the vials, blotted and air dried as above, mounted on 0.8mm thick glass slides with DEPEX and examined by DGM and BFM using X 40 objective. The normal inclusions stained by Giemsa have a green or golden "autofluorescing", perinuclear reticulate appearance by DGM and a mauve cytoplasmic vesicle with a definite boundary filled with chlamydial bodies when viewed by BFM

iii) IMMUNOFLUORESCENT STAIN.

a) CONJUGATE. Anti-human IgG conjugated with fluorescein label (Wellcome laboratories) was used. It was titrated and diluted in phosphate buffered saline (Dulbecco's formula, with no magnesium and calcium).

b) SERUM ANTIBODY. A pool of sera from patients with high titres of chlamydial antibody, titrated against the conjugate, was used.

c) STAINING METHOD. Cells were fixed in methanol for 10 minutes. The methanol was replaced with a suitable dilution of positive serum and incubated at 37°C for 1 hr. The cells were washed 3 times in phosphate buffered saline (PBS) and stained with conjugate diluted in PBS laced with Evans blue (1:10,000 Evans blue in PBS). The cells were washed in PBS 3 times, gently knocked out of the vials and mounted in fluorescence mounting fluid on a 0.8 mm thick glass slide and examined by incident light (X 40 objective). Normal inclusions are apple green and reticulate in structure.

iv) ACRIDINE ORANGE STAIN.

a) STAIN. The stain used was a 0.1% stock solution of Acridine orange (0.1 g in 100 mls of distilled water) diluted to 0.01% in McIlvaine buffer just before use.

b) BUFFER. McIlvaine phosphate buffer (pH 3.8)

Stock Solution A 0.2 M Na_2HPO_4

Stock Solution B 0.1 M Citric acid

64.5 mls of solution B + 35.5 mls of solution A made up freshly before use.

c) STAINING METHOD. Cells were fixed in acetic acid (1 part glacial acetic acid + 2 parts absolute alcohol) for 10 minutes, washed once in the buffer, stained in 0.01% acridine orange solution for 4 minutes followed by 2 washes in McIlvaine buffer and mounted in buffer and examined by fluorescence microscopy. The normal inclusions are seen as vesicles filled with orange (RNA), green (DNA) or a mixture of both colour stained bodies.

3.1.5. PREPARATION OF INFECTED McCOY CELLS FOR ELECTRON MICROSCOPY (EM).

The SA2f strain of C.trachomatis was grown in IUDR treated McCoy cells in small bottles in the presence or absence of antibiotics. After incubation, cultures were fixed and stained for EM as follows:

i) FIXING.

a) BUFFER (Phosphate Buffer for Electron Microscopy). The following were mixed to obtain Phosphate buffer pH 7.3:

0.2 M NaHPO4	450 mls
0.2 M K2HPO4	150 mls
Distilled water	381 mls

b) FIXATIVES.

Gluteraldehyde: 4% gluteraldehyde prepared in Phosphate buffer.

Osmium tetroxide: 1% osmium tetroxide prepared in phosphate buffer.

c) FIXING METHOD. Infected cells were scraped off the bottles using a rubber tipped glass rod and transferred to a sterile tube and centrifuged at 1000 rpm for 5 minutes. The resultant pellet was washed in cold phosphate buffer, mixed with an equal volume of 4% gluteraldehyde and left for 2-3 hrs at room temperature (RT). The cells were then washed in cold phosphate buffer and left at 4°C for up to 2 weeks before use. When convenient, the cells were fixed in 1% OsO_4 (Osmium tetroxide) for 1 hr 30 mins at 4°C and washed twice for 5 minutes in cold buffer.

ii) DEHYDRATION. The cells were taken through a process of dehydration in ascending concentrations of alcohol as follows:

Alcohol%	Minutes
50	5
70	5
80	2x5
90	2x5
95	2x5
100	3x20

At the end of this process the cells were transferred into glass tubes.

iii) CLEARING AND EMBEDDING PROCESS. The cells were washed twice for 10 minutes each time in propylene oxide (1,2 Epoxy propylene), then in a 50/50 solution of Araldite and propylene oxide for 1 hr, and centrifuged at 2000 rpm for 10 minutes. The supernatant fluid was removed and replaced with a solution of 100% Araldite. After 2 hrs at RT, the cells were transferred into a Beem capsule and centrifuged for 10 minutes at 2000 rpm. The supernatant fluid was removed and replaced with fresh 100% Araldite. A strip of paper with the particulars of each preparation was inserted into the capsules and the resin cured at 60°C in a hot oven for 48hrs.

The blocks were removed from the capsules, trimmed, and thin sections (0.5μm in thickness) and ultra thin sections (90nm in thickness) were cut onto water with glass knives using a Reichert OMU2 ultramicrotome.

iv) STAINING THE SECTIONS.

a) BRIGHT FIELD MICROSCOPY (BFM). Toluidine blue stain at a concentration of 0.25% of toludine blue (stock solution made in 1% Borax) was used. 0.5µm thick sections recovered from the water bath were placed on clean microscope glass slides and heated on a hot plate for 30 minutes. The slides were then immersed in 15% H_2O_2 for 2 minutes, rinsed with distilled water, dried on the hot plate and sections stained with 0.25% boiling toluidine blue for 10 seconds. They were then rinsed in distilled water, differentiated in acetone and mounted in Histomount.

b) ELECTRON MICROSCOPY (EM). 90nm thick sections were cut in water and transferred to a 300 mesh copper grid. The grids were blotted on a clean piece of filter paper and the sections were then stained by:

Uranyl acetate - Enough uranyl acetate powder was added to 50% ethanol to achieve saturation. The solution was then centrifuged until clear, and the supernatant fluid used for staining. Saturated solution of Uranyl acetate in 50% ethanol was made up fresh and a drop put on a piece of dental wax. The grid was dropped on the stain for 3 minutes with the section facing down. The stain was then washed off, first in 50% ethanol for 10 seconds and then in distilled water for 10 seconds. The surplus water was blotted off and the grid left to dry in air.

Lead citrate - 1.33 g of lead nitrate and 1.76 g of sodium citrate were added to 30 mls of distilled water and shaken. 8 mls of normal NaOH was added to this mixture and the volume was then made up to 50 mls with distilled water. This solution was kept in an air-tight flask, stored at 4°C and an aliquot was centrifuged

before use.

A drop of the clear supernatant fluid was placed on a clean piece of dental wax in a Petri dish containing a few tablets of NaOH. The grid (already stained with uranyl acetate) was dropped face down onto the lead citrate solution for 3 minutes, washed in dilute NaOH for 10 seconds and then in distilled water for another 10 seconds. The surplus water was blotted off and the grid left to dry in air. All the grids were kept at RT until examined in a JOEL JEM 1200 transmission electron microscope.

3.2. DEVELOPMENT OF A NOVEL CELL CULTURE TECHNIQUE.

3.2.1. PREPARATION OF McCOY CELL CULTURE.

A monolayer of McCoy cells was trypsinised and a 100 mls suspension of $2.7X10^4$ cells/ml made in McCoy growth media (MGM).

36 mls of rehydrated stock solution (2.5 g/100 mls PBS) of Cytodex 2 microcarriers was washed and concentrated in warm (37°C) MGM and added to the cell suspension. This mixture of cells and cytodex beads was added to a one litre sterile pre-siliconized Techne bottle (fig 3.2.). The bottle was sealed and placed on a magnetic stirring machine in a 37°C incubator. The contents were stirred at 30 minute intervals for 2 minutes at 30 rpm over a 3 hr period. A further 200 mls of warm MGM was added to the cells and stirred continuously at 30 rpm for 3-5 days. A sample of cytodex beads was removed and examined microscopically to ensure the successful growth of the cells on the beads.

3.2.2. INOCULATION OF THE CELLS WITH C.TRACHOMATIS.

The Techne bottle was allowed to stand at RT and the supernatant fluid removed aseptically, using a sterile plastic pipette. A 300 mls inoculum of C.trachomatis, containing 600-1,000 IFU/ml and 1 µg/ml cycloheximide in CTM, was added to the beads. The bottle was sealed and incubated at 37°C with no stirring for 30 minutes and then with continuous stirring at 30 rpm for 2 days. A sample of the original inoculum was taken and cultured [see controls (a) and (b) p. 94].

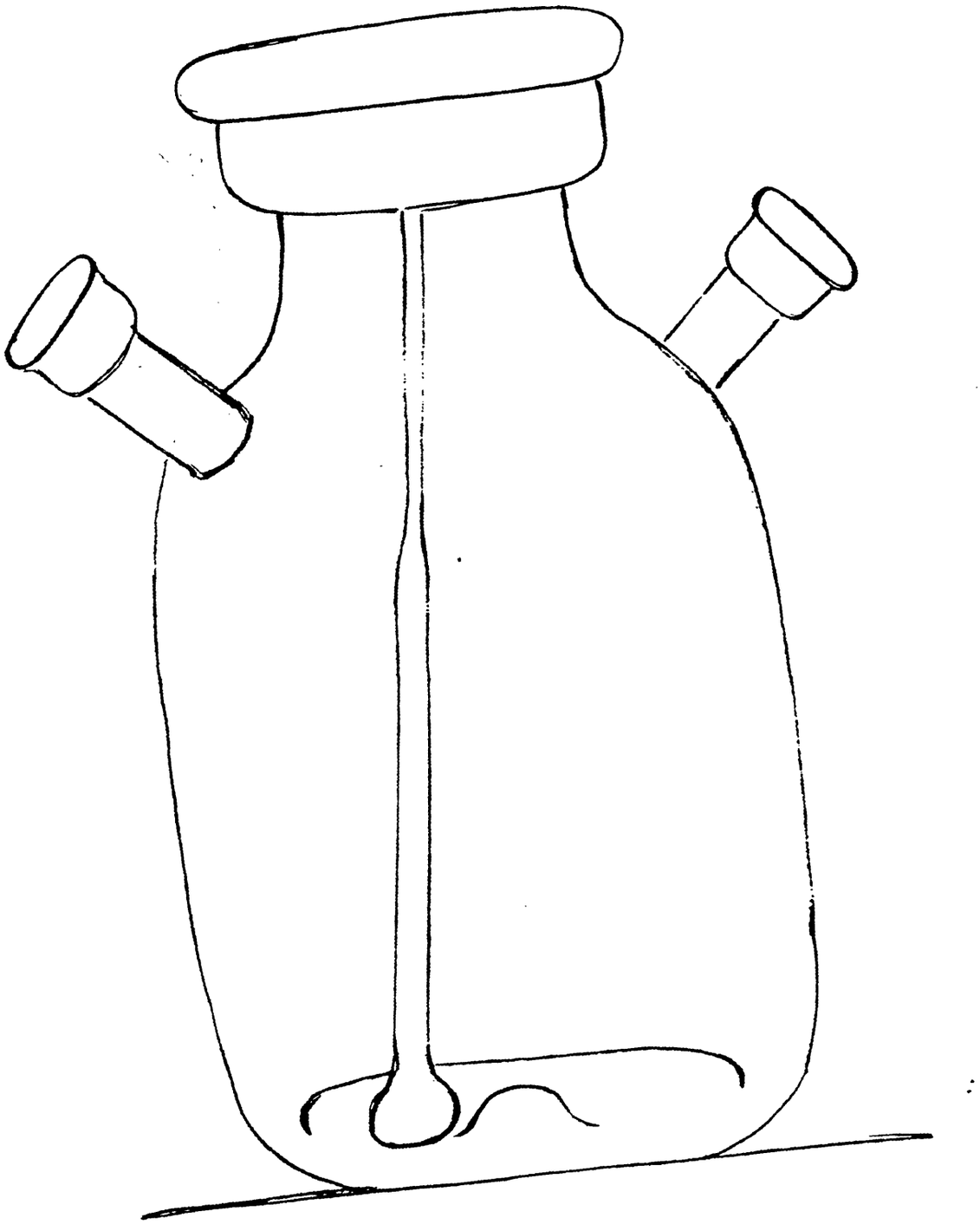

fig 3.2. Techne bottle.

3.2.3. HARVEST.

The Techne bottle was allowed to stand at RT until the beads had settled. The supernatant fluid was removed aseptically and stored at 4°C until the end of the harvest. The beads were centrifuged in sterile plastic tubes at 1,000 rpm, for 5 minutes at 10°C and the supernatant fluid harvested and kept as above. The beads were then washed in warm PBS by centrifugation at 500 rpm for 10 minutes. A 5 mls solution of 0.25% trypsin/versene (TV) was added. The mixture was incubated at 37°C for 5 minutes with occasional agitation. The action of TV was neutralised by the addition of 20 mls of fresh CTM. The bottle was shaken, the beads allowed to settle and the supernatant fluid harvested. All the harvested fluids and cellular materials were pooled together, and a sample removed [harvests (c) and (d) p.159]. The remaining harvested materials were stored at -70°C.

3.2.4. CONTROLS.

(a) An aliquot of the original inoculum was placed in an empty sterile bijou bottle and kept alongside the Techne bottle for the entire incubation period. This sample was then inoculated into a sterile vial containing a coverslip monolayer of cycloheximide treated McCoy cells. The vial was centrifuged at 3,000 g at 36°C for 1 hr and incubated at 36°C for 2 days. This control was set up to demonstrate the failure of Chlamydia to survive outside the cell system.

(b) 1 ml of the original inoculum was immediately inoculated onto a cycloheximide treated McCoy cell monolayer and processed as above. This control was set up to show the number of live organisms ordinarily available to the system.

(c) Samples of harvested material were inoculated onto 2 monolayers of McCoy cells as above, except that one set of these tubes was incubated without prior centrifugation. This control was set up to show the increase in yield obtained by the experiment compared with control (b). All inoculated monolayers were fixed with methanol and stained with 10% iodine. These were then examined microscopically and an inclusion count performed.

The organisms: Three isolates of C.trachomatis were grown in the microcarrier system. These were SA2f and two clinical isolates (isolates number 25088 and 81591) which were grown in cell culture and passaged 5-6 times to obtain high titres.

3.3. SENSITIVITY OF C.TRACHOMATIS TO ANTIMICROBIAL AGENTS.

3.3.1. DETERMINATION OF MIC.

The technique deployed for the determination of MIC has been described by Ridgway et al (1976). In brief, doubling dilutions of the antimicrobial agents in antibiotic free medium are prepared. One ml of each dilution is added to duplicate flat bottomed plastic tubes containing coverslip monolayers of IUDR treated McCoy cells. A suspension of Chlamydia containing approximately 300 IFU/ml is added to each tube. After centrifugation at 3,000 g for one hr, the tubes are incubated for 38-40 hrs at 36°C. The cell monolayers are fixed with methanol, and stained with iodine or Giemsa. Both staining methods are used in case some of the antimicrobial agents selectively affect the staining properties of C.trachomatis. The MIC is taken to be the lowest concentration of antimicrobial which completely inhibits inclusion formation with either staining method. The studies presented here were conducted on at least two different occasions.

3.3.2. DETERMINATION OF MBC.

This involved making double dilutions of the antimicrobial agents and inoculation with SA2f as above, except that four sets of tubes were used for each dilution. After centrifugation at 3,000 g for 1 hr at 35°C, the tubes were incubated at 36°C for 38-40 hrs. Two tubes from each dilution were fixed and stained with iodine and Giemsa and examined microscopically. The MIC of the drug was noted. The contents of the third and fourth tubes from the corresponding negative Giemsa or iodine stained monolayer were passaged onto two fresh IUDR treated McCoy cell monolayers. The tubes were centrifuged as above, the

supernatant fluid replaced with fresh CTM and incubated as above. One set of these tubes was fixed with iodine and the results noted. The duplicates of all the negative iodine tubes were passaged into two tubes and processed as above. This process was repeated for up to 10 passages over a period of 3½ weeks. The MBC was taken as being the concentration which completely inhibited the growth of SA2f after 10 passages. These studies were done on at least two different occasions.

3.3.3. MIC DETERMINATION WITH RECENT CLINICAL ISOLATES.

This involved the isolation of new isolates of C.trachomatis from patients attending the Department of Genito Urinary Medicine at University College Hospital. The isolates were passaged 5 or 6 times to obtain high titres of the organisms and titrated in coverslip monolayers. The MIC of each drug against individual isolates was determined as described above for SA2f.

3.3.4. PREPARATION OF ANTIMICROBIAL DRUGS.

i) TETRACYCLINE.

Stock solutions of oxytetracycline and doxycycline at a concentration of 10,000 µg/ml were prepared in distilled water. These were kept at -20°C for further use. The stock solutions were then further diluted in distilled water to give 128 µg/ml. All subsequent dilutions were made in CTM.

Minocycline was initially diluted in 0.02 M HCL to 1 mg/ml and further diluted to 128 µg/ml in distilled water. All subsequent dilutions were made in CTM. This antibiotic was made up fresh from powder each time before use.

ii) MACROLIDES.

Compound ICI-187642, josamycin and ER-42859 were dissolved in 10% DMSO to initially obtain a concentration of 1,000 µg/ml and further diluted in distilled water to 128 µg/ml. All subsequent dilutions were made in CTM.

Roxithromycin was disssolved in 50:50 methanol and water to 32 µg/ml and then further diluted in CTM.

Miocamycin, erythromycin and spiromycin were dissolved in pure methanol to 1000 µg/ml. Erythromycin and spiromycin were further diluted in water and CTM as above. Miocamycin was diluted in pure methanol to obtain 400 µg/mL. This solution was then diluted in water to 40 µg/ml and subsequent dilutions were made in CTM.

Compound CP-62993 (azithromycin) was initially diluted in methanol to 1 µg/ml and further diluted in 50:50 methanol and water to 128 µg/ml. Further dilutions were made in CTM.

iii) QUINOLONES.

Quinolones (except for Abbott 56619, Abbott 56620, C1934 and NY198) were made up from powder in alkaline distilled water to 1,000 µg/ml and then further diluted in water to 128 µg/ml. All subsequent dilutions were made in CTM.

Abbott 56619 (difloxacin) and Abbott 56620 were made up in hot water to 1,000 µg/ml and then further diluted in water and CTM as above.

Compound CI934 was dissolved in water to 1,000 µg/ml and then

further diluted in water and CTM as above. This compound is lightsensitive and was therefore protected from direct light throughout the experiment. Compond NY198 (lomefloxacin) was diluted in water to 128 µg/ml. All subsequent dilutions were made in CTM.

iv) BETA-LACTAM ANTIBIOTICS.

Amoxycillin, clavulanic acid and augmentin (amoxycillin/clavulanic acid) were dissolved in sterile distilled water to give stock concentrations of 1,000 µg/ml. Further dilutions to 128 µg/ml were also made in water. All subsequent dilutions of these agents were made in CTM.

CEPHALOSPORINS: Cefotetan, RO 158074 and cephtriaxone were dissolved in water to obtain concentrations of 1,000 µg/ml. All subsequent dilutions were made in CTM.

v) AMINOCYCLITOLS.

Trospectomycin and spectinomycin were dissolved in water to obtain stock solutions of 1,000 µg/ml. All subsequent dilutions were made in CTM.

vi) PSEUDOMONIC ACID.

The stock solutions of the compounds BRL 35390 and BRL 4910A were prepared from powder in 10% DMSO to obtain concentrations of 1,000 µg/ml. All subsequent dilutions were made in CTM. All dilutions were made in an exhaust protective cabinet with tubes wrapped in foil to protect them from direct light.

vii) IMIDAZOLES.

Six imidazoles tested in this work were dissolved in appropriate

solvents as follows:

Imidazoles SC 38344, SC 38390 and SC 37211 were dissolved completely in 10% DMSO to obtain stock solutions of 1,000 µg/ml. The agents were further diluted in water to 128 µg/ml and all subsequent dilutions made in CTM.

Imidazoles SC 37154, SC 38833 and SC 38911 were found to be poorly soluble in DMSO and stock solutions of 1,000 µg/ml were made in methanol. These were further diluted in water to 128 µg/ml and subsequent dilutions were made in CTM.

viii) NOVOBIOCIN.

Novobiocin was dissolved in 10% DMSO to obtain a stock solution of 1,000 µg/ml. This solution was diluted in water to 128 µg/ml and all subsequent dilutions were made in CTM.

A solution of coumermycin was made as follows:
8 mg of the drug was added to 1 ml of DMSO so as to obtain a clear solution. 9 mls of water was then added to give a stock solution of 800 µg/ml. This solution was further diluted down with water to 128 µg/ml and all subsequent dilutions were made in CTM.

ix) THIAMPHENICOL.

Thiamphenicol was initially dissolved in a small amount of methanol to obtain a clear fluid and then further diluted in water to 1000 µg/ml and later to 128 µg/ml. All subsequent dilutions were made in CTM.

x) RIFAMYCINS.

Rifampicin and rifapentin (DL473) were initially dissolved in a small amount of DMSO and further diluted in water to obtain concentrations of 1,000 µg/ml and 128 µg/ml . All subsequent dilutions were made in CTM.

xi) FOLIC ACID PATHWAYS.

Solutions of sulphamethoxazole and sulfadoxine were made in alkaline distilled water to obtain a concentration of 1,000 µg/ml. Pyrimethamine and trimethoprim were dissolved in a small volume of DMSO and diluted to 1,000 µg/ml in distilled water. All subsequent dilutions were made in CTM.

3.3.5. INDUCTION OF IN-VITRO RESISTANCE OF SA2f TO ROXITHROMYCIN.

A variation of the technique described by Jones et al (1983) was used. In brief this involved the inoculation of two flat sided culture bottles containing large coverslip monolayers of IUDR treated McCoy cells with 3.5 mls of 3,0000 IFU/ml suspension of SA2f. 3.5 mls of CTM containing the antibiotic at its MIC was added to each bottle to obtain a concentration equal to half the MIC. After incubation at 36°C for 48 hrs, the coverslip from one bottle was fixed and stained with iodine for examination of the inclusion formation. The contents of the other bottle were passaged into 4 bottles containing fresh McCoy cell monolayers. Two of these bottles contained the initial concentration of the antimicrobial, whilst the remaining two contained twice the initial concentration. After further incubation as above, one bottle from each pair was examined. If it was found that the bottle containing the higher concentration contained inclusions, then the contents of the second bottle of this pair were passaged into another two bottles containing the same antimicrobial concentration as in the previous bottles, and also into two bottles containing twice this concentration. If only the bottle containing the lower concentration contained inclusions, then the second bottle of that pair was passaged into 2 bottles containing the same concentration of the antimicrobial as in the parallel bottle and also into 2 bottles containing half the concentration. This process was repeated until no further increase in resistance could be achieved.

3.3.5. STUDY OF THE POSSIBLE TOXICITY OF RIFAPENTIN (DL473) AND RIFAMPICIN FOR McCOY CELLS.

Both rifampicin and rifapentin were initially dissolved in a small volume of DMSO and then in distilled water to obtain a concentration of 10,000 µg/ml. This was done with the rigorous use of a vortex mixer to avoid precipitation and gelatination of these drugs. Further dilutions down to 2,000 µg/ml were made in water and thereafter in CTM to cover the range 500-8 µg/ml. This study was performed in 3 parts.

In part 1, the effect of long term exposure (44 hrs) of different concentrations of the two drugs (500-8 mg/l) on free growing uninhibited McCoy cells was studied. Observations using direct bright field microscopy of Giemsa and iodine stained monolayers were made. Total cell counts demonstrating the percentage of dead and live cells were performed.

Four sets of tubes, containing monolayers of uninhibited McCoy cells ($2.5X10^5$ cells/ml dispensed in sterile vials containing a 12 mm coverslip and incubated overnight at 36°C), were incubated for 44 hrs with 1 ml of different dilutions of each antibiotic covering a range of 500-8µg/ml. Another set, without any antibiotic was also set up to serve as control. At the end of part 1 of the study all the coverslips of McCoy cells were examined by:

(a) Direct light microscopy to observe any change in the appearance of the intact cells prior to fixation and staining.

(b) One set of tubes containing the antibiotics was stained with Giemsa whilst another was stained with trypan blue.

(c) The two remaining sets of antibiotic treated cells were trypsinised and using trypan blue, a total count of (dead and live) cells was performed and an average taken.

In part 2, the effect of short term exposure of McCoy cells (both IUDR inhibited and uninhibited free growing cells) to the antimicrobial agents was studied.

Five sets of uninhibited and 5 sets of IUDR inhibited McCoy cell monolayers, prepared as described previously, were pre-treated with high concentrations (200-50 µg/ml) of the antimicrobials for 3 hrs. The cells were washed with antibiotic free medium prior to incubation for 2 days. The possible toxic effect on the cells by this short exposure of drugs was studied by performing a vital stain cell count on two sets of trypsinised cells by using trypan blue stain, and on one set each by using iodine, Giemsa and trypan blue stains.

In part 3, the effect of short term exposure of McCoy cells (both IUDR inhibited and uninhibited free growing cells) to the antimicrobial agents on the eventual growth and morphology of SA2f was studied.

Two sets of untreated monolayers of McCoy cells as well as 2 sets of IUDR treated McCoy cell monolayers (prepared as above) were subjected to contact with either 1 ml of rifampicin or of rifapentin in varying concentrations covering a range of 50-200 µg/ml for 3 hrs, so as to allow the uptake of the antimicrobials by the cells.

The monolayers were then washed 3 times with antimicrobial free medium to remove the excess of extracellular drug prior to inoculation with a rifampicin-resistant variant of SA2f (resistant to 240 µg/ml rifampicin). The cells were then centrifuged at 3,000 g for 1 hr at 35°C and incubated at 36°C for 2 days. Control tubes containing cells not pretreated with the drugs were included in the experiment. One set of each dilution was stained with Giemsa and the other set with iodine. The stained cells were then examined by bright field microscopy.

3.3.6. THE STUDY OF SYNERGISTIC OR ANTAGONISTIC ACTIVITIES OF COMBINATIONS OF SULPHAMETHOXAZOLE/TRIMETHOPRIM(COTRIMOXAZOLE) AND SULFADOXINE/PYRIMETHAMINE(FANSIDAR) AGAINST SA2f.

The MIC and MBC of each of the antimicrobials were determined using the techniques described earlier. A nine by nine chequerboard was set up in duplicate for each combination of the drugs, working above and below the individual MICs in two-fold dilutions of antimicrobial agents. For this, double dilutions of the drugs at 4 times the required final concentrations were made. 0.25 ml of each dilution of the antimicrobial was then added to appropriate tubes. The tubes were inoculated with 0.5 ml of a suspension (300 IFU/ml) of SA2f, centrifuged for 1 hr at 35°C and incubated for 38-40 hrs at 35°C. Coverslips from one chequerboard were then fixed and stained with iodine for examination by BFM.

The contents of each tube from the second chequerboard were passaged into duplicate tubes containing antimicrobial free medium. These tubes were incubated as previously and one of each pair of tubes fixed and stained. Each duplicate tube was further passaged into 2 tubes. This process was continued for 10 passages. For each passage, results observed were recorded as growth or no growth on a nine by nine chart (fig 3.3.)

On completion of 10 passages, the combined inhibitory concentrations for both passages 1 and 10 were calculated from the results obtained.

In order to use a severe test of synergy, the recommendations of Krogstad and Mollering were followed. The fractional inhibitory

concentrations (FIC) for all combinations containing the MIC of the combined drugs were determined. From these values the FIC index (FICI) for each effective combination was calculated, and the mean ($\overline{FICI}$) taken (fig 3.3.). Synergy was defined as $\overline{FICI}$ <0.5, indifference as >0.5<2.0 and antagonism as >2.0.

		DRUG A (mg/l)					
		1	2	4	8	16	32
DRUG B	0.06	+	+	+	-	-	-
					a		
	0.12	-	-	-	-	-	-
		d	c	b			
	0.25	-	-	-	-	-	-

$$FICI = \frac{\text{MIC COMB}}{\text{MIC A}} + \frac{\text{MIC COMB}}{\text{MIC B}}$$

$$FICa + b + c + \ldots n = \text{SUM OF FICI}$$

$$\frac{FICI}{n} = \overline{FICI}$$

fig 3.3. Calculation of mean Fractional Inhibitory Concentration Index ($\overline{FICI}$)

CHAPTER 4

RESULTS

4.1. OBSERVATIONS ON THE LIFE CYCLE OF CHLAMYDIA

4.1.1. DEVELOPMENTAL CYCLE (LIGHT MICROSCOPY).

Iodine stain (BFM X40 dry objective):

There were no inclusions visible 16 hrs post inoculation (T16) and only very scanty, tiny ones at 24 hrs post inoculation (T24). At 30 hrs post inoculation (T30), a considerable number of larger inclusions were seen and at 42 hrs post inoculation (T42), a large number of well defined and well developed inclusions with a brown glycogen matrix, reticulate in structure, were visible (fig 4.1- 4.3.). The latter was taken as the reference (normal) appearance, against which all other iodine stains are compared.

Giemsa stain (BFM x40 and DGM x40 dry objective):

At T16, there were no inclusions visible by DGM. Some inclusions which were very tiny, were just visible by BFM (fig 4.4-4.5.). At T24, very poorly auto fluorescent inclusions were visible by DGM, whereas small, well defined inclusions were seen by BFM (fig 4.6-4.7.). At T30, some larger inclusions exhibiting autofluorescence were seen and near normal size inclusions were observed by BFM (fig 4.8-4.9.). At T42, inclusions were well defined, well developed, reticulate in structure and exhibited strong autofluorescence by DGM. Giemsa BMF showed intracytoplasmic, perinuclear inclusions. The contents of the inclusions appeared as delicate mauve-pink coloured speckles in the phagocytic vesicles. All other Giemsa stains are compared with this stage of the life cycle (figs 4.10. and 4.11.).

Fluorescent stain (X40 oil immersion):

At T16, very scanty, tiny perinuclear inclusions were seen. As the cycle of infection progressed, the inclusions became larger, better defined and visibly reticulate in structure (figs 4.12-4.14.). At T42, considerable numbers of large inclusions which were reticulate in structure, showing intense apple green fluorescence, were observed. All other immunofluorescence work is compared with the appearance of inclusions at this time (fig 4.15.).

Acridine orange stain (x40 UV):

At T16, there were small, orange, granular inclusions. The number of these inclusions had increased by T24 but their appearance remained more or less the same. At T30, however, there were both orange and green particles present and at T42 larger numbers of inclusions, filled with predominantly green bodies, were present (figs 4.16.-4.18.). All acridine orange stain work is compared with the T42 data (fig 4.19.).

4.1.2. DEVELOPMENTAL CYCLE (EM)

At T24, the inclusions stained with toluidine blue were small. They were visible by BFM, could be marked and found on the corresponding grid for EM examination. These inclusions were filled with several large bodies, some in the process of binary fission (fig4.20.).

At T30, larger inclusions were visible by toluidine blue. By EM, the inclusions were seen to be filled with a mixture of several electron dense particles as well as a few ghost particles. Several intermediate bodies undergoing fission were also present (fig 4.21.).

At T42, much larger inclusions could be seen by toluidine blue stain. These inclusions, when examined by EM, were filled with a mixture of elementary bodies, ghost bodies and some intermediate dividing particles. These inclusions also contained some rosettes of glycogen. It was against this stage of the life cycle that all other EM work is compared (fig 4.22.).

NOTE MAGNIFICATION ON ALL LIGHT MICROSCOPY FIGURES IS X400.

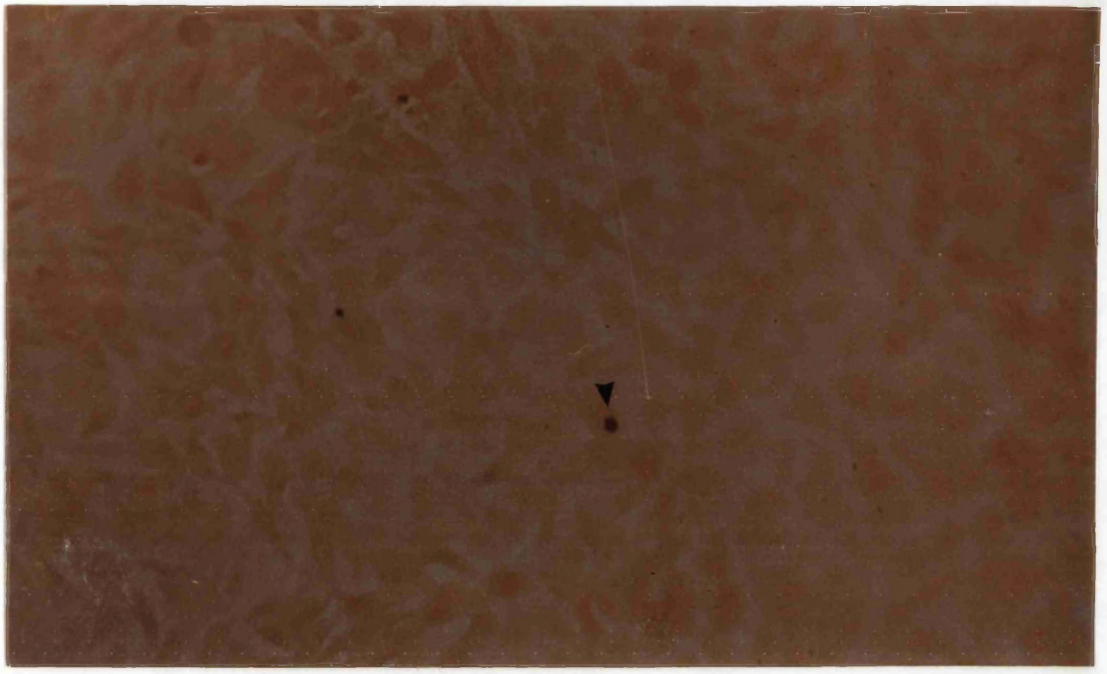

fig 4.1. Small iodine stained inclusion at T24.

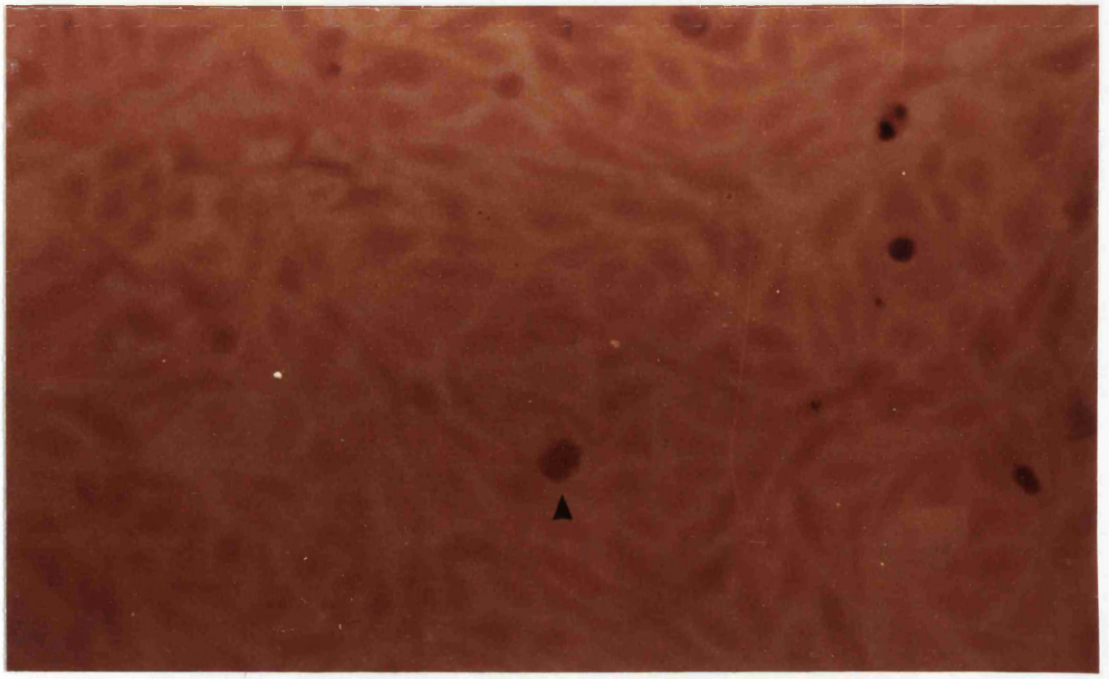

fig 4.2. Iodine stained inclusion at T30.

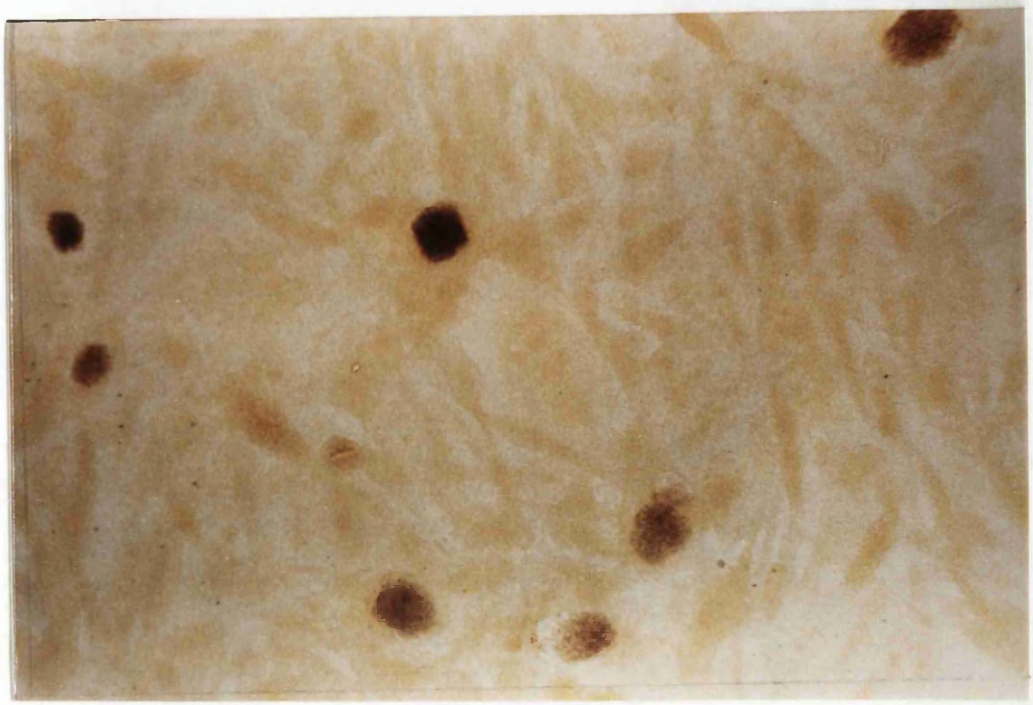

fig 4.3. Iodine stained inclusions at T42.

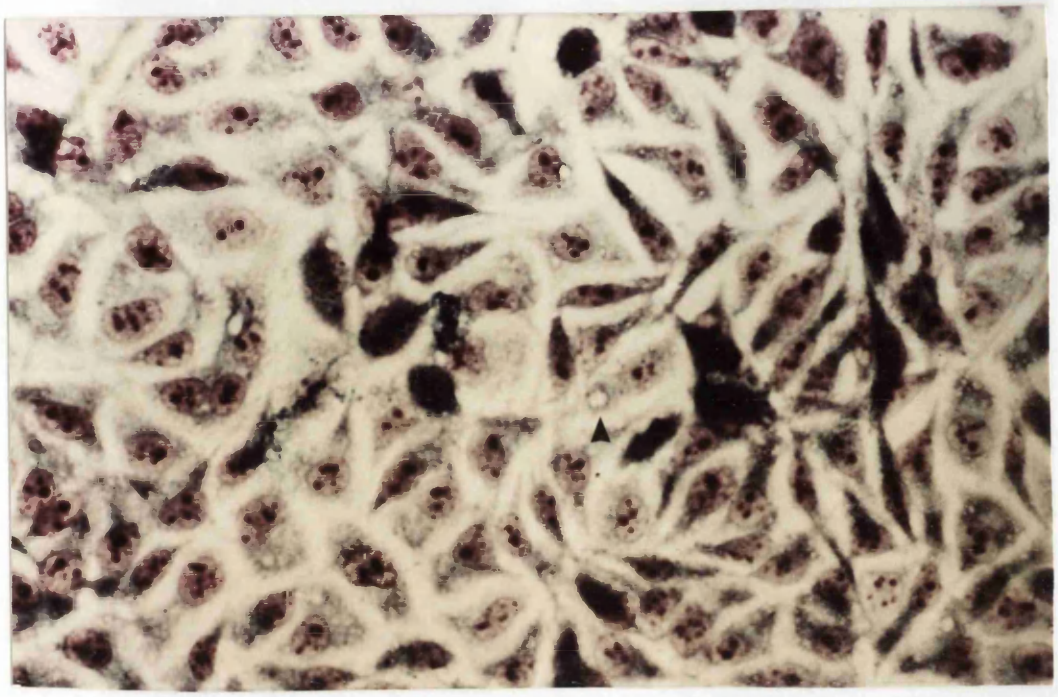

fig 4.4. Tiny Giemsa stained inclusion at T16 viewed by BFM.

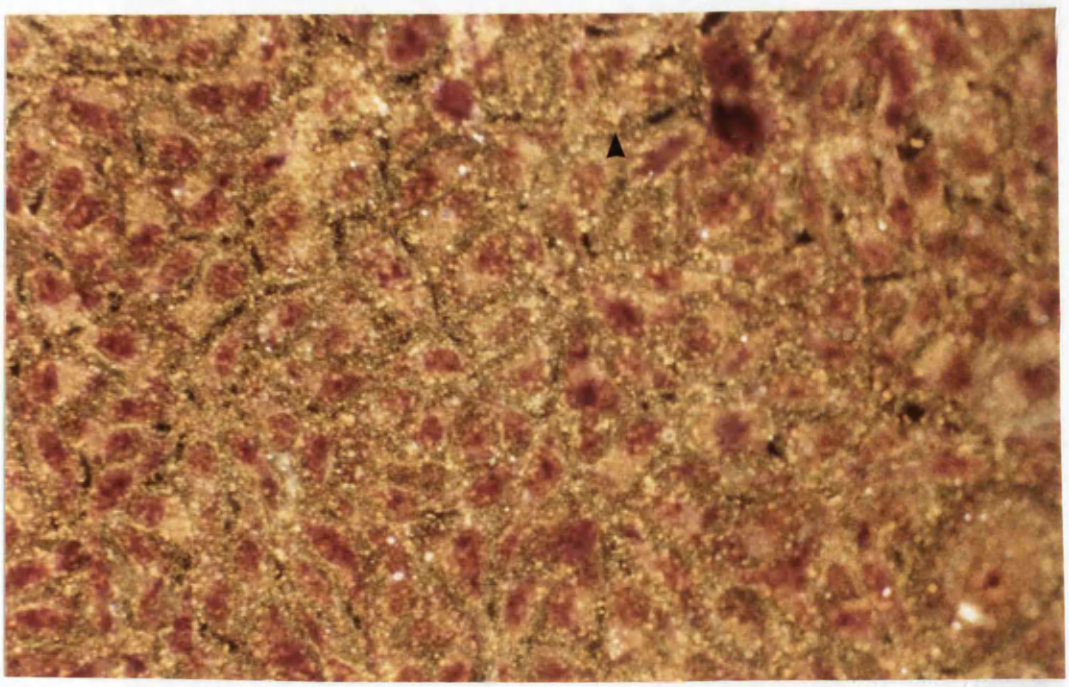

fig 4.5. Tiny Giemsa stained inclusion at T16 viewed by DGM.

No autofluorescence observed.

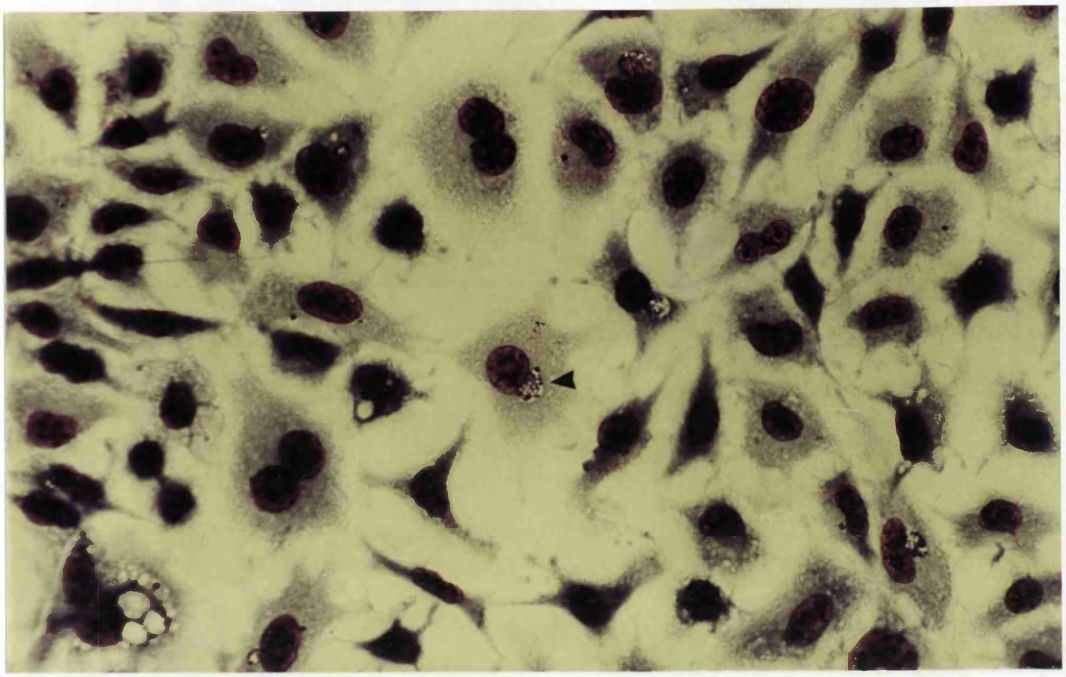

fig 4.6. Small Giemsa stained inclusion at T24 viewed by BFM.

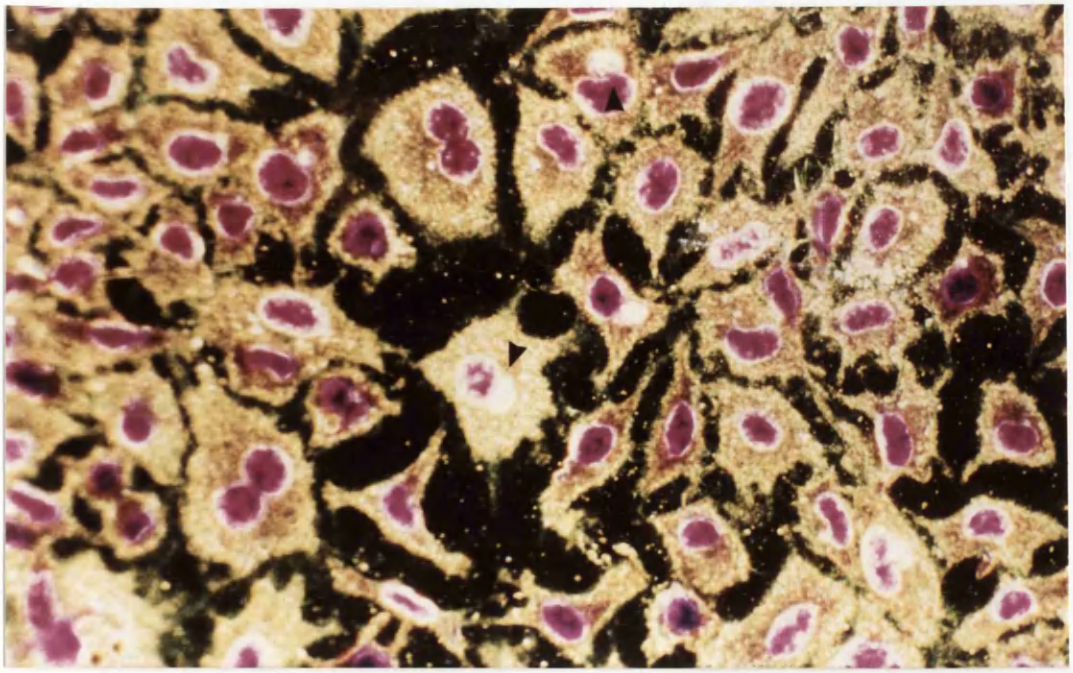

fig 4.7. Small Giemsa stained inclusion at T24 viewed by DGM.

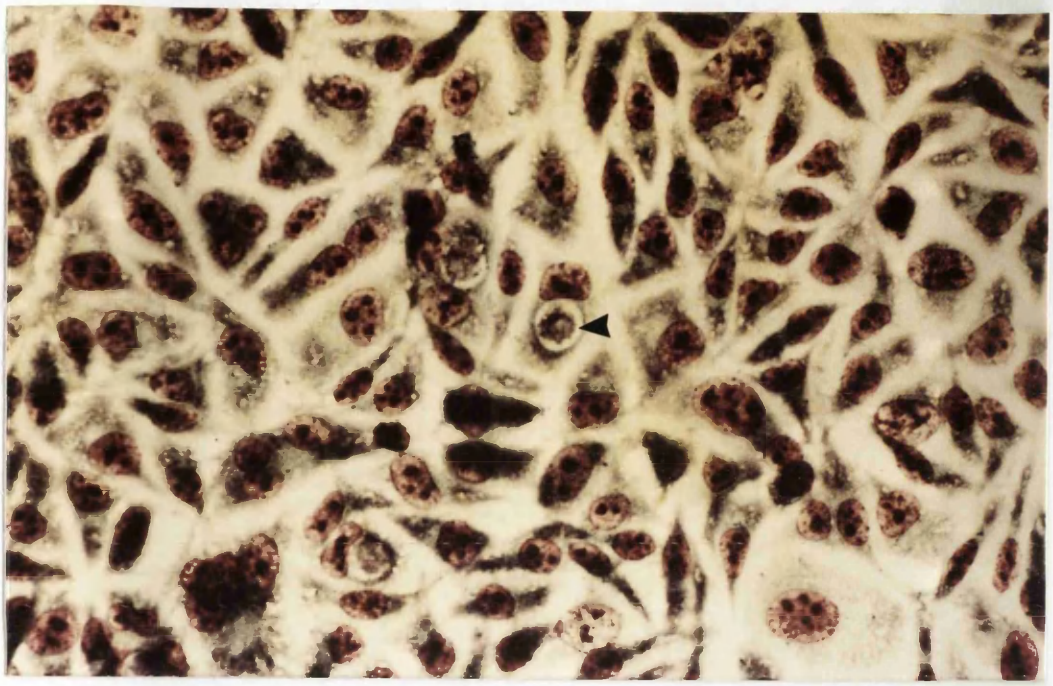

fig 4.8. Giemsa stained inclusion at T30 viewed by BFM.

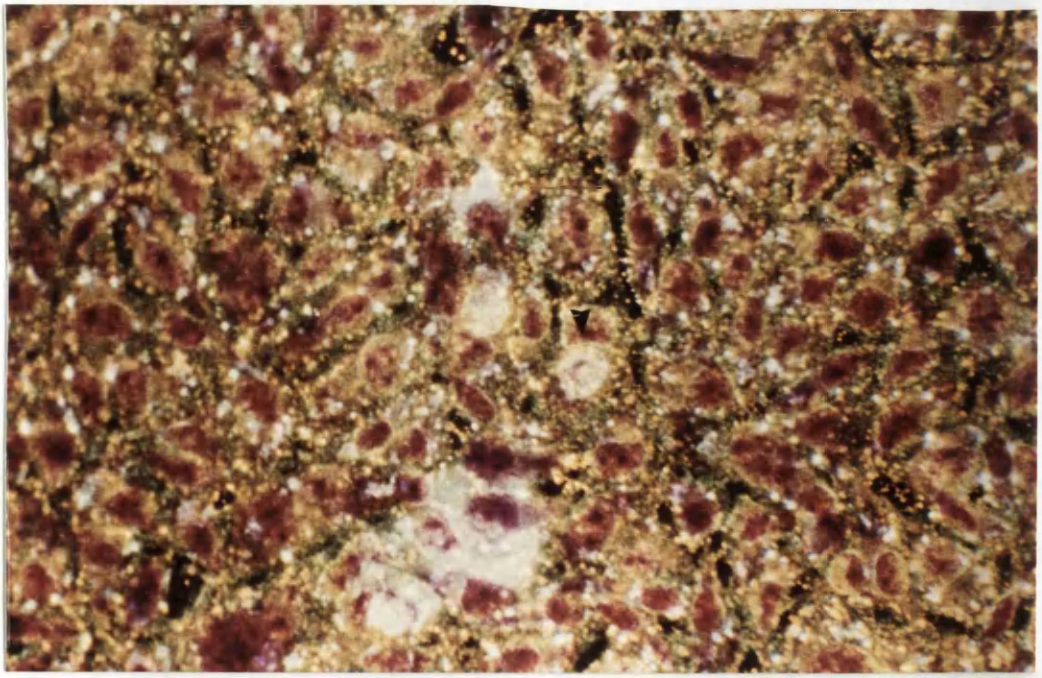

fig 4.9. Giemsa stained inclusion at T30 viewed by DGM.

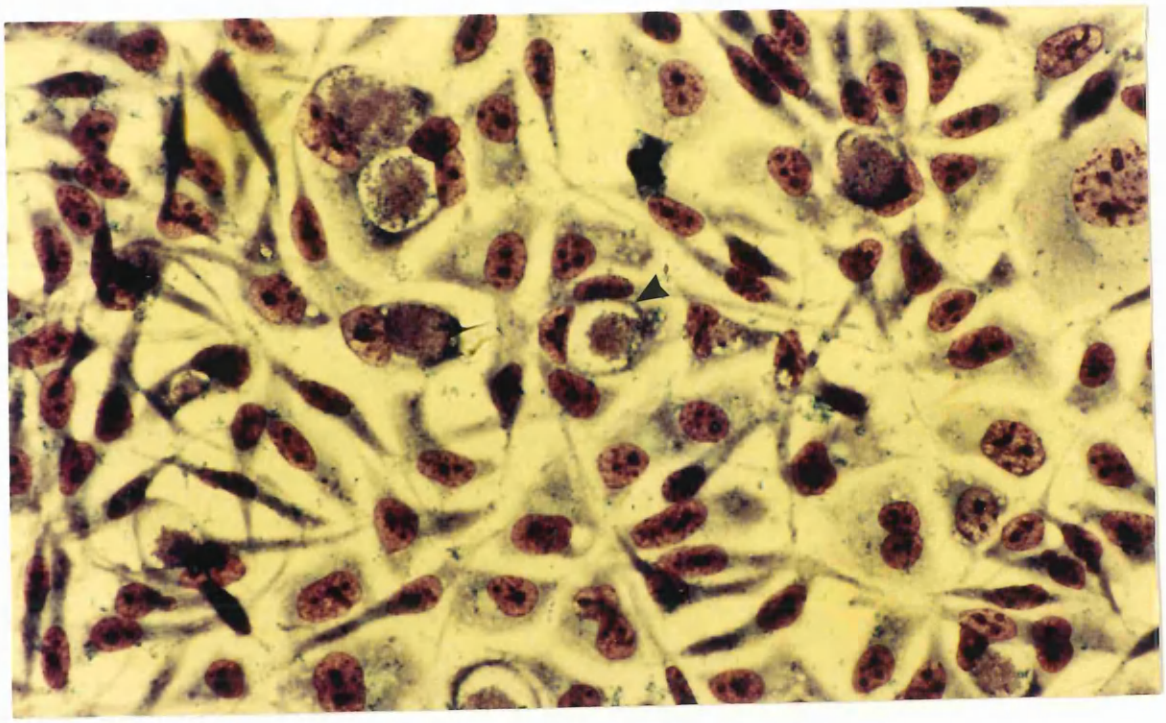

fig 4.10. Giemsa stained inclusion at T42 viewed by BFM.

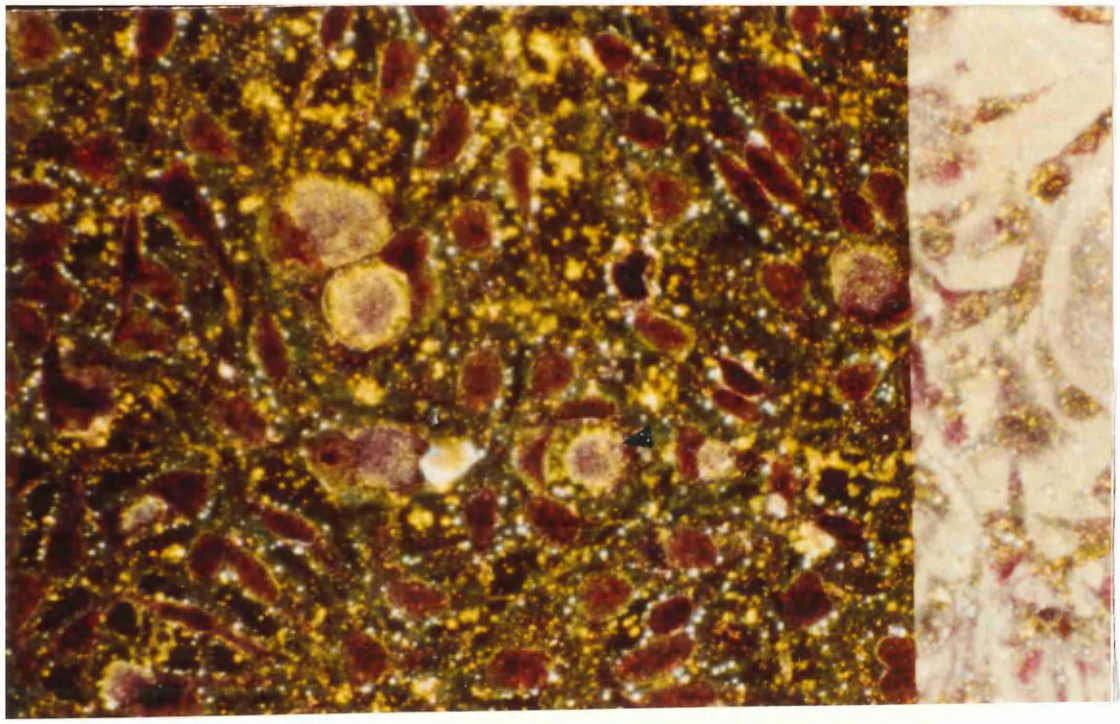

fig 4.11. Giemsa stained inclusion at T42 viewed by DGM.

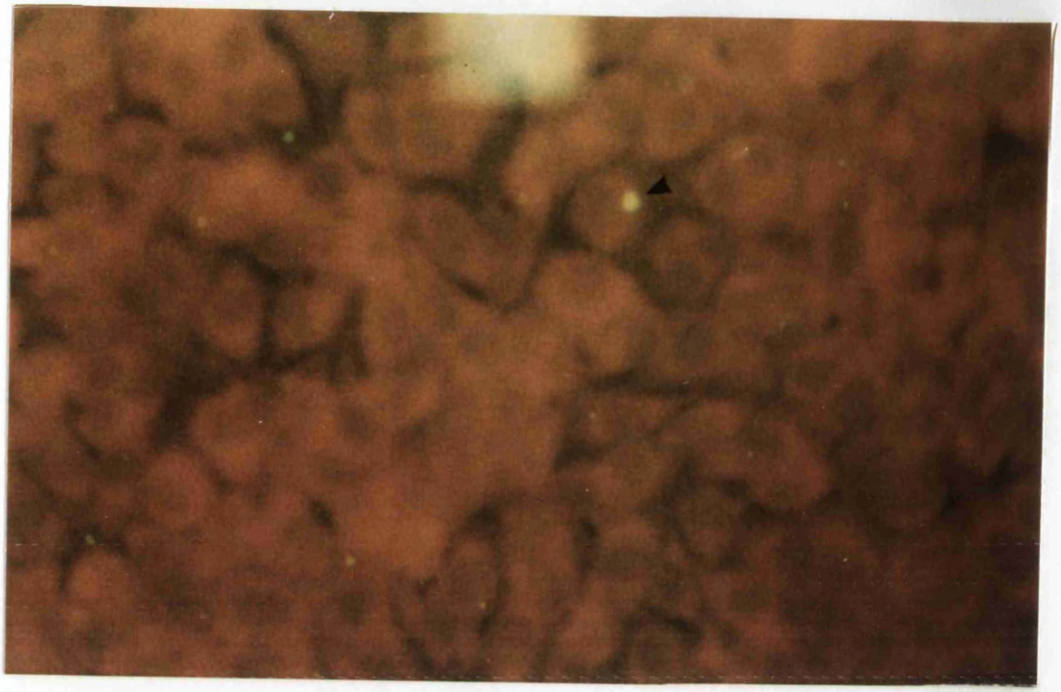

fig 4.12. Tiny fluorescent stained inclusion at T16.

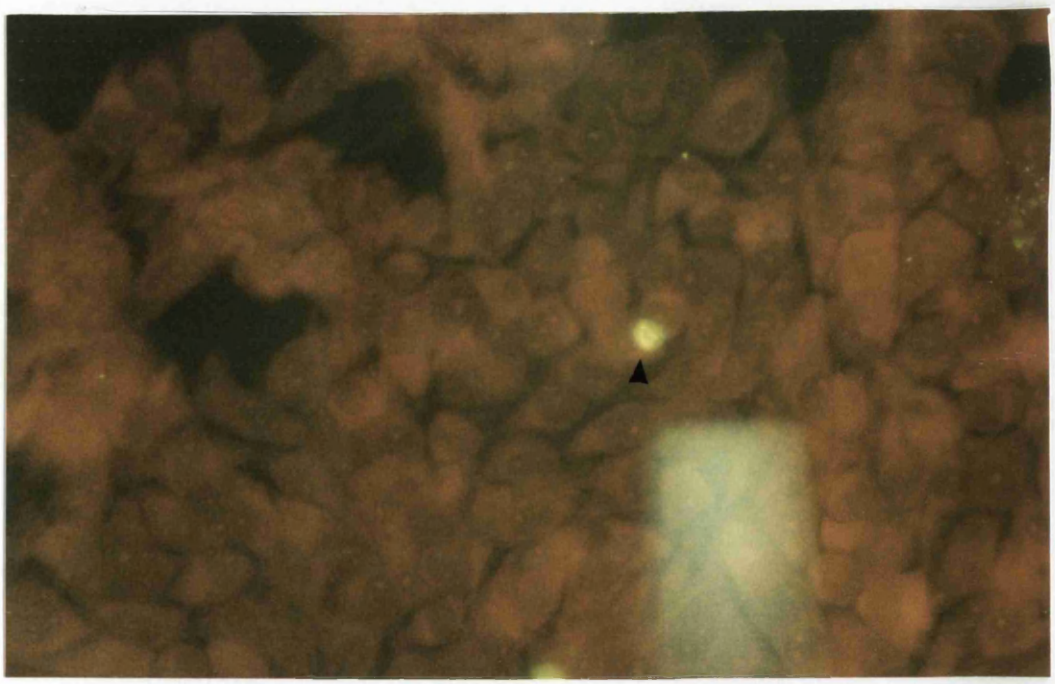

fig 4.13. Small fluorescent stained inclusion at T24.

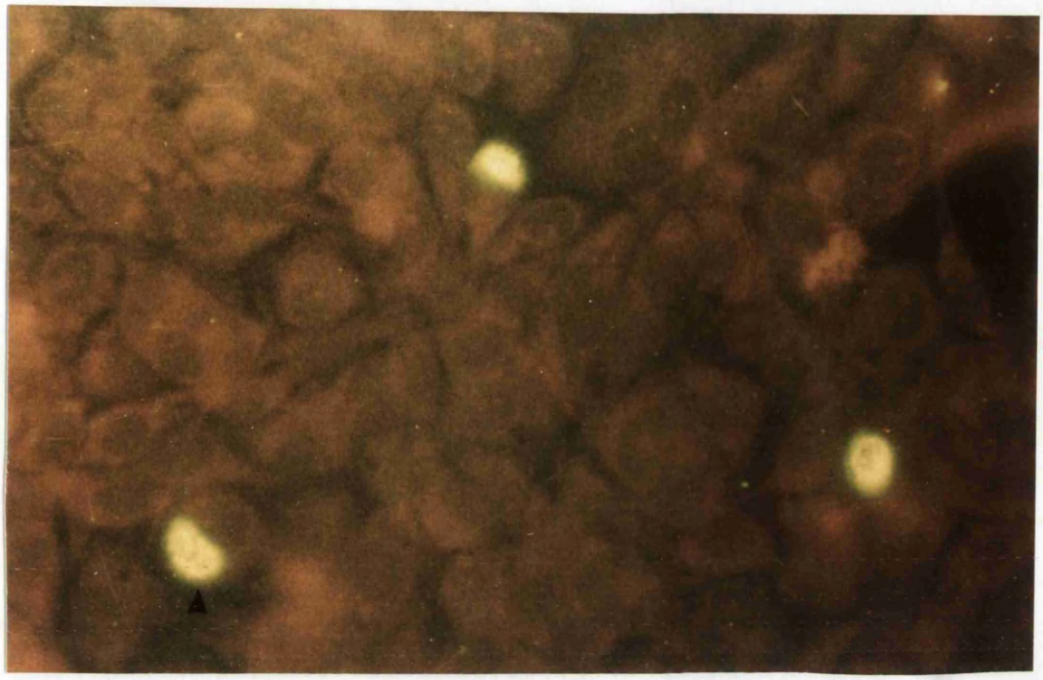

fig 4.14. Fluorescent stained inclusions at T30.

Inclusions are reticulate in structure.

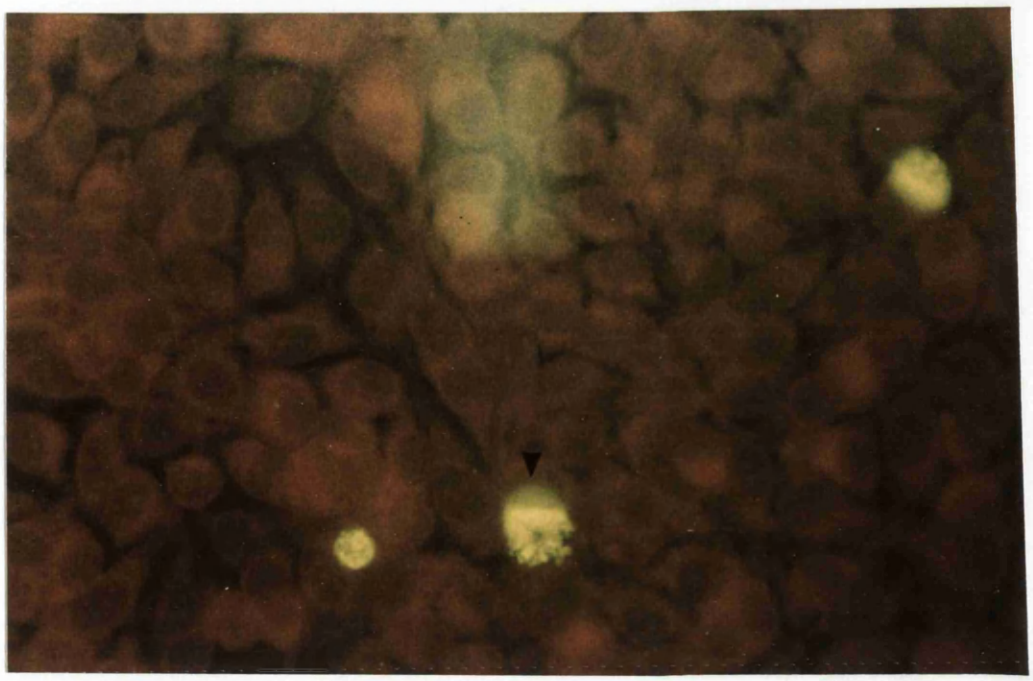

fig 4.15. Fluorescent stained inclusions at T42.

Inclusions are reticulate in structure.

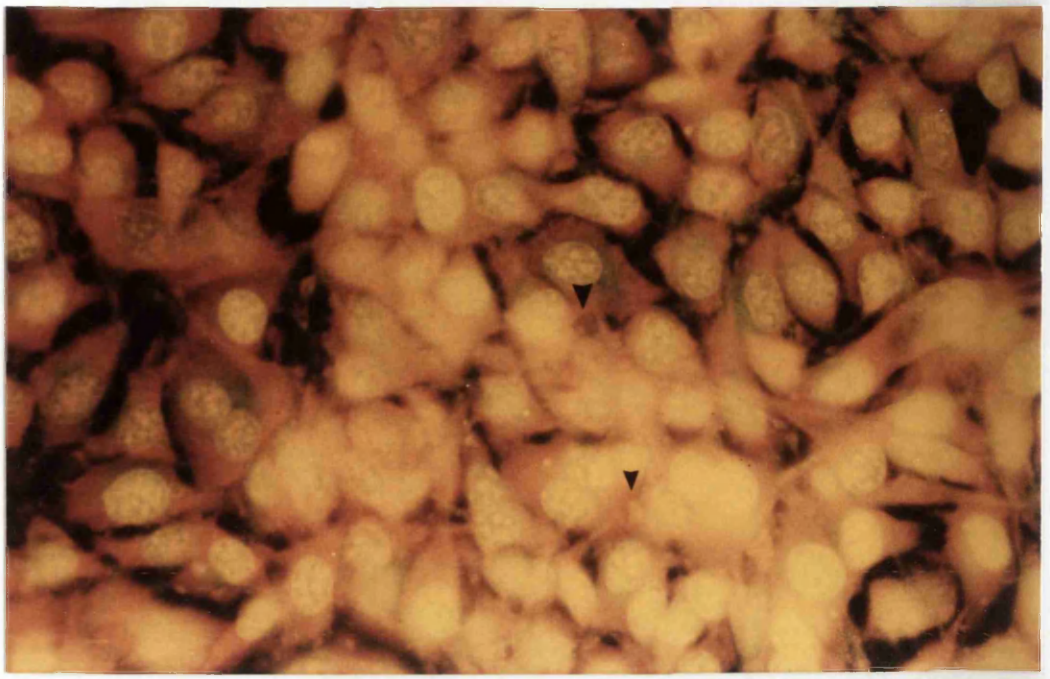

fig 4.16. Tiny acridine orange stained inclusions at T16.

The inclusions contain orange stained material (RNA).

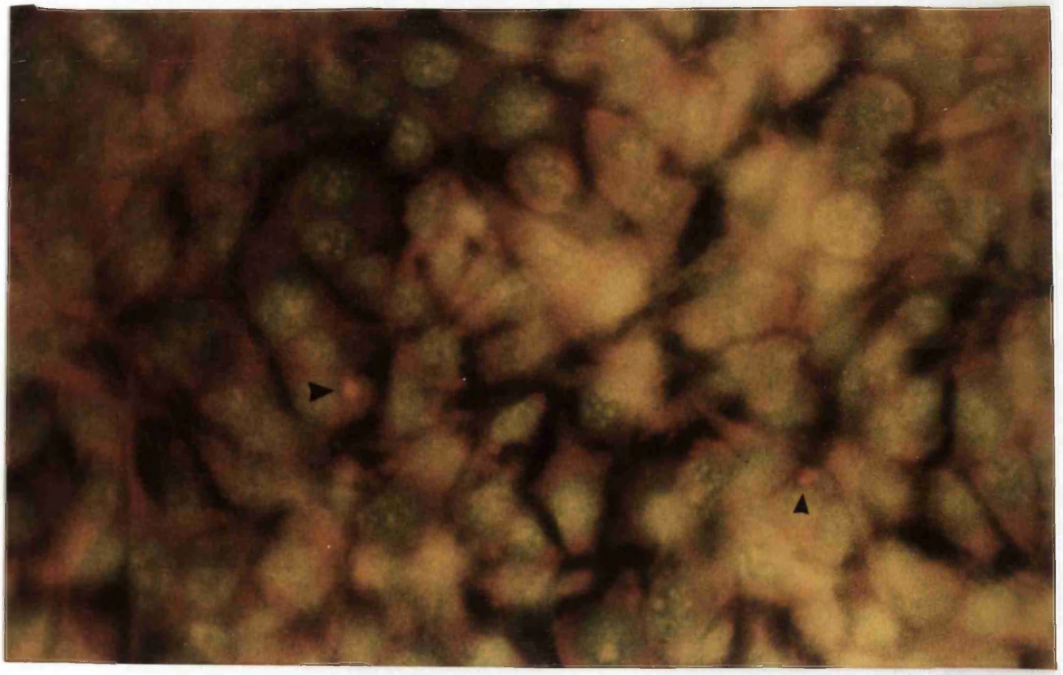

fig 4.17. Small acridine orange stained inclusions at T24.

The inclusion contains orange stained material (RNA).

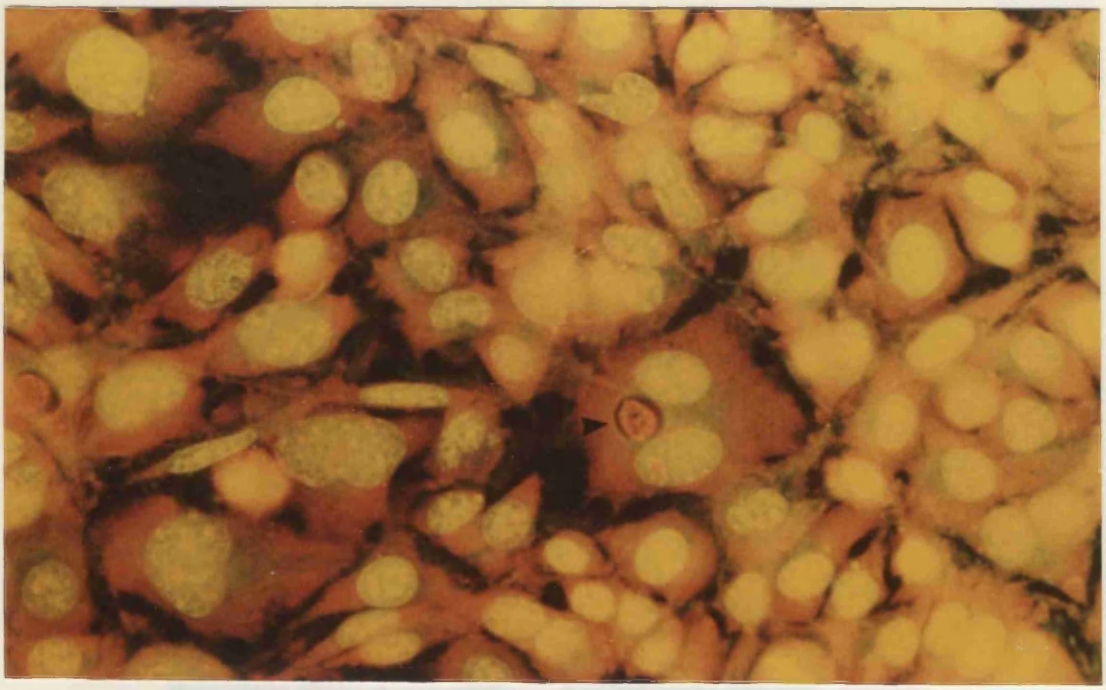

fig 4.18. Acridine orange stained inclusion at T30. The inclusion contains orange stained (RNA) as well as a small amount of green stained (DNA) material.

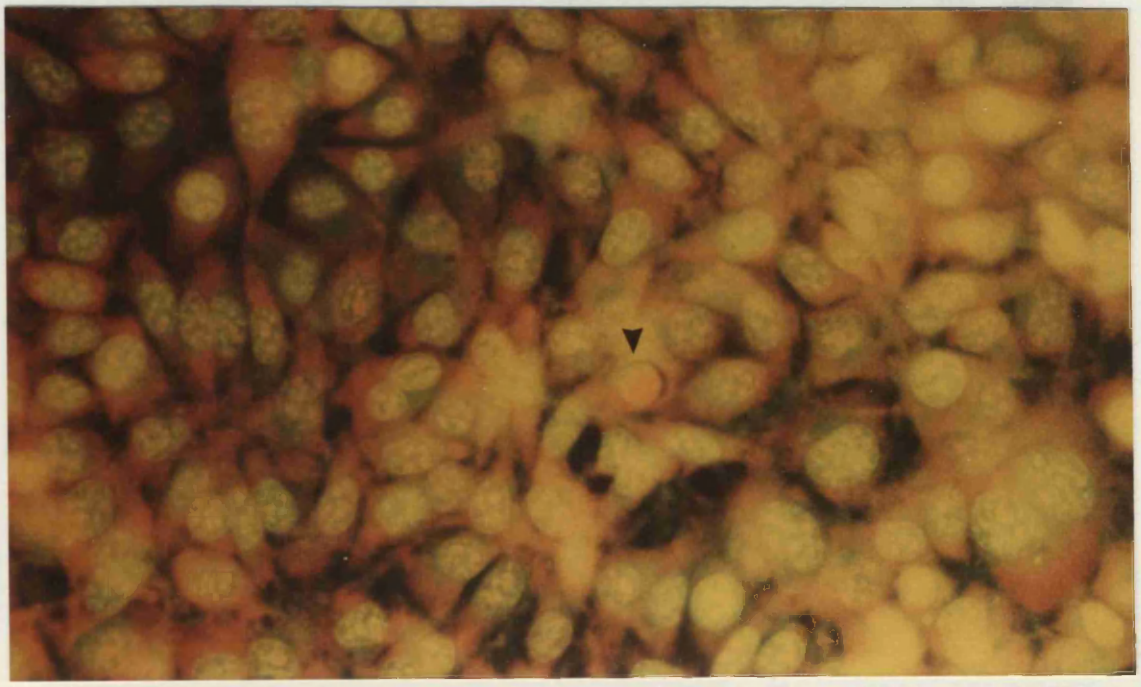

fig 4.19. Acridine orange stained inclusion at T42. The inclusion contains both orange (RNA) and green (DNA) stained material.

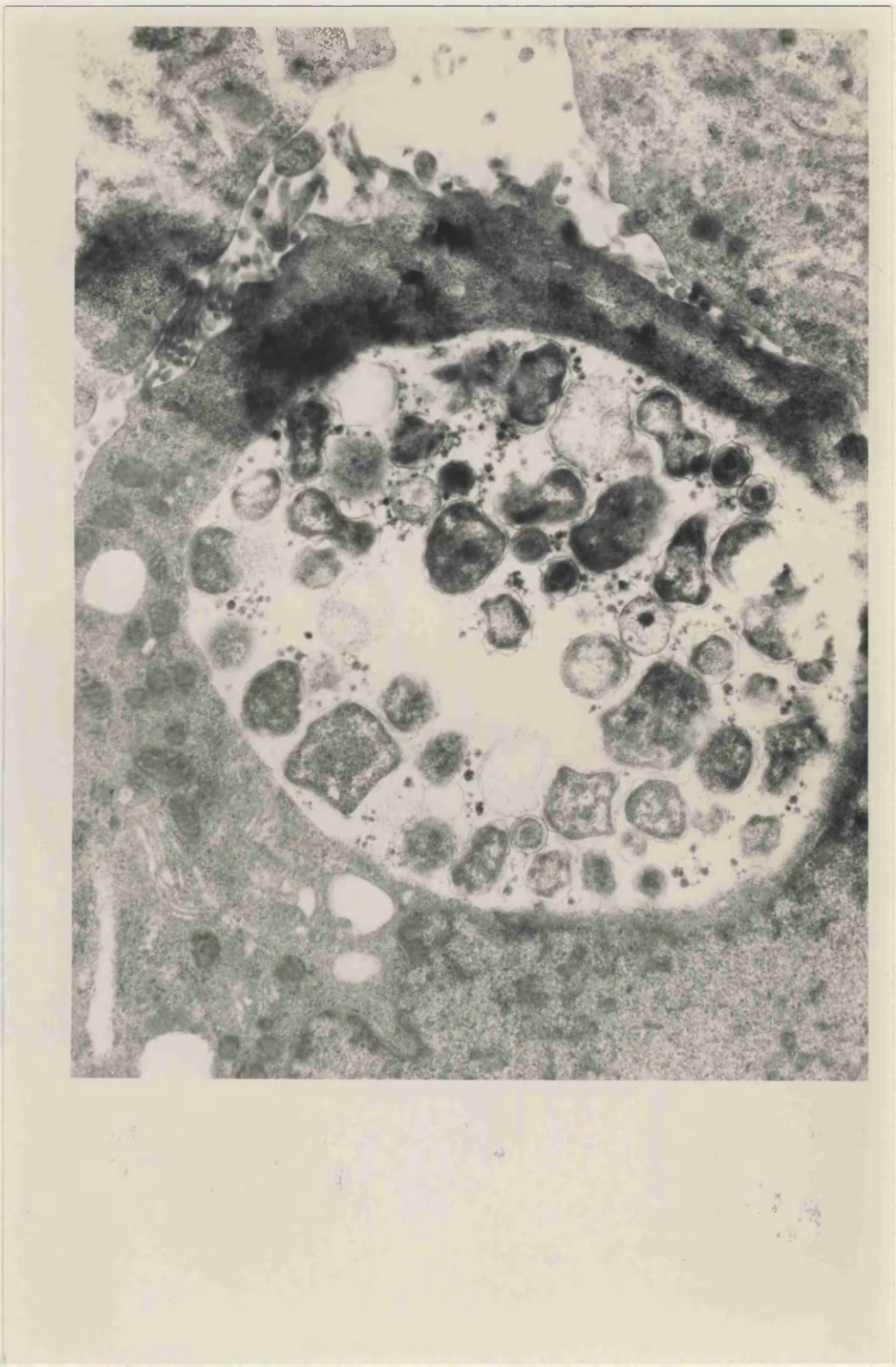

fig 4.20. Electron micrograph of the contents of an inclusion at T24. Reticulate and intermediate bodies as well as rosettes of glycogen are seen (X 20000).

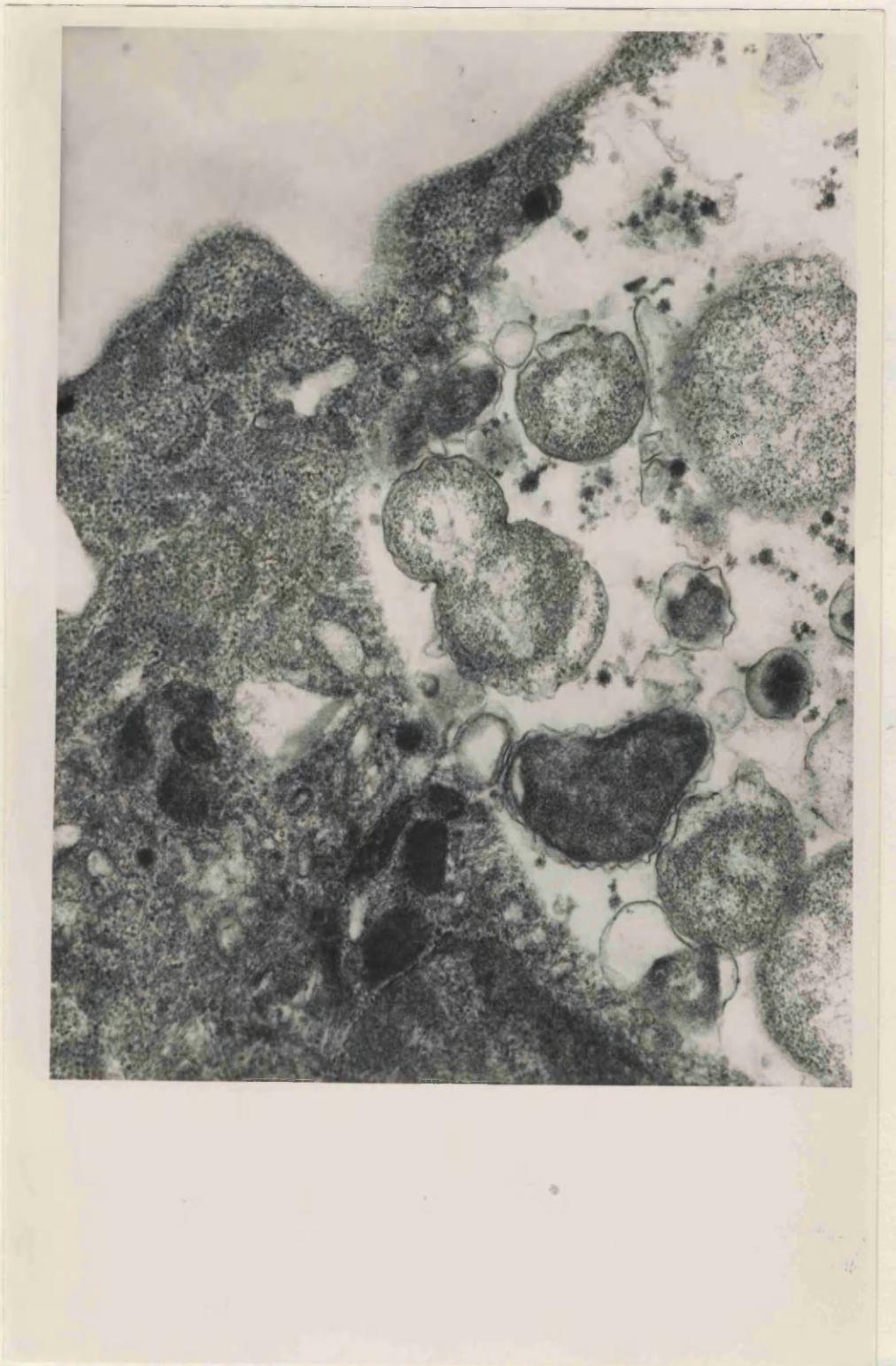

fig 4.21. Electron micrograph of the contents of an inclusion at T30. One reticulate body in the process of binary fission is seen. The membranes of the reticulate bodies are undulated and loose. Rosettes of glycogen are also present (X40000)

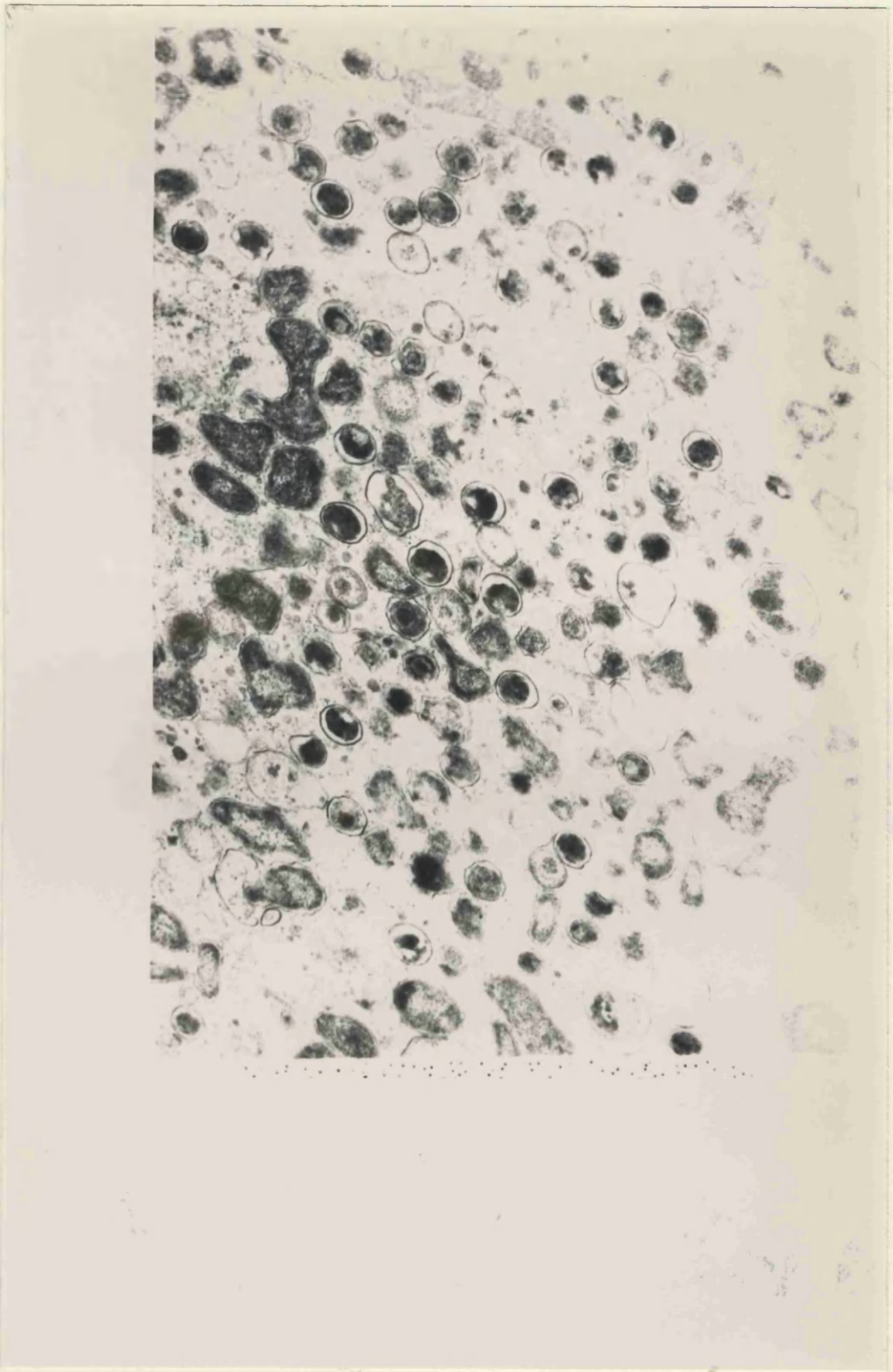

fig 4.22. Electron micrograph of the contents of an inclusion at T42. The inclusion is filled predominantly with elementary bodies. Simultaneous occurrence of the three types of particles (elementary, reticulate and intermediate bodies) are seen in the inclusion (X20000).

4.1.3. EFFECT OF PENICILLIN ON THE DEVELOPMENTAL CYCLE (BFM).

Using different staining techniques, the effects of the addition of penicillin at different intervals during the life cycle of SA2f were noted.

Iodine stain (BFM x40 objective):

For PT0, the inclusions seen were scanty, small, irregular and smooth in appearance (fig 4.23.). For PT16, the inclusions were larger, irregular, smooth and they appeared to be comprised of several hollow discs. This gave a globular appearance to the inclusions (fig 4.24.). For PT24, the inclusions seen were less globular as compared with those for PT16. Some very pale inclusions were also present (fig 4.25.). For PT30, the inclusions were scanty, large, well defined and exhibited a cotton wool appearance (fig 4.26.). For PT40, the inclusions were well defined and normal (fig 4.27.).

Giemsa stain (BFM x40 and DGM x40 dry objective):

For PT0, the inclusions seen by BFM were scanty, small and irregular. Some did not exhibit autofluorescence when observed by DGM (figs 4.28.-4.29.). For PT16, the inclusions observed by BFM were scanty, though much larger in size as compared with those for PT0 and their contents had a beaded appearance. No autofluorescence was observed by DGM (figs 4.30.-4.31.). For PT24, the inclusions seen by BFM were greater in number and much bigger than those for PT16 and their contents had a beaded appearance. Very scanty, poorly autofluorescing inclusions were revealed by DGM. Inclusions showing a beaded appearance by BFM did

not exhibit strong autofluorescence (figs 4.32.-4.33.).
For PT30, inclusions present by BFM were of greater numbers, bigger, and beaded and/or mottled. Only those inclusions with a mottled appearance exhibited autofluorescence by DGM (figs 4.34.-4.35.). For PT40, the inclusions seen were of normal size when viewed by BFM and DGM. These inclusions gave an appearance of being filled with blue cotton wool when examined by BFM (figs 4.36.-4.37.).

Acridine orange stain (x40 UV):

For PT0, small, globular inclusions which stained orange, a characteristic of RNA containing material, were seen (fig 4.38.). For PT16 the inclusions seen were larger than those for PT0 with a very globular appearance. These globules which were better defined, were stained orange (fig 4.39.). For PT24, the inclusions were larger than those for PT16 and contained large numbers of globular bodies, all stained orange. Small numbers of tiny, mottled, green structures characteristic of DNA containing material were also present (fig 4.40.). For PT30, the inclusions seen were numerous, large, and contained green (DNA) and orange (RNA) stained particles. The RNA material was still very globular (fig 4.41.). For PT40, the inclusions observed were large with a mixture of RNA and DNA materials. The RNA material appeared to be granular but <u>not</u> globular (fig 4.42.).

Fluorescent stain (x40 oil immersion):

For PT0, the inclusions observed were small and smooth (fig 4.43.). For PT16, the inclusions were larger as compared to

those for PT0, and globular in appearance (fig 4.44.). For PT24, the inclusions seen were globular, in greater numbers and of larger size than those for PT16 (fig 4.45.). For PT30, the inclusions seen were in greater numbers still, very large, globular, and some of these also gave a granular appearance (fig 4.46.). For PT40, large numbers of normal inclusions were seen (fig 4.47.).

4.1.4. EFFECT OF PENICILLIN ON THE DEVELOPMENTAL CYCLE (EM).

Using EM, the effects of the addition of penicillin at different intervals during the life cycle of SA2f were noted.

For PT0, small inclusions containing scanty particles were seen. A well defined inclusion with one abnormal body showing abnormal division was noted (fig 4.48.). For PT16, some giant particles with granular cytoplasm were present (fig 4.49.). For PT24, giant particles with granular cytoplasm were present. One abnormal giant body containing an electron transparent area was noted. Rosettes of glycogen were also present (fig 4.50.). For PT30, large inclusions filled with hollow discs were seen. Particles with ringed sections of electron dense and tranparent areas, giving a bull's eye appearance, were observed. Rosettes of glycogen were also present (fig 4.51.). For PT40, inclusions filled with particles, the same variety as those of normal inclusions, were present. Rosettes of glycogen were also noted (fig 4.52).

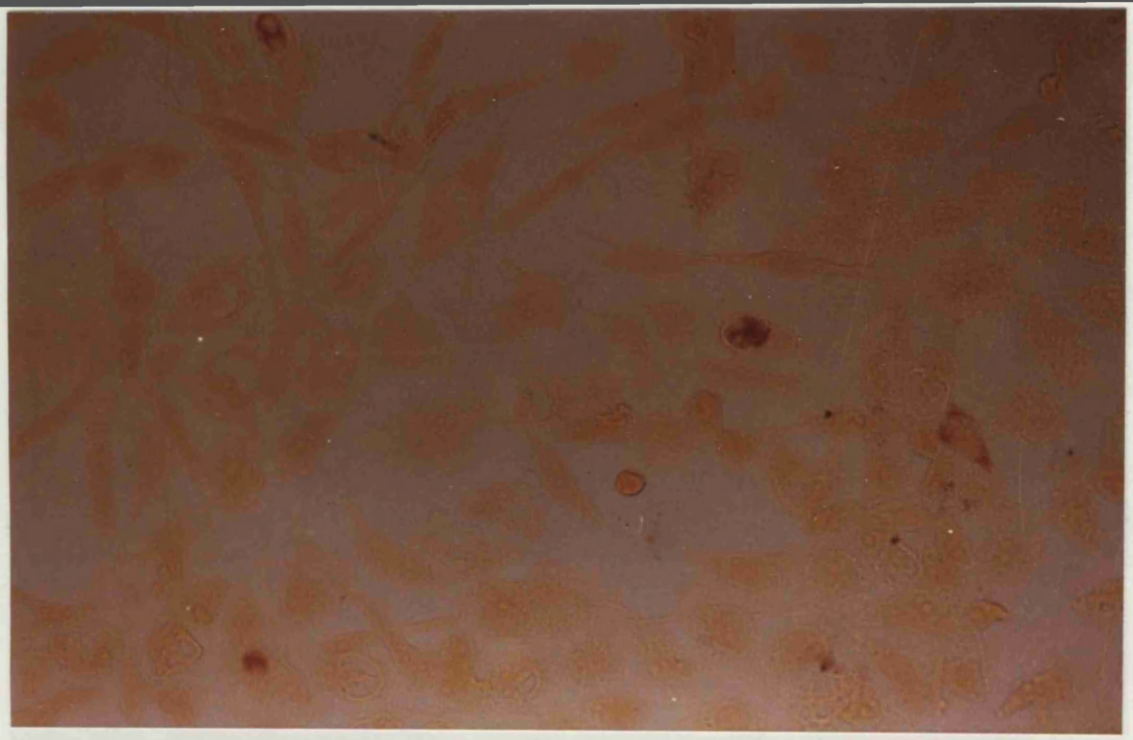

fig 4.23. Iodine stained inclusions with 0.125µg/ml penicillin added at PTO. Inclusions are small, smooth and globular in structure.

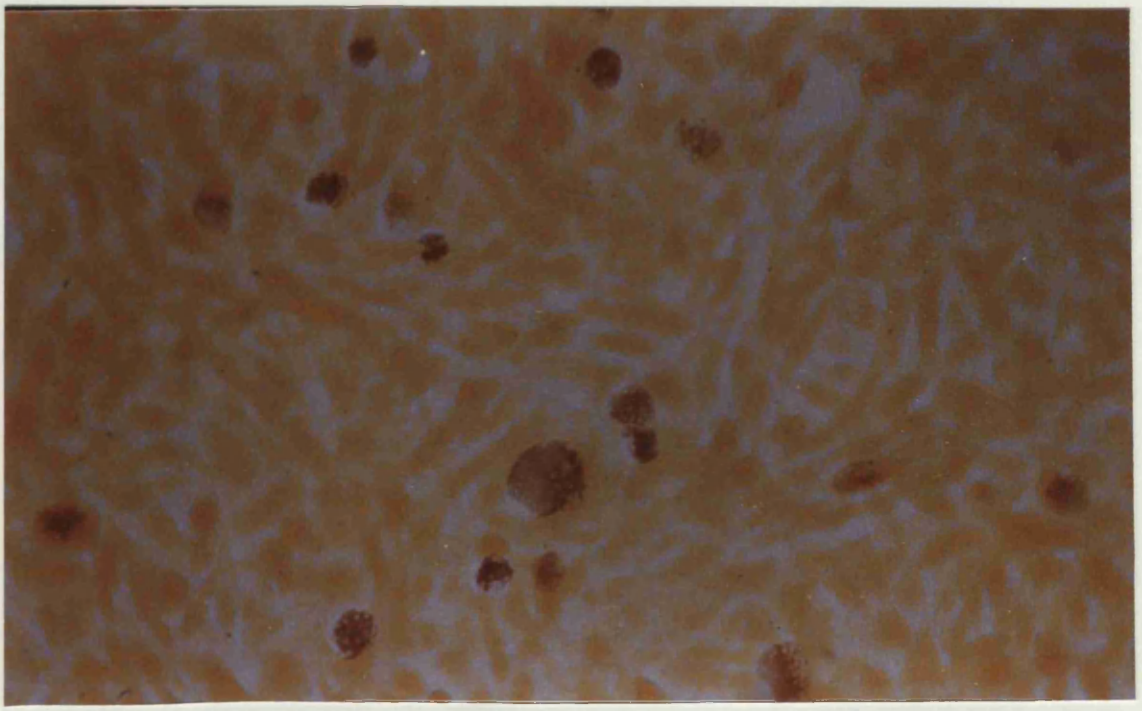

fig 4.24. Iodine stained inclusions with 0.125µg/ml penicillin added at PT16. Inclusions appear to be made up of small globules.

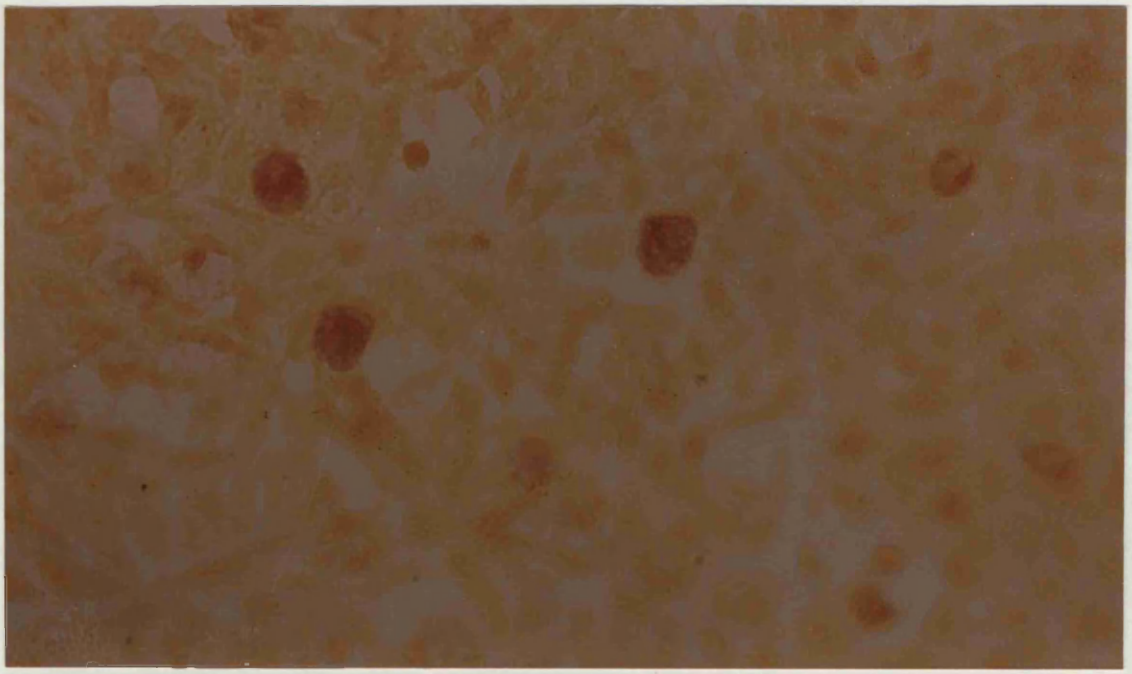

fig 4.25. Iodine stained inclusions with 0.125µg/ml penicillin added at PT24. Smooth, globular and some very pale inclusions are present.

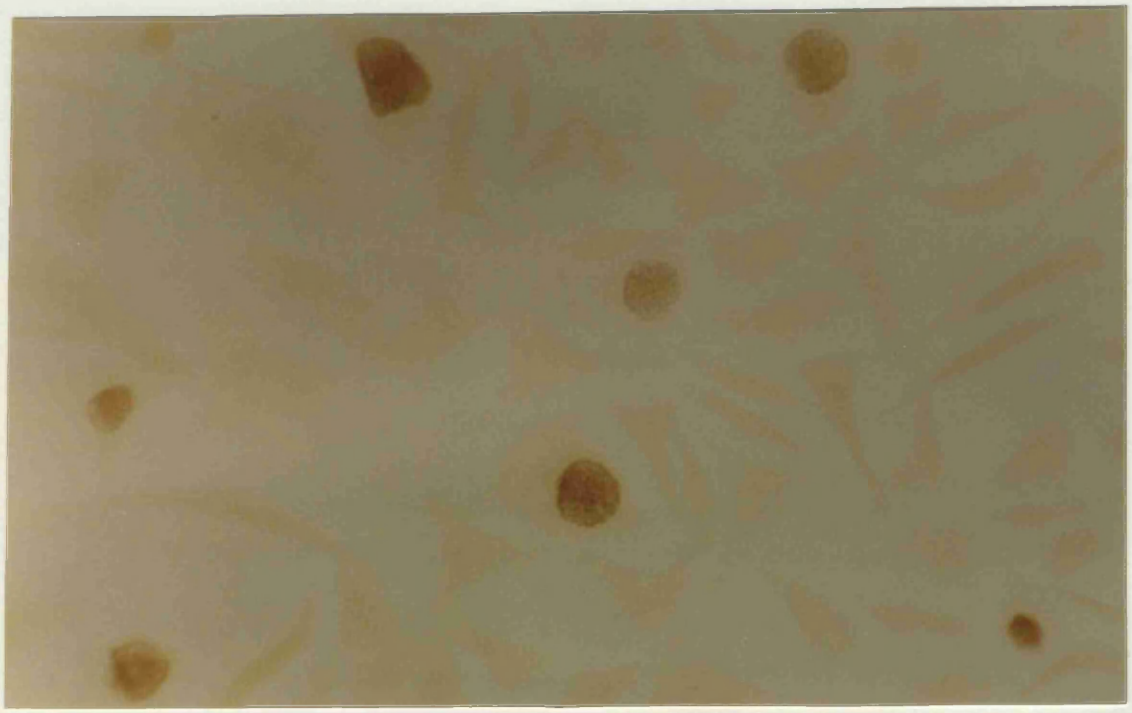

fig 4.26. Iodine stained inclusions with 0.125µg/ml penicillin added at PT30.

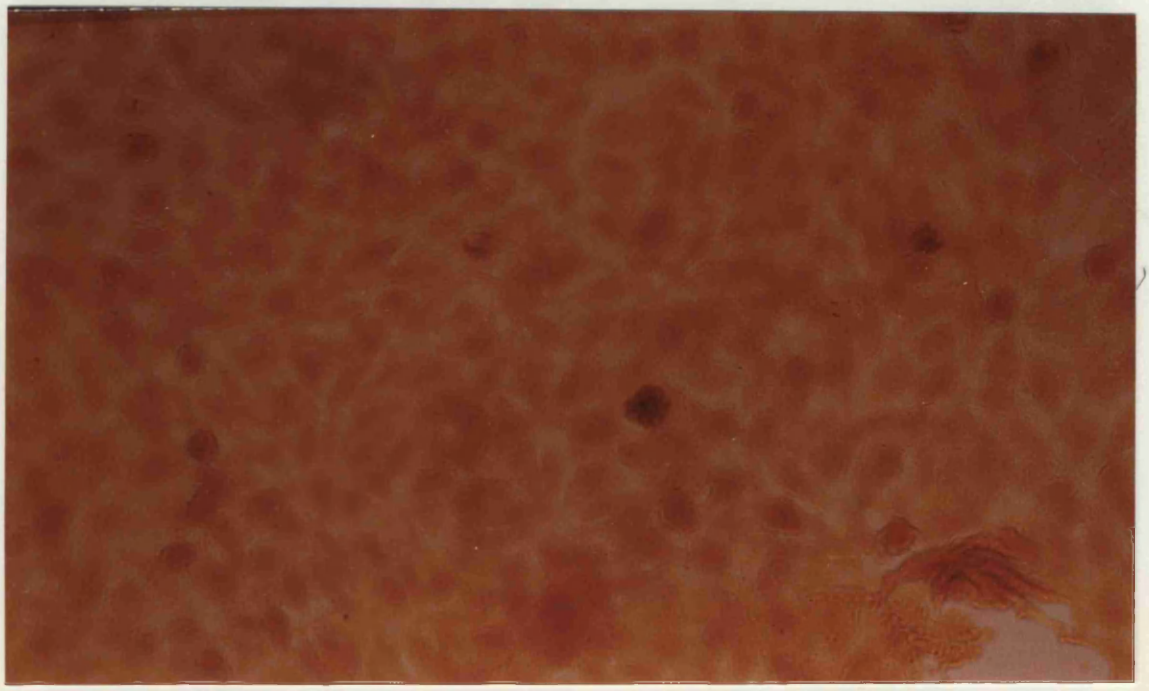

fig 4.27. Iodine stained inclusion with 0.125µg/ml penicillin added at PT40.

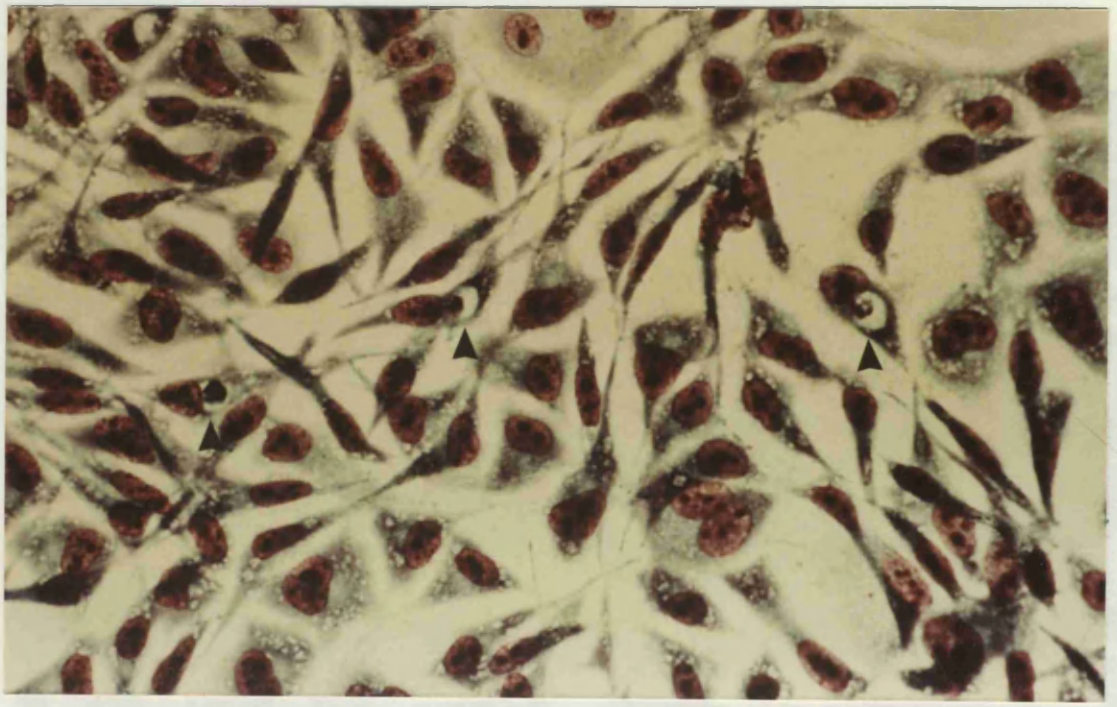

fig 4.28. Giemsa stained inclusions (BFM) with 0.125µg/ml penicillin added at PTO. The inclusions are small and well defined.

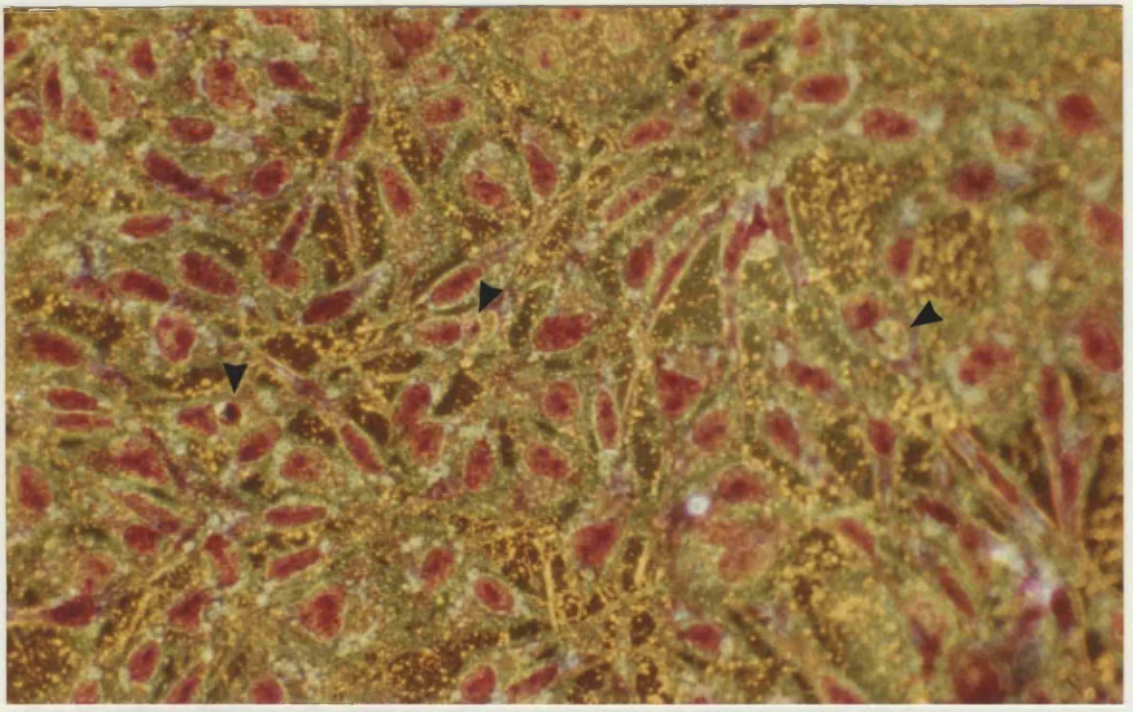

fig 4.29. Giemsa stained inclusions (DGM) with 0.125 µg/ml penicillin added at PTO. Some small inclusions do not exhibit autofluorescence.

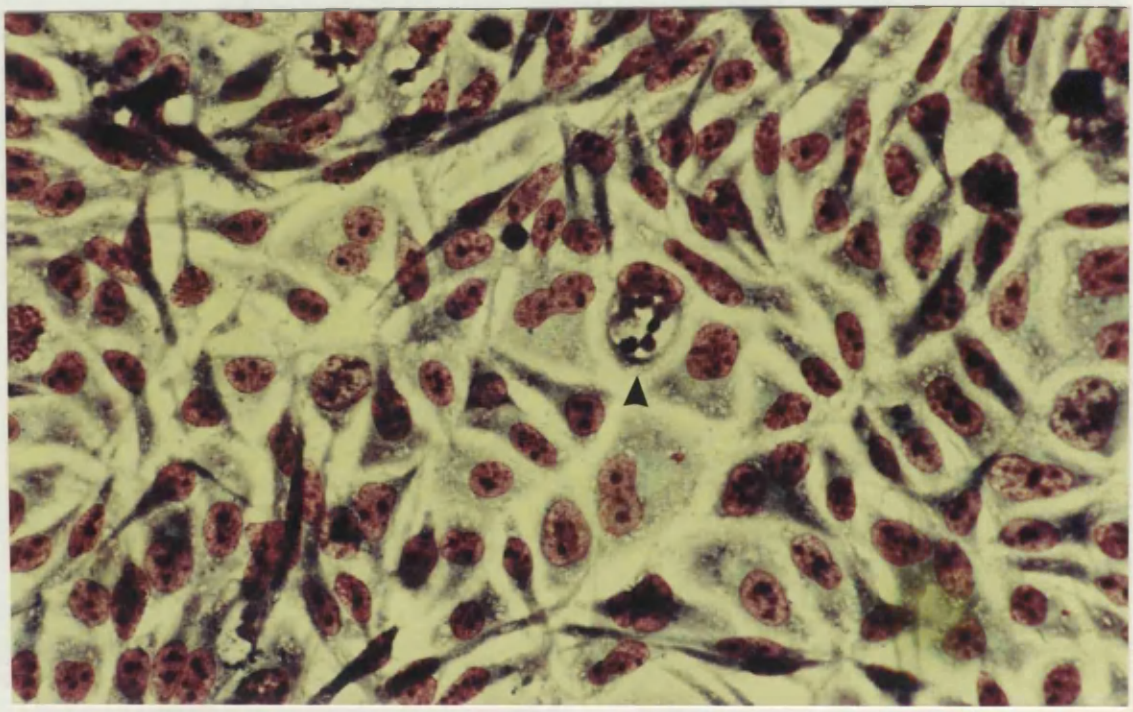

fig 4.30. Giemsa stained inclusion (BFM) with 0.125µg/ml penicillin added at PT16. The inclusion is large, well defined and contains large beaded particles.

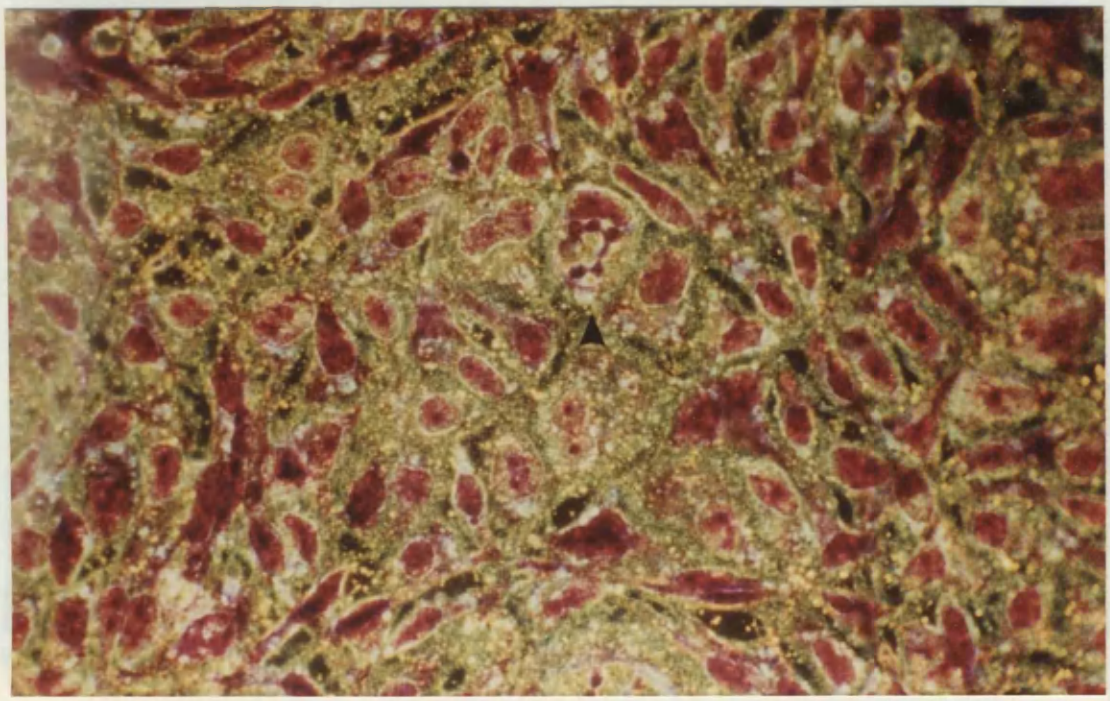

fig 4.31. Giemsa stained inclusion (DGM) with 0.125µg/ml penicillin added at PT16. Beaded particles do not autofluoresce.

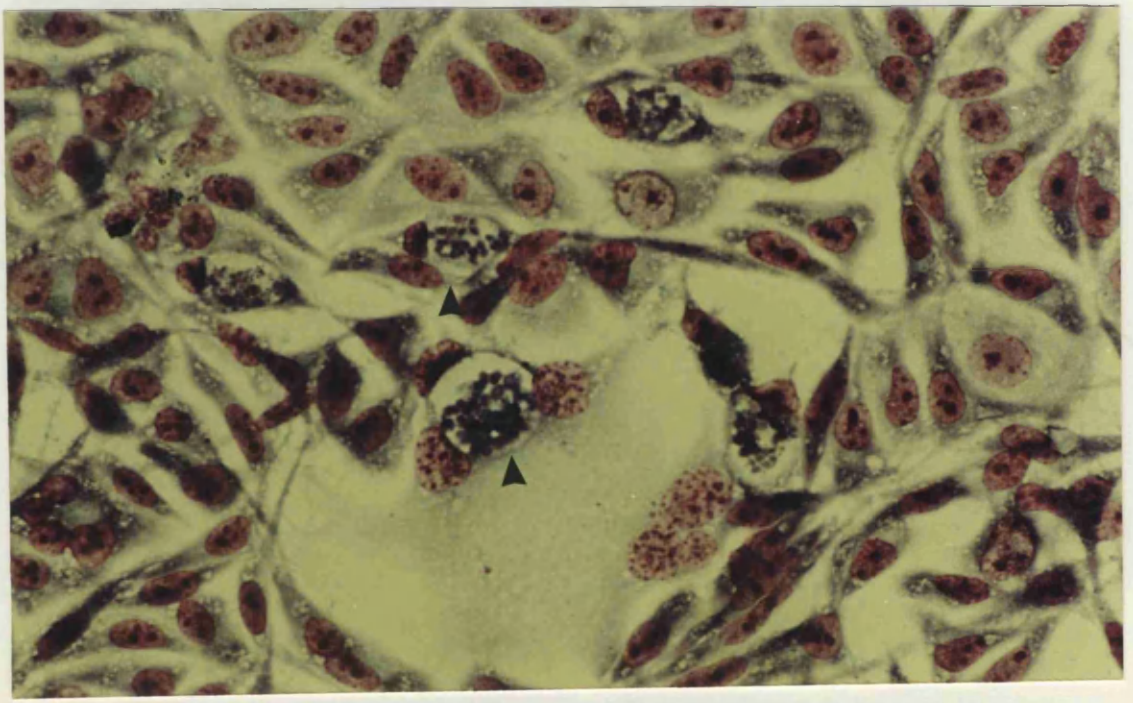

fig 4.32. Giemsa stained inclusions (BFM) with 0.125µg/ml penicillin added at PT24. Large and well defined inclusions containing beaded particles are present.

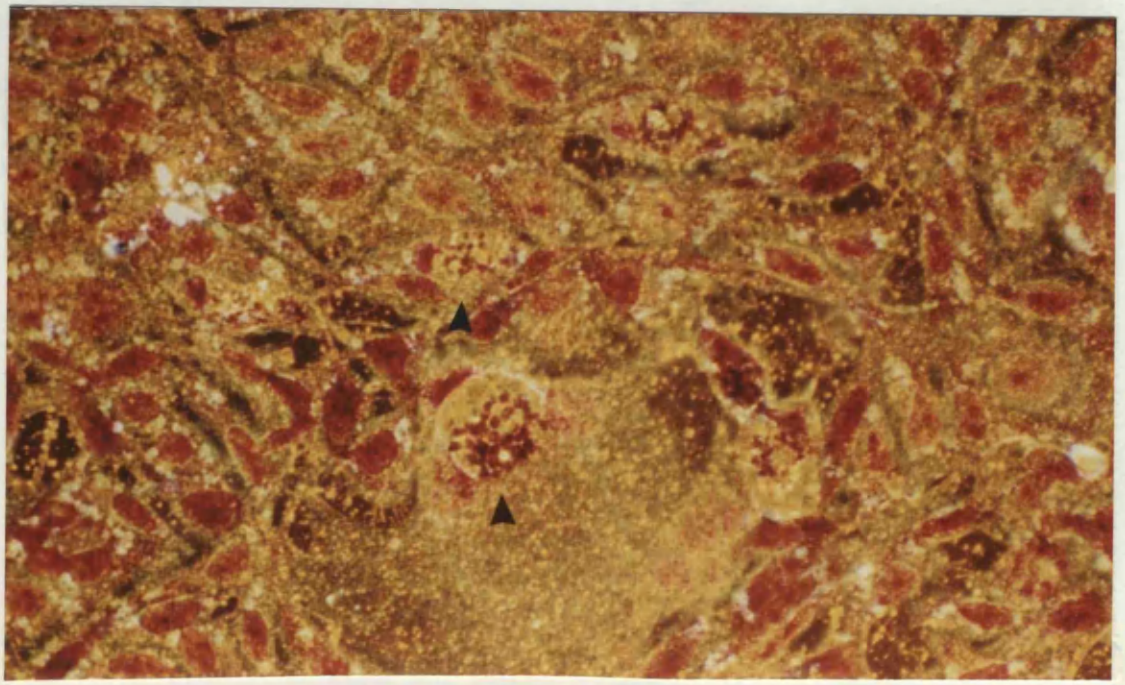

fig 4.33. Giemsa stained inclusions (DGM) with 0.125µg/ml penicillin added at PT24. Beaded particles do not autofluoresce.

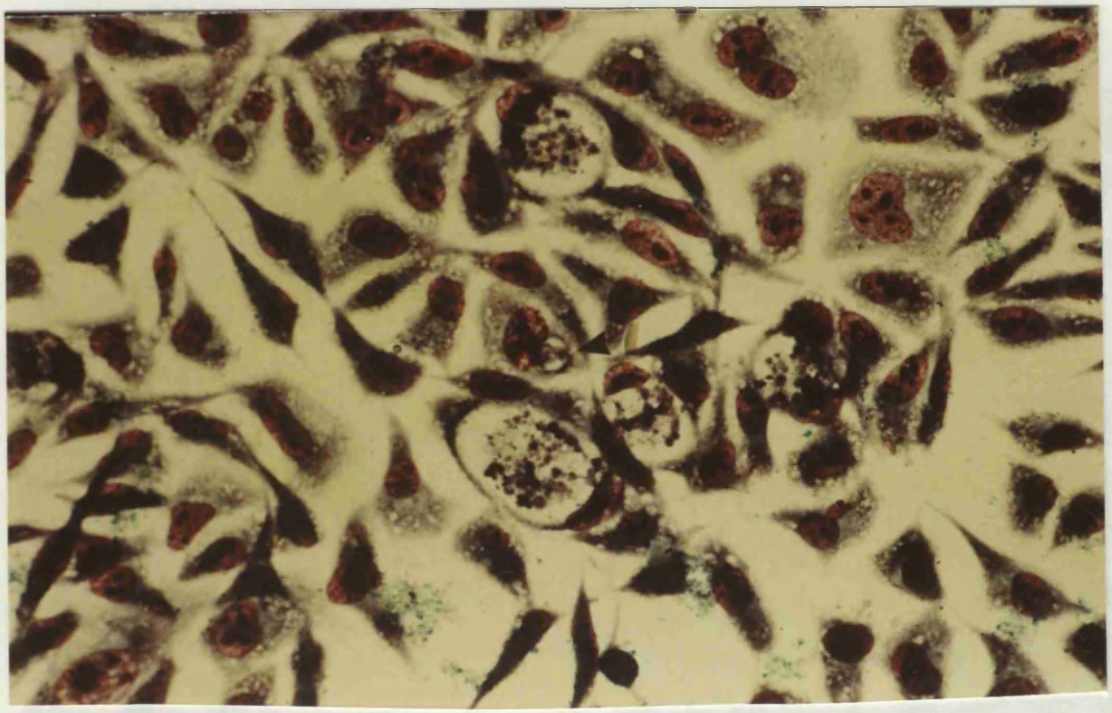

fig 4.34. Giemsa stained inclusions (BFM) with 0.125µg/ml penicillin added at PT30. Very large inclusions with beaded/mottled appearance are present. Arrow head shows a small inclusion with a smooth appearance.

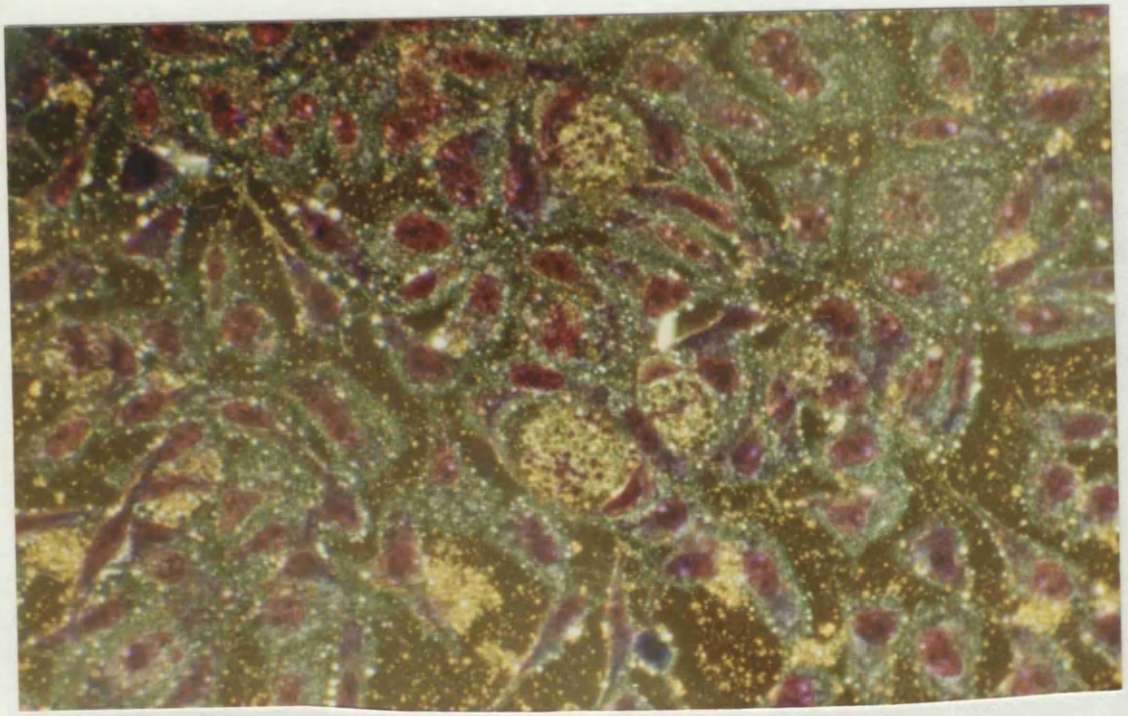

fig 4.35. Giemsa stained inclusions (DGM) with 0.125µg/ml penicillin added at at PT30. Only the mottled particles autofluoresce. The small inclusion seen by BMF does not autoflouresce.

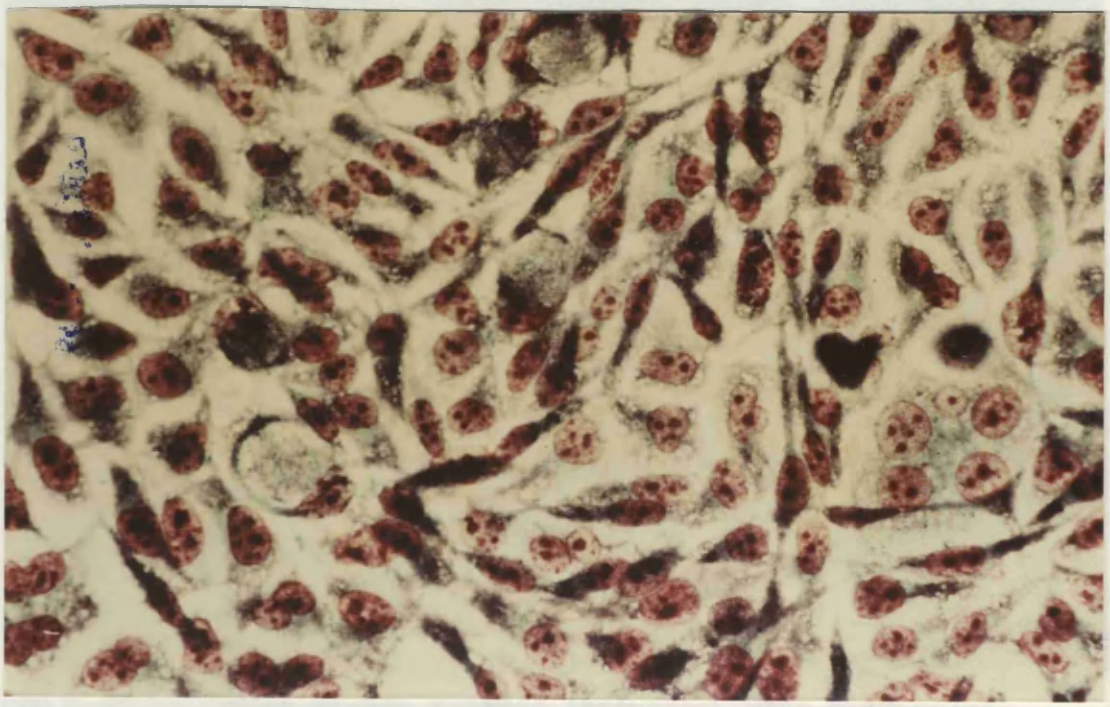

fig 4.36. Giemsa stained inclusions (BFM) with 0.125µg/ml penicillin added at PT40. Inclusions are well defined with a cotton wool appearance.

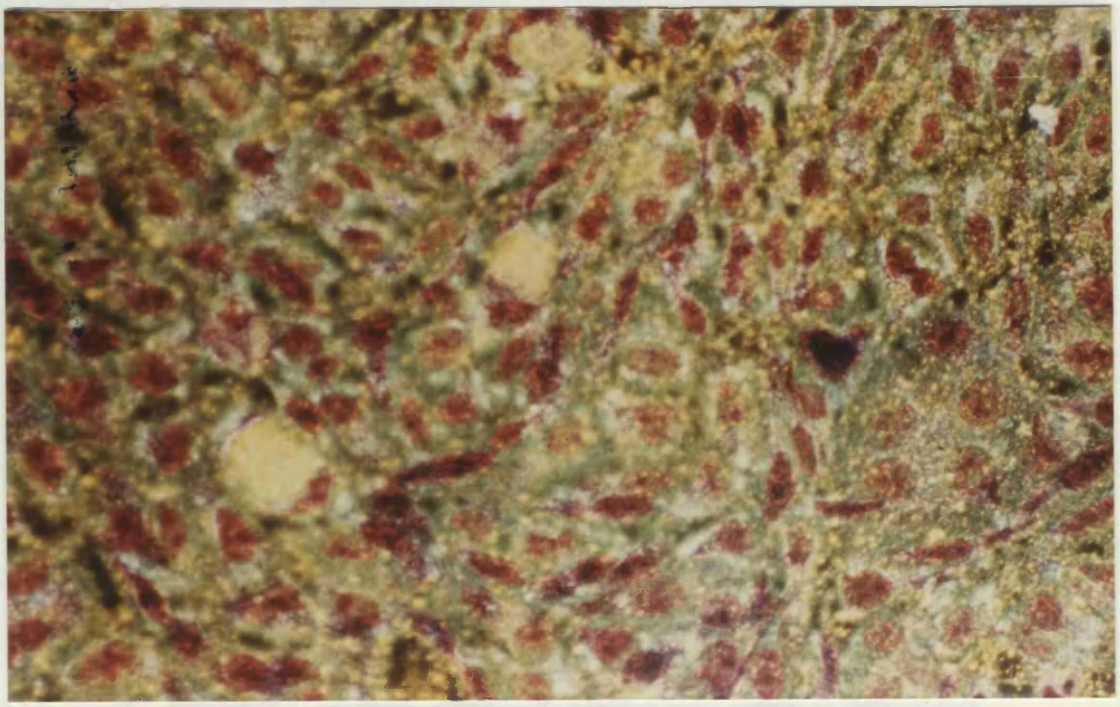

fig 4.37. Giemsa stained inclusions (DGM) with 0.125µg/ml penicillin added at PT40. Inclusions exhibit autofluorescence.

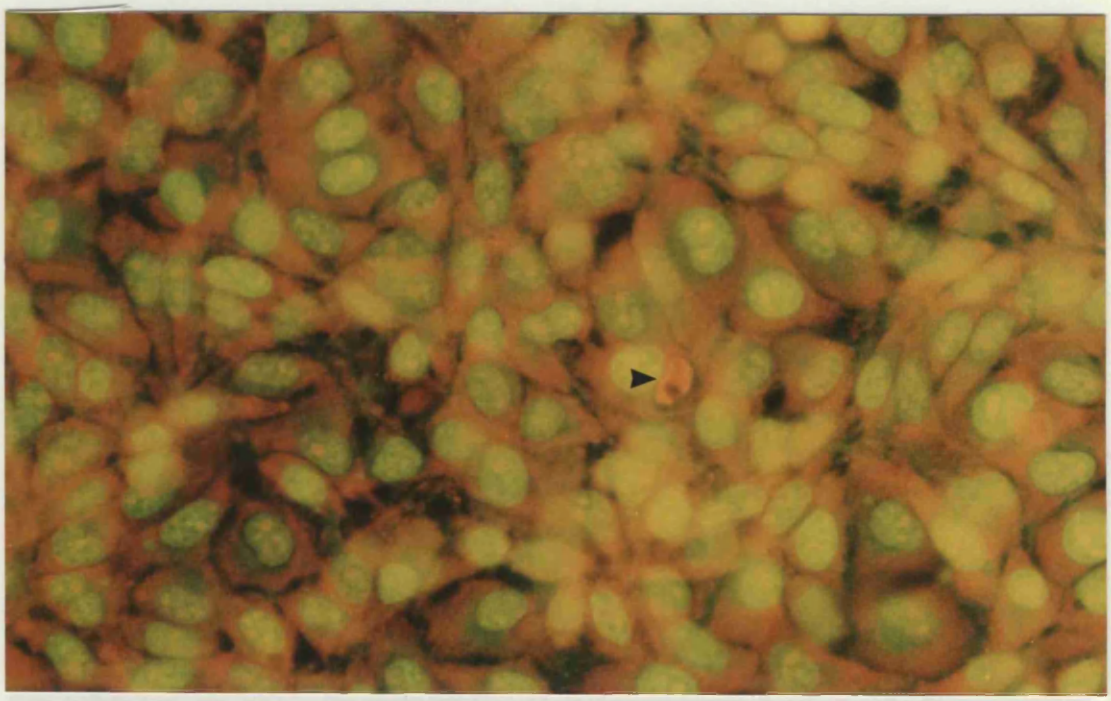

fig 4.38. Acridine orange stained inclusions with 0.125µg/ml penicillin added at PT0. Inclusions are small and smooth in appearance containing unevenly orange (RNA) stained material.

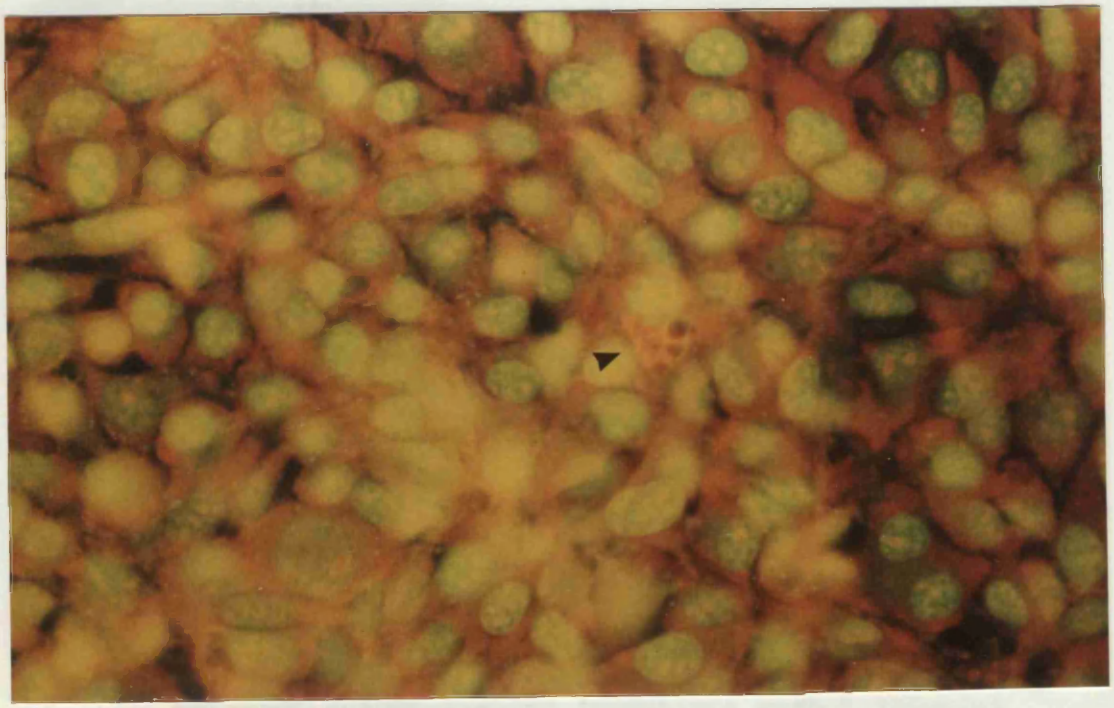

fig 4.39. Acridine orange stained inclusion with 0.125µg/ml penicillin added at PT16. Inclusions are large and smooth in strucure containing unevenly stained material.

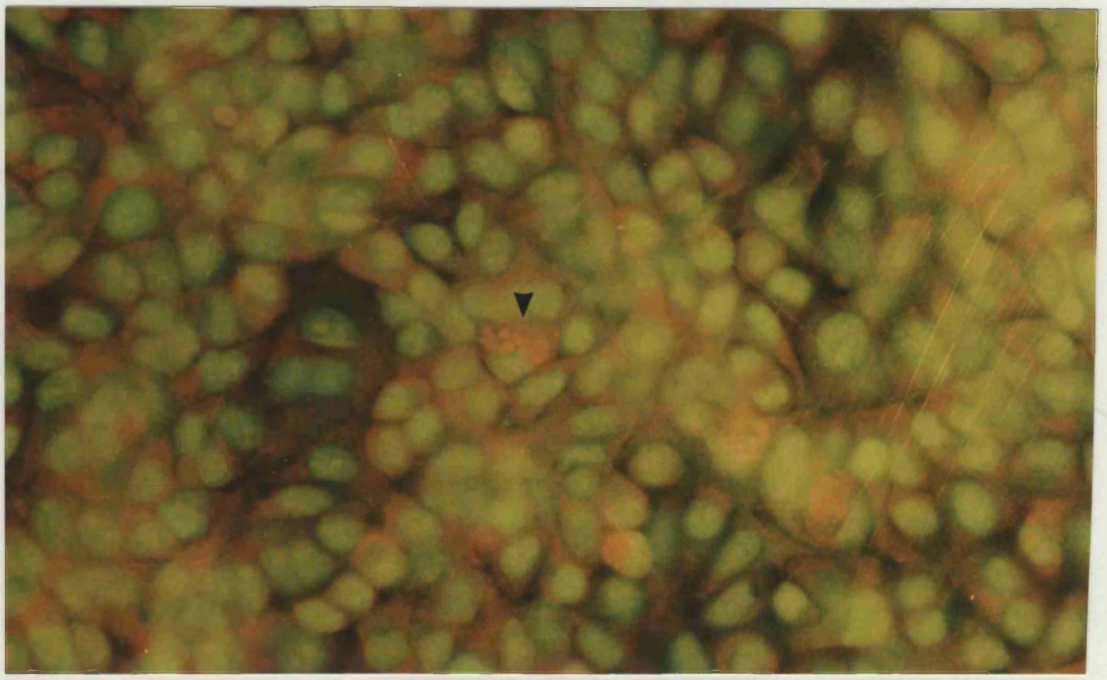

fig 4.40. Acridine orange stained inclusions with 0.125µg/ml penicillin added at PT24. Inclusions are large and globular and contain orange (RNA) and a small amount of green (DNA) stained material.

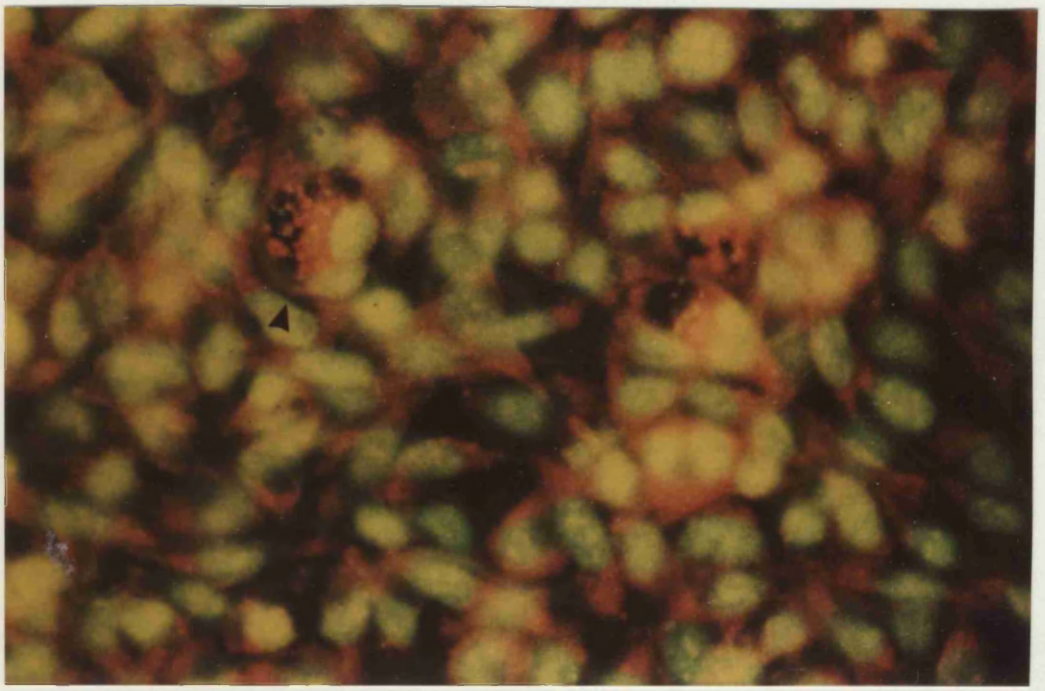

fig 4.41. Acridine orange stained inclusions with 0.125µg/ml penicillin added at PT30. Inclusions are large and beaded in appearance containing both orange (RNA) and green (DNA) stained material.

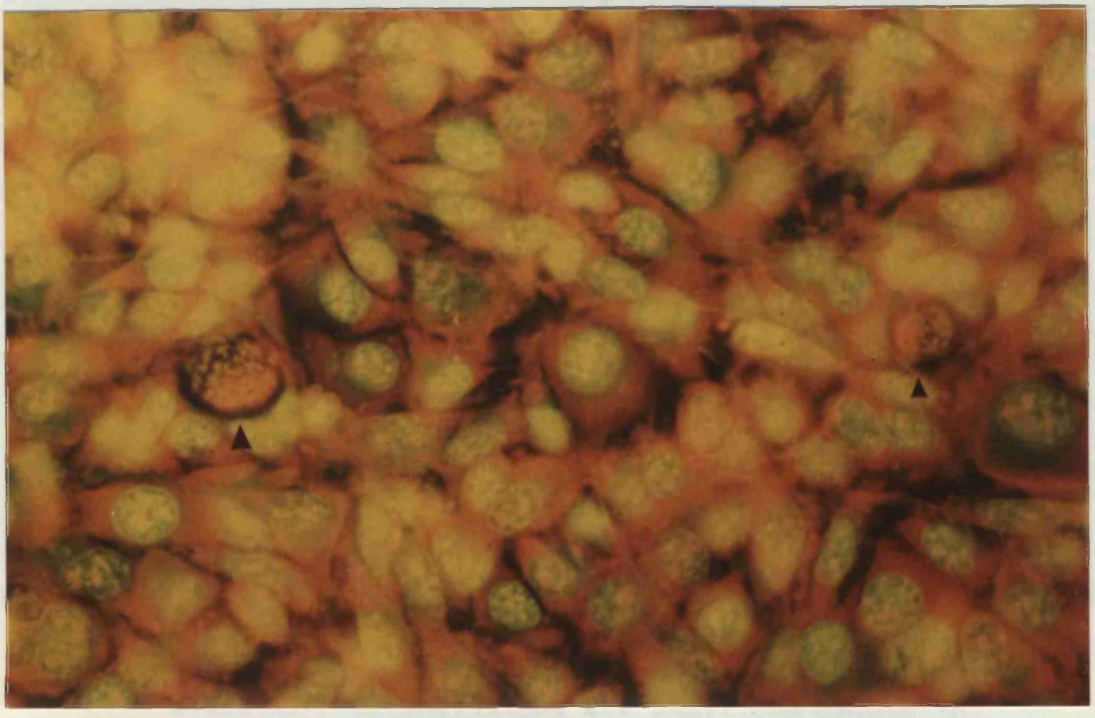

fig 4.42. Acridine orange stained inclusions with 0.125µg/ml penicillin added at PT40. Inclusions are normal in appearance having both RNA and DNA materials.

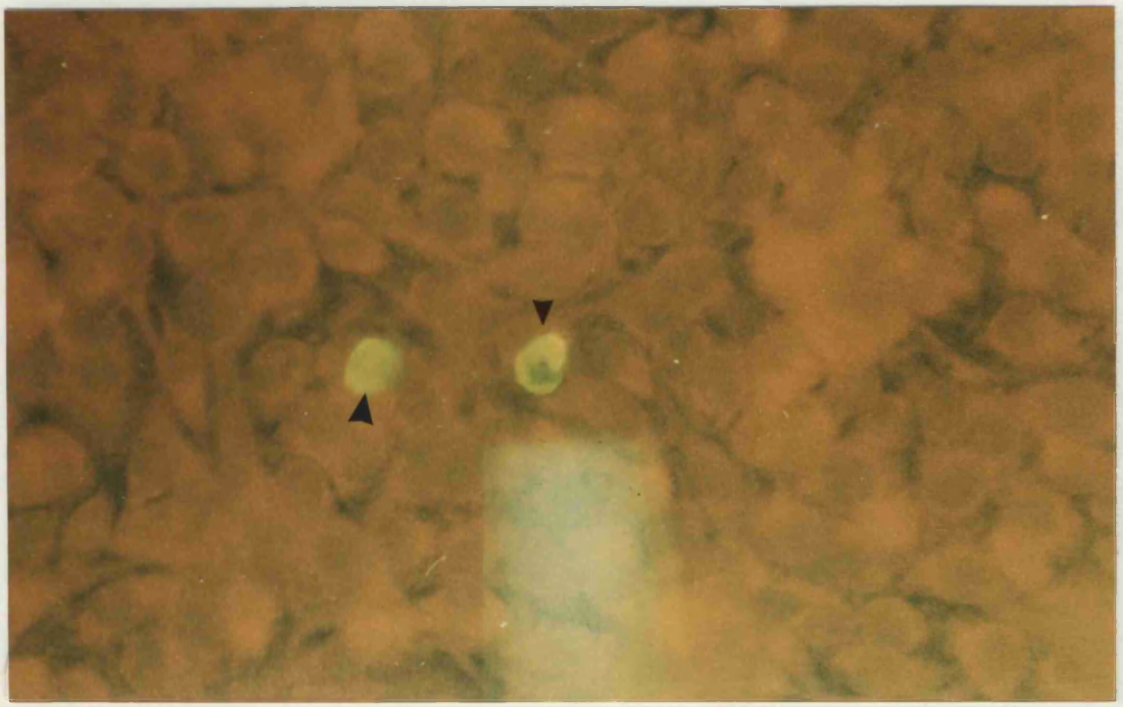

fig 4.43. Fluorescent stained inclusions with 0.125µg/ml penicillin added at PTO. Inclusions are small and smooth in appearance.

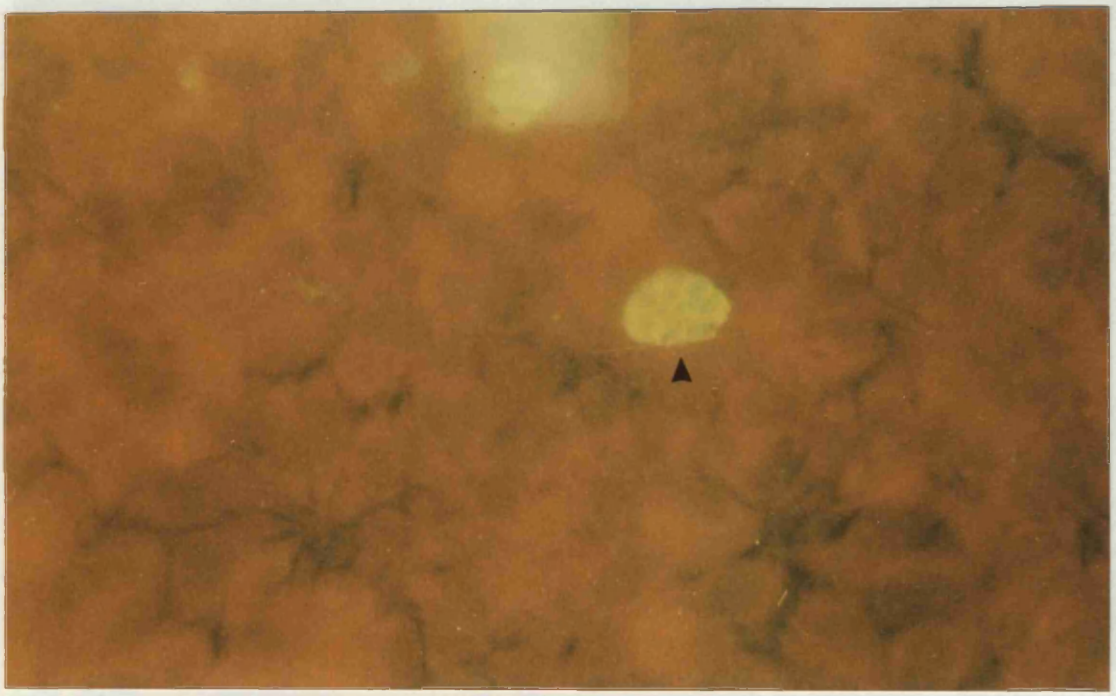

fig 4.44. Fluorescent stained inclusions with 0.125µg/ml penicillin added at PT16. Inclusions are large and appear to contain several unevenly stained areas.

fig 4.45. Fluorescent stained inclusions with 0.125µg/ml penicillin added at PT24. Inclusions are large with globular appearance.

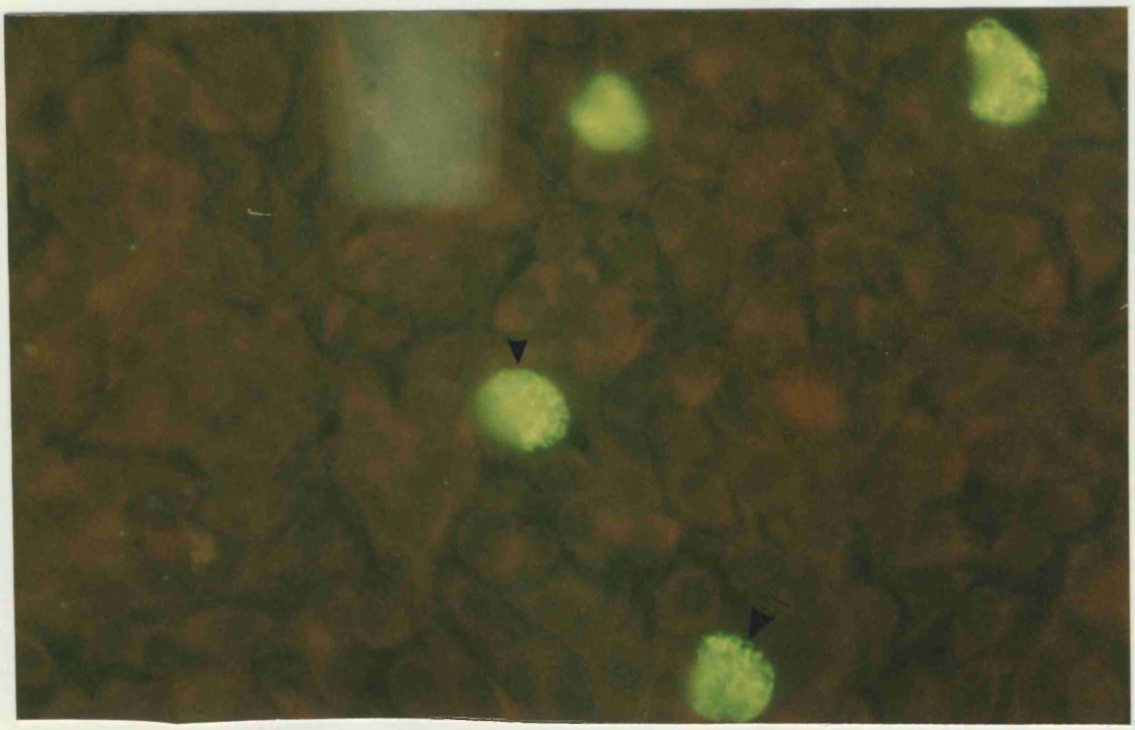

fig 4.46. Fluorescent stained inclusions with 0.125µg/ml penicillin added at PT30. Large inclusions appear to be made up from very small hollow discs.

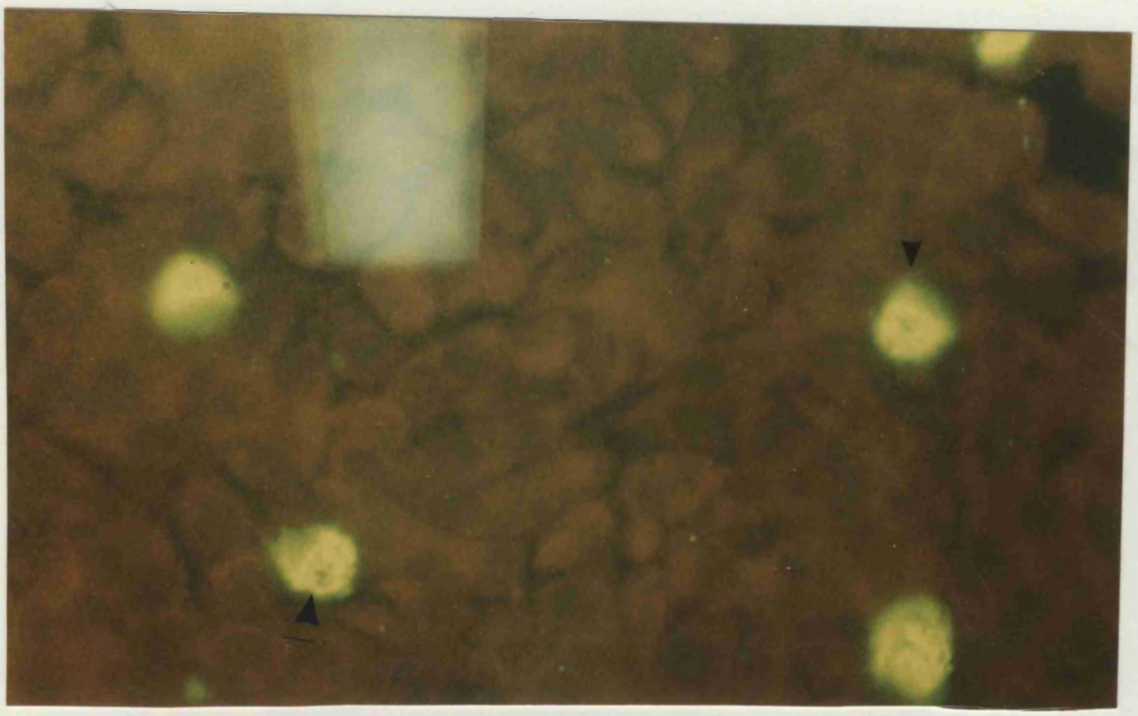

fig 4.47. Fluorescent stained inclusions with 0.125µg/ml penicillin added at PT40. Inclusions are large showing reticulate structure.

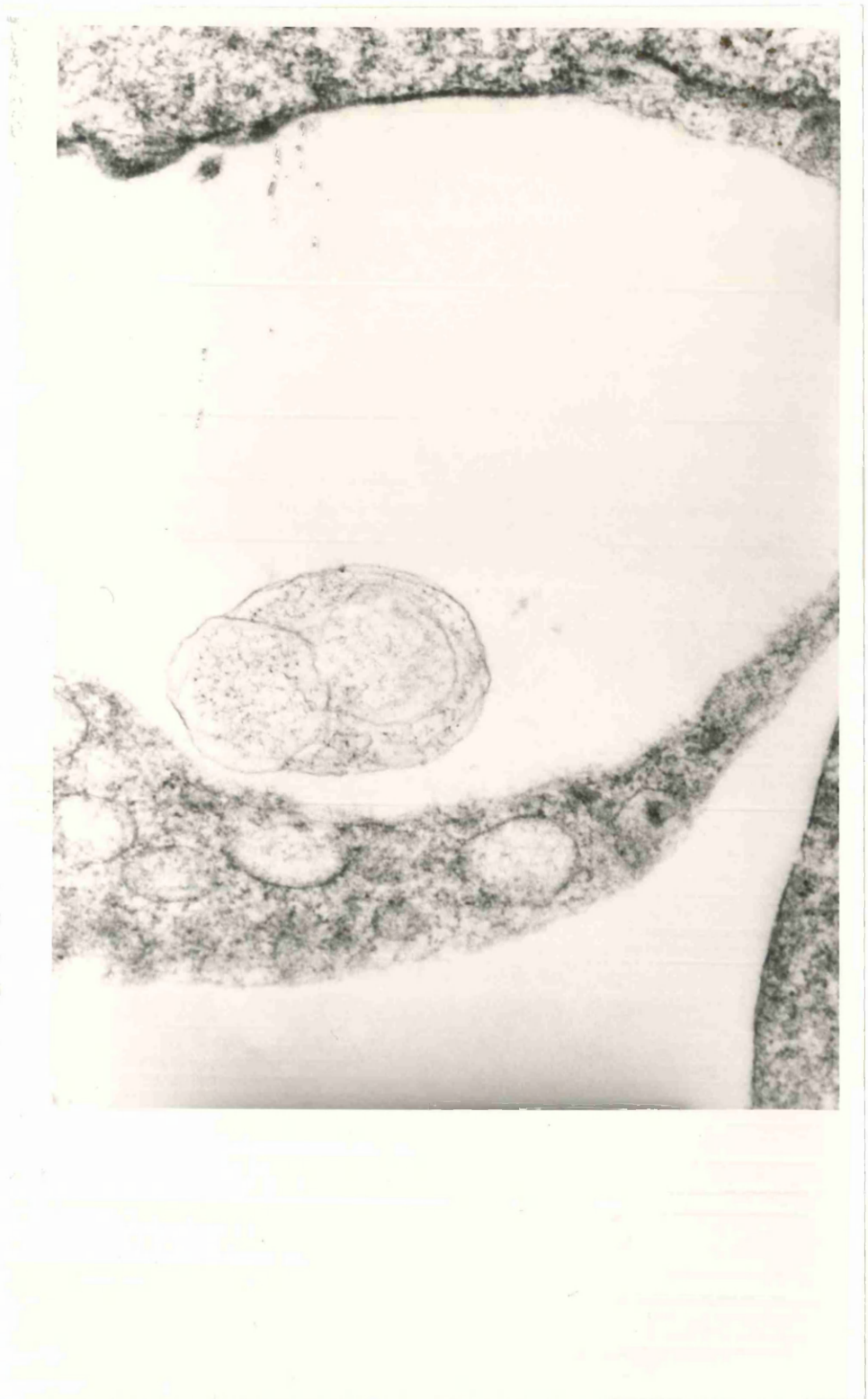

fig 4.48. Electron micrograph of the content of an inclusion with 0.125µg/ml penicillin added at PTO. The reticulate body in this inclusion shows abnormal division (X40000).

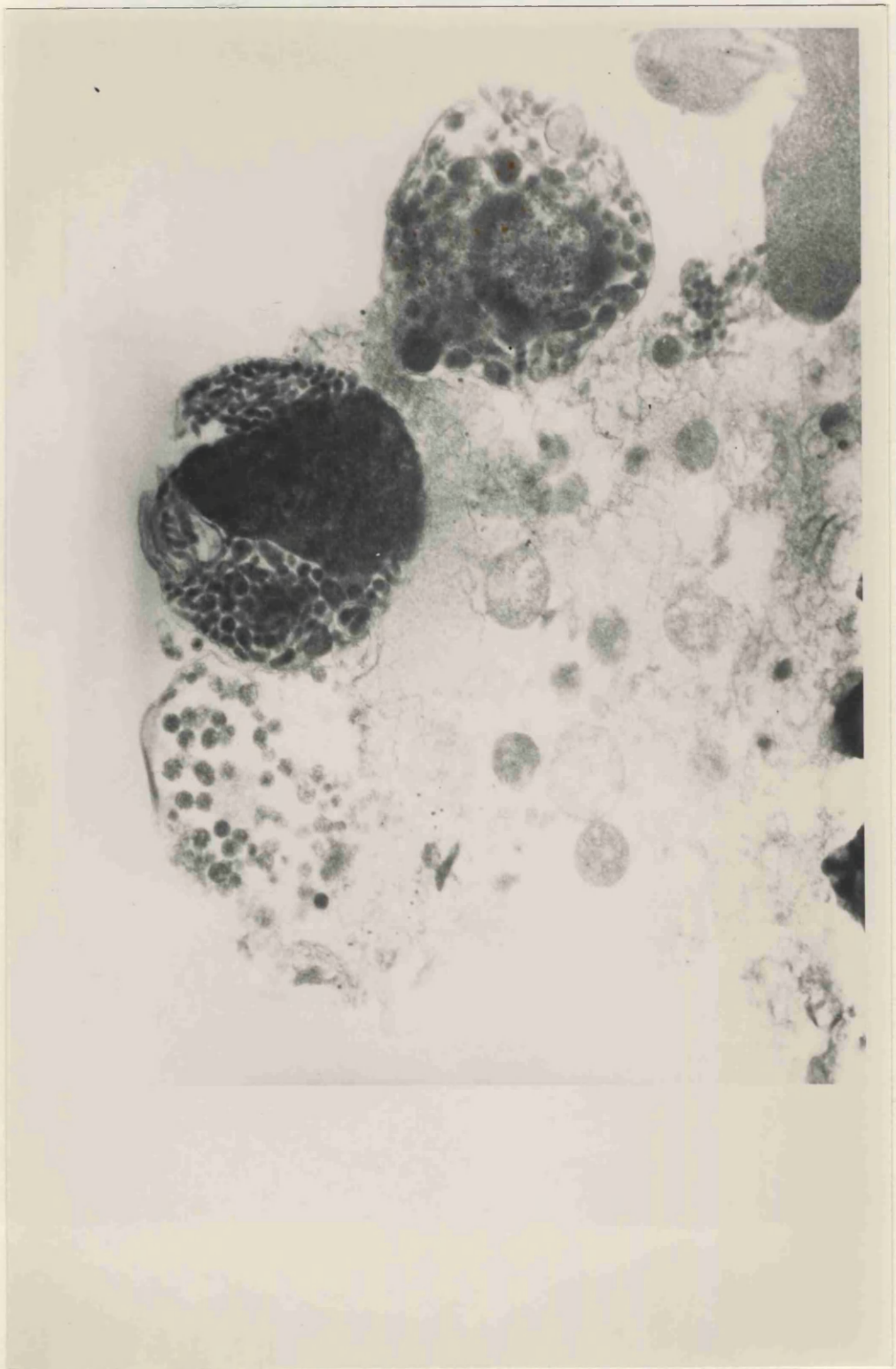

fig 4.49. Electron micrograph of the contents of an inclusion with 0.125µg/ml penicillin added at PT16. Giant bodies with granular cytoplasm are present (X20000).

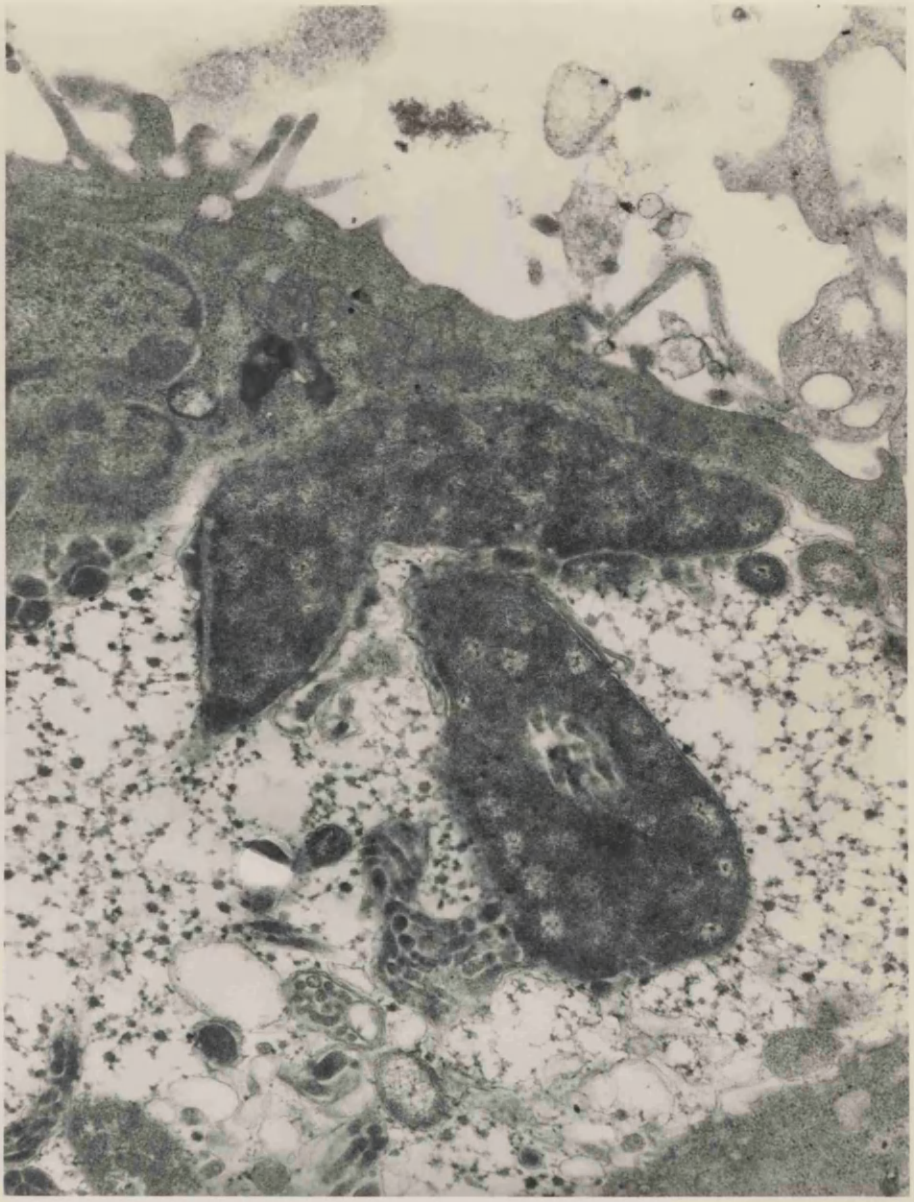

fig 4.50. Electron micrograph of the contents of an inclusion with 0.125µg/ml penicillin added at PT24. Very globular giant bodies are present. One giant body contains a large electron transparent area. Rosettes of glycogen are also seen (x30000).

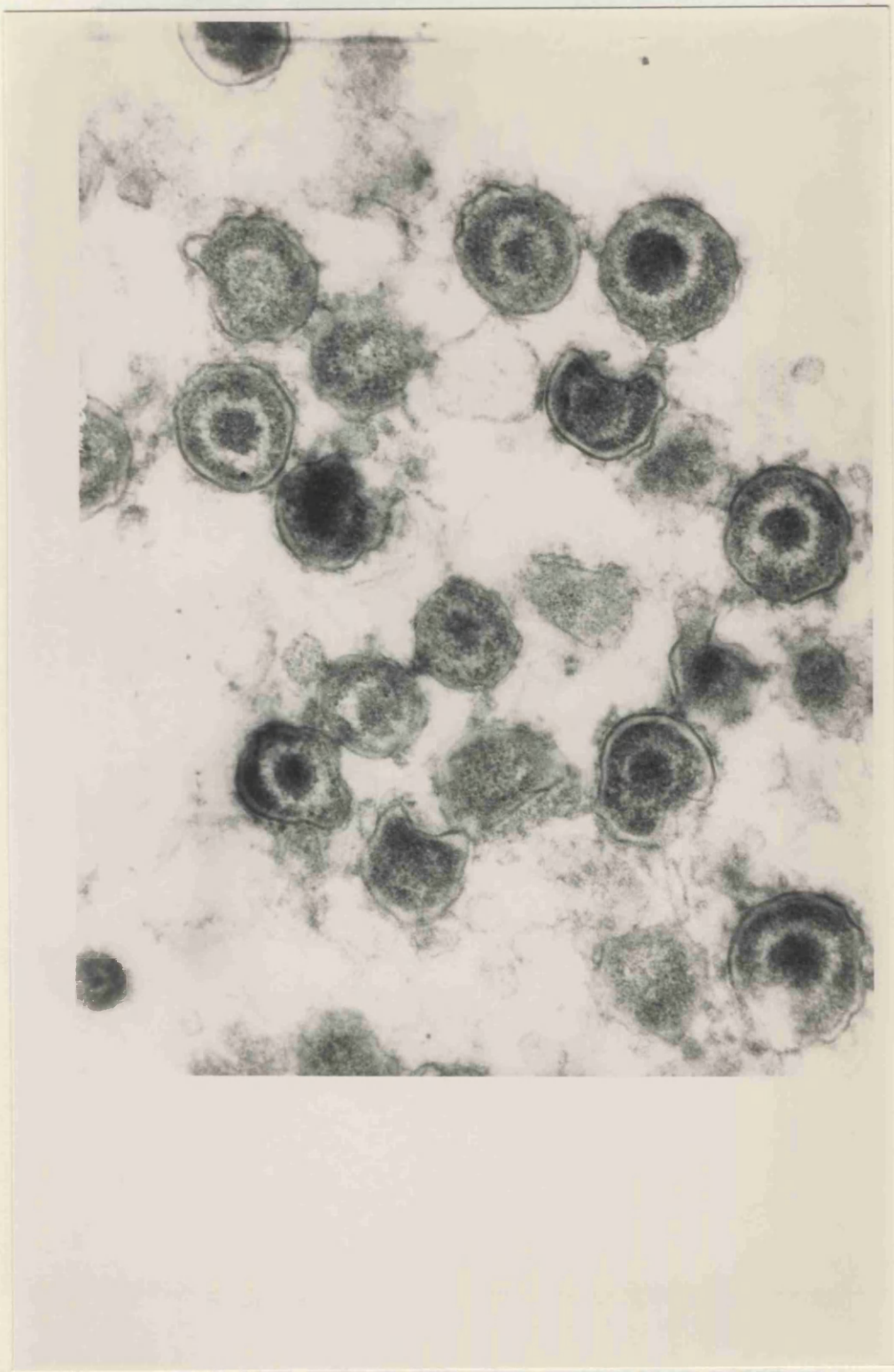

fig 4.51. Electron micrograph of the contents of an inclusion with 0.125µg/ml penicillin added at PT30. A large number of intermediate bodies exhibiting bull's eye appearance are present (X600000).

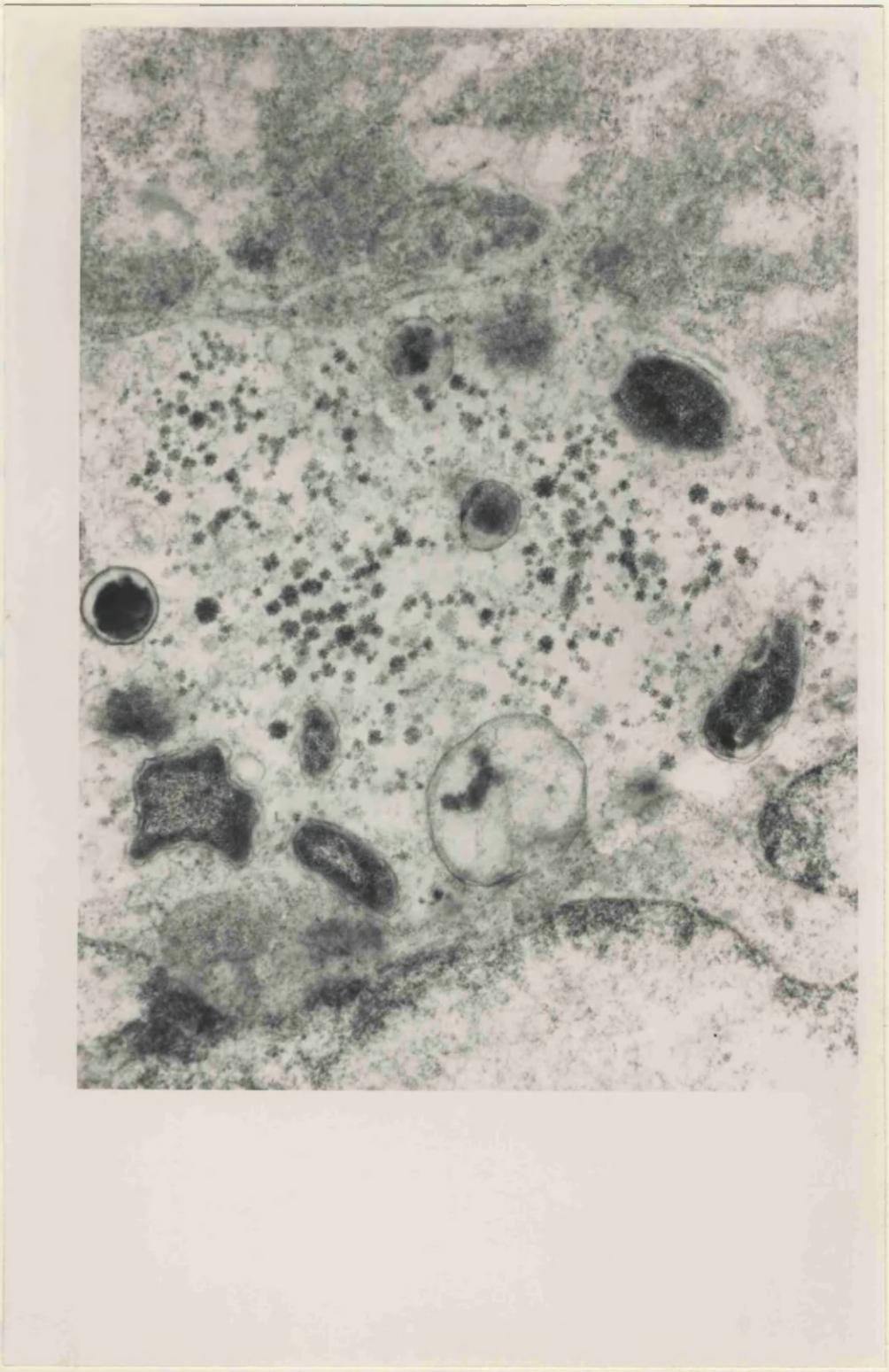

fig 4.52. Electron micrograph of the contents of an inclusion with 0.125µg/ml penicillin added at PT40. The inclusion contains a large number of glycogen rosettes as well as some elementary and intermediate bodies (X40000).

4.1.5 TIME LAPSE PHOTOGRAPHY.

Every 3 minutes, one photograph of the infected cells was taken. The difference in the time from the moment of the appearance of the inclusion to its bursting, was calculated by counting the number of photographic frames. Table 4.1. shows the time taken for six different inclusions from appearance to burst.

INCLUSION NUMBER	NUMBER OF FRAMES FROM APPEARANCE TO BURST	TIME TAKEN FROM APPEARANCE TO BURST hr.min
1	576	28.48
2	560	28.00
3	480	24.00
4	1072	53.36
5	336	16.48
6	1031	51.30

TABLE 4.1. THE NUMBER OF FRAMES AND THE TIME TAKEN FOR 6 INCLUSIONS FROM APPEARANCE TO BURST.

The time taken for the actual process of bursting was also calculated, and is shown in table 4.2. This time was calculated from the instant there was a visibly dramatic change in the morphology of the cells.

INCLUSION NUMBER	NUMBER OF FRAMES TAKEN FOR BURST	TIME TAKEN FOR BURST min
1	1	<3
2	3	9
3	4	12
4	14	42
5	16	48
6	62	183

TABLE 4.2. TIME TAKEN IN MINUTES FOR 6 DIFFERENT INCLUSIONS TO BURST.

fig 4.53. The initial appearance of an inclusion, captured by time lapse photography (TLP). The inclusion, indicated with an arrow head, is seen as a small dimple on the surface of the cell.

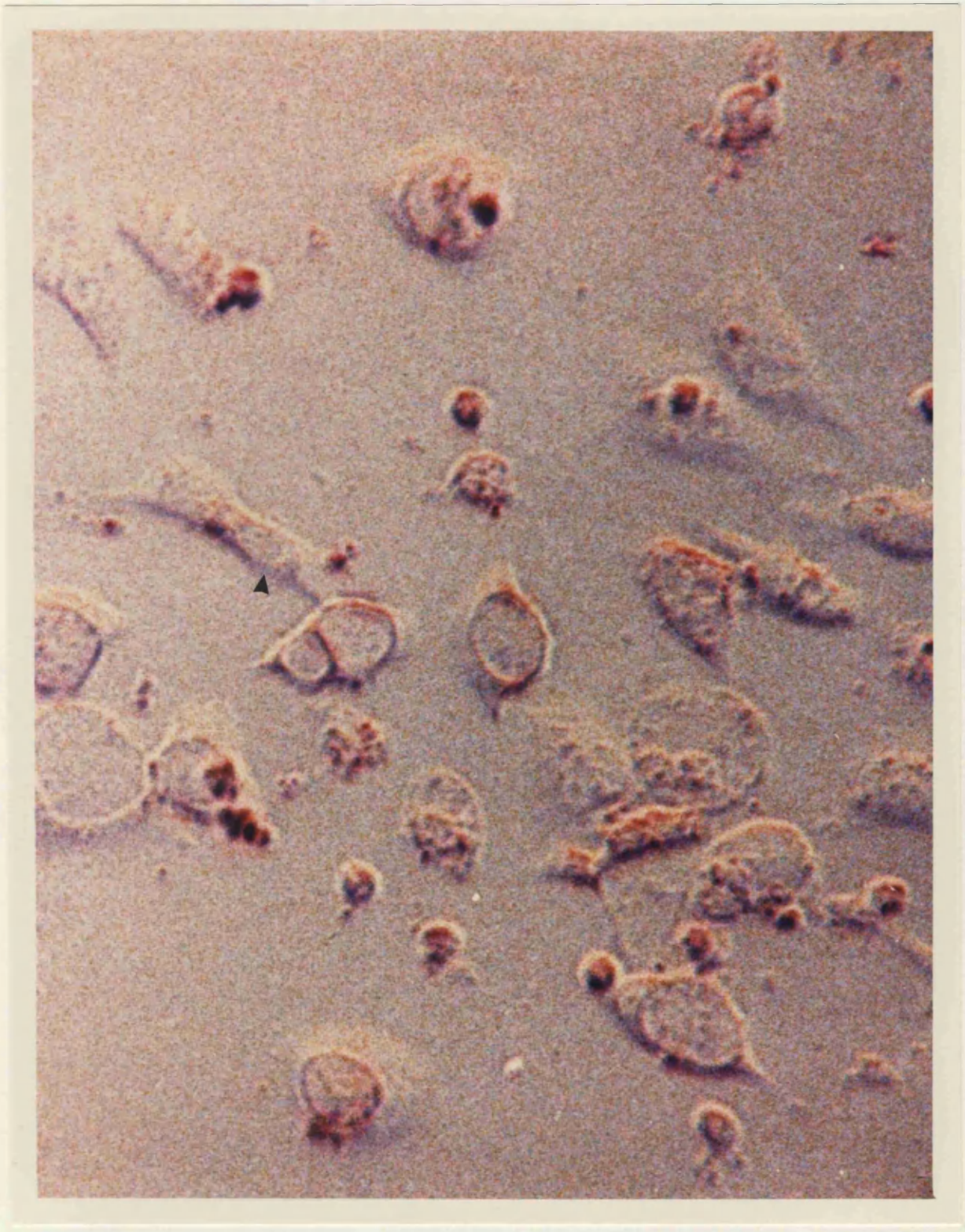

fig 4.54. TLP of the same inclusion at a later stage of it's life cycle. The inclusion is larger and easily recognisable.

fig 4.55. TLP. Later stage in the life cycle of the same inclusion.

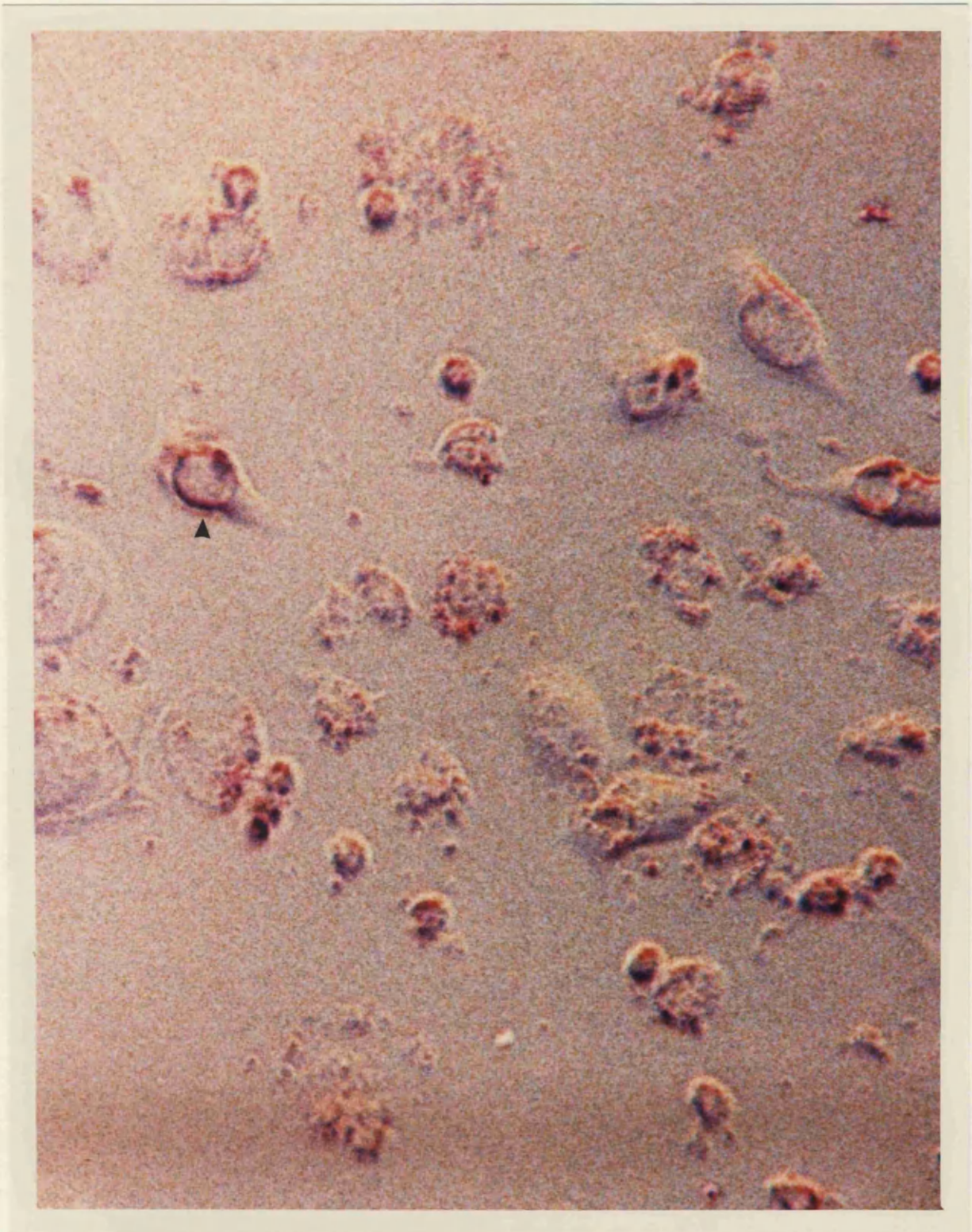

fig 4.56. TLP during the pulsating stage of the inclusion showing the beginnings of a drastic morphological change prior to burst.

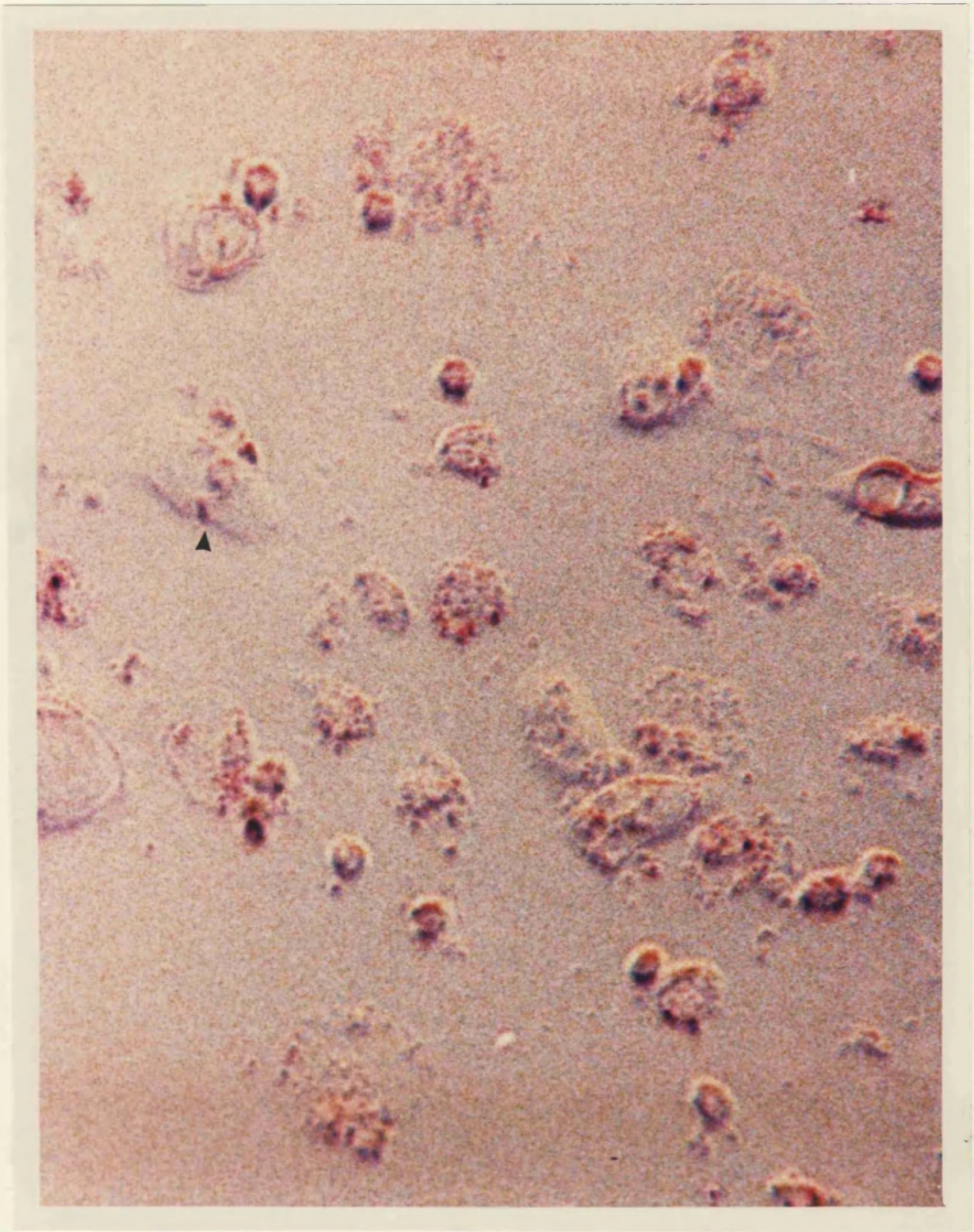

fig 4.57. TLP of the cell containing the inclusion at the moment of burst.

4.2. RESULTS OF THE NOVEL CELL CULTURE TECHNIQUE.

For the purposes of this study, the tissue culture adapted strain of LGV2, SA2f, was selected so as to examine for the first time the practicality of the microcarrier system. This strain grew very well in the system giving a high yield (over 3200x the original inoculum).

Isolate number 25088 yielded over 4X the original inoculum, whilst isolate 81591 gave almost similar numbers of inclusions as originally inoculated. See table 4.3.

	CONTROL a LEFT AT 37°C FOR 48 HRS CENTRIFUGED	CONTROL b ORIGINAL INOCULUM CENTRIFUGED	c HARVEST MATERIAL 1 CENTRIFUGED	d HARVEST MATERIAL 2 NOT CENTRIFUGED
	IFU/ML	IFU/ML	IFU/ML	IFU/ML
SA2F	0	200	>2000 (AT 1:320 DILUTION)	ND
ISOLATE 25088	0	600	>2500 (NEAT)	37
ISOLATE 81591	0	540	550	5

TABLE 4.3. INCLUSION COUNT OF C.TRACHOMATIS BEFORE AND AFTER MICROCARRIER CULTURE.

Key: ND = Not done.

4.3. RESULTS OF STUDIES ON SENSITIVITY TO ANTIMICROBIAL AGENTS.

4.3.1. TETRACYCLINES.

Table 4.4. shows the MICs of minocycline, doxycycline and oxytetracycline against SA2f. Table 4.5. shows the MICs of these agents against SA2f and 10 clinical isolates compared with erythromycin.

	ANTIBIOTIC CONCENTRATION (µg/ml)					
	.015	.03	.06	.125	.25	
ANTIBIOTICS						MIC
OXYTETRACYCLINE	+	+	-	-	-	.06
MINOCYCLINE	+	-	-	-	-	.03
DOXYCYCLINE	+	+	-	-	-	.06

TABLE 4.4. THE MICs OF OXYTETRACYCLINE, MINOCYCLINE AND DOXYCYCLINE AGAINST SA2f.

ANTIBIOTICS	ANTIBIOTIC CONCENTRATION (µg/ml) .015	.03	.06	.125	TOTAL NUMBER OF STRAINS
OXYTETRACYCLINE	0	0	6*	5	11
MINOCYCLINE	5	6*	0	0	11
DOXYCYCLINE	0	0	4*	6	11
ERYTHROMYCIN	0	2	9*	0	11

TABLE 4.5. THE IN-VITRO ACTIVITY OF 3 TETRACYCLINES AND ERYTHROMYCIN AGAINST 10 CLINICAL ISOLATES OF C.TRACHOMATIS.

Key: * = Includes control strain (SA2f)

4.3.2. MACROLIDES.

The MICs of eight macrolides (ICI-187642, josamycin, roxithromycin, miocamycin, erthromycin, azithromycin, ER-42859 and spiramycin) were estimated against SA2f. These results are shown in Table 4.6.

Table 4.7. shows the results of MBC of roxithromycin against SA2f.

Table 4.8. shows the results of MBC of miocamycin against SA2f.

Table 4.9. shows the in-vitro activities of oxytetracycline, azithromycin, miocamycin, roxithromycin and erythromycin against 10 clinical isolates of C.trachomatis.

ANTIBIOTICS	ANTIBIOTIC CONCENTRATION (µg/ml)						MIC
	.015	.03	.06	.125	.25	.5	
OXYTETRACYCLINE	+	+	-	-	-	-	.06
ICI-187642	+	-	-	-	-	-	.03
JOSAMYCIN	+	-	-	-	-	-	.03
ROXITHROMYCIN	+	-	-	-	-	-	.03
MIOCAMYCIN	+	+	-	-	-	-	.06
ERYTHROMYCIN	+	+	-	-	-	-	.06
AZITHROMYCIN	+	+	+	-	-	-	.125
ER-42859	+	+	+	+	-	-	.25
SPIRAMYCIN	+	+	+	+	+	-	.5

TABLE 4.6. ACTIVITIES OF EIGHT MACROLIDES AND OXYTERACYCLINE AGAINST SA2f.

Key: + = Inclusions seen by iodine and/or Giemsa stain.

- = No inclusions seen.

PASSAGE NUMBERS	ANTIBIOTIC CONCENTRATION (µg/ml)								
	.015	.03	.06	.125	.25	.5	1	2	MBC(*)
1	+	-	-	-	-	-	-	-	
2		+	-	-	-	-	-	-	
3		+	-	-	-	-	-	-	
4				-	-	-	-	-	
5				-	-	-	-	-	
6				-	-	-	-	-	
7				-	-	-	-	-	
8				-	-	-	-	-	
9				-	-	-	-	-	
10				-	-	-	-	-	.06(2)

TABLE 4.7. MBC OF ROXITHROMYCIN AGAINST SA2f.

Key: (*) = Stable after passage number.

PASSAGE NUMBERS	ANTIBIOTIC CONCENTRATION (µg/ml)								
	.015	.03	.06	.125	.25	.5	1	2	MBC(*)
1	+	+	+	+	-	-	-	-	
2	+	+	+	+	+	+	-	-	
3	+	+	+	+	+	+	-	-	
4							-	-	
5							-	-	
6							-	-	
7							-	-	
8							-	-	
9							-	-	
10							-	-	1(2)

TABLE 4.8. MBC OF MIOCAMYCIN AGAINST SA2f.

Key: + = Inclusions seen by iodine and/or Giemsa stain.

- = No inclusion seen.

(*) = Stable after passage number.

ANTIBIOTICS	ANTIBIOTIC CONCENTRATION (μg/ml) .015	.03	.06	.125	.25	TOTAL NUMBER OF STRAINS
OXYTETRACYCLINE			6*	5		11
AZITHROMYCIN			2	4*	5	11
MIOCAMYCIN			9*	2		11
ROXYTHROMYCIN	1	7*	3			11
ERYTHROMYCIN		1	10*			11

TABLE 4.9. IN-VITRO ACTIVITIES OF OXYTETRACYCLINE, AZITHROMYCIN, MIOCAMYCIN, ROXITHROMYCIN AND ERYTHROMYCIN AGAINST 10 CLINICAL ISOLATES OF C.TRACHOMATIS

Key: * = Includes the control strain SA2f.

Resistance development to roxithromycin:

The experiment with roxithromycin was performed on two occasions.

On the first occasion, the organism grew in 0.03 µg/ml roxithromycin at passage 5. Subsequent passages at this concentration yielded no visible inclusions. Passage 7 was then passed into both 0.03 µg/ml and 0.015 µg/ml of antibiotic. Inclusions (passage 8) were found in 0.015 µg/ml, but not in 0.03 µg/ml of the antibiotic. The experiment was, therefore, terminated and repeated.

On the second occasion, the organism was growing poorly at passage 2 in 0.06 µg/ml of roxithromycin. However, it could not be maintained in this concentration. Indeed, subsequent passages at 0.015 µg/ml were required to recover the organism before it again became possible to maintain it in 0.06 µg/ml of antibiotic (passage 11). The organism survived passages 12 and 13 at this concentration, but was growing poorly with few inclusions. Passage 14 contained no inclusions in 0.06 µg/ml, and very scanty inclusions in 0.03 µg/ml of antibiotic. Growth of the organism in 0.012 µg/ml (MBC) of roxithromycin could not be achieved. Table 4.10. summarizes the results of this experiment.

	INITIAL MIC	HIGHEST INHIBITORY CONCENTRATION	*NUMBER OF PASSAGES
	(µg/ml)	(µg/ml)	(DAYS)
Roxithromycin (EXPERIMENT 1)	0.03	0.06	8(18)
Roxithromycin (EXPERIMENT 2)	0.03	0.12	14(32)

TABLE 4.10. DEVELOPMENT OF RESISTANCE OF C.TRACHOMATIS (SA2f) TO ROXITHROMYCIN

Key: * = Total number of passages to reach highest non-inhibitory concentration.

4.3.3. QUINOLONES.

Table 4.11. shows the MIC results of Abbott 56619, Abbott 56620, ofloxacin, C1-934, ciprofloxacin, RO 23-6240 (fleroxacin), NY198 (lomefloxacin), S25930, enoxacin, pefloxacin, WIN 49375, rosoxacin, S25932, norfloxacin, flumequine, WIN 35610 and nalidixic acid. The inclusions of Chlamydia, near the MIC values of all quinolones, exhibited small "pin head" appearance.

Table 4.12. shows the MBC results for ofloxacin, CI-934, ciprofloxacin, lomefloxacin and rosoxocin.

Table 4.13. shows the results of the MICs of ofloxacin, ciprofloxacin, lomefloxacin and rosoxacin against 10 clinical isolates of C.trachomatis.

ANTIBIOTICS	ANTIBIOTIC CONCENTRATION (µg/ml) .03	.06	.125	.5	1	2	4	8	16	32	64	MIC
ABBOTT 56619	+	+	-									.125
ABBOTT 56620	+	+	+	-								.5
OFLOXACIN	+	+	+	-								.5
CI-934	+	+	+	-								.5
CIPROFLOXACIN	+	+	+	+	-							1
RO 236240	+	+	+	+	+	-						2
LOMEFLOXACIN	+	+	+	+	+	+	-					4
S 25930	+	+	+	+	+	+	+	-				8
ENOXACIN	+	+	+	+	+	+	+	-				8
PEFLOXACIN	+	+	+	+	+	+	+	-				8
WIN 49375	+	+	+	+	+	+	+	-				8
ROSOXACIN	+	+	+	+	+	+	+	-				8
S 23932	+	+	+	+	+	+	+	+	-			16
NORFLOXACIN	+	+	+	+	+	+	+	+	-			16
FLUMEQUINE	+	+	+	+	+	+	+	+	+	+	-	64
WIN 35610	+	+	+	+	+	+	+	+	+	+	+	>64
NALIDIXIC ACID	+	+	+	+	+	+	+	+	+	+	+	>64

TABLE 4.11. MICs OF 17 QUINOLONES AGAINST SA2f.

Key: + = Inclusions seen by iodine and /or Giemsa stain.

- = No inclusions seen.

The MBCs of the compounds ofloxacin, CI-934, ciprofloxacin and rosoxacin were estimated and recorded on charts similar to the one shown for roxithromycin and miocamycin. The summary of these results is shown below.

ANTIBIOTICS	µg/ml MBC	STABLE AFTER PASSAGE NUMBER
OFLOXACIN	.5	1
CI-934	.5	1
CIPROFLOXACIN	1	1
LOMEFLOXACIN	4	1
ROSOXACIN	8	4

TABLE 4.12. SUMMARY OF THE MBC RESULTS OF 4-QUINOLONES AGAINST SA2f.

The MICs of ofloxacin, ciprofloxacin, lomefloxacin and rosoxacin against 10 clinical isolates of C.trachomatis was estimated and the results are shown in the table below.

	ANTIBIOTIC CONCENTRATION (µg/ml)					
ANTIBIOTICS	.5	1	2	4	8	TOTAL NUMBER OF STRAINS
OFLOXACIN	11*					11
CIPROFLOXACIN		6*	4			11
LOMEFLOXACIN			4*	7		11
ROSOXACIN				6	5*	11

TABLE 4.13. IN-VITRO ACTIVITIES OF 4-QUINOLONES AGAINST 10 CLINICAL ISOLATES OF C.TRACHOMATIS

Key: * Includes control strain SA2f.

4.3.4. BETA-LACTAMS.

i) AMOXYCILLIN AND CLAVULANIC ACID.

Table 4.14. shows the results of the MICs of each of Amoxycillin and clavulanic acid and combinations of clavulanic acid and amoxycillin.

	µg/ml						
ANTIBIOTICS	.25	.5	1	2	4	8	MIC
AMOXYCILLIN	±	±	±	!	!	!	2
CLAVULANIC ACID	±	±	±	±	!	!	4
AMOXYCILLIN + CLAVULANIC ACID	±	±	±	!	!	!	2
AUGMENTIN	±	±	±	!	!	!	2
OXYTETRACYCLINE							.06

TABLE 4.14. ACTIVITIES OF AMOXYCILLIN, CLAVULANIC ACID AND AUGMENTIN AGAINST SA2f.

Key: ± = Scanty number of inclusions by iodine and/or Giemsa stain.

! = A large drop in the number of inclusions and persistence of abnormal inclusions.

ii) CEPHALOSPORINS.

Cefotetan and RO 158074 behaved in a similar manner in producing typical granular inclusions characteristic of beta-lactams throughout the dilution range. Cephtriaxone, on the other hand, produced a sharp drop in the inclusion count around the MIC point with a small number of bizarre forms persisting throughout the dilution range. Table 4.15. summarizes the results of these agents against SA2f.

	ANTIBIOTIC CONCENTRATION (µg/ml)					
	4	8	16	32	4096	
ANTIBIOTICS						MIC
CEFOTETAN	+	+	+	+	+	>4000
RO 15074	+	+	+	+	ND	>32
CEPHTRIAXONE	+	!	!	!		8

TABLE 4.15. MICs OF THREE CEPHALOSPORINS AGAINST SA2f.

Key: + = Inclusions seen by iodine and/or Giemsa stain.

! = Persistence of abnormal inclusion.

ND = Not done.

4.3.5. AMINOCYCLITOLS.

Table 4.16. shows the MICs of the two agents as well as of the control oxytetracycline and the MBC result of trospectomycin.

	ANTIBIOTIC CONCENTRATION (µg/ml)	
	MIC	MBC(*)
ANTIBIOTICS		
TROSPECTOMYCIN	8	16 (2)
SPECTINOMYCIN	128	ND
OXYTETRACYCLINE	.06	

TABLE 4.16. ACTIVITIES OF TROSPECTINOMYCIN, SPECTINOMYCIN AND OXYTETRACYCLINE AGAINST SA2f.

Key: (*) = Stable after passage number.

ND = Not done

Table 4.17. shows the results of the MICs of 10 clinical isolates against trospectomycin.

	MIC (µg/ml)			
	8	16	32	
ANTIBIOTIC				TOTAL NUMBER
TROSPECTOMYCIN	9	2	0	11*

TABLE 4.17. IN-VITRO ACTIVITY OF TROSPECTOMYCIN AGAINST 10 CLINICAL ISOLATES OF C.TRACHOMATIS.

Key: * = Includes the control organism SA2f.

4.3.6. PSEUDOMONIC ACIDS.

Table 4.18. shows the results of the MICs of these agents against SA2f.

	ANTIBIOTIC CONCENTRATION (µg/ml)						
	4	8	16	32	64	128	
ANTIBIOTICS							MIC
BRL 35390	+	+	+	+	+	+	>128
BRL 4910A	+	+	+	+	+	+	>128

TABLE 4.18. MICs OF PSEUDOMONIC ACIDS AGAINST SA2f.

Key: + = Inclusions seen by iodine and/or Giemsa stain.

4.3.7. IMIDAZOLES.

The results of the MICs of these agents are shown in table 4.19.

ANTIBOTIC	(µg/ml) MIC
SC 37154	16
SC 37211	32
SC 38344	32
SC 38390	32
SC 38833	16
SC 38911	16
OXYTETRACYCLINE	.06

TABLE 4.19. MICs OF SC37154, SC37211, SC38344, SC38390, SC 38833, SC38911 AND OXYTETRACYCLINE AGAINST SA2f.

4.3.8. NOVOBIOCIN AND COUMERMYCIN.

Table 4.20. shows the results of the activities of these two compounds against SA2f.

ANTIBIOTICS	µg/ml MIC
NOVOBIOCIN	>32
COUMERMYCIN	.5

TABLE 4.20. MICs OF NOVOBIOCIN AND COUMERMYCIN AGAINST SA2f.

4.3.9 CHLORAMPHENICOL/THIAMPHENICOL.

Table 4.21. shows the MIC and MBC results of thiamphenicol compared with those of oxytetracycline, erythromycin and chloramphenicol against SA2f (Ridgway personal communication). The results of MICs of oxytetracycline and thiamphenicol against 15 clincal isolates of C.trachomatis are shown in table 4.22.

ANTIBIOTICS	MIC (µg/ml)	MBC (µg/ml)	NUMBER OF PASSAGES TO STABLE MBC
OXYTETRACYCLINE	.06	.25*	2
ERYTHROMYCIN	.06	.5*	3
THIAMPHENICOL	.5	>8*	6
CHLORAMPHENICOL	4	16*	3

TABLE 4.21. THE MICs AND MBCs OF OXYTETRACYCLINE, ERYTHROMYCIN, CHLORAMPHENICOL AND THIAMPHENICOL AGAINST SA2f.

Key: * = G.L.Ridgway (personal communication).

ANTIBIOTICS	ANTIBIOTIC CONCENTRATION (μg/ml) .06	.12	.25	.5	1.0	TOTAL NUMBER OF CLINICAL ISOLATES
OXYTETRACYCLINE	9*	7	0	0	0	16*
THIAMPHENICOL	0	0	0	14*	2	16*

TABLE 4.22. IN-VITRO ACTIVITIES OF OXYTETRACYCLINE AND THIAMPHENICOL AGAINST 15 CLINICAL ISOLATES OF C.TRACHOMATIS.

key: * = Includes SA2f.

4.3.10. RIFAMYCINS.

i) MICs AND MBCs. Table 4.23. shows the results of in-vitro activities of these agents against isolates of Chlamydia.

	ANTIBIOTIC CONCENTRATION (µg/ml)						
ANTIBIOTICS	.003	.007	.015	.03	.06	.12	TOTAL NUMBER OF ISOLATES
RIFAMPICIN	1	9*	3				13
RIFAPENTIN				5*	7	1	13
ERYTHROMYCIN				5	8*		13
OXYTETRACYCLINE				4	7*	2	13

TABLE 4.23. IN-VITRO ACTIVITIES OF RIFAMPICIN, RIFAPENTIN, ERYTHROMYCIN AND OXYTETRACYCLINE AGAINST 12 CLINICAL ISOLATES OF C.TRACHOMATIS.

key: * = Includes the control organism SA2f.

ii) RIFAMYCIN TOXICITY STUDY.

This study was performed in three parts and the results are shown below.

For part 1, tables 4.24. and 4.25. summarize the results of the effects of 44 hrs exposure of varying concentrations of each of the agents on McCoy cells.

For part 2, table 4.26. summarises the results of the cell counts of the IUDR inhibited and uninhibited McCoy cells pretreated with rifamycin drugs.

For part 3, tables 4.27. and 4.28. summarize the observations on the effects of 3 hrs exposure of different concentrations of the antibiotics on the inclusions in the IUDR treated and untreated McCoy cells.

EFFECTS OF VARYING CONCENTRATIONS OF RIFAMPICIN ON McCOY CELLS:

µg/ml	500	250	125	64	32	16	8	control
DIRECT OBSERVATION	DC++	GR	H	H	H	H	H	H
TRYPAN BLUE	DC++	GR	H	H	H	H	H	H
GIEMSA	DC++	GR	H	H	H	H	H	H
AVERAGE CELL COUNT X10^4								
LIVE	8	50	77	42	34	51	60	56.6
DEAD	8.5	3.5	5	1.5	1.5	2.5	2.5	2.5
TOTAL	16.5	54	83	44	35.5	54	64	59
% DEAD CELLS	51.8	6.5	6.1	3.4	5.9	4.6	4	4.2

TABLE 4.24. EFFECT OF 44 HOURS EXPOSURE OF RIFAMPICIN ON McCOY CELLS.

Key: DC ++ = Large number of dead cells.

GR = Granular cells.

H = Healthy cells.

EFFECTS OF VARYING CONCENTRATIONS OF RIFAPENTIN ON McCOY CELLS:

µg/ml	500	250	125	64	32	16	8	control
DIRECT OBSERVATION	DC++	DC+	DC+	GR	H	H	H	H
TRYPAN BLUE	DC++	DC+	DC+	DC±	H	H	H	H
GIEMSA	DC++	DC+	DC+	GR	H	H	H	H

AVERAGE CELL COUNT X10^4							
LIVE	0	1.5	2.5	33	61.5	54.5	46.5
DEAD	12	11	6.5	5	2	2	1.5
TOTAL	12	12.5	9	38	63.5	56.5	48
% DEAD CELLS	100	88	72.2	13.2	3.1	3.5	3.1

TABLE 4.25. EFFECT OF 44 HOURS EXPOSURE OF RIFAPENTIN ON McCOY CELLS.

Key: DC = Dead cells. ++ = Large numbers.
GR = Granular cells. + = Some.
H = Healthy cells. ± = Scanty.

						% DEAD CELLS			
µg/ml		200	100	50	CONTROL	200	100	50	CONTROL
IUDR CELLS									
PRETREATED WITH RIFAMPICIN	(L)	28	33	31		1.8	0	3.2	0
COUNT $X10^4$	(D)	0.5	0	1					
UNINHIBITED CELLS									
PRETREATED WITH RIFAMPICIN	(L)	77	68	64		0	1.4	1.5	1.5
COUNT $X10^4$	(D)	0	1	1					
IUDR CELLS									
PRETREATED WITH RIFAPENTIN	(L)	36	32	46	45	1.4	3	0	0
COUNT $X10^4$	(D)	0.5	1	0	0				
UNINHIBITED CELLS									
PRETREATED WITH RIFAPENTIN	(L)	58	45	57	95	2.5	0	.8	1.5
COUNT $X10^4$	(D)	1.5	0	o.5	o.5				

TABLE 4.26. CELL COUNTS OF THE IUDR INHIBITED AND UNINHIBITED McCOY CELLS PRETREATED WITH THE RIFAMYCIN DRUGS.

Key: (L)= Live cells. (D)= Dead cells.

RIFAMPICIN PRETREATED	µg/ml 200	100	50	CONTROL	
IUDR INHIBITED CELLS	N+	N+	N+	N+	IODINE STAIN
	N+	N+	N+	N+	GIEMSA STAIN
UNINHIBITED CELLS	S+	S+	S+	S+	IODINE STAIN
	S+	S+	S+	S+	GIEMSA STAIN

TABLE 4.27. EFFECT OF 3 HOURS PRETREATMENT OF McCOY CELLS WITH RIFAMPICIN ON THE APPEARANCE OF SA2f INCLUSIONS.

RIFAPENTIN PRETREATED	µg/ml 200	100	50	CONTROL	
IUDR INHIBITED CELLS	S+	N+	N+	N+	IODINE STAIN
	S+	N+	N+	N+	GIEMSA STAIN
UNINHIBITED CELLS	S+	S+	S+	S+	IODINE STAIN
	S+	S+	S+	S+	GIEMSA STAIN

TABLE 4.28. EFFECT OF 3 HOURS PRETREATMENT OF McCOY CELLS WITH RIFAPENTIN ON THE APPEARANCE OF SA2f INCLUSIONS.

Key: + = Expected number of inclusions.

N = Normal inclusions.

S = Small inclusions

4.3.11. FOLIC ACID INHIBITORS.

The MICs and MBCs for sulphamethoxazole, trimethoprim, sulfadoxine and pyrimethamine are shown in table 4.29.

ANTIBIOTICS	MIC (µg/ml)	MBC (µg/ml)	MBC STABLE AFTER PASSAGE NUMBER
SULPHAMETHOXAZOLE	4	32*	5
TRIMETHOPRIM	128	1024*	4
SULFADOXINE	4	64	2
PYRIMETHAMINE	4	64	1

TABLE 4.29. MICs AND MBCs OF FOLIC ACID PATHWAY INHIBITORS AGAINST SA2f.

Key: * G.L.Ridgway personal communication

The MICs of folic acid pathway inhibitors against Chlamydia are difficult to determine owing to the persistence of tiny inclusions throughout the dilution range. However, the MICs recorded here are the dilutions at which there is a large drop in the number of inclusions coupled with the appearance of abnormal inclusions as observed by Giemsa and iodine stains.

Tables 4.30.- 4.34 illustrate the FICI results for each combination at passage 1 and passage 10.

SULPHAMETHOXAZOLE/TRIMETHOPRIM COMBINATION (PASSAGE 1)

	FIC (T)	+	FIC (S)	=	FICI
a	8/128	+	0.06/4	=	0.08
b	4/128	+	0.125/4	=	0.06
c	2/128	+	0.125/4	=	0.04
d	1/128	+	0.125/4	=	0.04

SUM OF FICI = 0.22

$\overline{FICI}$=.022/4 = 0.055

TABLE 4.30. CALCULATION OF THE $\overline{FICI}$ FOR SULPHAMETHOXAZOLE/TRIMETHOPRIM COMBINATION AT PASSAGE 1.

SULPHAMETHOXAZOLE/TRIMETHOPRIM COMBINATION (PASSAGE 10)

	FIC (T)	+	FIC (S)	=	FICI
a	32/1024	+	0.06/32	=	0.031
b	16/1024	+	0.12/32	=	0.018
c	8/1024	+	0.12/32	=	0.019
d	4/1024	+	0.12/32	=	0.006
e	2/1024	+	0.25/32	=	0.008
f	2/1024	+	0.5/32	=	0.016
g	1/1024	+	1/32	=	0.031

SUM OF FICI = 0.129

$\overline{FICI}$= 0.129/7 = 0.018

TABLE 4.31. CALCULATION OF THE $\overline{FICI}$ FOR SULPHAMETHOXAZOLE/TRIMETHOPRIM COMBINATION AT PASSAGE 10.

SULFADOXINE/PYREMETHAMINE COMBINATION (PASSAGE 1)

	FIC (P)	+	FIC(S)	=	FICI
a	8/4	+	0.06/4	=	2.015
b	4/4	+	0.12/4	=	1.03
c	4/4	+	0.24/4	=	1.06
d	4/4	+	0.5/4	=	1.125
e	2/4	+	1/4	=	0.75
f	1/4	+	1/4	=	0.5
g	0.5/4	+	1/4	=	0.375
h	0.25/4	+	1/4	=	0.031
i	0.12/4	+	1/4	=	0.28
j	0.06/4	+	2/4	=	0.515
k	0.03/4	+	2/4	=	0.507
			SUM OF FICI	=	8.467
			$\overline{FICI}$ = 8.467/11	=	0.769

TABLE 4.32. CALCULATION OF THE $\overline{FICI}$ FOR THE SULFADOXINE/PYRIMETHAMINE COMBINATION AT PASSAGE 1.

SULPHADOXINE/PYRIMETHAMINE COMBINATION (PASSAGE 10)

	FIC (P)	+	FIC (S)	=	FICI
a	8/64	+	1/64	=	0.14
b	4/64	+	1/64	=	0.075
c	2/64	+	1/64	=	0.075
d	2/64	+	2/64	=	0.067
e	1/64	+	4/64	=	0.063
f	0.5/64	+	4/64	=	0.061
g	0.25/64	+	4/64	=	0.063
h	0.125/64	+	4/64	=	0.061
i	0.06/64	+	4/64	=	0.067
j	0.03/64	+	4/64	=	0.060
			SUM OF FICI	=	0.732
			$\overline{FICI}$ = 0.732/10	=	0.073

TABLE 4.33. CALCULATION OF THE $\overline{FICI}$ FOR THE COMBINATION SULFADOXINE/PYRIMETHAMINE AT PASSAGE 10.

	MEAN FICI	
SULPHAMETHOXAZOLE/TRIMETHOPRIM (PASSAGE 1)	0.06	SYNERGY
SULPHAMETHOXAZOLE/TRIMETHOPRIM (PASSAGE 10)	0.018	SYNERGY
SULFADOXINE/PYRIMETHAMINE (PASSAGE 1)	0.769	INDIFFERENCE
SULFADOXINE/PYRIMETHAMINE (PASSAGE 10)	0.073	SYNERGY

TABLE 4.34. MEAN FICI VALUES FOR THE COMBINATION OF SULPHAMETHOXAZOLE/TRIMETHOPRIM AND SULFADOXINE/PYRIMETHAMINE AGAINST SA2f.

CHAPTER 5

DISCUSSION

The pace of study of the genus Chlamydia has been slow. This is because these organisms have specialised growth requirements which have hampered the unravelling of their life cycle, laboratory diagnosis and isolation. Similarly, the study of their role in the diseases of man and animals, and the search for suitable antimicrobial agents, have also been slow. The present work is an attempt to expand on some facets of these studies.

LIFE CYCLE OF CHLAMYDIA.

To gain insights into the behaviour of these organisms, conventional staining techniques (Bedson & Bland 1932, 1934, Gordon & Quan 1965a, Starr et al 1968) and electron microscopy (de La Maza et al 1982, 1984), either individually or in combination, have previously been employed. This study goes further, in that Giemsa, iodine, fluorescence and acridine orange staining techniques, plus electron microscopy were used concurrently at different stages during the cycle of growth. Moreover, time lapse photography has been used for the first time. The observations presented in this thesis give new insights on various stages of the growth cycle of these organisms. Comments on these observations are given.

At T16, iodine stained inclusions were not visible as the glycogen matrix is not produced at this stage of the life cycle. Although a few tiny pin point inclusions were visible by Giemsa BFM, they did not exhibit autofluorescence by DGM suggesting that in its infection cycle the autofluorescent property of Chlamydia is not established by this time. Immunofluorescence microscopy showed some tiny inclusions, demonstrating the presence of chlamydial antigen and confirming the results obtained by Giemsa DGM. Acridine orange stain

demonstrated inclusions which contained predominantly orange/yellow entities suggesting that the organisms are engaged in high metabolic activity.

At T24, iodine stained inclusions were visible, suggesting that the glycogen matrix had begun to be produced sometime between 16-24 hrs post infection. Using Giemsa DGM, the inclusions were just visible, exhibiting autofluorescence. This suggests that whatever the cause of this phenomenon, and like the glycogen matrix, autofluorescence does not begin to manifest itself until sometime between 16-24 hrs post infection. The organisms, therefore, have to be at a certain stage in their life cycle before they are able to exhibit autofluorescence. Inclusions stained by Giemsa and examined by BFM, and those stained with immunofluorescent stain, were larger than those seen earlier in the life cycle. This was expected because, as the growth cycle progresses, there is an increase in the numbers of reticulate bodies and hence a larger amount of antigen is available inside the inclusions. Acridine orange stained inclusions behaved in the same way at T24 as at T16 except that the inclusions were larger. This finding, coupled with the fact that the inclusions were still exhibiting the presence of only RNA material (orange staining reaction), is indicative of an increased metabolic activity at this stage.

At T30, the inclusions were larger still and a greater number of them could be seen by iodine stain. As the initial inoculum was the same for all the tubes, this increase in numbers suggests that either not all inclusions produce the glycogen matrix between 16-24 hrs, or that some of the elementary bodies, phagocytosed by the cells, remain inactive for a period of time and are slow to initiate infection and

therefore many of the inclusions are not in phase with one another in their growth cycle. Giemsa BFM showed larger inclusions than before and Giemsa DGM exhibited good autofluorescence. These results were consistent with findings from the iodine stain. The immunofluorescence staining showed larger, better defined inclusions that were reticulate in structure. Acridine orange stain exhibited both DNA and RNA material suggesting a reduction in metabolic activity and a reorganization of the inclusion contents. It is submitted that this finding and the Giemsa DGM observation indicate that the appearance of the DNA material, which predominates in the elementary body, and the autofluorescing property of the mature inclusions are caused by the elementary bodies. Support is given to this hypothesis by the fact that observation of a more intense autofluorescence is seen at T42 as well as there being an increased DNA content of the inclusions at this time.

When the above results were compared with the electron microscopy observations, it was noted that at T24 inclusions were also small and contained both initial and intermediate bodies. This observation confirms the findings from the acridine orange stain. At T30, the inclusions were larger, containing an increased number of initial or intermediate structures, some showing the process of binary fission. The presence of electron dense particles is indicative of the beginnings of the process of rearrangement by the particles into becoming elementary bodies. This observation is supported by the presence of the green stained material seen in the acridine orange stain. The picture changed at T42 in that the majority of the particles in the inclusions were elementary bodies. This leads to the conclusion that the appearance of the elementary bodies coincides with more intense autofluorescence by Giemsa DGM, and green stained material

(DNA) by acridine orange stain.The increased amount of antigen observed by immunofluorescence was consistent with the increase in the number of chlamydial particles inside the inclusion. The results obtained from EM work confirm the observations made by BFM.

EFFECT OF PENICILLIN-

When low concentrations of penicillin (0.125µg/ml) were added during different stages of the life cycle (PT), and the inclusions examined at 42 hrs post infection and compared with T42, the morphological differences between the inclusions noted were as follows: A small number of tiny globular inclusions with iodine stain indicated that the addition of penicillin at the beginning of the life cycle stopped enlargement of the inclusions. This effect was still apparent when penicillin was added as late as 24 hrs post infection. Though small, the inclusions were easily recognisable. Becker et al. 1962, showed that when a high concentration of penicillin (1000 U/ml) was added during the early stages of the life cycle, "Lugol's" stained material appeared 48 hrs after infection but in only a small number of inclusions. In the work presented here, it is clearly demonstrated that a low concentration of penicillin added at the beginning of the life cycle drastically affects the development of the inclusions and interferes with their enlargement. However, glycogen production is not affected. Additional work using 6 µg/ml (10 U/ml) of penicillin added at the beginning of the life cycle produced only a small number of abnormal inclusions after an incubation period of 40 hrs. This number increased as the developmental cycle progressed. Therefore, the addition of penicillin in large concentrations delays glycogen production. Moreover, the presence of penicillin in a concentration as low as 0.125µg/ml still resulted in the formation of giant abnormal

bodies inside the inclusions.

The effect of penicillin on the morphology of the contents of inclusions was best seen by Giemsa BFM and DGM. These effects manifest themselves through the appearance of beaded bodies inside the inclusions when the drug was added during the early stages of the life cycle. The beaded bodies lack the ability to autofluoresce. The addition of penicillin at later stages of the life cycle does not grossly interfere with autofluorescence. This means not only that elementary bodies possess the ability to autofluoresce but also that penicillin arrests the maturation process of the initial bodies. This hypothesis is further strengthened by the fact that, coinciding with the appearance of DNA material observed by acridine orange stain, a more intense autofluorescence was seen by Giemsa DGM.

The electron microscopy findings of the effects of the addition of penicillin at different times during the life cycle support the observations made by light microscopy. The detrimental effects of penicillin added during early stages of the chlamydial life cycle is evident by the presence of a large number of giant abnormal bodies.

Penicillin acts on bacteria by inhibiting peptidoglycan synthesis. The morphological effect on affected organisms (eg, E.coli) is seen as an inhibition of cell division and formation of long filamentous cells. For Chlamydia this effect was seen in the production of large irregular bodies inside the inclusions which gave an overall globular or beaded appearance to the inclusions, interfering with maturation of the inclusions and their autofluorescent property. These effects were most pronounced when the antibacterial was added at the beginning of the growth cycle, and were still found when penicillin

was added as late as 30 hrs post infection.

TIME LAPSE PHOTOGRAPHY-

In 1953, Abercrombi and Heaysman pioneered the use of tissue culture cinematography in their studies on the locomotion of chick heart fibroblasts. Using phase contrast microscopy, a number of workers have since then documented cellular movement and have compared morphological and cytopathic changes occurring in infected and uninfected cells (BMA film library 417, 1966 and 568, 1972).

Here, employing time lapse photography and using cycloheximide treated McCoy cells, the life cycle of SA2f was sequentially captured on film for the first time. Normaski's system of differential interference-contrast, as opposed to phase contrast, was used. The reason for employing Normaski's system for the examination of unstained live cells was that it produces three dimensional images with enhanced clarity. The behaviour of the inclusions regarding their time of appearance and the time taken for the cells to burst varied considerably. As the cytoplasm of the cell is in constant motion, and though the overall appearance of the preparation remains in focus, the focus of any given point in the moving cytoplasm is constantly changing. Consequently, the precise instant for the appearance of the inclusions, which look like tiny dimples on the cells, is difficult to ascertain. However, it was noted that most inclusions appeared between 18-24 hrs post infection. Some appeared much later, with one particular inclusion becoming visible almost 18 hrs after its neighbouring inclusion had burst, indicating the possibility of secondary seeding. Of the six inclusions studied in detail (tables 4.1-4.22.), the time taken from appearance to bursting of inclusion no. 5 was about 17 hrs,

making the total cycle time of this inclusion to be about 40 hrs. On the other hand, inclusion no. 4 took 53 hrs 36 mins from initial appearance to bursting, making the total cycle time of this inclusion to be more than 72 hrs. Other inclusions had life cycles of about 48 hrs. These results support the general belief that inclusions in a given in-vitro environment do not all appear at the same time and that the life cycles of these organisms vary within a range of 40 to 70 hrs. The time taken for the actual process of bursting also varied. For some the process was almost instantaneous, taking less than 3 minutes. Other inclusions took much longer (eg. 183 minutes). The actual timing of these slow bursting cells was calculated starting from the moment when a visible morphological change in the appearance of the cell, indicating cytoplasmic disturbance, was detected. The cells at the beginning of the process seemed to be constantly in turmoil, twisting and turning, contracting and expanding. Those which had burst more quickly did so with little or no dramatic changes in their morphology. Some cells with larger inclusions remained intact for a longer period and some with smaller inclusions underwent more dramatic morphological changes before bursting. Two inclusions recorded on film which were adjacent to one another and almost identical in size, showed markedly different bursting times. Cytopathic effects of herpes virus have similarly been demonstrated by the shrinking and rounding off of the cells with nuclear and locomotor changes clearly being visible (BMA film library 413, 1966). Todd and Storz (1975), using ultrastructural cytochemical evidence, have shown that lysosomal enzymes play a major role in the bursting of the cells which coincides with the process of C.psittaci maturation. On the other hand, Todd and Caldwell (1985) infected Hela cells with serotype D of C.trachomatis and, using

transmission and scanning electron microscopy along with a fluorescence antibody test, demonstrated that chlamydial antigen is expressed on the surface of the infected cells. These workers suggested that quantitative studies were needed to definitely show that cells infected with the SA2f strain of Chlamydia remain viable during and after the release process. If movement of cells is taken as a sign of life, then, on the basis of the time lapse photography evidence presented here, it can be argued that many cells harbouring SA2f inclusions are very much alive and undergo dramatic morphological changes before bursting. Also, though Todd and Caldwell suggest that the mature bodies may exit by exocytosis, the work presented here clearly shows that lysis of the infected cells also occurs which inevitably results in the release of mature bodies. Whether the burst of a cell in this system is an inevitable outcome due to age, or due to deterioration in the health of the cell, or the result of increased lysosomal activities in the infected cell requires further investigation. What is apparent is that:

1. Some inclusions appear earlier than others.
2. Inclusions in the cells are of differing sizes.
3. Some cells containing inclusions burst suddenly with a minimum of visible morphological changes.
4. Some cells undergo dramatic morphological changes before bursting.
5. The lengths of time taken by infected McCoy cells containing chlamydial inclusions to burst are different.
6. The life cycle of C.trachomatis in McCoy cells is about 2 to 3 days.

HIGH YIELD CULTIVATION STUDY.

The initial stages in the development of rapid diagnostic techniques for the detection of chlamydial antigen or antibody currently require the preparation of large amounts of chlamydial antigen. This is used for the production of antichlamydial antibody in animals, or for the pre-coating of slides or solid phases for use in microimmunofluorescence or EIA antibody detection techniques. The antigen required is conventionally grown in eggs or in large volumes of tissue cultures. Although microcarriers have elsewhere been used for obtaining large volumes of cells, cell products and viruses (van Wezel 1967), their use for the production of chlamydial antigen on a large scale has not been previously explored.

Preliminary results presented in this thesis for the production of chlamydial antigen using the cytodex 2 microcarrier system are encouraging. The experiment was conducted with SA2f which, being a laboratory adapted strain, gave a much higher yield than the clinical strains. Indeed, the total gain was of the magnitude of x3200. The two clinical strains used were isolated from two females who had attended the Department of Genito- Urinary Medicine at UCH. The isolates were chosen randomly. Isolate 25088 yielded 4 times as much antigen as the starting inoculum but, although grown successfully (as was apparent when compared with control no. 1), the yield of isolate 81591 remained the same, neither increasing nor decreasing.

The fact that such a system supports the growth of clinical isolates of C.trachomatis is of some importance, particularly bearing in mind the need for centrifugation of the clinical isolates on to the

cell monolayers for the routine isolation of these organisms in tissue culture systems. At this time the precise reason for the success of the system are a matter for conjecture. It is possible that the electrostatic charges on the surface of the cytodex beads play a role in the adsorption process. The constant stirring of the cells grown on the cytodex beads may create a dynamic collision force making the interaction between the infectious particles and the cells more profound. The increased surface area may play a part in reducing surface tension between the infecting bodies and the cells. Whatever the exact mechanism might be, the fact that this system has supported the growth of C.trachomatis justifies further work so as to establish the full potential of the system in achieving higher yields of chlamydial antigen.

SENSITIVITY OF C.TRACHOMATIS TO ANTIMICROBIAL AGENTS.

TETRACYCLINES-

Oxytetracycline is the drug of choice for the treatment of C.trachomatis infections. It has been most widely used either topically against trachoma or systemically for the treatment of genital chlamydial infection. Because of their activity against M.hominis and some strains of N.gonorrhoeae, tetracyclines are also one of the drugs of choice for cases of PID. However, they are ineffective against most anaerobic organisms associated with this condition.

Many clinical trials have shown the superiority of tetracyclines over other antimicrobial agents for the treatment of chlamydial infections (80% cure rate). Reisolation of the organism (in up to 20% of patients) post tetracycline treatment has been demonstrated. Oriel (1986) has suggested that this may be due to reinfection or non-compliance, and calls for more controlled trials with prolonged follow up.

The results for the MICs of the three tetracyclines tested in this work have been found to be similar. Hence in-vitro activities of these drugs have not demonstrated any superiority by one over another. Therefore, other factors have to be considered when a choice between one of these drugs has to be made in the treatment of chlamydial infections. Oxytetracycline is used as the first-line antibiotic in the treatment of chlamydial infection. Doxycycline and minocycline are more expensive but have a longer half life. Because of its better compliance coupled with a lack of need for dietary restrictions regarding dairy products, doxycycline may be given in cases of oxytetracycline treatment failure. Minocycline is similar to oxytetracycline for the treatment of chlamydial infection and may be given twice daily as

opposed to four times a day.

MACROLIDES-

At the time of this work, erythromycin was the only macrolide which had been evaluated and used for the treatment of mucosal chlamydial infections. It is used in cases of treatment failure with tetracyclines and is given systemically to newborns and lactating mothers. However, like tetracyclines, the success rate for the treatment of chlamydial infection is of the order of 80%, and there is a real need for other members of this family of drugs to be tested and evaluated for the treatment of these infections.

The in-vitro sensitivities of the eight macrolides tested in this work were very similar. With the exception of spiromycin (MIC = 0.5 µg/ml), the MICs of the others lay between 0.03 µg/ml and 0.125 µg/ml. Preliminary studies with roxithromycin show encouraging results for the bioavailability and tolerence of this antimicrobial agent. These findings, coupled with the good in-vitro activity shown against C.trachomatis, make it a credible candidate for inclusion in clinical trials of chlamydial infections. Indeed, the results of trials obtained in some centres have been encouraging (Worm et al 1988). However, not all clinical results were good, possibly because of the differences in the antigen detection systems used for evaluation (personal communication with Dr.G.L.Ridgway). Therefore, some contradictory findings have cast doubts on the future of this agent in the field of antichlamydial chemotherapy. The latest data from Italy (Negosanti et al 1988), describe the use of this agent in the treatment of NGU. These workers, using culture technique for the test of cure, reported a 94% cure rate in Chlamydia positive patients (16/17

patients) and 92% with complete remission of the symptoms of NGU (35/38 patients). The discrepant results obtained by different workers in trials of this drug alone clearly show a need for the standardization of laboratory procedures for the test of cure and the setting up of clear guidelines regarding a recommended period of time post treatment when such a test may be performed.

In this study, investigations into the development of resistance to roxithromycin were conducted on two separate occasions. The findings are similar to those of the experiment with erythromycin (Jones et al 1983) and suggest that roxithromycin has a bactericidal activity against C.trachomatis at 0.12 µg/ml, which is the MBC value observed in this work. Therefore, it is not possible to induce resistance in C.trachomatis against this agent in-vitro, using the technique described. These results further strengthen the stated view that better controlled clinical trials are needed to realise the full potential of this agent.

Compound ER-42859 was found to be 4-fold less active than erythromycin (MIC = 0.25 µg/ml) against SA2f. Data available on its activity against M.hominis and U.urealyticum are also not encouraging. As such, it does not seem to be a useful drug in the treatment of these genital pathogens. Azithromycin and miocamycin have MICs of 0.125 µg/ml and 0.06 µg/ml respectively against SA2f. In addition, the data available on their activity against M.hominis and U.urealyticum are comparable with those of erythromycin and tetracycline and therefore these compounds may be of some use in the treatment of concomitant infections with these organisms. Results of toxicity and bioavailability studies, and clinical trials are awaited.

For the treatment of tetracycline failures, there is a need for a macrolide to be found which has better compliance factors, less toxicity than erythromycin, and which is also safe for newborns and lactating mothers. Josamycin has an MIC of 0.03 µg/ml. Clinical trial results reported at the European Society for Chlamydia Research (1988) demonstrated a 91% cure rate with 500 mg/day for 8 days in a group of pregnant women (Soltz-szots et al 1988), a 96.4% cure rate with 2g/day for 10 days in a high risk group (Primiero et al 1988) and a 97% cure rate with 2 g/day for 15 days in women undergoing infertility studies (Lucisano et al 1988). These workers concluded that josamycin is a highly effective drug for the treatment of chlamydial infection in these groups of patients, confirming the preliminary laboratory findings indicated in this work. However, it is recommended that additional investigations are carried out so as to establish the role of this agent in the treatment of other sexually transmitted pathogens such as mycoplasmas.

QUINOLONES-

The newer 4-quinolones have a wider antimicrobial spectrum and greater activity than nalidixic acid. They are active against N.gonorrhoeae including the beta-lactamase producing strains. In order to assess their potential for treating other sexually transmitted pathogens, their activity against Chlamydia and mycoplasmas is of some importance. Accordingly, the MICs and MBCs of seventeen 4-quinolones against the SA2f strain of C.trachomatis were determined. The MIC and MBC values of the 4-quinolones for Chlamydia are exactly the same or within a two-fold dilution. Thus these agents are bactericidal at their MIC.

Ciprofloxacin inhibits C.trachomatis (MIC = 0.5 µg/ml), M.hominis (MIC = 0.25 µg/ml) and Ureaplasma urealyticum (MIC = 1 µg/ml) in-vitro. Ciprofloxacin is highly active against gonococci in-vitro, and clinical studies have shown a 100% cure rate, with few or no side effects (Loo et al 1985). However, despite the encouraging results initially obtained by in-vitro studies in this work, its activity against C.trachomatis infection has been found to be disappointing. In using ciprofloxacin in single dose therapy for gonorrhoea, Loo et al have reported its failure to prevent PGU caused by C.trachomatis. Felmingham et al (1985) have used ciprofloxacin for the treatment of NGU in 40 men administering a dosage of 500 mg twice daily (21 men) or 3 times a day (19 men) over a period of a week. Clinical cure of NGU was achieved in only about half these patients. Also, from amongst the originally infected patients, C.trachomatis and U.urealyticum were reisolated 1-2 weeks after completion of therapy in 3/22 and 2/12 cases respectively. Other studies on the value of ciprofloxacin for the treatment of urethritis in men (Arya et al 1986 and Sholtz et al 1986)

have shown various degrees of success. Recently,Teisala et al (1988) treated 12 women with ciprofloxacin IV 200 mg twice daily during the first two days, followed by 750 mg orally twice a day for 14 days. Six of these patients were Chlamydia positive, 2 had chlamydial as well as gonoccocal infections and 4 were non-chlamydial PID. Ciprofloxacin treatment was successful in all of these patients. Of 15 patients in a parallel control group treated with doxycycline plus metronidazole, 2 had chlamydial PID, 3 had chlamydial-gonoccocal PID and 10 had nonchlamydial-gonoccocal PID. The patients in the control group had a 40% failure rate owing to unsuccessful treatment of gonococcus and nongonococcal-nonchlamydial PID (6/15 patients). These workers concluded that ciprofloxacin, as opposed to doxycycline and metronidazole, is an effective and safe drug in the treatment of chlamydial PID. These data suggest that although ciprofloxacin has some value in the treatment of mixed gonococcal and chlamydial infections when given in high dosage for 1-2 weeks, further clinical trials in both sexes are needed to justify the use of this drug in the treatment of chlamydial infection. Moreover, the success of this drug in the treatment of NGU and PGU is not absolute, though its relative nontoxicity makes it a useful agent for problematic cases of NGU following treatment failure with conventional antibiotics.

Ofloxacin has an MIC of 1 µg/ml against C.trachomatis as well as against U. urealyticum. In a trial organised at UCH, the clinical efficacy of ofloxacin in the treatment of NGU was studied. The drug was given orally to both sexes at a dosage of 200 mg for 7 days. The results were compared with those obtained by administering oxytetracycline 500 mg four times a day for 7 days (Nayagam et al 1988). In the ofloxacin study, 69% of the men were clinically cured and C.trachomatis was not reisolated from any of the 16 previously positive

patients. This treatment compared favourably with that using oxytetracycline. In women with cervical chlamydial infection, the cell culture technique for the exclusion of Chlamydia at the end of treatment, and again 2 weeks post therapy, showed a 100% cure rate. The side effects reported in this study, which included fatigue, headache and dizziness, were mild and transient, occurring in only 13% of males and 16% of females. These facts, coupled with the good activity of ofloxacin against the gonoccocus, and a sufficiency of using only two daily doses without any dietary restrictions as opposed to four doses of oxytetracycline, make it a potentially useful antimicrobial agent for the treatment of genital chlamydial infection.

Of the other quinolones listed in table 4.11, clinical studies of NGU patients treated with norfloxacin (Bowie et al 1986) were unsuccessful. The two Abbott products show good in-vitro activity in cell culture and as much merit further investigations. Abbott 56619 is not undergoing clinical trials. The results of clinical trials with fleroxacin, CI-934, Abbott 56620 and lomefloxacin are not yet available.

BETA-LACTAMS-

The effects of amoxycillin and augmentin (amoxycillin plus clavulanic acid) were studied in-vitro against SA2f, and the results are recorded in table 4.14. The MICs of these compounds were taken to be those concentrations of the drugs which produced a sharp fall in the number of normal inclusions. Augmentin has good activity against the gonococcus and, as such, is a potentially useful candidate for inclusion in the studies of activity against C.trachomatis. Clavulanic acid is a beta-lactamase inhibitor and there is no evidence that C.trachomatis produces this enzyme. No significant difference was found in the MICs of augmentin (commercial or in-house preparation) or in its individual components, although it is noteworthy that the results of in-vitro studies presented here show that clavulanic acid has some activity in inhibiting SA2f. Kramer et al (1979) used amoxycillin to prevent C.trachomatis infection in mice. These workers concluded that amoxycillin was approximately 8 times more active than either ampicillin or oxytetracycline in achieving its goal. They speculated that this success of amoxycillin may be due to its better rate of absorption in these animals. Bowie et al (1981) successfully used amoxycillin to eradicate C.trachomatis infection from urethras of men with NGU. The patients seemed to be free of the organism 24-48 days post treatment. However, because of its lack of activity against U.urealyticum, these workers concluded that amoxycillin is not an efficient therapy in cases of NGU. Alexander et al (1982) treated a small group of Chlamydia positive pregnant women with either erythromycin or amoxycillin. This study was undertaken to establish a strategy for the prevention of chlamydial infection in infants born to such mothers. They suggested that both medications were effective. On

the other hand, Bell et al (1982) used amoxycillin in a similar group of women and found it to be ineffective. Amoxycillin, being a bacteristatic drug, like penicillin, may suppress but not eradicate chlamydial infection. Therefore, as is evident by these in-vitro studies, the cessation of therapy may result in a relapse which may only be observed through efficient follow up studies.

The cephalosporins (RO-158074 and ceftriaxone) showed no in-vitro activity against C.trachomatis (table 4.15), and hence are of no obvious use in the treatment of chlamydial infections.

AMINOCYCLITOLS-

Trospectomycin and spectinomycin have good activity against the gonococcus. The activity of trospectomycin (MIC = 8 µg/ml) against C.trachomatis shown in this work (table 4.16) is some 8 times greater than spectinomycin (MIC = 128 µg/ml). The results of the MICs of the 10 clinical isolates studied here (table 4.17) are also similar to the control organism. The outcomes of clinical trials will be of interest particularly for the treatment of gonococcal and chlamydial infections.

PSEUDOMONIC ACIDS AND IMIDAZOLES-

Pseudomonic acids studied in this work showed no in-vitro activity against SA2f (table 4.18) and so are of no obvious value in the treatment of chlamydial infections. Similarly, the imidazoles studied in this work (table 4.19) showed only limited activity against Chlamydia.

COUMERMYCIN-

Coumermycin has an MIC of 0.5 µg/ml against SA2f and is therefore over 64 times more active than novobiocin (table 4.20). This result is

very encouraging and further investigation into the activity of this antimicrobial against C.trachomatis is called for.

THIAMPHENICOL-

The MIC of thiamphenicol against SA2f was found to be 0.5 µg/ml. From out of a total of 15 clinical isolates looked at in this work, 13 also showed an MIC of 0.5 µg/ml. This agent is 8 times more active than chloramphenicol in-vitro and, as such, warrants closer examination in the treatment of chlamydial infections. This is especially so, as the activity of thiamphenicol against N.gonorrhoeae, including the beta-lactamase producing strain, has been found to be encouraging. Workers at UCH found the MIC of this drug against 276 isolates of N.gonorrhoeae to be <4.0 µg/ml (Ridgway et al 1984). In a clinical trial reported by Mascaro and Capdevila (1984), thiamphenicol produced satisfactory results for patients with gonorrhoea after single dose therapy.

In view of the fact that chloramphenicol suppresses but may not eradicate chlamydial infection, there is room for speculation that the same may apply to thiamphenicol especially as it has an MBC >8. However, a recent report by Schlapfer et al (1988), on a group of 25 men with urethritis caused by C.trachomatis, has shown that treatment with this agent for 10 days results in a 100% cure not only of Chlamydia positive patients but also of those who additionally had M.hominis infection. The reported side effects were mild gastrointestinal upset and tiredness. More clinical trials are recommended to establish the value of this agent in the treatment of concomitant gonorrhoea and chlamydial infection.

RIFAMYCINS-

The in-vitro activities of rifampicin and rifapentin against SA2f and 12 clinical isolates were studied. Of all the drugs studied in this work, rifampicin showed the highest activity (MIC = 0.007 µg/ml). This compound is 8 times more active than rifapentin and 16 times more active than tetracycline and erythromycin. Coufalik et al (1979) treated men with NGU with either rifampicin or minocycline. Some 2% of the Chlamydia positive patients in each group remained positive after treatment. However, only 37% of patients in the rifampicin group, as compared to 68% in the minocycline group, recovered from NGU symptoms. Although rifampicin was found to be comparable with minocycline in the treatment of chlamydial infection, its use in the treatment of nonchlamydial NGU was not satisfactory. The clinical value of rifapentin in the treatment of chlamydial infection awaits clinical trials.

Rifampicin has been used in the treatment of ocular chlamydial infection (Darougar et al 1977) and also in the treatment of LGV (Menke et al 1979) with no adverse effects. However, laboratory studies, involving concentrations of these agents above 200 µg/ml, have been difficult to interpret owing to the toxic effect observed on the cells. The design of the toxicity study in this work was comprised of 3 parts.

In part one, the effects of 44 hrs exposure of different concentrations (8-500 µg/ml) of each drug on the morphological appearance and health of uninhibited McCoy cells was observed using a Giemsa or iodine stain, and a total dead and live cell count was performed. Cells showed signs of degeneration when concentrations of rifampicin and rifapentin exceeded 125 µg/ml and 32 µg/ml respectively.

In part two, the effects of a short exposure (3 hrs) of each drug on McCoy cells were studied. Following further incubation in antimicrobial free medium, the percentages of dead cells in both treated and control tubes were recorded. No overwhelming destruction of the cells was observed. This was further confirmed by the fact that cells stained with iodine, Giemsa and trypan blue also looked normal, showing no signs of toxicity. These findings indicate that the removal of the drugs from McCoy cells, after a short exposure, leaves no apparent adverse effects on the well-being of the cells.

In part three, the effects on the development of the inclusions were examined after exposing the cells to the antimicrobial agents for a brief period of 3 hrs. Though small inclusions at a concentration of 200 µg/ml of rifapentin treated cells were observed, the cells from the other tubes yielded the expected number of inclusions with normal morphology.

Therefore, on the basis of the above findings, it would appear that when McCoy cells are subjected to long-term treatment with either drug, there is a pronounced toxic effect which is demonstrated by an increase in the number of dead cells. Shorter treatment of the cells with the drugs has little adverse effect on their well-being. However, the adverse effect of high concentrations of rifapentin on McCoy cells is obvious, being demonstrated by the appearance of inclusions which are smaller than expected. Hence, the problems of testing high concentrations of rifamycins against a resistant strain of C.trachomatis (MIC >200 µg/ml) in McCoy cells remain unsolved particularly as further increases in the concentrations of the drugs result in the death of the cells, limiting the findings on the final MICs.

FOLIC ACID INHIBITORS-

In the past, the results of in-vitro studies of the combination of sulphamethoxazole/trimethoprim against C.trachomatis with fixed ratios of 1:1, 5:1 and 20:1 of sulphamethoxazole to trimethoprim have been reported by Johannison et al (1979) and Hammerschlag (1982). These workers demonstrated an additive response in these combinations. In these reports, at no time were the ratios of the two antimicrobial agents reversed, resulting in the concentration of trimethoprim exceeding that of sulphamethoxazole.

In contrast, How et al (1985) used a chequerboard titration similar to the one described in this work, allowing the ratios to be reversed. They found that the combination of the two agents was synergistic when the ratios of trimethoprim to sulphamethoxazole were 64:1 or 4:1. However, from their report the criteria used for the determination of the end point after a single cycle of growth at 72 hrs are unclear.

Folic acid pathway inhibitors are essentially bacteristatic against Chlamydia, as seen by the need for multiple passages to reach a stable end point. In order to overcome this problem, the experiment presented in this work was extended. The results were examined and calculations made for passage one (P1), and again, following multiple passages, for a further period of 4 weeks at passage 10 (P10). A stable end point was reached and maintained after 6 passages in the sulphamethoxazole/trimethoprim combination as well as in that of sulfadoxine/pyrimethamine. The FICI for each combination at P1 and P10 was then calculated, taking the MIC of each drug into consideration at P1 and at P10 respectively.

The results obtained show a synergistic activity between sulphamethoxazole and trimethroprim at P1 as well as at P10. Indifference was demonstrated with sulfadoxine and pyrimethamine at P1, but at P10 activity was synergistic. The indifference observed with sulfadoxine and pyrimethamine after a single cycle of growth is somewhat subjective, as an end-point reading of only a 2 fold difference alters the MIC calculation towards synergy. This further demonstrates the necessity for multiple passages in a study of combinations of these drugs against chlamydiae. The synergy between sulphamethoxazole and trimethoprim is most striking when the concentration of trimethoprim exceeds that of sulphamethoxazole by a factor of 8, and that of sulfadoxine and pyrimethamine when the concentration of pyrimethamine exceeds sulfadoxine by a factor of 32. However, cotrimoxazole (sulphamethoxazole:trimethoprim at 5:1) is occasionally used in the treatment of chlamydial infections. This compound produces a plasma level of 20:1 and, therefore, it is unlikely that tissue concentrations of the two drugs will ever be optimal for the succesful treatment of chlamydial infections. The same conclusion applies in the case of the proprietary preparation of sulfadoxine/pyrimethamine if used in the treatment of chlamydial infections.

The in-vitro activities of a total of 51 antimicrobial drugs have been examined in this thesis. Only a few of these drugs have undergone clinical trials, with varying degrees of success. However, on their own, none of the drugs clinically tested can give a 100% cure rate for concomitant genital gonococcal, chlamydial and mycoplasmal infections. Penicillin and tetracycline resistant gonococci already exist, and the possibility of the emergence of tetracycline or erythromycin resistant strains of chlamydiae cannot be ruled out. With an increasing awareness of the importance of genital chlamydial infections coupled with a high prevalence of these organisms worldwide, it is obvious that there is an urgent need to find safe and effective antimicrobial agents against these organisms.

There is also a clear need to standardize methods of testing in-vitro sensitivy of these organisms. Currently, several different methods are employed. These, though similar in principle, vary in such detail as the choice of cell lines and inhibitors, the size of the inoculum, speed of centrifugation and, finally, the staining methods and the criteria employed for the determination of the end point (Blackman et al 1977, Ridgway et al 1978, Baily et al 1984 and Asche and Hutton 1988). However, it should be noted that in-vitro studies aimed at finding an active drug provide only initial guidelines and the ultimate test for the effectiveness of these drugs has to rest with the results of clinical trials. Several clinical trials for the treatment of chlamydial infections with the newer antimicrobials have produced inconsistent results. This has arisen largely as a result of the lack of an agreed protocol regarding a test of cure, coupled with an agreed laboratory procedure for such a test. Therefore the need for well organized and controlled clinical trials with efficient follow up and standardized laboratory investigations to assess the effectiveness of

new antimicrobial agents against infections with Chlamydia is clear.

The results of several of the in-vitro investigations studied here have been promising, and indeed some in-vivo studies were conducted at UCH based on these findings. However, there are still a number of these active drugs which await clinical trials, and some of these may prove to be useful alternatives to conventional therapy used against chlamydial infection.

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ACKNOWLEDGEMENTS

I would like to express my thanks to the following:

The Microbiology Special Fund for partly supporting this work through a grant.

Dr. N. Taylor of London Scientific Films for his generosity in making available his expertise and resources in time lapse photography.

Mr. G. Mcphail of the Histopathology Department at UCH for teaching me the sectioning techniques and photographic procedures necessary for the electron microscopy work.

Mr. A. Cremer of the Microbiology department at UCH for helping me with the photography related to the light microscopy work.

Dr. G.L.Ridgway, my supervisor, and Prof. J.R.Pattison for all their advice and guidance.