

THE EPIDEMIOLOGY OF CHLAMYDIAL  
INFECTIONS IN SCOTLAND WITH PARTICULAR  
REFERENCE TO CHLAMYDIA TRACHOMATIS

BY

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TO MARGARET AND COLIN

TABLE OF CONTENTS

Page No.

ACKNOWLEDGEMENTS

i

SUMMARY

ii

CHAPTER 1

EARLY PHASES OF CHLAMYDIAL  
INVESTIGATION

1.1 Introduction

1

1.2 Phase 1

4

1.3 Phase 2

11

CHAPTER 2

CELL CULTURE TECHNIQUES FOR  
C. TRACHOMATIS ISOLATION

2.1 Introduction

16

2.2 Cell Culture Contaminants

17

2.3 Strains of Cells Used For In  
Vitro Cultivation

20

2.4 Enhanced Sensitivity to Infection  
of Continuous Cell Strains

23

CHAPTER 3

ISOLATION RATES ASSOCIATED  
WITH DISEASE ENTITIES

3.1 Non-Gonococcal Urethritis

39

3.2 Epididymitis

44

3.3 Reiter's Syndrome

47

3.4 Cervicitis

50

3.5 Salpingitis

54

		<u>Page No.</u>
3.6	Prenatal or Intrapartum Infections	56
CHAPTER 4	SEROLOGICAL TESTS FOR <u>C. TRACHOMATIS</u>	
4.1	Group and Type Specific Antigens	60
4.2	Screening Human Sera	70
4.3	Chemical Composition of Cell Wall Components	70
CHAPTER 5	PARAMETERS AFFECTING INFECTIVITY TITRATIONS OF <u>C. TRACHOMATIS</u> IN CELL CULTURE - MATERIALS AND METHODS	
5.1	Introduction	73
5.2	General Requirements for Cell Culture	74
5.3	Chlamydial inoculum	79
5.4	Centrifugation.	82
5.5	Staining Procedures	84
5.6	Counting Procedures	85
5.7	Isolation Techniques	86
5.8	Statistical Analyses	89
CHAPTER 6	RESULTS OF INFECTIVITY ASSAY CONDITIONS	
6.1	Introduction	93

6.2	Comparison of Different Isolation Techniques for <u>C.trachomatis</u>	98
6.3	Comparison of Different Media For The Maintenance of Chlamydiae at Different Storage Temperatures	100
TABLES	6.1 - 6.8.4	103
FIGURES	6.1 - 6.5	112
CHAPTER 7	PREPARATION OF CHLAMYDIAL ANTIGENS - MATERIAL AND METHODS	
7.1	Introduction	118
7.2	Chick Embryo Inoculation Procedures	119
7.3	Urografin	124
7.4	Enzyme-Immunoassay Procedures	124
7.5	Preparation of Slide Antigens for the Micro-IF Technique	126
7.6	Staining Procedures for the Indirect FAT	127
7.7	Complement Fixation Test	128
7.8	Titration of Pooled Reference Serum	131
7.9	Purification of <u>C.trachomatis</u> by Centrifugation.	132

7.10	Purification of Cell Culture Grown <u>C. trachomatis</u>	135
7.11	Growth of IC CAL8 in 80 cm <sup>2</sup> Culture Flasks	137
CHAPTER 8	RESULTS OF PURIFICATION PROCEDURES FOR <u>C. TRACHOMATIS</u>	
8.1	Purification of Yolk-sac grown organisms	140
8.2	Preparation of Micro-IF Chlamydial A Antigens	141
8.3	Purification of Cell Culture Grown Organisms	142
8.4	Purification of Organisms from 80 cm <sup>2</sup> Culture Flasks	145
8.5	CF Antigen	146
8.6	Comparison of CFT and Micro-IF to Detect Elevated Antibody Levels in Chlamydia infected patients	146
8.7	Characterization of the EIA Coating Antigen	147
FIGURES	8.1 - 8.11	149
TABLES	8.1 - 8.3	154
CHAPTER 9	AN EPIDEMIOLOGICAL INVEST- IGATION OF CHLAMYDIAL INFECTION IN WEST SCOTLAND	

		<u>Page No.</u>	
	9.1	Introduction	157
9.2	9.2	Patients	158
	9.3	Isolation and Serological Procedures	162
	9.4	Isolation Results	163
	9.5	Perinatal Infection Rates	164
	9.6	Prevalence of Chlamydial Seroreactors	165
	TABLES	9.1 - 9.13	170
	FIGURES	9.1 - 9.6	177
CHAPTER 10		DISCUSSION	
	10.1	Introduction	184
	10.2	Cell Culture Procedures	184
	10.3	Preparation of Chlamydial Antigens for Serology	193
	10.4	Serological Techniques - Complement Fixation	198
	10.5	Micro-IF	202
	10.6	Frequency of Chlamydial Infection in Different Groups of Patients:-	
		Female patients	204
	10.7	Male patients	216
		APPENDIX 1	222

Page No.

APPENDIX 111

229

REFERENCES

230



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## SUMMARY

Cell culture was found to be the most useful system for investigation of different aspects of C.trachomatis infection. Chlamydial infectivity was shown to be influenced by slight changes in the physiologic condition of the culture medium including transport medium used to collect clinical specimens. In this respect optimal conditions were sought that would maximize the infectivity titre. Several isolation procedures developed by others using cell culture techniques were available for a large scale epidemiological study. These were found to vary in their relative sensitivities; best results were obtained from cycloheximide treated McCoy cells.

A recently modified complement fixation test (CFT) and micro-immunofluorescent (micro-IF) technique were both used to estimate the prevalence of positive seroreactors to chlamydial antigens in different categories of patient. Cell culture grown organisms were used in the CFT and infected yolk-sac suspensions of serotypes D-K (inclusive) for immunofluorescence. Both types of chlamydial preparation were concentrated and purified by centrifuging through a cushion of diatrizoate (Urografin). Urografin was selected from other solutes examined in the experimental work. Antigenic (whole organism) purity was measured using an enzyme-immunoassay (EIA) developed for this purpose.

Finally, different groups of patients were examined for incidence

of both past and present chlamydial infection. Among female patients the highest rate was found among an unselected group attending a Genito-urinary Medicine Clinic (GUM). These patients tended to be infected at a relatively young age (63 per cent were under 24 years of age) and it was postulated that this may be associated with the subsequent development of cervical dysplasia in older women.

The infection rate in pregnant women was significantly lower than similar studies elsewhere. This appeared to be related to the stage at which women were examined during pregnancy. With evidence from other published data it was suggested that the level of ovarian hormones may influence the outcome of isolation attempts.

Chlamydial conjunctivitis was found in a proportion of neonates, but there appeared to be no evidence of an associated pneumonitis syndrome.

Different groups of male patients and their consorts were examined. The current infection rate in patients with Reiter's syndrome (RS) was related to the number of previous recurrences of symptoms. These patients differed from a control group who had recurrent non-gonococcal urethritis (rNGU) in that a higher proportion of RS patients had the HLA - B27 antigen, also more of the consorts of the RS group appeared to have serological evidence of chlamydial infection.

The infection rate of male attenders at a G.U.M. clinic was similar to results found elsewhere. It was suggested that a small proportion of patients with NGU eventually become sub-fertile. However a low incidence of current infection was found in a group of subfertile male patients examined.

## CHAPTER 1

### EARLY PHASES OF CHLAMYDIAL INVESTIGATION

1.1

#### INTRODUCTION

Psittacosis, ornithosis, lymphogranuloma venereum (L.G.V.), trachoma and inclusion conjunctivitis are well recognised human diseases caused by microorganisms of a group which is widespread in nature. At a conference on Trachoma and Allied Diseases, Meyer (1967) gave a detailed and comprehensive review of the worldwide distribution of these organisms and the broad ranges of their parasitism. Among the mammals found to be carriers of Chlamydia, and found to be suffering from chlamydia-induced diseases are: mice, cats, cattle, sheep, goats, dogs, rats, and many other rodents. Meyer stated that 130 species of birds have been found to be infected and there is no doubt that this list will be extended as more bird species are studied.

Epidemiological investigations in the past, have been successful when the infecting chlamydial strain has been isolated in the laboratory with relative ease, or when serum antibody is detectable in the host specific for the group antigen. This is particularly true for the wide range of infections in birds and mammals caused by one of the two chlamydial species, Chlamydia psittaci (C.psittaci)

In contrast to the broad range of hosts parasitized by C.psittaci, naturally occurring infections associated with Chlamydia trachomatis (C.trachomatis) are restricted to the human.

Excluding the strains of C.trachomatis associated with L.G.V., organisms belonging to this species are generally less virulent in vivo and in ovo than C.psittaci. This has been technically inconvenient and has temporarily restricted scientific investigations into C.trachomatis. A useful but laborious procedure for the isolation of C.trachomatis in the yolk-sac of embryonated hen's eggs was developed by the 1960's and provided impetus for a surge of epidemiological investigations. It is now recognised that C.trachomatis is a major cause of morbidity in tropical and semi-tropical regions. Trachoma is an ocular infection of varying degrees of severity which can eventually result in serious blindness. Grayston (1975) reported that the worldwide impact of trachoma had diminished significantly since the Second World War. C.trachomatis still remains one of the most common infectious agents in the world, partly because of its tendency to produce long-term infectious processes, its almost universal distribution in 'third world' countries and the lack of adequate health care in these countries. It has been estimated that infected individuals worldwide number hundreds of millions.

In addition to the ocular infections, C.trachomatis has recently been implicated as the aetiological agent for a spectrum of urogenital infections including non-gonococcal urethritis and cervicitis. These infections are not associated with the production of significantly high titres of antibody against the group antigen and investigations based on isolating the microorganism have until relatively recently, been unproductive.

The diagnosis of all chlamydial infections in Scotland has for the

last 30 years depended on clinical, epidemiological and serological investigations and has been concerned mainly with C.psittaci strains. Attempts to isolate chlamydial strains on a larger scale have not been made. Accurate diagnosis has ultimately depended on serological tests which in the infected individual indicates the presence of complement fixing antibodies specific for the chlamydial group antigen. The complement fixation test utilizing the group antigen provides a simple, sensitive and rapid test for L.G.V. and psittacosis. The superficial ocular and uro-genital infections fail to evoke a diagnostically significant antibody response.

A technological breakthrough occurred in 1963 when Gordon and his associates isolated C.trachomatis in cell culture. The new technique greatly reduced the time and effort required to isolate the organism and started a phase of interest which continues to this present time.

This thesis is not primarily concerned with psittacosis or trachoma although they are mentioned when appropriate. The experimental work and discussion concentrates on the strains of C.trachomatis which have recently been implicated in uro-genital and neonatal infections. A broad epidemiological survey has been carried out utilising various laboratory diagnostic and screening procedures. Certain selected populations of patients with distinctive and suspicious clinical symptoms have been examined for the presence of viable infecting chlamydiae and for specific antibodies of the IgG class. The precise details of this work are discussed fully in chapters 6,7 and 9.

The technical advances associated with these topics were pioneered

during two major periods of investigation. The first phase was initiated in the early 1930's in response to the need to control a pandemic of psittacosis. The second extended to the late 1960's and was attributed to a concerted eradication programme against trachoma world-wide, sponsored by WHO and governmental agencies.

These periods of investigation will first be reviewed in detail as they produced our fundamental knowledge and provided the frame-work around and on which this work was based.

1.2.1

### PHASE 1

#### The P-L Group

In 1929, cases of a serious and peculiar pneumonia among the inhabitants of Cordoba and Buenos Aires (Argentina) appeared in epidemic proportions (Bedson, S.P. 1930). The outbreak was associated with the importation of 5,000 psittacine birds (parrots, parakeets and budgerigars) to Argentina from Brazil. Smears of virulent material obtained from naturally occurring human and avian cases revealed very small pleomorphic and basophilic bodies which were visible with difficulty using the light microscope known to have a resolution of 200 nm.

Further laboratory investigations of the agent of psittacosis demonstrated their ability to pass through the finer grades of Berkefeld and Chamberlain filters, and their inability to grow on non-living media. These characteristics partially fulfilled the definition of viruses. However, unlike viruses, chlamydiae stain readily by methods which had earlier been used to identify rickettsiae (Castaneda and Giemsa). Perhaps the most significant early observation



was the characteristic of the psittacosis agent to undergo a complex intracellular developmental cycle. (Bedson, S.P. 1959). This characteristic is unique amongst microorganisms and has important taxonomic implications.

At about the same time the agent of psittacosis was being characterised, the aetiology of a long recognised clinical entity, 'climatic bubo', was determined. Findlay (1933) showed that the infectious agent present in inguinal buboes was filterable and mice inoculated intracerebrally succumb 2-30 days later. Two years after these observations, Miyagawa and his colleagues (1935) demonstrated the staining and microscopic characteristics of this agent and the similarity to the previously identified agent of psittacosis. MacKenzie (1936) showed that the cycle of intracellular development was similar to the psittacosis agent described 6 years earlier. The term 'climatic bubo' has been replaced by lymphogranuloma venereum (L.G.V.)

#### 1.2.2. IN VITRO CULTIVATION OF C.PSITTACI

Early cultivation of the agents of psittacosis and lymphogranuloma (P-L group) for diagnostic purposes were carried out in mice. Specimens containing sputum samples in the cases of psittacosis, and pus aspirated from fluctuant nodes in cases of L.G.V. were injected intraperitoneally. The infected mouse died several days after inoculation and at autopsy showed enlarged spleen, liver necroses and a serofibrinous peritoneal exudate with abundant chlamydiae present in the cytoplasm of reticuloendothelial cells. Sterility of cultures in the usual bacteriology medium prove the chlamydial

nature of the infectious process.

The development of egg cultivation techniques provided a more satisfactory alternative to the use of experimental animals. Fertile eggs are readily available, easier to look after and have little likelihood of carrying endogenous chlamydiae. Rake et al (1940) isolated a chlamydial agent from a case of L.G.V. in the yolk-sac of chicken embryos. Depending on the concentration of the inoculum, embryos die 3 to 14 days after inoculation. The endothelial lining cells of the yolk-sac, which are the primitive haemopoetic tissue, are susceptible to all agents of the P-L group.

Susceptibility of the yolk-sac endothelial cells is attributed to its special kind of metabolism (Moulder, 1953). It has been postulated that the unusual size of the cells permit the development of much larger inclusions than do other tissues (Weiss, 1949). The folded structure of the yolk-sac increases the proximity of the endothelial cells to each other and may favour ready passage of chlamydiae from cell to cell. Once chlamydial replication has taken place to the extent of cellular rupture, infectious particles are released into the yolk-sac. The yolk itself is probably a favourable medium for survival (Allen, 1952).

1.2.3

#### TAXONOMY

Classification of the family Chlamydiaceae alongside the families Rickettsiaceae and Bartonellaceae in the order Rickettsiales was first made in Bergey's 6th edition (1948). The reasons for placing

Chlamydiaceae in the order were not explained in earlier editions but by 1957 it appeared that this order had accumulated several groups of very diverse organisms, most of which shared only one characteristic; they could not be cultivated outside of living animal cells. The concept of placing the Chlamydiaceae in a separate order was initially suggested by Storz. This notion was unanimously endorsed by members of the Subcommittee on Chlamydiaceae of the Taxonomy Committee of the American Society for Microbiology (1971).

The principal argument for establishing the new order was that the intracellular developmental cycle of chlamydiae as originally described by Bedson and Bland, is unique, not only amongst organisms of the other families in the order Rickettsiales but is without parallel among organisms in all other orders of bacteria.

#### 1.2.4 INTRACELLULAR DEVELOPMENT OF C.PSITTACI

Numerous cell culture systems have been developed to study in greater detail features of the chlamydial developmental cycle. The first system was successfully employed by Bland and Canti (1935). Primary cell monolayers were obtained from 12 - 14 day old chick embryos. Leg muscle fibroblasts and lung epithelial cells were suitable for the intracellular development of the psittacosis agent (C.psittaci). A laboratory strain of C.psittaci, which had originally been isolated from a parrot and passaged subsequently in laboratory mice was used for the experimental work. The details of colony formation within the cellular cytoplasm could be viewed in live preparations by dark ground illumination. No repetition of

the cycle appeared to occur even in cultures kept for 1 week or more.

Since those initial experiments in cell culture, C.psittaci has been adapted to grow in a wide range of established cell lines. Storz has listed many of the lines that have been used for studies on the isolation, cultivation and host-parasite interaction of chlamydiae (Storz, 1971). Not all strains however grow well in cell culture and production of large amounts of infectious particles in this manner has been difficult.

In more recent years study of growth cycles has been investigated using a successful cell culture technique developed by Higashi, (1959) (1960). The Cal-10 strain of C.psittaci was adapted to grow in suspended cultures of mouse fibroblasts (L cells). The organism grows well on both monolayer and in cell suspension; in the latter system 10 - 100 mg. amounts of highly purified organism can be routinely prepared.

Using the Cal-10/L-cell system, electron micrographs show that populations of chlamydiae are heterogeneous in size and internal structure. In spite of a continuous morphological spectrum and variability in size, it is convenient to regard C.psittaci as being composed of two main particle types: one relatively small, about 300 nm. diameter with a dense central body, the other relatively large, 1000 nm., and with no central body. Detailed electron microscopy at different stages of the infection cycle show that the growth cycle is usually initiated by the smaller elementary body (EB). The EB envelope resembles the cellular envelope of Gram-negative bacteria in a number of respects, such as

amino acid composition and the presence of a carbohydrate-lipopolysaccharide complex (Manire and Tamura, 1967) (Narita and Manire, 1976). It has been suggested that the EB is specialised for a brief extracellular journey and invasion of a new cell. The EB's resistant rigid cell wall has a demonstrable haemagglutinin in its envelope which may be involved in the process of attachment and penetration of the host cell (Gogolak and Ross, 1955) (Hilleman et al, 1951). Friis (1972) concluded that the entry of C.psittaci into L cells was brought about entirely by the phagocytic activity of host cells. Dead chlamydiae were ingested just as fast as live ones. L cells treated with phagocytosis-inhibiting drugs or chilled to low temperatures failed to take up chlamydiae.

After penetration of host cell membrane, the EB begins to undergo changes in its envelope, shown by its increasing sensitivity to sonic treatment. The dense central part of the EB disperses to form a homogeneous cytoplasm in which strands of nucleic acid and many ribosomes can be seen. The mass of the organism enlarges and the second form, the reticulate body, (RB), is complete. The cell envelope of the RB is very fragile and contains no haemagglutinin (Tamura and Manire 1967) (Tamura and Manire 1974). The RB divides and multiplies within host cell vacuoles (phagosomes), giving rise to a characteristic inclusion body. At 20 - 25 hours post-infection, some of the RB's begin to undergo a conversion process in which there is a central condensation of the cytoplasmic contents and a typical EB is formed. The process of release of organisms from the cells normally occurs 48 - 72 hours after infection. Little is known of this process, infected cells probably dying 40 - 60 hours after infection followed by autolysis.

The complexity of the antigenic structure of chlamydiae was first recognised by Bedson (1933) (1936). Sera from patients convalescent from psittacosis, fixed complement in the presence of a "psittacosis virus". Initial observations by Bedson showed that C.psittaci contained two antigens; one heat labile, the other resisting boiling or even autoclaving at 120°C. Convalescent sera contained antibodies to both antigens. This could be demonstrated by complement fixation tests made with unabsorbed sera and with serum that had been absorbed separately with the whole chlamydial particle or with its heat stable component. Bedson concluded that all members of the P-L group possessed the heat stable antigen. Also, C.psittaci must possess specific antigenic components and these must be found in the heat labile fractions. Sera from patients convalescent from L.G.V. reacted almost completely with the unheated "psittacosis virus" and completely with heat (100°C) treated particles.

A more detailed analysis of the antigenic components of the chlamydial particle was not undertaken until 1957, when Ross and Gogolak used C.psittaci from cases of psittacosis and feline pneumonitis in their investigations. Chlamydiae were grown in the allantoic fluid of 8 day old chicken embryos, purified by fractional centrifugation, disrupted by sonic vibration and lyophilised. Two complement fixing antigens were extracted from these preparations, one ether soluble, the other alkali soluble. The latter possessed both specific and group activity, whereas the ether soluble fraction was only group specific.

This specificity was shown by the effect of treatment with potassium periodate and lecithinase which completely abolished the activity of the ether soluble antigen but narrowed the activity of the alkali soluble fraction to one of species specificity. The specific antigen of this latter preparation could then be destroyed by papain, but not trypsin. Ross and Gogolak (1957) suggested that both the group and specific serological activities of chlamydial particles reside in a lecithin nucleoprotein complex.

1.3.1

### PHASE 2 - C. TRACHOMATIS

In 1907 Halberstaedter and Prowazek made the first important aetiological observation when they found cytoplasmic inclusion bodies in epithelial scrapings from cases of trachoma. Two years later Lindner found identical inclusions in cases of gonococcus-free neonatal conjunctivitis. This bacterial-free conjunctivitis of the newborn was defined by the term inclusion blenorrhoea. Most of the pioneering studies of chlamydial genital-tract infections were performed by ophthalmologists such as Lindner and later Thygeson (Thygeson 1936, 1942). The infant with inclusion blenorrhoea was shown to have a chlamydial infection. Subsequently, the cervix of the mother was shown to be infected as was the urethra of the father. Lindner produced disease in a macaque and a baboon with genital secretions of a mother whose baby had inclusion blenorrhoea and found numerous inclusions in scrapings from the diseased conjunctiva of the baboon. Previously however, Nicolle et al (1913) passed the agent of trachoma through Berkefeld filters, inoculated a chimpanzee and a barbary ape (Macaca innus) and produced an experimental disease for which a trachomatous

nature was established by transmission to the conjunctiva of a human volunteer.

On the basis of the morphological similarity of their developmental cycle, the agent of trachoma and inclusion blenorrhoea were accepted as members of the P-L group. The validity of this concept was strengthened by the demonstration by Rake (1940) of a serological relationship through the possession of a common group complement fixing antigen and finally by the cultivation of these agents in the yolk-sac of embryonated hen's eggs - a group characteristic.

### 1.3.2 IN VITRO CULTIVATION OF C. TRACHOMATIS

The history of cultivation attempts for cases of trachoma is a long one. Thygeson commented in 1962: "Looking back on the problem of cultivation in the light of recent yolk-sac inoculations one wonders at the scant attention given this medium compared with the great deal of attention given the chorioallantois and tissue culture. Previously, in 1957, using material from untreated stage II trachoma cases, T'ang and his colleagues isolated the agent of trachoma in the yolk-sac of 6 - 8 day old embryonated eggs. The findings of the Peking investigators were confirmed and extended in 1958 by Collier and Sowa (1958). These initial successes of isolating the trachoma agent were soon followed by isolations in Saudi Arabia and Egypt (Murray 1960). In the same year, a number of strains were isolated by Bernkopf in Israel (1959).

T.'ang's yolk-sac method for the isolation of C. trachomatis was applied to genital-tract infections; the sequence of



investigations started with cases of neonatal conjunctivitis followed by related cervical and urethral infections. In 1959 isolation of the organism from the cervix was reported by Jones and Collier, and in 1964, isolation of chlamydiae had been obtained from men with urethritis and women with cervicitis (Jones and Collier 1959) (Jones et al, 1964) (Rose and Schachter, 1964). These results were obtained from egg cultures, a procedure that could take up to 6 weeks before readily identifiable chlamydiae could be detected.

### 1.3.3 DEVELOPMENT OF SEROLOGICAL TECHNIQUES FOR C.TRACHOMATIS

Of the diseases associated with C.trachomatis, trachoma attracted most attention. Treatment programmes using sulphonamides were ineffective in endemic areas. The failure of these programmes was attributed to:- (1) The long treatment time required and the consequent lack of patient cooperation. (2) The frequency of reinfection in trachoma endemic areas. In 1962 it was estimated that approximately 400 million of the world population suffered from trachoma.

A number of studies were aimed at developing vaccines for the control and possible eradication of trachoma (Grayston, 1967) (Nichols et al, 1966). While it was possible to modify the course of experimental trachoma and to prevent trachoma in the monkey model, the difficulty and expense of preparing high titred purified antigens and the limited period of protection have suggested that the techniques of vaccine production employed were not practical for the control of trachoma (Grayston 1971).

Perhaps a more serious difficulty associated with the vaccination experiments, was the observation that trachoma often tended to be more severe in vaccinated persons. This phenomenon may be due to hypersensitivity reactions. Similar incidents have been experienced with killed measles virus, respiratory syncytial virus and Mycoplasma pneumoniae vaccines (Craighead, 1975). It was found that immunisation with an inadequate antigenic mass or with a heterologous strain results in a more severe disease (Wang et al 1967).

If human vaccination is to be pursued further with thoughts of preventing the disease, it is necessary to determine how many immunological types exist and whether or not geographical distribution is important. The complement fixation test and other serological tests before 1967 failed to provide adequate specificity for classification. McComb and Bell (1967) succeeded in demonstrating some specificity with the immunofluorescence technique. Purified chlamydial antigens were prepared and inoculated into rabbits by a single intracardiac inoculation. Seven to ten days later the animal was bled. Immune serum was titrated against 5% infected crude yolk-sac preparations by immunofluorescence. A method for calculating specificity differences between strains was used to distinguish three serotypes associated with trachomatous infections in hyperendemic areas.

Following on from McComb and Bell's work, a similar system was developed by Wang et al, utilizing immune serum against chlamydial serotypes raised in mice (Wang and Grayson 1970, 1975). Each laboratory isolate was passaged in embryonated eggs until a high

infectivity titre was obtained. Serum specific for each yolk-sac isolate was collected from mice that had been immunised by intravenous inoculation. A two-way test was carried out for each isolate. Immune serum was tested against known yolk-sac grown chlamydial antigens and each yolk-sac isolate was titrated for homogeneity against serum from known serotypes. In this way it was possible to distinguish between 14 different stable C.trachomatis serotypes (immunotypes). Wang and Grayson examined 300 isolates obtained from different parts of the world and from varying types of infection. A pattern of serotype distribution emerged. Certain serotypes (A, B and C) were associated with endemic trachoma regions and other serotypes (D, E, F, G, H, I, J and K) with uro-genital and some types of ocular infections. Organisms isolated from cases of L.G.V. were typed into a further three distinct serotypes designated: LI, LII and LIII. Infected yolk-sac material is dotted onto a glass slide in such a way that each known serotype is repeated eight times on the slide. The antigen plaques are no larger than 1 mm. in diameter and, for this reason, the tests became known as the micro-immunofluorescent technique (micro-IF).

All further discussions relate to strains of C.trachomatis associated with uro-genital and neonatal infections (Serotypes D - K inclusive).

## CHAPTER 2

### CELL CULTURE TECHNIQUES FOR C.TRACHOMATIS ISOLATION

2.1

#### INTRODUCTION

Although the Chlamydia are recognised as highly evolved host dependent bacteria (Moulder, J.W.; 1964), the techniques required to isolate these organisms have been developed in virology laboratories. In 1947 Enders, Walker and Robbins reported that poliovirus could be grown in cultured non-neural cells with the production of recognisable histological changes. Since then a large number of animal viruses have been grown in cultured cells. These initial observations were made at a time when cell culture techniques had undergone some remarkable developments. With the added practical interest, the number of people involved in cell and tissue culture increased and the whole subject evolved rapidly between 1950 and 1960.

Cell culture has been greatly aided by the development of chemically defined media containing almost all the nutrients required for cell growth. All media used in cell culture have as a basis a synthetic mixture of inorganic salts known as a Balanced Salt Solution (BSS). The function of this salt solution is to maintain the pH and osmotic pressure in the medium and also to provide an adequate concentration of essential inorganic ions. All physiological salt solutions have been derived from the salt solution originally described by Ringer.

Salt solutions can be divided into two types: those intended to equilibrate with air (e.g. Hank's BSS) and those designed to equilibrate with a gas phase containing a high carbon dioxide tension of the order of 5 per cent (e.g. Earle's BSS).

Rota and Nichols (1973) studied the ability of several commercially available synthetic media to support C.trachomatis infections in cell culture. Earle's BSS with 10 per cent horse serum at pH 7.4 proved favourable for the development of intracytoplasmic inclusions. The medium required gassing, and when serum was omitted, more inclusions were observed at pH 7.0 than at pH 7.4. Hank's BSS was almost as effective as Earle's BSS but had the advantage of not requiring gassing and could be used successfully at the pH supplied by the manufacturers. All their work utilised McCoy cells for which it was suggested that a suitable combination of media would be 50 per cent Earle's BSS with 50 per cent Hank's BSS. The medium would be sufficiently buffered and would require neither gassing nor additional  $\text{NaHCO}_3$ .

2.2

### CELL CULTURE CONTAMINANTS

Media for the cultivation of cells are highly nutritious not only for mammalian cells but also for bacteria and fungi. The majority of these micro-organisms have more rapid growth-rates than eukaryotic cells and frequently produce toxins which are lethal to them. One of the preventative measures taken to avoid contamination in most cell culture laboratories, is the use of antibiotics in culture media.

The susceptibility of C.trachomatis to antimicrobial drugs has been confirmed in several laboratories. Treharne (1977) used a cell culture system to assay minimal inhibitory concentrations for a wide range of antimicrobial compounds against C.trachomatis (Serotype D). Penicillin and streptomycin are the antibiotics classically used in tissue culture. However, the low level of penicillin required to inhibit chlamydial growth (1 µg/ml) does not permit its use. For chlamydial isolation, vancomycin and streptomycin have been used (Gordon et al, 1962: Sowa et al, 1971). Treharne however pointed out that these antibiotics have a limited antibacterial spectrum and suggested the additional use of gentamycin to help reduce bacterial contaminants. This may be particularly relevant when attempting to isolate C.trachomatis from the genital tract or rectum.

Minimal inhibitory and minimal lethal concentrations of antimicrobial agents for C.trachomatis (Serotype D)

Taken from: (Treharne et al, 1977)

DRUG	MIC (µg/ml)	MLC (µg/ml)
Rifampicin	0.05 - 0.1	1.0
Oxytetracycline	0.02 - 0.5	0.5
Doxycycline	0.2	0.2
Microcycline	0.2	-
Spiramycin	0.5	0.5
Erythromycin	0.5	-
Sulphamethoxazole	0.5	-
Chloramphenicol	1.0	5.0

DRUG	MIC ( $\mu\text{g/ml}$ )	MLC ( $\mu\text{g/ml}$ )
Benzylopenicillin	1.0	-
Septrin	1.0	-
Ara-AMP	5.0	-
Trimethoprim	20.0	-
Novobiocin	50	-
Kanamycin	50	-
Soframycin	200	-
Gentamycin	500	-
Colistin	500	-
Vancomycin	1000	-
5-Fluorocytosine	50	-
Nystatin	500	-

Ordinary microbial contamination has now been reduced to a minor role in cell culture work. However, despite the use of antibiotics two categories of micro-organisms have been detected as contaminants. One of these groups of possible contaminants is viruses found in animal serum and latent in primary cell cultures, against which antibiotics are ineffective. Kajima (1967) used electron microscopic techniques to examine batches of McCoy cells that had been stored frozen in liquid nitrogen in 10 per cent glycerin. He found virus-like particles associated with the McCoy cells similar to type A (doughnut-like particles) and type C (particles with a large nucleoid) murine tumour viruses. Cylindrical structures were also seen among the spherical (100 nm diameter) virus-like particles and have been observed previously by others in tissues of leukaemic

rodents. It was suggested in discussion, that these particles may be morphological representatives of an abnormal cell secretion. The McCoy cell line is the most frequently used for C.trachomatis isolation. In the absence of overt cytopathology, it is unusual to find circulating viruses in passaged heteroploid cell lines such as McCoy cells. It is possible that Kajima's work was carried out on cells passaged only a few times from their original source.

Viral contamination has been found much less frequently in serially passaged cultures than have contaminants, by the second category of micro-organism, members of the genus Mycoplasma. Hayflick (1965) estimated that about 60 per cent of established cell lines were contaminated with mycoplasmas. The presence of mycoplasmas in the great majority of contaminated cell cultures cannot be detected by any macro- or microscopic changes in appearance of the culture medium or of the cells themselves (Krule and Patterson, 1973). Generally, the presence of mycoplasmas in cell cultures can only be made by demonstrating 100  $\mu$ m. diameter colonies giving their characteristic appearance in the appropriate agar, (PPLO agar supplemented with 20 per cent unheated horse serum). Recently Cheu (1977) developed an in situ detection technique of mycoplasma contamination in cell cultures by using a fluorescent stain. Cells are grown on cover-slips, fixed, air dried and stained with a DNA specific fluorescent stain (Hoechst 33258).

### 2.3 STRAINS OF CELLS USED FOR IN VITRO CULTIVATION

Culture of cells in monolayer is the most common method used in



diagnostic virology. Cell suspensions are prepared from tissue or from stock cultures by using one of the dispersing agents. The suspension is then diluted in "growth" medium to contain the number of cells required and dispensed in suitable containers.

When cells are taken freshly from animals and placed in culture, the culture consists of a variety of cell types most of which are capable of a very limited growth in vitro. However, these so called primary cell lines have been shown to support the replication of a wide range of viruses.

A further category of cell strains frequently used for the isolation of viruses is the established (continuous, heteroploid) cell line. These lines consist of cells of a single type that are capable of indefinite propagation in vitro. Such "immortal" lines usually originate from cancers, or by transformations occurring in diploid cell lines. They often no longer bear any resemblance to their cell of origin; they are aneuploid and probably undergo many sequential mutations. The great advantage of established cell lines over the other category is that they can be propagated indefinitely by sub-culturing at regular intervals. Furthermore, they retain viability for many years when suspended in a medium containing dimethyl sulphoxide (10 per cent) and stored in the vapour phase of liquid nitrogen ( $-170^{\circ}\text{C}$ ).

Hobson (1976) in discussing some of the difficulties of growing C.trachomatis in replicating established cell lines, suggested that the problem is fundamental rather than procedural. Cells

from established lines tend to be relatively small and have rapid cell cycles in complete medium. The types of cells previously shown to be susceptible to infection by Chlamydia, the entodermal cells of the yolk-sac and chick fibroblast explants, had opposite characteristics. They were relatively large and appeared to be "slow" growing.

Hobson tested primary cultures of human thyroid epithelium. This source of cells provided confluent monolayers of large slowly growing cells with abundant cytoplasm. Another characteristic, not mentioned in Hobson's report, is the particularly active endocytosis shown by these cells (Silverstein, Steinman and Cohn, 1971). Endocytosis is a widespread cellular function that regulates the uptake of exogenous material from the cell environment. Chlamydia, in common with other obligate intracellular parasites, utilize endocytosis pathways to penetrate and replicate within their host cells. Hobson's results showed that inclusion counts in infected thyroid cells were about eight times greater than respective replicating McCoy cell controls.

Primary human amnion epithelial cells have also been evaluated for their susceptibility to C.trachomatis infection (Harrison et al, 1979). Work with this cell line may have particular relevance in the clinical situation where amnionitis and associated complications may be possible consequences of chlamydial cervical infection. Results showed that these cells were as susceptible to C.trachomatis infection as McCoy cell controls.

## 2.4 ENHANCED SENSITIVITY TO INFECTION OF CONTINUOUS CELL STRAINS.

A recent approach in order to find a sensitive cell system for the primary isolation of C.trachomatis from clinical specimens, has been to render established cell lines susceptible to infection by using special "enhancing" techniques. The "enhancing" techniques considered in this review are:-

- (1) Centrifugation.
- (2) X-irradiated cells.
- (3) DEAE-dextran treated cells. (Diethylaminoethyl-dextran).
- (4) Chemical assault of cell monolayers to inhibit cell replication, but to allow cytoplasm to grow relative to nucleus. (Idoxuridine and Cycloheximide).

### 2.4.1

#### CENTRIFUGATION

Prompted by the earlier observation of Macdougald's (1953), that chick entodermal cells can withstand exceedingly high centrifugal forces and by work undertaken by Gey et al (1954) who effectively sedimented eastern equine encephalomyelitis virus onto cells, Weiss and Dressler investigated similar techniques for the isolation of Rickettsia prowazeki, the "psittacosis virus" and Coxiella burneti (Weiss et al, 1954). By this procedure, infection of cells with R.prowazeki was enhanced about 10,000 fold, "psittacosis virus" 100 fold and C.burneti showed a moderate increase.

Gordon and Quan (1960) used a similar technique to isolate a strain (TW10) of C.trachomatis, isolated originally from a case of trachoma in the yolk-sac of hen's eggs. The chlamydial isolate was partially purified by a procedure adapted from methods for

rickettsias (Allan et al, 1954). Cells were prepared by explanting the entodermal layer of 4 day old chick embryos to coverslips at 36°C under Hank's BSS and 25 per cent chicken serum. A monolayer of large epithelial cells developed within a few days. A small volume of partially purified suspension was centrifuged at 1,800 g for 1 hour at 18°C onto the cell monolayer.

A comparison of the efficiency of isolating C.trachomatis from the yolk-sac of embryonated hen eggs with the cell culture technique was undertaken. Entodermal cells were replaced with McCoy cells in this work. A monkey (*Macacca mulatta*) was inoculated with a strain of C.trachomatis (TRIC/USA-Cal) and attempts to isolate the same organism from the right tarsal conjunctiva were made. The cell culture system proved three to four times more sensitive than the isolation of C.trachomatis in the yolk-sac or the detection of inclusion bodies in smears (Gordon et al, 1969).

The centrifugal force used in these experiments was of the order 1,800 g for one hour at 18°C. The choice of 18°C appears to have been made as a compromise between thermal inactivation of C.trachomatis and inhibition of host-parasite interactions (Weiss and Dressler, 1960). The sensitivity of the procedure was increased significantly when centrifugation was carried out at 37°C. Thermal inactivation was not a problem and it was suggested that the increase in sensitivity was related to enhanced host-parasite interaction, particularly endocytosis, a temperature dependent process. There is a

gradual increase in infectivity to 15,000g but at this level of centrifugation, batch preparation of large numbers of specimens is not possible. For practical purposes centrifugation at 2,500g is effective. Centrifugation at 2,500g is not sufficient to physically deposit single chlamydial particles onto the cell monolayer. There have been few published investigations concerned with the effects of centrifugal forces on cell membranes. Pearce and Allan (1979) have investigated some aspects of centrifuge assisted infection. Their results strongly suggest centrifuge induced cell surface changes affecting susceptibility to infection and that successive transient cell states develop which affect both organism attachment and ingestion.

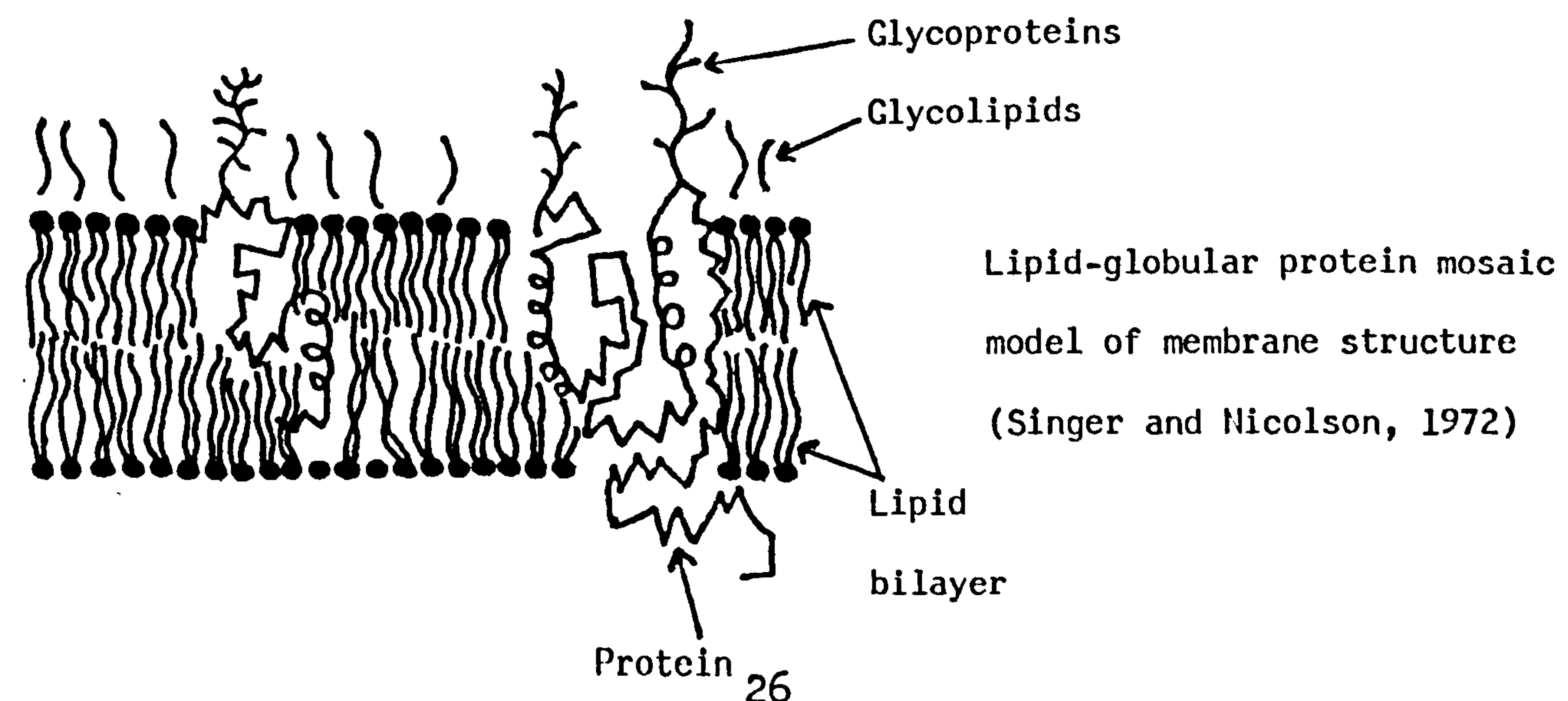
During centrifuge assisted infection C.psittaci, strain guinea pig inclusion conjunctivitis, showed two kinds of attachment to cell monolayers. In the first, 'production binding', organisms attached to cells and then entered and infected them. In the second, 'unproductive binding', organisms attached to cells but were not ingested. Their presence could be demonstrated by treating the monolayers with trypsin which stripped off extracellular organisms and titrating the trypsin supernatant on fresh monolayers. When the trypsin treated cells containing productively bound organisms were re-attached to cover slips, they gave the normal infectivity titre.

Infectivity could be increased if monolayers were cold-shocked and re-centrifuged after the initial centrifuge-assisted infection. Analysis showed that this procedure increased infectivity as a

result of entry and infection of previously unproductively bound organisms.

A comparison of infection curves (productive binding and cold-shock enhanced binding) showed that productive and unproductive binding took place sequentially and not simultaneously. Over the first 30 minutes, binding was slowly productive. By this time, cells appeared to have entered a refractory state during which no attachment occurred although many organisms were present in the supernatant. At about 45 minutes, further attachment took place indicating the appearance of a new cell state. This latter state was different from the initial state developing after the onset of centrifugation since wholly unproductive binding occurred.

Singer (1972) has examined in considerable detail several models of the gross structural organisation of membranes, in terms of thermodynamics of macromolecular systems. From this analysis it was concluded that a mosaic structure of alternating globular proteins and phospholipid bilayer was the only membrane model among those analysed that was simultaneously consistent with thermodynamic restrictions and with all the experimental data available.



The mosaic appears to be a fluid or dynamic one and for many purposes it is best thought of as two-dimensional orientated viscous solution. Allan and Pearce suggest that the compressive effects of pressure and directional force exerted by centrifuging may induce cell surface movement. Equally, sedimentation of extracellular structures might indirectly affect membrane receptors via cytoskeletal linkages. The successive transient nature of the changes is suggestive of an elastic response which might recur during prolonged centrifugation. Cold-shock may bring about the depolymerization of microtubules without the need for organism detachment.

#### 2.4.2

#### IRRADIATION OF CELL MONOLAYERS

Several workers have shown that irradiation of cells before inoculation with virus enhances susceptibility of that cell line. Levine (1962) showed that although X-irradiation of established cell lines was followed by the formation of 'giant' cells, irradiation of primary cells was not. Irradiation of cells consistently increased poliovirus yield per cell in established lines but not in primary monkey kidney, which suggested a relationship between the formation of giant cells and the yield of virus.

Levine calculated the cell volume of irradiated and non-irradiated cells. The cell monolayers were trypsinized and the diameters of cells measured with a calibrated ocular micrometer. Both the major and minor axes of epithelial cells were measured. Since in the series studied the axial ratio never exceeded 1:4,

the volumes were calculated as though the epithelial cells were spheres with diameters equal to the mean of the major and minor axes. This resulted in an over estimation of no more than 25 per cent.

Number of days <u>after plating</u>	Cell Volume	Cell Volume
	<u>Non-irradiated</u>	<u>Irradiated</u>
6	$(1.68 \pm 0.82) 10^4 \mu^3$	$(9.6 \pm 1.71) 10^4 \mu^3$
7	$(1.75 \pm 0.57) 10^4 \mu^3$	$(7.45 \pm 1.35) 10^4 \mu^3$

Anita and Matumoto (1967) found that replication of measles virus was enhanced in FL cells (human foetal tonsil) that had received certain levels of ultra-violet irradiation and suggested that this effect was attributable to the failure of the irradiated cells to produce some inhibitory factor such as interferon. A similar enhancement of viral growth was seen in cells treated with actinomycin D (Matumoto, 1965).

Using the McCoy cell line, Gordon (1972) showed that it was as susceptible as any of several cell lines tested to yolk-sac established chlamydial strains. It was noted that a small proportion of McCoy cells, several times larger than the normal cell and often containing multiple nucleii, occurred among the mononucleated polygonal cells in an incidence estimated at one in several hundred. It was also suggested that a greater proportion of these 'giant' cells in infected cultures, contained inclusions than did the smaller cells. Pomerot (1957) had previously shown that the proportion of 'giant' cells within a specified measurable area could be greatly increased by irradiating McCoy cell cultures. Using these cells, Fernandez (1959) demonstrated



the greater susceptibility to infection by blue-tongue virus (Orbivirus).

Gordon calculated that the incidence of inclusion per cell in complete monolayers could theoretically be 40 to 80 times greater in irradiated cells than in non-irradiated. A combined enhanced effect of centrifugation and irradiation was readily observed.

Effect of centrifugation of inoculum (C.trachomatis, Cal-1 strain) onto monolayers of non-irradiated and irradiated cells; inclusion counts per 54 fields:

<u>Procedure</u>	<u>Non-irradiated cells</u>	<u>Irradiated cells</u>
Inoculum not centrifuged	1	52
Inoculum centrifuged	88	652

Although the factors for greater susceptibility are obscure, the physical nature of the large flat cells resulting from irradiation is probably significant. The large flat cells obtainable from explants of 4-day chick embryo entoderm were found to be especially suitable for growth of chlamydiae (Weiss et al, 1954).

A further consideration for the enhancing effects of irradiation is that host-cell DNA replication is damaged resulting in the accumulation of intermediary metabolites especially precursors of DNA including ATP. Although a range of substrates are thought to be metabolised by Chlamydia (glucose-6-phosphate, 6-phosphogluconate, pyruvate,  $\alpha$ -ketoglutarate, aspartate and isoleucine) the most surprising finding of experiments carried out

by Moulder (1964), is the complete dependence of the agent of meningopneumonitis (C.psittaci), on large quantities of exogenously supplied ATP for the utilisation of glucose. Moulder commented, ..... "the requirement of exogenous ATP by the agent to my knowledge is without parallel among bacteria or rickettsia".

A similar type of "metabolic cooperation" has been observed in cells of low colony formation efficiencies ( 1 per cent). These cells can be "helped", if they are supported by "feeders":- viz, cells that have been X-irradiated or treated with mitomycin C. Such cells, are incapable of division but they retain the ability to help the growth of sparse viable cells either by "conditioning" the medium or by making direct contacts with the isolated cells and supporting them by the process of "metabolic cooperation" described by Subak-Sharpe (1966) (e.g. the growth of Vibrio cholerae in similarly "conditioned" liquid culture medium).

McCoy cells have been used extensively in C.trachomatis propagation, mainly as a consequence of these initial irradiation experiments. The origin and description of the McCoy cell line is not clearly documented. Originally regarded as an established human cell line derived from synovial cells, evidence has accumulated from several laboratories suggesting they are mouse cells. Kajima (1967) first raised the possibility and an attempt has been made to collate evidence to support this hypothesis. The following criteria suggest murine origins:-

(1) Karyotype examination

- (2) Not infected by poliovirus type 1.
- (3) McCoy cells were susceptible to action of macrophages from mice immunized with L (C<sub>3</sub>H mouse) cells.
- (4) Mixed agglutination tests performed by Coomb indicate mouse.
- (5) Positive immune adherence test with antiserum prepared in Balb C x (C<sub>57</sub>B<sub>1</sub>) F<sub>1</sub> against C<sub>3</sub>H (H - 2K) mice having anti 211 specificity.
- (6) Mouse interferon reduced chlamydial growth in McCoy cells.

#### 2.4.3 DIETHYLAMINOETHYL-DEXTRAN (DEAE-D) TREATED CELLS

The enhancing effect of DEAE-d has been reported for the culture of viruses in cell culture as well as for Chlamydia (Harrison, 1970; Rota and Nichols, 1971). The optimal concentration of DEAE-d for enhancement was found by Harrison to be 20 µg/ml. It is interesting to note that the optimal concentration of DEAE-d for enhancement of cellular transformation by avian and murine sarcoma viruses is 25 µg/ml.

Interest in high molecular weight polyions resulted from the observation that some animal viruses under agar overlays were inhibited in their ability to form plaques (Wallis and Melnick, 1968). This inhibitory action is believed to result from the binding of viruses to polyanions such as sulphated polysaccharides present in agar. Anionic polymers have also been reported to enter cells and interfere with virus replication. The addition of polycations to agar showing inhibitory effects, neutralizes poor plaque formation and leads to enhanced production of viable virus particles.

The addition of DEAE-d to agar overlays also enhanced infectivity in the absence of detectable amounts of inhibitors. The enhancing effect has been shown to be early in infection; probably attachment (adsorption) and penetration. This was determined from experiments with avian sarcoma viruses (Nguyen, 1968). The polycation - enhanced adsorption of certain avian sarcoma viruses to host cells may be explained by assuming that these viruses possess negatively charged surfaces and therefore adsorb poorly under normal conditions to the likewise negatively charged cell surface. Polycations would be expected to neutralize this electrostatic repulsion. Polyanions like heparin, adsorbed to viral or cellular surfaces would increase the electrostatic repulsion. This may account for the virus inhibitory effect which has been observed with heparin (Loh, 1978).

Further evidence to support the electrostatic nature of DEAE-d enhancement has been shown with mouse sarcoma virus (MSV)

( Duc-Nguyen, 1968). Sequential electron microscopic examination within the first few hours after MSV infection of mouse cells pre-exposed to DEAE-d revealed numerous C type particles which were either in contact with the host-cell membrane or lodged in several peripheral cytoplasmic vesicles. In contrast, there were only rare virus particles on the surface of cells in untreated controls inoculated with an equal dose of MSV.

The model that emerges from the published data, suggests that in the case of Chlamydia, some viruses and certain types of infectious RNA, a net negative charge prevails under specific conditions and is repulsed by negative charges associated with sialic acid residues

on cell membrane surfaces. This effect can be neutralized by the addition of polycations to either virus or cellular surface.

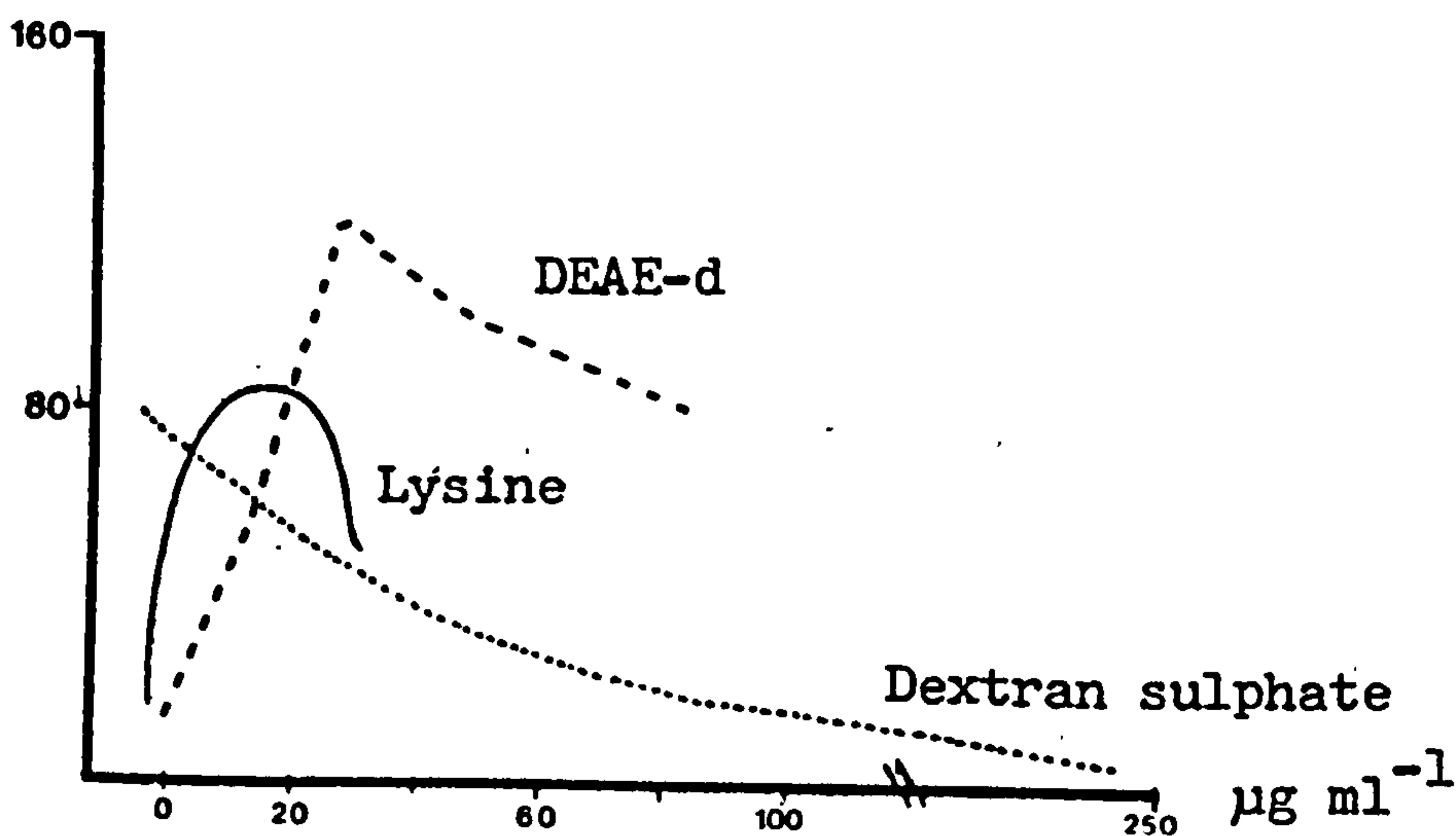
A phenomenon which complicates the mode of action of DEAE-d has been recently reported (Loh, 1978). Although results showed that DEAE-d enhanced Reovirus adsorption, such treatment interfered with the production of whole viable particles. Corollary studies using other polycations such as the basic polypeptide poly-L-lysine and the synthetic polybrene showed that not all polycations behaved alike in their interactions. These last two polymers did not affect Reovirus production.

Work studying the effects of DEAE-d on infectious RNA, suggested that in the case of poliovirus RNA, enhancement was due to an increased number of ribosomal subunits present in polycation treated cells (Smull, 1962; Pagano, 1965).

The use of polycations in cell culture has been known for over twenty years. Their mode of action is not yet fully understood and recent evidence suggests that they modify intracellular events as well as cell membrane potentials.

Effect of polycations, polyanions on the infectivity of trachoma-inclusion conjunctivitis and L.G.V. organisms in HeLa cells (Kuo, Wang, and Grayston, 1973):-

Inclusions  
per  
30 fields



The enhancing effect of DEAE-d was utilized by Kuo et al (1972). for the primary isolation of C.trachomatis from clinical specimens. HeLa 229 cells were pre-treated with a dilute solution of DEAE-d. Their results showed that this system is as sensitive as the irradiated McCoy cell technique for the isolation of C.trachomatis from the genital tract.

A similar comparison was made by Darougar (1973). However, these results indicated that irradiated McCoy cells were more sensitive than HeLa 229 cells treated with DEAE-d. The treatment of irradiated cells with DEAE-d failed to further increase the sensitivity of the system in McCoy cells. In the case of specimens from genital tract and conjunctivae with lower numbers of infective particles, McCoy cells were significantly more sensitive.

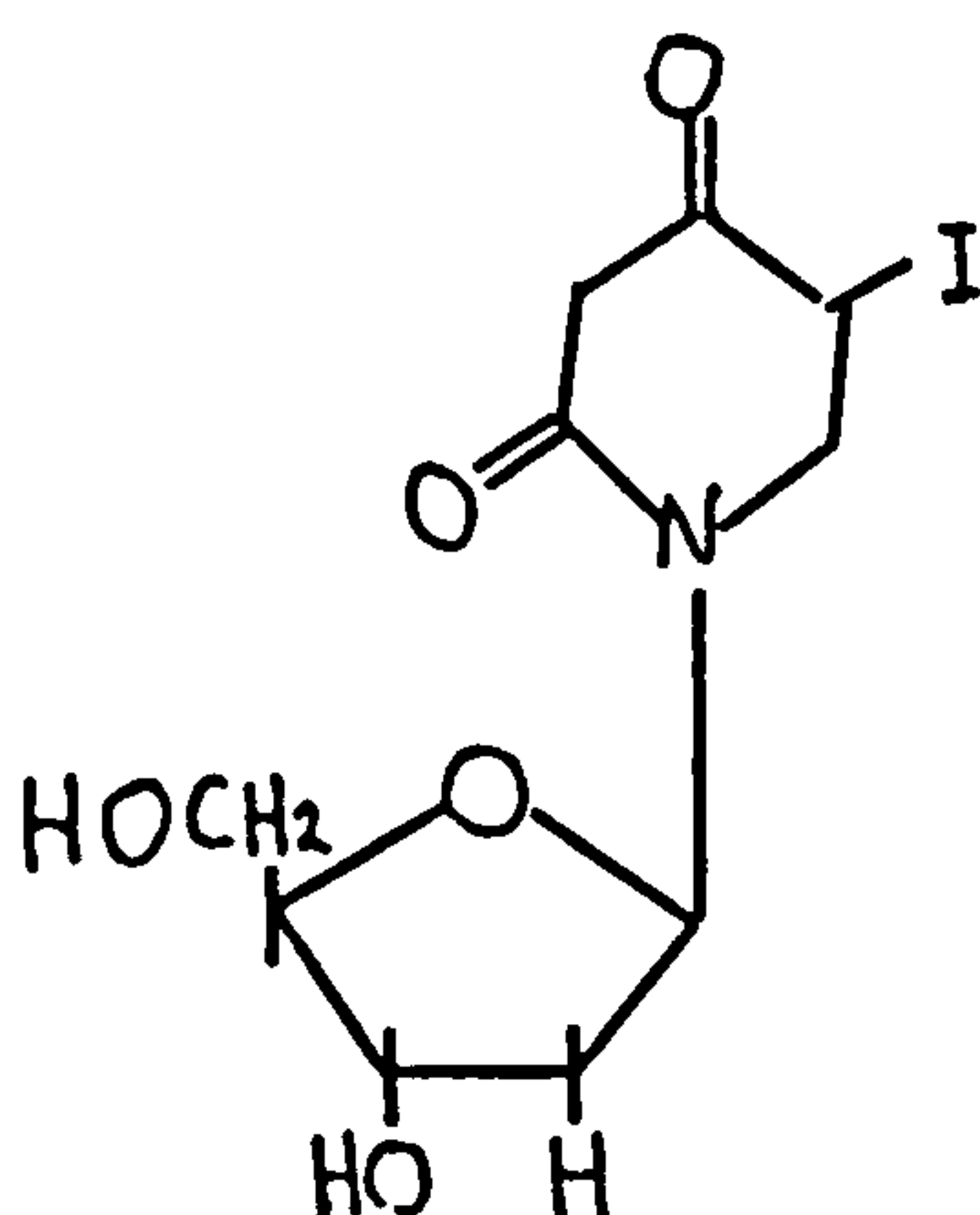
The use of polycations for the enhancement of chlamydial infectivity in vitro provides a more practical alternative to the irradiation techniques. Centrifugation is required in both procedures. There seems to be a discrepancy in published data on the relative sensitivities of both techniques. This may be accounted for by differences in general isolation procedure:- viz differences in techniques for collecting and transporting specimens and variations in the types of medium components used for cell culture.

2.4.4

CHEMICAL TREATMENT OF CELL MONOLAYERS

(a) 5-iodo-2-deoxyuridine. (IUdR)

The advantages of the irradiated cell technique described above were often outweighed by the difficulty in obtaining an irradiation source. Wentworth and Alexander (1974) investigated the substitution of chemical assault upon cells for that of irradiation. For this purpose they chose two halogenated pyrimidines: 5-iodo-2-deoxyuridine (IUdR) and 5-bromo-2-deoxyuridine (BUdR).



IUdR

Monolayers of McCoy cells were prepared on round glass cover-slips and propagated in a medium containing 25 µg/ml IUdR.

Results showed that at 3 - 7 days after seeding, cells were as sensitive to C.trachomatis infection as were irradiated McCoy cells. BUdR treatment of cells appeared to be ineffective at the various concentrations tested.

The mechanisms for the enhanced susceptibility of McCoy cells pretreated with IUdR remains obscure. Gerber (1972) accidentally showed that thymidine analogues (BUdR and IUdR) can induce the production of Epstein-Barr virus in non-permissive cells. In an attempt to select BUdR resistant lymphoid cells that were EBV negative for cell fusion experiments, it became apparent that BUdR treated cells showed the presence of EBV antigens. Further studies indicated that induction of virus synthesis was reproducible. Gerber noted that inhibition of BUdR activation could be achieved with thymidine. This strongly suggested that incorporation of the analogue into DNA may be an essential step in the induction process.

In discussing the possible mechanism of the enhanced susceptibility of IUdR treated cells to infection, Wentworth and Alexander (1974) suggested that restricted infectivity in untreated McCoy cells is due to the digestive action of host enzymes. The altered RNA translation in BUdR treated cells results in altered enzymes that have lost the capacity to digest chlamydial proteins. BUdR which can be incorporated into DNA was not an effective sensitizing agent at the concentrations tested. It is possible that smaller concentrations of this analogue may be more effective.



Reeve (1975) compared the sensitivity to chlamydial infection of McCoy cells pretreated with IUdR and irradiated cells. Specimens for this study were collected from the cervix of a group of women who were sexual contacts of men with non-gonococcal urethritis. The results showed that there was no significant difference in sensitivity between the two cell culture systems. Specimens were collected from 81 women. Isolates of C.trachomatis were obtained from 26 women using IUdR-treated cells and from 24 women using irradiated cells.

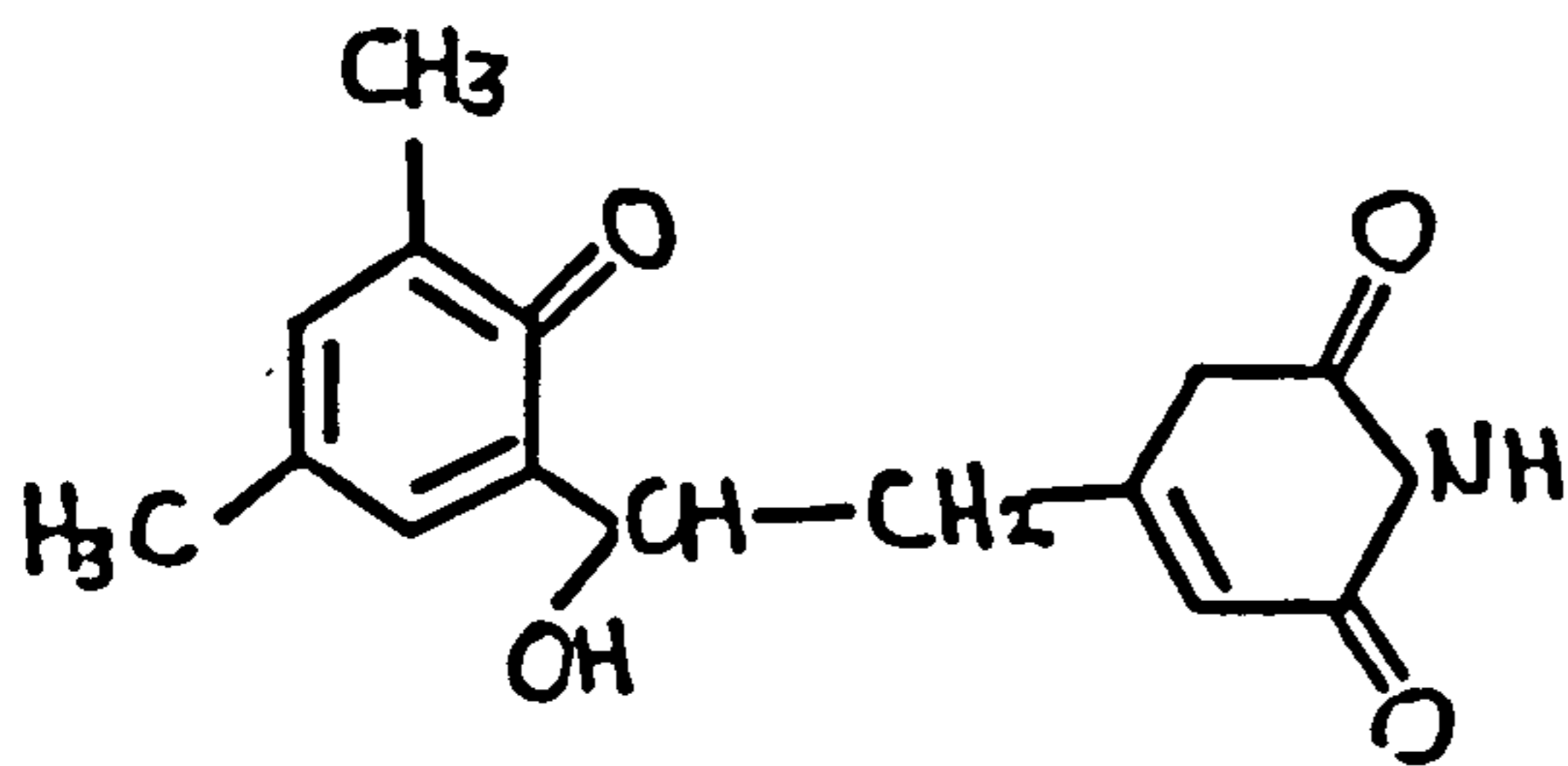
One of the problems with the continual use of base analogues in cell culture medium may be the adverse effects they have on chlamydial infectivity. Base analogues of this type can reasonably be expected to be incorporated into the DNA of host and chlamydial cells. The analogues differ from the normal bases in their distribution of hydrogen atoms which creates a greater tendency for improper pairing. The most potent mutagens among these analogues are 5-bromouracil and BUdR which are analogues of thymine and thymidine respectively (Hayes, 1974).

#### (b) Cycloheximide

Ripa and Mårdh (1977) developed a new simplified culture technique which used cycloheximide-treated McCoy cells and which did not involve pretreatment of the cells.

Cycloheximide is a glutarimide antibiotic that inhibits protein synthesis in eukaryotic cells both in vivo and in vitro (Ennis and Lubin, 1964; Waltstein et al, 1964). This antibiotic prevents

detachment of ribosomes from polysomes and the incorporation of amino acids into nascent polypeptide chains.



CYCLOHEXIMIDE

Cycloheximide had previously been used in metabolic studies of chlamydiae (Alexander, 1968; Hatch, 1975) but had not until the work of Ripa and Mårdh, been employed in the isolation of chlamydiae from clinical specimens. They found cycloheximide to be of potential value when used in concentrations not completely inhibiting the metabolism of McCoy cells, but only depressing it to such an extent that the intracellular "energy" parasite would be at an advantage.

There is evidence that cycloheximide has other sites of action in addition to inhibiting protein synthesis. Using a mutant of Physarum polycephalum shown to possess cytoplasmic ribosomes resistant to cycloheximide, it was possible to show that levels of 5 and 10 µg/ml lowered the uptake of amino acids by 80 per cent.

Another mode of action of cycloheximide was suggested when it was observed that the molecular weight of glutamate dehydrogenase in vitro and in vivo changed to form units about the quarter of the original weight.

## CHAPTER 3

### ISOLATION RATES ASSOCIATED WITH DISEASE ENTITIES

#### INTRODUCTION

The application of the cell-culture techniques described in the preceding chapter, has in large part led to the current interest in sexually transmitted chlamydiae. This chapter reviews the evidence that is presently available implicating chlamydiae as the aetiological agent for a range of genital-tract and related infections.

#### 3.1.1 NON-GONOCOCCAL URETHRITIS (NGU)

Non-gonococcal urethritis (non-specific urethritis) is a term used to describe a very common condition seen in men presenting clinically as a purulent urethral discharge occurring a few days to a few weeks after sexual intercourse. The term non-specific genital infection (NSGI) has been introduced to include with NGU in the male, the clinically less clearly defined infection in the female, who may have neither symptoms nor easily detected signs (Robertson, McMillan and Young, 1980). In discussing general aspects of NGU, Oriel and Holmes (1977) questioned the validity of an imprecise term like NSGI particularly with reference to women.

In the differential diagnosis of NGU, gonococcal urethritis (GU) must be ruled out. The clinical characteristics of gonococcal urethritis differ from those of NGU and have been reviewed by Swartz

(1977). The patient's history, symptoms and signs may suggest the diagnosis of either GU or NGU: the incubation period of GU is about 48 hours and is dose dependent. However, the clinical impression is confirmed by laboratory evaluation of the urethral secretions. This is normally achieved by examining smears of urethral exudate microscopically and by inoculating material obtained by urethral swab on to Thayer-Martin selective media. The identification of typical Gram-negative diplococci (GND) within polymorphonuclear cells (PMN) correlates with the diagnosis of GU in the great majority of cases (Swartz, 1977). Failure to find GND either within or outside PMN correlates with the diagnosis of NGU. Evaluation of patients with dysuria and discharge may also require the differentiation between urethritis and prostatitis.

One of the major problems in diagnosing NGU has been the use of non-comparable case definitions. Oriel (1977) suggested that although the United Kingdom statistics could reflect an over-diagnosing of NGU, they are consistent from one year to the next and do reflect a real increase. Since 1967 NGU has been more common than GU. In 1973 there were 4508 cases of NGU recorded in Scotland.

### 3.1.2 ISOLATION RATES ASSOCIATED WITH URETHRITIS

There have been several studies in the United Kingdom which investigated the frequency of C.trachomatis in men suffering from NGU. The results are summarised below:-

STUDY	NGU: Number chlamydia cultures positive/Number examined (%)
Dunlop et al; London 1972	44 / 99 (44)
Oriel et al; London 1972	49 / 135 (36)
Richmond et al; Bristol 1972	40 / 103 (39)

Dunlop's report included a comparison of different specimen-taking techniques. In tests of urethral material containing C.trachomatis from 52 men, meatal swabs gave positive results in 32 cases (67 per cent). This compared with positive results from 47 of 51 scrapings with a curette (92 per cent) and 28 of 33 endourethral swabs (85 per cent). Results with the endourethral swab were sufficiently good to allow it to be used for further investigations.

The London-St. Thomas Study (Oriel, 1972) was restricted to men during their first episode of urethritis.

In the Bristol Study, the chlamydia isolation rate in a group of men presenting with NGU was compared with the isolation rate of:-

- (i) men with GU
- (ii) men without urethritis, matched as far as possible for age and sexual promiscuity

The following isolation rates were obtained from each category:-

- NGU : 40/103 - 39 per cent
- (i) GU : 32/99 - 32 per cent
- (ii) no urethritis : 5/92 - 5 per cent

Chlamydiae were isolated from significantly greater numbers of men with NGU who had a long-standing untreated urethral discharge than from men who appeared at the clinic early.

After treatment for GU with the penicillins or aminoglycosides, up to 50 per cent of men can be expected to develop post-gonococcal urethritis (PGU) (Vaughan-Jackson et al, 1977).

In Richmond's (1972), study it was found that patients with GU and who were also found to have a chlamydial infection were more prone to develop PGU than the chlamydia-negative patients.

Because of the high rate of chlamydial isolation in cases of gonorrhoea, not significantly different from the rate with NGU, Richmond postulated that C.trachomatis might be a secondary pathogen to an unknown agent in NGU as it appeared to be to N.gonorrhoea urethritis.

Holmes (1975) investigated the prevalence of five organisms in patients suffering from NGU. These organisms had previously been implicated as possible causal agents of NGU. As controls, two further groups of men were investigated:-

- (i) men with GU (all had positive urethral cultures for N.gonorrhoea)
- (ii) men with no urethritis and had negative cultures for N.gonorrhoea

This latter category attended the clinic either because of external genital lesions including genital herpes, venereal warts, or Phthirus pubis infestation, or for routine examination. The results are summarised as follows:-

Pretreatment Isolation of C.trachomatis, M.hominis, T-Mycoplasma, HSV and CMV from the anterior urethra of patients with NGU, GU and no urethritis (Taken from Holmes 1975).

Organism Isolated	Number of Isolations/number of patients tested (per cent)		
	NGU	GU	No urethritis
C.trachomatis	48/113 (42)	13/69 (19)	4/58 (7)
Mycoplasma hominis	16/112 (14)	8/49 (16)	26/62 (42)
T-Mycoplasma	70/112 (63)	23/49 (47)	36/62 (58)
Herpes Simplex	2/115 (2)	3/53 (6)	2/62 (3)
Cytomegalovirus	0/60 (0)	0/28 (0)	0/20 (0)

Holmes pointed out that the control group with no urethritis were carefully described and compared with the cases for a number of characteristics, including promiscuity. Only 19 per cent of GU patients had concomitant chlamydial infection. This contrasts significantly with Richmond's 32 per cent.

### 3.1.3 ROLE OF UREAPLASMAS IN CASES OF NGU

Holmes did not discuss the significance of a 63 per cent isolation rate for T-mycoplasmas in NGU patients: controls with no urethritis showed a 58 per cent isolation rate. Bowie et al (1976) reported the results of using either sulphonamides or aminocyclitols to treat NGU. Sulphonamides active against chlamydiae but not T-mycoplasmas (Ureaplasmas), were effective in treating chlamydia-positive cases and aminocyclitols, active against ureaplasmas but not chlamydiae,

were successful in ureaplasma cases. These results give some support to the idea that ureaplasma do cause some cases of NGU.

Taylor-Robinson (1977) reviewed the possible role of ureaplasmas in NGU and concluded:- "The results of (i) ureaplasma isolation studies on subjects with or without NGU, (ii) attempts to detect specific antibody responses, (iii) serotyping the ureaplasmas isolated and (iv) organ culture experiments have not suggested a significant causal relationship between ureaplasma infection and NGU. On the other hand, the results of (i) certain microbiological investigations during antibiotic treatment of NGU, (ii) experiments in animals, and (iii) intraurethral inoculation of human subjects indicate that ureaplasmas of human origin are able to cause urethritis."

These contradictory lines of investigation may be explained by the persistence of these organisms in the genital tract.

Ureaplasmas have been isolated from the urogenital tract of volunteers working in the Antarctic, who had no sexual contact for about 1 year. It is likely that only some strains of these organisms are pathogenic and others are non-pathogenic and may remain in the urogenital tract longer than others. Suitable serotyping techniques, antibiotic investigations and the use of animal models may elucidate the extent to which ureaplasmas may be implicated in NGU.

### 3.2

#### EPIDIDYMITIS

There is growing evidence that in some patients C.trachomatis



infection may ascend from a primary site in the anterior urethra to settle in tissues in and around the posterior urethra. The epididymis is involved most commonly. There is a problem in implicating chlamydiae in epididymitis, particularly when the majority of men have urethritis and other microorganisms are also recovered. To avoid contamination from the urethra, Berger et al (1978) obtained aspirates directly from the epididymis and isolated chlamydiae from patients who were all under 35 years of age.

Clinical observations relating NGU to idiopathic epididymitis prompted Harnisch (1977) to define the association of acute epididymitis with those agents implicated as causes of NGU, namely:- C.trachomatis, Herpes simplex, and U.urealyticum. The results are summarised:-

Microorganisms Isolated from the Urethra of 19 Men under  
the Age of 32 Years with Epididymitis (Harnisch, 1977)

<u>Organism</u>	<u>No. of patients</u>	<u>No. with Urethritis</u>
N.gonorrhoea isolated	7	7
N.gonorrhoea only	3	
N.gonorrhoea and C.trachomatis	1	
N.gonorrhoea and U.urealyticum	2	
N.gonorrhoea and HSV	1	
N.gonorrhoea not isolated	11	7
C.trachomatis only	4	2
C.trachomatis and U.urealyticum	2	2
U.urealyticum only	2	1
U.urealyticum and CMV	1	1
<u>No. isolate</u>	2	1

It is still uncertain from these studies whether the organisms which initiate urethritis actually reach the epididymis or whether the production of urethritis per se allows reflux of urine and/or urethral commensal organisms into the epididymis. Harnisch . cites evidence from similar investigations of ascending infections in women. These studies have shown that women with salpingitis who have gonoccal infections of the endocervix, may have tubal or peritoneal infections with N.gonorrhoea alone, N.gonorrhoea plus vaginal commensals or vaginal commensals alone. The fact that C.trachomatis and U.urealyticum have been isolated in the absence of other organisms from the endosalpinx of women with non-gonoccal salpingitis suggests these organisms might cause

genital adnexal infections in men as well.

### 3.3. Sexually Acquired Reactive Arthritis (Reiter's Syndrome)

A small proportion of men with NGU develop sexually acquired reactive arthritis (SARA) or Reiter's Syndrome. This syndrome is characterised by the close temporal association of genitourinary signs of infection with arthritis and either conjunctivitis or the characteristic mucocutaneous lesions of circinate balanitis or keratoderma blennorrhagica. Non-gonoccal urethritis is the most common form of urinary tract inflammation and responds to treatment with tetracyclines. In a series of 144 cases of Reiter's Syndrome associated with urethritis, the urethritis associated with the initial episode was:-

GU	:	17 per cent	
NGU	:	43 per cent	
GU + NGU	:	36 per cent	
Undiagnosed	:	4 per cent	(Csonka, 1960)

The findings in 22 patients suffering from Reiter's Syndrome were reported by Schachter et al (1966). Isolates of chlamydiae in yolk-sacs had been attempted in eight cases, with positive results from urethral, conjunctival and synovial material in five. Similar results were obtained by Harper et al (1967). However, none of the isolates was obtained on the first passage in yolk-sac. It was indicated that there was a possible danger of cross-contamination during these isolation attempts that rendered all but first passage isolates as possibly suspect.

Vaughan-Jackson et al (1972) utilised the irradiated McCoy cell technique to investigate 29 patients suffering from Reiter's syndrome. Positive chlamydial isolates were obtained from 3 separate urethral specimens. Nineteen patients in this group had recent anti-chlamydial preparations. If the Reiter's patients who did not have anti-chlamydial treatment are considered separately, the overall isolation rate was then 30 per cent. No positive isolates were obtained from the conjunctiva or synovial fluid. More recent evidence has shown that chlamydiae have been isolated from the urethra of untreated patients with Reiter's Syndrome as frequently as from patients with uncomplicated NGU (Keat et al 1978).

At the present time, there appears to be no direct substantial evidence to support the view that chlamydiae are causally related to the extra-genital manifestations of the syndrome. There is general agreement that the extragenital manifestations of the disease are "aseptic" and that the organism causing the precipitating infection cannot be cultured from the joints, eyes or mucocutaneous lesions. Consequently indirect pathogenic mechanisms such as toxins or aberrant immunological reactions have been implicated as a means whereby the organisms could produce aseptic lesions.

Clinical relationships can be demonstrated between Reiter's Syndrome and a group of six other diseases collectively known as the sero-negative spondarthritides:-

1. Negative test for rheumatoid factor
2. Absence of (rheumatoid) subcutaneous nodules
3. Inflammatory peripheral arthritis

4. Radiological sacro-illitis with or without classical ankylosing spondylosis
5. Evidence of clinical overlap between members of the group
6. A tendency to partial aggregation, that is, the presence of two or more examples of the same disease and/or two or more different diseases of the group within a single family (Moll et al, 1974)

Seven diseases fulfill these criteria:-

1. Idiopathic ankylosing spondylitis
2. Psoriatic arthritis
3. Reiter's syndrome
4. Ulcerative colitis
5. Crohn's disease
6. Whipple's disease
7. Behçet's Syndrome

A single histocompatibility antigen (HLA-B27) is found in approximately 90 per cent of patients with Reiter's Syndrome, suggesting a genetic predisposition to the disease (Brewerton et al 1973). In 1975, Brewerton et al demonstrated that 72 of 75 patients with idiopathic ankylosing spondylitis also possessed the antigen in their tissues, whereas only three of 75 controls possessed HLA-B27. Eighty per cent of individuals who possess the tissue type HLA-B27 have however, no clinical or radiological evidence of any disease whatsoever.

Only when there is radiological evidence of spondylitis in patients

suffering from psoriatic arthritis, Crohn's disease or ulcerative colitis is there a significant difference from the control group in the possession of HLA-B27. So there seems to be a "central position" of ankylosing spondylitis in this group of diseases. The mechanism whereby specific HLA characteristics could predispose to diseases are unknown, although they are under intense study at this time. The current hypothesis involve closely associated immune response genes, common antigenicity between organisms and HLA antigens, or cell-surface HLA-determined responses for pathogenic microorganisms. Unfortunately, these hypotheses have not been adequately defined at this time to assist in the understanding of Reiter's Syndrome.

#### 3.4.1

#### CERVICITIS

Assessment of the pathogenic role of C.trachomatis in the uterine cervix is important for three reasons:-

- (i) The effect on the foetus - prenatal or intrapartum - with documented evidence of premature labour and foetal morbidity,
- (ii) The effect on fertility of ascending infection to the fallopian tubes.
- (iii) The possible incorporation of part of the DNA into epithelial cells and the implication of this for future carcinogenicity, (Rees et al 1977).

A major difficulty in investigating these three different aspects of chlamydial infection in women is that several potentially pathogenic microorganisms can often be isolated from cervical

specimens.

### 3.4.2 CONCOMITANT CERVICAL INFECTION

Ridgeway and Oriel (1977) considered the incidence and inter-relationship of four commonly isolated microorganisms, N.gonorrhoea, C.trachomatis, T.vaginalis and C.albicans. Specimens were collected from a group of women attending a department of genito-urinary medicine and examined using appropriate culture systems. Statistical methods were then used to seek associations between pairs of organisms. Chlamydial isolations were carried out in IUdR-treated McCoy cells.

#### Frequency of Multiple Isolations of Pathogens from 1136 Patients (Taken from Ridgeway et al, 1977)

<u>Number of Pathogens</u>	<u>Number of Patients</u>	<u>Percentage</u>
0	566	49.8
1	428	37.7
2	128	11.3
3	14	1.2

Ridgeway's results showed that N.gonorrhoea was significantly associated with C.trachomatis. In a previous study, Hilton et al (1974) recorded a group of 40 women with gonorrhoea, of whom 25 (62 per cent) were infected by C.trachomatis. Two combinations of pathogens were uncommon in both C.trachomatis positive and negative cultures:-

N.gonorrhoea and C.albicans, and T.vaginalis and C.albicans.

In discussing his results, Ridgeway noted that the enhancement or diminution of the isolation of one microorganism in the presence of another is due to the operation of several simultaneous factors:- the direct action of one organism against another, environmental conditions which selectively favour the growth of the organism, the differential operation of host factors and epidemiological considerations.

### 3.4.3 ISOLATION RATES ASSOCIATED WITH CERVICITIS

There has been a tendency to associate chlamydiae with cervical erosion. However, Schachter (1978) points out that the term "erosion" is not a precise one and may have different meanings to different observers. There is no proof at the present time that chlamydiae cause cervical erosion. Cervical erosions or extensions of the columnar epithelium into the area normally covered with squamous epithelium, could simply provide more sites for replication of chlamydiae. There is evidence that C.trachomatis cannot infect squamous cells but grows only in columnar cells. The existence of erosions may mean greater clinical susceptibility to chlamydial infection. Cervical ectopy is generally not eradicated by a simple course of tetracycline.

Paavonen et al (1978) reported the result of colposcopy on a series of premenopausal women attending gynaecological outpatient clinics. They isolated C.trachomatis from the cervix of 13/144 ( 9 per cent) women but although colposcopy or biopsy examinations revealed non-specific inflammatory changes in the cervix of those



who harboured chlamydiae, specific changes that could be ascribed to these organisms were not found. These workers also showed that chlamydiae are recovered from the cervix of a much greater proportion of women who are partners of men with chlamydia positive NGU than of those who are partners of men with chlamydia negative NGU (1978).

Dunlop et al (1966) were the first to associate chlamydial infections of the cervix with lymphoid follicle formation and likened this to the follicles seen on the conjunctivae in trachoma and inclusion conjunctivitis. Follicular cervicitis was observed in 90 per cent of mothers whose babies were suffering from chlamydial neonatal conjunctivitis.

Rees and colleagues (1977) examined clinically and recorded the vaginal cervix (ectocervix) under the following categories:-

- (i) no erosion
- (ii) simple erosion (ectopic columnar epithelium)
- (iii) hypertrophic erosion (oedematous and congested erosion)
- (iv) chronic cervicitis (lacerated with ectropian or nabothian follicles)

The endocervical content was also assessed:-

- (a) clear mucus
- (b) cloudy mucus
- (c) mucoid discharge (white)
- (d) mucopus (yellow)

The clinical findings suggested that hypertrophic erosion and mucopurulent discharge are associated with chlamydial infection of the cervix in a proportion of cases and that these signs regress after treatment.

Isolation rates of C.trachomatis from cervical specimens of women attending departments of genito-urinary medicine range from 12 per cent to 60 per cent. These surveys have been reviewed by Taylor-Robinson and Thomas (1980). Rates from other types of clinics have also been obtained and are summarized below:-

CLINIC	NO. CHLAMYDIA CULTURES POSITIVE/ NO. EXAMINED (PER CENT)	
	Family Planning Hilton et al (1974)	2/63
Gynaecology Schacter et al (1975)	7/90	(8)
Gynaecology Paavonen et al (1978)	13/144	(9)
Gynaecology Ripa et al (1978)	31/161	(19)

### 3.5 SALPINGITIS - ascending infection to the fallopian tubes

In gonococcal and non-gonococcal salpingitis not associated with surgical operations, the inflammatory process affects mainly the endosalpinx, the organisms ascending by way of the endometrium where only mild inflammation may be induced. Destruction of tubal epithelium occurs and a purulent exudate fills the lumen. Pus may escape from the fimbriated end of the tube and may track down to the pouch of Douglas (rectovaginal pouch) where a pelvic abscess may form. With continued inflammation, the ostia become

occluded by oedema and pus collects in the cavity of the tube forming a pyosalpinx. If untreated, fibrous adhesions eventually form within the tube. Occasionally a hydrosalpinx (an accumulation of serous fluid in the tube) is found. It's pathogenesis is uncertain but may result from recurrent episodes of subclinical salpingitis.

Although inflammation of the uterine tubes is clinically the most prominent feature, the supporting structures, mainly the broad ligament of the uterus, share in the inflammation to a greater or lesser extent. Therefore pelvic inflammatory disease (PID) is strictly the more accurate term (Robertson, McMillan and Young, 1980).

Several factors predispose to ascending infections. Surgical operations in the uterus may lead to the development of PID in less than 5 per cent of cases. Women using intra-uterine contraceptives (IUCD) have a greater risk of developing PID (Westrom, et al, 1976). Infertility may follow in about 20 per cent of women who have been treated for PID (Westrom, 1975).

A study by Rees et al (1977) suggested a possible role for C.trachomatis in PID. A high incidence, 16/24 (66 per cent), of PID with an onset of pain between 13 and 38 days post partum was found in mothers of babies with chlamydial conjunctivitis. Mårdh (1970) isolated Mycoplasma hominis and Ureaplasma urealyticum from 10 per cent and 4 per cent respectively of women with acute PID but the role of these organisms is not clear. The consensus however tends to be against their playing a

pathogenic role.

With the exception of N.gonorrhoea, it has not yet been possible to prove a causative relationship between organisms found in the cervical flora and those recovered from the fallopian tubes. It is not always possible to determine the aetiology because it would be necessary to obtain cultures directly from the uterine tubes at laparoscopy or laparotomy, or from the pelvic peritoneal fluid by culdocentesis.

Mårdh et al (1977) obtained specimens from fallopian tubes and the pouch of Douglas under visual control during laparoscopy. The specimens from twenty women with acute PID were inoculated into McCoy cells that had been treated with cycloheximide.

C.trachomatis was isolated from 6/20 tubal specimens (30 per cent).

Of seven patients from whom C.trachomatis was isolated from the cervix and from whom tubal specimens were also obtained, the organism was recovered from the fallopian tubes in all but one. Specimens from the pouch of Douglas were all negative. Five tubal specimens from controls matched for age with acute salpingitis, but without symptoms and signs of genital infections were negative for C.trachomatis.

### 3.6.1 PRENATAL OR INTRAPARTUM INFECTION

The presence of an inflammatory process in the cervix of a mother may affect the foetus in the prenatal, intrapartum, or neonatal period. Chandler and Alexander (1977) performed a prospective trial on development of chlamydial infection in neonates. They studied 142 unselected pregnant women and found

18 (12.7 per cent) to have chlamydial infection of the cervix. Of the 18 infants born to these women, conjunctivitis developed in 9 (50 per cent).

A similar survey was carried out by Schachter et al (1979).

C.trachomatis was recovered from the cervices of 36/900

(4 per cent) of unselected pregnant women tested. 20 infants born through chlamydia-infected cervices were followed-up for a year, as were 18 infants born to chlamydia negative mothers.

A statistically significant excess of conjunctivitis and pneumonia was found in infants exposed to chlamydiae. The attack rate for inclusion conjunctivitis was 7/20 (35 per cent). None of the 18 unexposed infants showed evidence of chlamydial infection. Chlamydiae were isolated in McCoy cells treated with IUdR.

In considering chlamydial cervicitis in pregnancy, the relative incidence of prematurity not associated with general factors affecting maternal health, such as toxæmia, may be important. In a series of twenty-nine chlamydia positive babies, twelve were premature (Rees et al, 1977):-

Gestation period (weeks)	
30 - 32	5 babies
33 - 34	4 babies
35 - 37	3 babies

Nine mothers developed pelvic infection after discharge from hospital 13 - 30 days post partum - 9/24 (37.5 per cent). 11 fathers of chlamydia positive babies were examined. All were

asymptomatic; 4/10 were positive for C.trachomatis

In discussing their results, Rees et al suggest that the possibility of ascending cervical infection being a contributory factor to premature rupture of membranes should be considered. The possible infection in utero of the baby delivered by caesarean section lends support to the hypothesis that chlamydial infection may ascend from the cervix and, therefore, may be directly involved not only in chorioamnionitis but also in the development of PID. Postnatal transmission of C.trachomatis by fingers of the attendants cannot with certainty be excluded.

### 3.6.2

#### NEONATAL PNEUMONITIS

Recently, C.trachomatis has been identified as a possible respiratory pathogen in infants with pneumonia (Schachter et al, 1975). Beem and Saxon (1977) conducted a prospective study of 12 infants with a distinctive pneumonia syndrome in which C.trachomatis was isolated from conjunctivae, nasopharynx or trachea, alone or in combination. They used McCoy cells pretreated with IUdR., for isolation of chlamydiae.

A report by Frommel et al (1977) seems to confirm the association between lower respiratory tract disease and C.trachomatis. In an eight week old infant with chronic pneumonia, C.trachomatis (type F) was isolated from an open lung biopsy. Neither the mother or her baby had a history or clinical evidence of conjunctivitis. Although inclusion conjunctivitis may precede

development of lower respiratory tract symptoms (Schachter, 1975), the absence of conjunctivitis does not preclude C.trachomatis as a cause of pneumonia.

In the prospective study of chlamydial infection in neonates by Schachter et al (1979), the attack rate for chlamydial pneumonia was 4/20 (20 per cent). They estimated an incidence rate of 8 cases of pneumonia per 1,000 live births.

## CHAPTER 4

### 4.1.1 SEROLOGICAL TESTS FOR C.TRACHOMATIS

Over a long space of time the association between a parasite and its host moves towards a balanced partnership in which the two exist together without seriously harming each other.

Microorganisms tend to mutate, and in the case of disease causing microbes, the less virulent mutant will be selected preferentially and will tend to persist. Were this not so, evolution of the host-parasite partnership might eventually result in destruction of the host, an outcome which would be equally detrimental to the parasite. So with time an infectious disease will tend to become milder.

A balanced partnership can exist in chlamydial infections where natural hosts to chlamydiae show a substantial degree of latent or inapparent infection. This situation is well recognised in avian psittacosis and ornithosis and in some of the mammalian infections. Apparently healthy birds may shed chlamydiae in their faeces, and such birds have been implicated as sources of human infection. A further example of inapparent infection may occur in a potentially useful animal model for human ocular and genital infections, naturally occurring guinea pig inclusion conjunctivitis. In this model, guinea pigs acquire early in life a chlamydial conjunctivitis that is probably contracted from the genital tract of the mother. However it is difficult to demonstrate infection in the naturally infected adult although the capability



of the genital tract to support the agent has been shown experimentally and artificial infection of the genital tract has led to disease in neonatal guinea pigs.

There is no evidence that chlamydiae persist in the intact host in a non-replicating form. It is more likely that latent or subclinical infections represent persistent low levels of multiplication held in check by host defence mechanisms.

Isolation techniques alone may not reflect the true prevalence of chlamydial infection within different sets of patients.

This may be more accurately estimated by measuring the levels of humoral antibodies to Chlamydia which may be said to represent "footprints", either faint or distinct, of an infection experienced in the remote or recent past (Paul, 1973). Of course antibody responses alone cannot necessarily be taken as evidence of a causal relationship, since infection might occur and antibody might develop without the organisms causing disease.

#### 4.1.2

#### COMPLEMENT-FIXING ANTIGENS

The classical approach to antigenic analysis of microorganisms is to fractionate the organism and then to purify certain biochemical components one at a time. These are then tested for serological activity against hyperimmune serum and finally against human sera for serological activity. The ultimate purposes of such investigations are to provide efficient serologic test antigens. At a later stage these may lead to development of vaccines.

An alternative approach is to use the whole organism for serodiagnosis

The difficulty in the antigenic analysis of chlamydiae has been attributed to the low yields of organism obtainable from cell culture systems, and the apparent resistance of chlamydiae to solubilization (Collins et al, 1970). The biochemical analysis of chlamydial antigens has only recently been attempted with the adaptation of "fast-growing" LGV serotypes to sensitive cultures (Caldwell, 1975). All serological tests employed, so far, depend on the use of whole organisms or their cell walls.

Complement fixation tests revealed four main antigens:- group-specific and strain-specific, each of which was associated with the EB and with particles smaller than the EB. Resistance to heat and various chemical agents differentiated the stable group-specific from the unstable strain-specific antigens (Bedson, 1936, 1949).

Chemical Nature of Principal Antigenic Components of "Psittacosis and L.G.V. Viruses" (Barwell, 1952)

<u>TREATMENT</u>	<u>EFFECT ON:-</u>	
	<u>Specific Antigen</u>	<u>Group Antigen</u>
Heat (100 - 130°C)	Destroyed	None
Phenol (0.5 - 0.2 per cent)	Destroyed	None
Papain	Destroyed	None
Trypsin	None	None
Ether	Destroyed	Extracted
KIO <sub>4</sub> (0.002M at 37°C)	None	Destroyed

The presence of group-specific and strain-specific antigens in the same preparations interfered with some of the serological reactions. Complement fixation tests with either human or animal sera in which fresh homologous or heterologous chlamydial suspensions were used as antigens yielded inconsistent results. Bedson eliminated strain specificity by boiling chlamydial suspensions which reacted equally well with homologous and heterologous sera. Strain-specific reactions could be demonstrated with fresh chlamydial suspensions and sera from which the group reactivity had been eliminated by absorption:-

Antigenic Composition of C.psittaci as Determined by Complement Fixation (Bedson, 1936)

<u>SERUM</u>		<u>ANTIGEN</u>							
<u>Treatment</u>	<u>Dilution (1/Log<sub>2</sub>)</u>	<u>Unheated</u>		<u>Boiled</u>					
Nil	4	+	+	+	+	+	+	+	+
		+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+
		+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+
		+	+	+		+	+	+	
	7	+	+			+	+		
		+				+			
8	+				+				
	-				-				
Absorbed with unheated antigen	4	+				+			
		-				-			
	5	-				-			
	6	-				-			
Absorbed with boiled antigen	4	+	+	+	+	+	+	+	+
		+	+	+	+	+	+	+	
	5	+	+	+	+				
		+	+	+	+	+			

Antigenic Composition of C.psittaci as Determined by Complement

Fixation

(Bedson, 1956)

<u>SERUM</u>		<u>ANTIGEN</u>	
<u>Treatment</u>	<u>Dilution (1/Log<sub>2</sub>)</u>	<u>Unheated</u>	<u>Boiled</u>
	6	+ + + + + + +	+ -
	7	+ + +	
	8	+ -	

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Further biochemical analysis has demonstrated that the heat-stable component is a lipopolysaccharide which reacts with antibodies to all members of the genus Chlamydia. The heat-labile component has recently been shown to be a protein with a molecular weight of approximately 30,000 daltons (Sacks, 1979).

The complement fixation test has found greater application in the diagnosis of infections caused by C.psittaci. Epidemiological surveys were previously limited in value by the fact that they described the antibody status of populations of domestic animals and humans in terms of the group antigen. Fraser (1965) described a procedure for producing large quantities of type-specific cell wall antigens, from infected yolk-sacs, and for using these antigens to group several isolates. The test antigen was purified first by blending a fluorocarbon (Genetron 113, trifluoro-trichloro-ethane) with the chlamydial suspension and then by light centrifugation. Lipids, proteins and fluorocarbon were deposited and the chlamydial particles remained in the aqueous supernatant phase. The purified antigen was subjected to further centrifugation at 10,000g and

treated with sodium deoxycholate and trypsin. The final preparation was designated cell wall type-specific antigen.

For each isolate purified in this way homologous guinea pig antiserum was obtained. Each specific antiserum was tested against known chlamydial isolates. To simplify evaluation of the homologous and heterologous titres observed, specificity differences (SPD) between pairs of antigens were calculated. The SPD expresses the difference between the homologous and heterologous reactions, and is defined by the formula:-

$$SPD = (Aa + Bb) - (Ab + Ba)$$

where Aa is the titre of antiserum A with its homologous antigen a, Ab is the titre of the heterologous reaction of antiserum A with antigen b, and Bb and Ba refer to the homologous and heterologous reactions with antiserum B. In cases of small or no differences in the specificity of two antigens, the sum of the homologous titres is about the same as the sum of the heterologous titres, and therefore the SPD = 0.

The chlamydial strains investigated by this procedure were isolates from a wide range of host species affected with a great variety of disease syndromes. It was possible to distinguish 7 sub-groups. Although this procedure seemed to offer greater sub-group specificity there has been little published data confirming Fraser's results. The identification of C.psittaci strains by serology has not been accomplished on a large scale and remains an area of current research.

The immunological relationship between strains of C.trachomatis associated with hyperendemic trachoma was considered vitally important if a useful prophylactic vaccine was to be produced. Initial successful attempts to serotype these strains were based on the mouse toxicity prevention test (MTPT). Manire et al (1950) had previously demonstrated that chlamydial strains killed mice consistently in less than 24 hours. These deaths could not be reasonably attributed to infection. Neither could early deaths be attributed to the effects of the diluent or yolk-sac tissue in which the chlamydiae were propagated. The toxic components of these strains cannot be separated from the elementary bodies by filtration or centrifugation. The extreme heat lability of the toxins, which are destroyed at incubator temperatures in a short time, and their detoxification by formalin without loss of antigenicity are not generally characteristics of bacterial endotoxins. In some characteristics, such as lability, detoxification, rapid effect in mice, the toxins resemble those found in studies with some of the Rickettsia which have been shown to resemble bacterial endotoxins.

The principle of the MTPT involves immunizing mice with three doses of concentrated formalin-inactivated chlamydial vaccine. Mice are subsequently challenged intravenously with a lethal dose of toxic chlamydiae using strains homologous or heterologous to the immunizing strain. Results are expressed as survivors per total inoculated.

For example:-	<u>VACCINE STRAIN</u>	<u>CHALLENGE STRAIN</u>	
	SA-2	SA-2	Eg-2
		106/157	0/59
	Eg-2	0/41	76/147

On the basis of these results, SA-2 and Eg-2 (strains of C.trachomatis from hyperendemic trachoma areas) are considered immunologically distinct.

Using this technique Bell and Theobald (1962) were able to distinguish two immunologically distinct groups. Altogether nineteen isolates from trachoma hyperendemic areas were tested. Ten chlamydial isolates fell into one immunological group, group 1, six into group 2, and three into an "uncertain" category.

The fluorescent antibody technique (FAT) was successfully used with C.psittaci grown in cell culture (Buckley et al, 1955). Nichols, and McComb (1962) applied the technique to identify:- (a) cytoplasmic inclusions of C.trachomatis in conjunctival smears from patients with active trachoma; (b) to detect elementary bodies in purified yolk-sac smears on glass slides. This latter observation was to have important consequences on future serotyping techniques. Purified and concentrated yolk-sac antigens appear as discrete, yellowish-green pin-points of light after staining by FAT and examined by appropriately filtered U.V. microscopy.

Bell et al (1967) described a direct FA technique giving a type-specific effect. Guinea pig antiserum was obtained after

a single intracardiac inoculation of antigen, this serum was utilised in a rapid test for typing strains of C.trachomatis associated with trachoma. Bell attributed the specificity of the reaction to the single massive intracardiac inoculation (1.0 ml) of highly purified antigen which results in antiserum being type-specific. The antiserum was believed to be primarily of the IgM class (19S pentameric gamma globulin) since titres obtained by the complement fixation test were markedly lowered by treatment of sera with ethanediol. Results using this type of test confirm those using the MTPT:- at least three immunologic types of C.trachomatis associated with trachoma.

The MTPT was extended in its application by Alexander et al (1967), to include C.trachomatis isolates from the genital tract. Three week old Swiss Webster mice were immunized intravenously with 0.5 ml of a 1 per cent yolk-sac suspension of a chlamydial isolate. One week later the immunization was repeated. After one further week, the mice were challenged intravenously with 0.5 ml of a crude yolk-sac suspension, which was estimated on previous toxicity titration to be about 1.5 LD<sub>50</sub>. Adrenaline (0.02 mg per mouse) was injected intraperitoneally five minutes before challenge to prevent mouse anaphylaxis. The toxicity of intravenous chlamydiae for mice is very sensitive to slight changes in toxicity of antigen, and is affected by the age and health of mice.

This test was used to examine antigenic relationships of 50 C.trachomatis isolates from various countries and various body sites. 62/64 ocular trachoma strains fell into one of three antigenically distinct types A, B and C. 16 strains associated



with a genital epidemiology fell into one of a further three types D, E or F.

As a direct extension of their own work with the MTPT, Wang and Grayston (1970) developed the sensitive micro-immunofluorescent technique. Their initial work on this subject dealt with strain typing C.trachomatis isolates. Hyperimmune sera collected from mice using the same immunization schedule as the MTPT was utilized. Sera was collected, pooled and absorbed with normal yolk-sac before use. Dip-pens were used to place antigen dots on glass slides. By testing the mouse serum against a range of chlamydial isolates in this way Wang was able to show that strain classification by immunofluorescence yield the same groupings as the MTPT.

LGV strains were formalized before being used for immunization. It was found that formalization of the antigen for the micro-IF test results in decrease of fluorescent staining. Non-formalized antigens proved to be highly effective.

Wang and Grayston demonstrated that a much lower titre of antigen, such as that produced in cell culture, is adequate to stimulate an antibody response in mice. This led to a simplified method for immunotyping isolates of C.trachomatis (Wang and Grayston, 1973). In this "one-way" test only mouse serum of the unknown strain is titrated against known yolk-sac grown material. There is a certain amount of cross-reaction between some serotypes and the hyperimmune mouse serum. At higher dilutions of serum the cross-reactivity is diminished. Determination of these antibody patterns with monotypic mouse antiserum has been of great assistance in the interpretation

of results of micro-IF antibody tests of human sera.

#### 4.2

#### SCREENING HUMAN SERA

Treharne et al (1977) further simplified the micro-IF test by using pools of serotypes which were "spotted" onto glass slides for screening serum specimens. Of the five systems investigated the following combinations of serotypes were considered most applicable:-

- POOL 1: All hyperendemic C.trachomatis serotypes (A, B or C)
- POOL 2: All serotypes associated with genital epidemiology (D - K inclusive)
- POOL 3: LGV serotypes
- POOL 4: C.psittaci serotypes arbitrarily selected

The titre of chlamydial antibody appears to correlate with the nature of the infection. Generally, women with genital tract infections appear to have higher levels than men. It has been suggested that in addition to having a larger area of infection women often remain asymptomatic for long periods of time, providing a larger and longer antigenic stimulation than usually occurs in men.

#### 4.3

#### CHEMICAL COMPOSITION OF CELL WALL COMPONENTS

Jenkin (1960) examined the chemical properties of intact particles and cell walls. The amino acid composition of cell walls was of particular interest because Gram-positive bacterial cell walls

contain as few as three amino acids, most frequently alanine, lysine, and glutamic acid, whereas Gram-negative walls yield most of the amino acids generally found in proteins. Twelve to fourteen amino acids were positively identified in hydrolysates of intact chlamydial particles and only nine to ten in cell walls.

A sensitive test for peptidoglycans revealed a trace amount of this amino sugar in both intact particles and cell walls ( 1 per cent dry weight). Peptidoglycans constitute a special class of biological heteropolymer. In Gram-positive unicellular bacteria peptidoglycans account for at least 50 per cent of the dry weight of the cell wall, but in other procaryotic groups, such as Gram-negative bacteria, it may constitute less than 10 per cent of the dry weight of the wall.

Jenkin concluded from his studies on the antigenic composition of the meningopneumonitis agent:- "it is obvious that the cell walls are far too complex in chemical composition to be compared with the cell walls of Gram-positive bacteria. They more closely resemble the cell walls of rickettsiae and Gram-negative bacteria". Caldwell (1981) has investigated the chlamydial outer membrane complex (COMC) and compared his findings with recent work on Gram-negative bacteria. A major outer membrane protein was isolated from C.trachomatis (L<sub>2</sub>) and found to represent approximately 60% of the COMC. This 39.5 K dalton protein appeared to have an important structural role in that its removal brought about collapse of the cell wall. Serologically it appeared to be a shared antigen common to a major serotype complex of C.trachomatis organisms. Similar matrix proteins have been found in Gram-negative

bacteria. The function of these proteins is currently a topic of intensive study. One of the functions attributed to matrix proteins is that they are transmembrane diffusion pores or 'porins'. They may also contribute to structural integrity because of their strong interaction with the peptidoglycan. Similar proteins have been found in the outer membrane of the genus Neisseria. The major outer membrane protein of Neisseria gonorrhoeae organisms is believed to be an important serotyping antigen and may play a role in virulence.

## CHAPTER 5

### MATERIALS AND METHODS I

#### PARAMETERS AFFECTING INFECTIVITY TITRATIONS OF C.TRACHOMATIS IN CELL CULTURE

##### 5.1

Since the first successful isolation of "slow-growing" strains of C.trachomatis (serotypes A - K inclusive), (Gordon et al, 1963) several modified techniques have been developed. One of the priorities in the present study was to compare the presently available isolation procedures for susceptibility to infection by C.trachomatis. After analysing the data, one procedure was selected and used for further investigation.

The following techniques described by others for the culture of C.trachomatis were investigated:-

- (a) Untreated McCoy cells in monolayer culture (Gordon et al, 1963)
- (b) Irradiated McCoy cells in monolayer culture (Gordon et al, 1963)
- (c) DEAE-dextran treated HeLa 229 cells in monolayer culture (Kuo, 1972)
- (d) IUdR treated McCoy cells in monolayer culture (Wentworth et al, 1974)
- (e) Cycloheximide treated McCoy cells in monolayer culture (Ripa et al, 1977)
- (f) Hydrocortisone treated McCoy cells in monolayer culture (Bushell et al, 1978)

Although the above investigations used either McCoy or HeLa 229 cells, this present study examined the effects of cell treatments on both cell lines. However, before such a comparison could be made, a suitable infectivity titration procedure was required and certain general requirements regarding cell culture must be fulfilled. The following is therefore a summary of the cell culture conditions employed and an examination of factors which may affect the accuracy and reproducibility of infectivity assays.

## 5.2 GENERAL REQUIREMENTS FOR CELL CULTURE

Glassware: Culture medium which is slightly alkaline may bring toxic material present in some types of glass into solution. Experience has shown that Pyrex (bromo-silicate glass) and soda glass is satisfactory. All glass bottles used to propagate cell lines had silicone lined screw caps.

Cleaning procedures: All glassware was washed in a programmable automatic washing machine. The following cycle was used:

- I Immerse in 1 per cent sodium hypochlorite (Chlorox, Durham Chemical Eng.)
- II Immerse in 0.9 N HCl for 1 hour (hr).
- III Immerse in 0.2 per cent Pyroneg for 1 hr.
- IV Rinse 6 times in tap water, emptying between rinses.
- V Rinse 3 times in deionised water.

After washing, glassware was dried in a hot air oven ( $160^{\circ}\text{C}$ ) and finally autoclaved at  $121^{\circ}\text{C}$  for 20 minutes (15 lbs. sq in<sup>-1</sup>).

Sterile, round glass coverslips (No. 1 13mm diameter; Chance Propper Ltd., Smethwick, U.K.) were used extensively in the experimental work. They had to be handled separately because of their small size and fragility. The coverslips were dropped one by one into a beaker of surface active agent (2 per cent Decon-90, Decon Lab. Ltd., Hove). Twenty-four hours later, the coverslips were removed, rinsed thoroughly in deionised water and soaked overnight in 95 per cent alcohol. Before each coverslip was inserted into a sterile plastic vial, they were flamed carefully over a gentle bunsen flame to evaporate excess alcohol.

The plastic vials (5ml stoppered vials, Redhill Surgical Co., Glasgow) used for coverslip cultures were sterilized, prior to seeding cells, by irradiation (2.5M rad using a cobalt source).

Cell culture media: The essential components of cell culture media have now been characterised. A classically defined synthetic medium was used throughout, Eagle's minimal essential medium with Earle's Salts and  $0.85\text{g l}^{-1}$   $\text{NaHCO}_3$  (pH 7.0) (Gibco Europe, Scotland). The medium was supplemented with 10 per cent foetal bovine serum (Gibco Europe, Virus and Mycoplasma screened).

Antibiotics: Many antibiotics have been used in cell culture as a safeguard against possible contamination by bacteria or fungi during handling. C.trachomatis is particularly sensitive to several antimicrobial agents in vitro. The minimal inhibitory concentrations (MIC) of a wide range of antimicrobial components has been investigated by Treharne et al (1977). Only those agents with an MIC  $\geq 500 \mu\text{g ml}^{-1}$  were considered for inclusion in cell

culture medium. Two antibacterial and one antifungal agent were finally used.

<u>Antimicrobial agent</u>	<u>MIC (<math>\mu\text{g ml}^{-1}</math> for <i>C.trachomatis</i>)</u>
Streptomycin	1000
Vancomycin	1000
Nystatin	500 units $\text{ml}^{-1}$

#### Cell lines:

1. McCoy cells (Flow Labs, Irvine, Scotland). These cells are generally polygonal in shape although fibroblasts appear. They are a robust continuous cell line and are readily propagated in the above growth medium.
2. HeLa 229 (Flow Labs). These cells were originally isolated from an epidermoid carcinoma of the cervix (Gey et al 1952). The 229 strain has previously been used for the isolation of *C.trachomatis* after treatment of the cell line with DEAE-dextran. HeLa 229 cells are polygonal but larger than McCoy cells.

Special methods were used to test these cell lines for contamination by *Mycoplasma* species. If the organism was cultivatable the following procedure was employed:-

- I 0.1 ml. of a cell suspension was spread on PPLO agar (Difco) supplemented with 20 per cent horse serum.
- II The plates were incubated in a 5 per cent  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$  for between 7 and 10 days.
- III *Mycoplasma* colonies were looked for with a magnifying lens.



The colonies are usually less than half a millimetre in diameter.

Giemsa stained preparations of coverslip cell cultures were examined regularly. High resolution light microscopy (X500-1000 magnification) of these cultures was a useful way of screening cells for microbial contamination including Mycoplasma species.

#### MANIPULATION OF CELL CULTURES

The procedures for passaging the above cell lines was as follows:-

- I Growth medium from a culture flask (Tissue culture flasks No 120, 120 cm<sup>2</sup>; Flow Labs) was decanted into sodium hypochlorite (chlorox) and replaced with a small volume of sterile phosphate buffered saline (PBS).
- II The cell monolayer was gently washed with PBS and then with a 1:1 (v/v) solution of trypsin and ethylene-diamine-tetra-acetic acid (EDTA). Cells were thoroughly immersed in this solution and the excess was discarded.
- III The cell monolayer was incubated on a warm plate for 2 - 5 minutes. After this time the cells would begin to disaggregate.
- IV 10 ml of growth medium was added to the culture flask and the cells were aspirated by pipette to obtain a monodispersed cell suspension.
- V The volume of cells was divided equally between 3 sterile culture flasks.

VI The pH of Eagle's MEME increases on exposure to the atmosphere, so gassing with 5 per cent CO<sub>2</sub> in air was required to preserve the pH. All synthetic media contained low levels of phenol red as a pH indicator.

Trypsin, a proteolytic enzyme, is used in this case at 0.025 per cent to remove eukaryotic cells from the sides of glass culture flasks.

EDTA:- The disodium salt of EDTA is a chelating agent which binds calcium and magnesium ions. These ions are important in the structure of "extracellular cement" which binds cells together. A 1:1 solution of trypsin and EDTA is more effective than the individual components at disaggregating cell monolayers.

#### PREPARATION OF CELL MONOLAYERS ON GLASS COVERSLEIPS

- I Cells from one 120 cm<sup>2</sup> flask were removed using trypsin and EDTA and suspended in 10 ml of growth medium as described.
- II 0.2 ml of the resulting cell suspension was diluted 1:2 with Trypan blue (0.4 per cent Gibco Europe, Glasgow). The surface membrane of living cells is able selectively to exclude Trypan blue whereas dead cells permit the blue dye to enter more easily. Using this dye it becomes simple to distinguish permeable cells.
- III A small volume of well mixed monodispersed cells was taken up by capillary action into the tip of a

Pasteur pipette. Cells were transferred from the pipette under a tightly placed coverslip of a Neubauer type haemocytometer chamber. Care was taken not to allow the chamber to overflow. The cells were allowed to settle for a few minutes before counting.

IV Cells in five  $1 \text{ mm}^2$  squares were counted. The average number of cells in  $0.1 \text{ mm}^3$  was calculated; the chamber depth is  $0.1 \text{ mm}$ .

V The total number of cells in  $1 \text{ ml}$  was calculated from the following formula:-

$$a.2.10^4$$

where

$a$  = the average number of cells in  $0.1 \text{ mm}^3$ .

$2$  = dilution factor accounted by adding Trypan blue.

$10^4$  = the number of  $0.1 \text{ mm}^3$  in one ml.

$$\text{in } 10 \text{ ml} \quad = \quad a.2.10^4.10$$

The optimal cell concentration for each vial containing a coverslip was determined by experiment (6.1.2); a 90 per cent confluent monolayer was required after 24 hours' incubation at  $37^\circ\text{C}$ . After diluting the  $10 \text{ ml}$  suspension of cells accordingly with growth medium,  $1 \text{ ml}$  aliquots were pipetted into each vial.

### 5.3. CHLAMYDIAL INOCULUM

One chlamydial isolate was used throughout the infectivity assays. Chlamydial strains have been shown to have phenotypic properties

conferred by the host chosen for propagation of the organism. Griffiths et al (1976) demonstrated that the agent of guinea-pig inclusion conjunctivitis showed greatly increased spontaneous infection (infectivity without centrifugation) after passage through cell monolayers than the same strain passed in the yolk-sac of embryonated hen's eggs.

To avoid using a prototype strain of C.trachomatis that had been repeatedly passaged in the laboratory over several years, a clinical isolate, Code Number 1/382, was used in infectivity assays. The first isolation attempt of this endocervical swab showed a high titre of infectivity:-

466 inclusion forming units (ifu) per 100 high powered (x500) microscope field;  $5.8 \times 10^4$  ifu ml<sup>-1</sup>.

Attempts to isolate this organism without centrifugation (spontaneous infection) produced no ifu per 100 fields.

The specimen labelled 1/382 was resuscitated after storage in a mechanical freezer (approximately -60°C) and passaged in replicate McCoy cell cultures. One further subculture was required to increase the infectivity titre and to provide a sufficient volume of viable chlamydiae required for the experimental work. Only two subcultures were carried out in McCoy cells and the final coding for this material was 1/382 P2M. This specimen was more representative of "wild type" strains likely to be isolated from clinical material than laboratory adapted prototype strains.

The following steps were taken to subculture the chlamydial isolate (1/382):

- I Growth medium covering the infected cell monolayer was discarded in Chlorox.
- II 0.2 ml PBS (pH 7.2) supplemented with 2 per cent foetal bovine serum and antibiotics was added to each of the 10 vials.
- III Infected cells were scraped off each coverslip using a sterile Pasteur pipette. Infected cell suspensions were pooled aseptically.
- IV The resulting suspension was subjected to 10 mins sonication in a Dawe Sonicleaner Type 64414. Iced water was used to reduce the temperature rise which occurs in this process. Vigorous vortex mixing (30 secs) finally disrupted the cell membrane and released infectious chlamydiae into PBS.
- V Cell debris was deposited by "light" centrifugation (x500g, 10 min at 4-10°C).
- VI The infectious supernatant was distributed in 0.2 ml amounts to a further 10 replicate cultures of McCoy cells and the infectious process initiated by centrifugation.

After 48 hours incubation at 35°C in growth medium containing glucose (5.4 mg ml<sup>-1</sup>) a count of ifu per 100 microscopic fields (x500 magnification) was made on one selected coverslip culture diluted 10<sup>-4</sup> in PBS:-

$$\begin{aligned} \text{ifu per 100 fields} &= 423 \\ &= 5.3 \times 10^8 \text{ ifu ml}^{-1} \end{aligned}$$

The remaining 9 vials were subjected to steps I - V this time using 2SP as diluent. 64 plastic vials (Cryotube 38 x 12.5 mm. polypropylene Nunc Inter Med Denmark) were each filled with 0.2 ml of 1/382 P2M diluted  $10^{-1}$  in 2SP and stored in the vapour phase of liquid nitrogen.

#### 5.4.1 CENTRIFUGATION OF THE CHLAMYDIAL INOCULUM

The following inoculation procedure for initiating infection of McCoy or HeLa cells with chlamydiae was carried out. The centrifugation step was essential for the propagation of all "slow-growing" strains of C.trachomatis.

- I Growth medium covering uninfected coverslip cultures was removed aseptically and discarded in Chlorox.
- II 0.2 ml of the specimen was inoculated into each vial.
- III Vials containing the cell monolayer and specimen were centrifuged for 1 hour at a speed and temperature determined by experiment (6.1.5).
- IV Isolation medium, which varied in its constituents depending on the technique used, was added to the centrifuged vials.
- V Chlamydial inclusion formation was interrupted after 48 hours incubation at  $35^{\circ}\text{C}$ .
- VI Infected cell monolayers were finally fixed, stained and an estimation of the number of inclusions present was made.

#### 5.4.2 CALCULATION OF INFECTIVITY TITRE

Assuming that one viable elementary body (EB) gives rise to a

single visible (by light microscopy) cytoplasmic inclusion, it is possible to calculate the number of inclusion-forming units (ifu) per ml or per 100 microscopic fields at a specified magnification. This applies to both McCoy and HeLa 229 cells.

(1) ifu per ml (x500 magnification) Wild M20 microscope.

A square graticule was present in one of the oculars whose dimensions were measured using a stage micrometer.

$$\begin{aligned} \text{Area of square graticule} &= 0.23 \times 0.23 \text{ mm}^2 \\ &= 0.0529 \text{ mm}^2 \end{aligned}$$

$$\text{Area of 13 mm diameter coverslip} = 132.665 \text{ mm}^2$$

$$\text{Number of fields per coverslip} = \frac{132.665}{0.0529} = 2508 \text{ fields}$$

$$\text{ifu per ml} = \frac{(\text{infected cells counted})}{(\text{number of microscopic fields counted})} \times \frac{\text{fields per coverslip}}{\text{volume of inoculum}}$$

The minimum number of fields counted to give an accurate estimate of the total number of inclusions per coverslip was determined by experiment (6.1.1). Only discrete inclusions present within the boundaries of the graticule were counted.

(2) ifu per 100 microscopic fields

ifu were counted per specified number of fields. The optimum number of fields to be counted was determined (6.1.1). 100 fields was suitable for most infectivity assays. Counts were made at x500 magnification using a graticule in one of the oculars.

## 5.5 STAINING PROCEDURES

### METHYLENE BLUE

- I Coverslip cultures were fixed in two changes of methanol for 3-5 minutes.
- II Methanol was replaced with an aqueous solution of methylene blue (5g litre<sup>-1</sup>) and stained for no longer than 5-7 minutes.
- III Stain was removed and cultures were briefly washed in tap water.
- IV Washing for 15 seconds in 0.025 per cent H<sub>2</sub>SO<sub>4</sub> was followed by a rapid rinse in tap water.
- V Dehydration in acetone for 15 seconds, followed by clearing in xylene for 15 seconds and mounting in DPX. (D.P.X. Mountant; B.D.H. Chemicals Ltd., Poole, England).

Nuclei stain pale blue with slightly darker chromatin. The cytoplasm stains a very pale blue. Inclusions in untreated McCoy cells appear as a deep blue mass.

### GIEMSA

- I Coverslip cultures were fixed in two changes of methanol for 3-5 minutes.
- II Methanol was replaced with 10 per cent Giemsa stain diluted with pH 6.8 buffer.
- III Stain was removed after 45 minutes and briefly washed in 0.025 per cent H<sub>2</sub>SO<sub>4</sub> followed by a rinse with pH 6.8 buffer (5 minutes).
- IV Dehydration in acetone and xylene is followed by mounting in DPX.



## IODINE

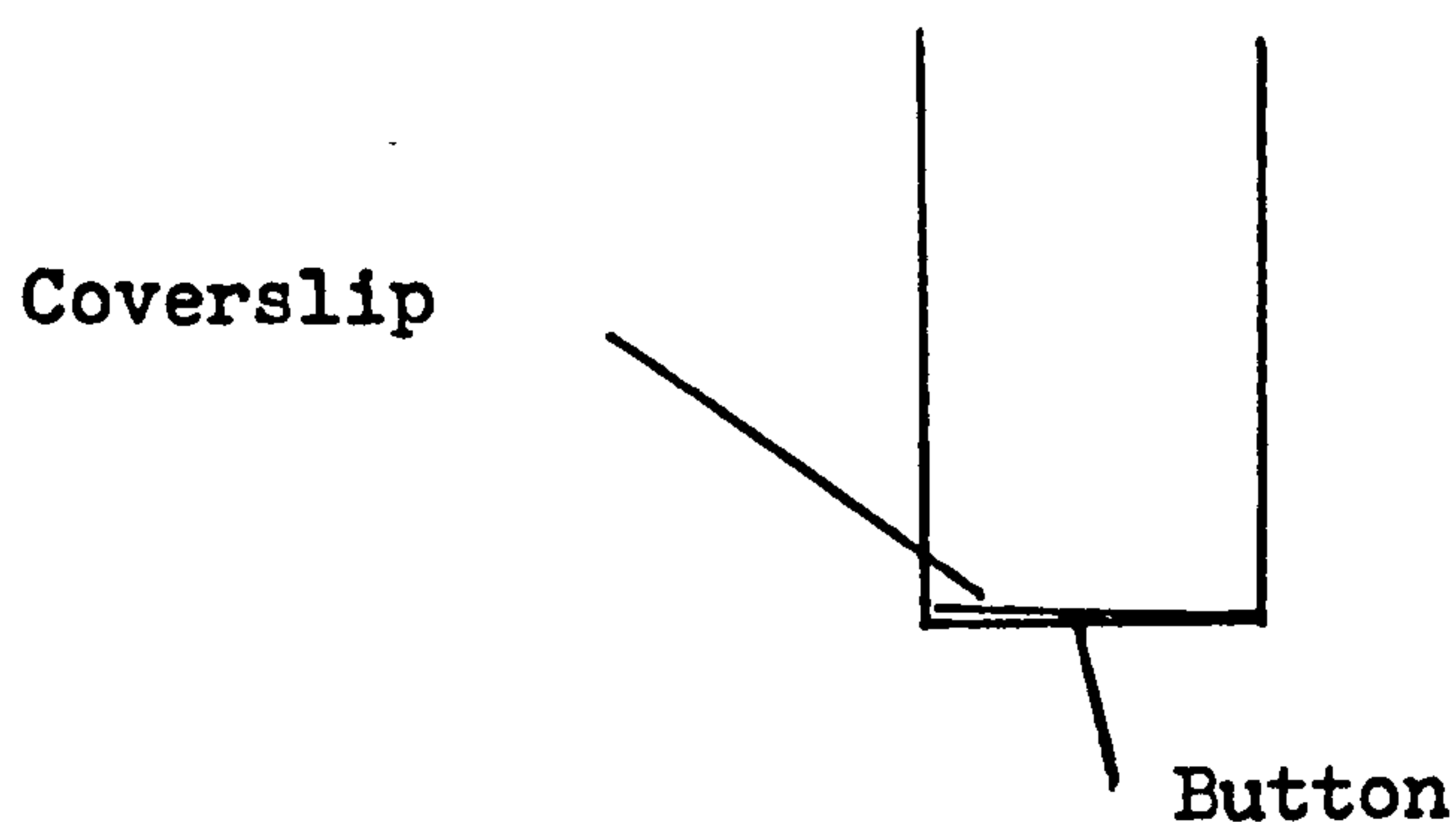
- I Coverslip cultures were fixed in methanol.
- II Methanol was replaced with a 5 per cent solution of iodine in 10 per cent potassium iodide solution for 3-5 minutes.
- III Coverslips were mounted in an iodine-glycerol solution.

Cytoplasmic inclusions appear as a reddish-brown mass commonly showing some granularity.

5.6

## COUNTING PROCEDURE

Plastic vials used in all infectivity assays were made of moulded plastic. During the manufacture of these vials a small button is formed on their inner surface. A glass coverslip lying at the bottom of the vial lies at a slight angle.



After seeding vials the effect of this very small angle was to produce more dense areas of cell growth than others on the coverslip. This was noticed visually after staining monolayers with Giemsa.

To ensure that this effect did not adversely affect the overall inclusion count, the following precautions were carried out:-

- (i) Before any batch of inoculations, one representative vial was selected, stained by Giemsa and examined by low power light microscopy for confluency. Only batches showing 90 per cent confluency were used.
- (ii) Microscope fields were selected randomly for inclusion counts in such a way that an equal number of fields were selected from each quadrant of the coverslip. This ensured that variation in inclusion-forming frequency which occurs over the coverslip is reduced by not concentrating on marginally dense or sparse areas of cells.

5.7

#### ISOLATION TECHNIQUES

##### CYCLOHEXIMIDE TREATED CELLS

Replicate cultures of McCoy and HeLa 229 cells were prepared by seeding  $2 \times 10^5$  cells  $\text{ml}^{-1}$  and incubating at  $35^\circ\text{C}$  for 24 hours. The stock chlamydial suspension (1/328 P2M) was diluted  $10^{-3}$  in PBS (supplemented with 2 per cent fbs and antibiotics) and 0.2 ml was inoculated per vial. After the centrifugation step, the inoculum was removed and the following medium was added to the infected cultures:- Eagles MEME, 10 per cent fbs, antibiotics, glucose ( $5.4 \text{ mg ml}^{-1}$ ) and cycloheximide ( $1.5 \mu\text{g ml}^{-1}$ ; BDH, Poole, Dorset).

### CORTISOL TREATED CELLS

The preparation of cell monolayers and the inoculation procedure were carried out in the same way as 5.7.1. However, after the centrifugation step, the following medium was added to the infected cell cultures:- Eagles MEME, 10 per cent fbs, antibiotics, glucose ( $5.4 \text{ mg ml}^{-1}$ ) and cortisol ( $1 \text{ } \mu\text{g ml}^{-1}$ ; Efcortisol, Glaxo Labs Ltd ).

### IUdR TREATED CELLS

Cells were seeded in sterile plastic vials as before but using a diluent growth medium containing 5-iodo-2-'deoxyuridine ( $30 \text{ } \mu\text{g ml}^{-1}$ ; Spodefell Ltd, London). Treated cell monolayers were used for chlamydial inoculation 3 days after seeding. After centrifugation the inoculum was removed and replaced with inoculation medium not containing IUdR.

### IRRADIATED CELLS

50 ml suspensions containing approximately  $5.5 \times 10^5$  cells  $\text{ml}^{-1}$  were subjected to 5000 centi-Grays X-irradiation. (Siemens Stabilipan 300 KV X-ray unit), hardened by passage through 1 mm copper filters (half-value thickness 2.5 mm). After irradiation, suspensions were diluted to  $1.0 \times 10^5$  cells  $\text{ml}^{-1}$  in growth medium and 1 ml portions were seeded into plastic vials containing coverslips. The cell monolayers were incubated at  $37^\circ\text{C}$  and used 4 to 8 days later.

### DEAE-DEXTRAN TREATED CELLS

Replicate monolayers of cells were prepared. Prior to inoculation, growth medium was decanted, and the cells were

rinsed three times with PBS pH 7.2. For DEAE-D treatment the buffer contained  $40 \mu\text{g ml}^{-1}$  of DEAE-D (mol. wt.  $2 \times 10^6$ ; Pharmacia, Uppsala, Sweden).

This solution remained on the monolayer for 20 - 50 minutes; it was withdrawn before addition of the inoculum. Adsorption of chlamydiae took place at  $37^{\circ}\text{C}$  for 1 hour followed by centrifugation. The monolayers were then rinsed twice with PBS, and 1 ml of inoculation medium was added.

THE F-TEST

The F-test was applied to test whether the sample variances  $S_x^2$  and  $S_y^2$  were significantly different. The assumptions made by this test are:-

1. Readings and samples must be independent.
2. Readings are normally distributed.

$$F = \frac{\text{Larger sample variance}}{\text{Smaller sample variance; with degrees of freedom } U_1 \text{ and } U_2}$$

The degrees of freedom are given by sample size less one,  $U_1 = N_L$  where  $N_L$  is the sample size of the larger variance. F-Tables indicate the level of significance of the calculated F statistic (Documenta Geigy, 1962).

## CHI-SQUARED TEST

This test compares the distribution of a discrete variable in a sample with the distribution of a discrete variable in another sample. To carry out the test a null hypothesis:- that there is no difference between the two distributions is made. This is what is tested by chi-squared. The analyses involves the use of a 2 x 2 contingency table which can be summarized as follows:-

	Number with Characteristic	Number Characteristic	Total
Population A	a	b	a + b
Population B	c	d	c + d
<hr/>			
Totals	a + c	b + d	n = a+b+c+d

The values obtained in this contingency table are applied to the formula:

$$\chi^2 = \frac{n(ad - bc)^2}{(a+b)(c+d)(a+c)(b+d)}$$

With a 2 x 2 contingency table there is one degree of freedom; the probabilities attached to selected  $\chi^2$  values are:-

Probability	0.05	0.01	0.001
$\chi^2$	3.841	6.635	10.83

## THE MANN-WHITNEY SUM OF RANKS TEST

This test can be used to check whether two populations have the same median, and it does not require the assumption of normality.

The statistic, the rank sum, is computed in four steps.

1. The two samples are combined into one, underlining the observations in the second sample to keep track of them.
2. The observations in the combined sample are arranged in order of increasing size.
3. The numbers  $1, 2, \dots, n_1 + n_2$  are written down in order. Ties are dealt with by averaging adjacent ranks.
4. From the average of the underlined numbers the average of the other is subtracted. The difference is called  $V$ .

The standard deviation is calculated from the following formula.

$$\sigma = \frac{(n_1 + n_2)^2 (n_1 + n_2 + 1)}{12n_1n_2}$$

Where  $n_1$  and  $n_2$  are the sizes of the population.

The null hypothesis is rejected if the standardized statistic  $V/\sigma$  exceeds  $t$

The upper 5 per cent point for the normal curve is 1.645.

## GEOMETRIC MEAN TITRES

Each titre was converted to the equivalent  $\text{Log}_2$  value. The mean of these logarithmic values was then calculated and the geometric mean titre was then obtained by taking the antilog of this mean value.

## STANDARD ERROR

The standard error of the mean of the sample is an estimate of the standard deviation that would be obtained from the means of a larger number of samples taken from that population.

$$\text{Standard error of the mean} = \frac{\text{SD}}{n}$$

When SD is the standard deviation of the sample and n the number of observations in the sample.



## CHAPTER 6

### 6.1 RESULTS OF INFECTIVITY ASSAY CONDITIONS

Successive counting experiments showed that if from a stock suspension of chlamydiae, the same volume of material was inoculated into a series of vials, a significant degree of variation in the number of inclusions occurred. The following steps in the infectivity assay were examined as possible sources of variation:-

- (a) Counting procedure.
- (b) Cell density for seeding coverslip cultures.
- (c) Type of pipette used.
- (d) Staining technique.
- (e) Centrifugation temperature.

#### 6.1.1 COUNTING PROCEDURE

An estimate was made of the minimum number of microscope-fields that might be counted to give an accurate estimate of the total number of ifu per coverslip.

A  $10^{-4}$  dilution of stock chlamydial suspension (1/382 P2 McCoy) in 2SP was made with PBS supplemented with 10 per cent fbs onto 3 replicate coverslip McCoy cell monolayers. Centrifugation (1890g), incubation and staining procedures were carried out as indicated in section 5.4.1.

Counts of ifu were made at regular microscope-field intervals.

The final mean count for 2000 fields was recorded and the minimum number of fields counted that would give a true estimate of this total was obtained (Table 6.1).

The calculated number of microscope fields (x500 magnification) was 2,508 (5.4.2). In this experiment 2000 separate fields were counted which was a good sample of the total. Results from Table 6.1 show that the mean ifu in 100 fields gave an accurate estimate of the total inclusion count in 2000 fields. All further infectivity assays were based on counts from 100 fields in three replicate cultures.

#### 6.1.2 CELL DENSITY FOR SEEDING COVERSIP CULTURES

The first step in preparing cell cultures for the isolation of chlamydiae is to seed plastic vials containing coverslips with a specified concentration of monodispersed cells. Once the cell suspension has been seeded, it settles, cells eventually adhere to the glass surface and multiply. A monolayer of cells varying in confluency depending on the concentration of cells seeded is established between 24 and 48 hours at 35°C.

An experiment was carried out to determine whether there is an optimal concentration of McCoy cells to measure infectivity. Only discrete ifu were counted.

Separate vials holding 13 mm diameter coverslips were seeded with varying cell concentrations. A cell monolayer was established after 24 hours incubation at 35°C. Each vial was inoculated

with 0.2 ml of  $10^{-3}$  dilution of the stock chlamydial suspension. No cell treatment procedure was used.

The results from Table 6.2 were plotted (Figure 6.1). The mean inclusion count was directly proportional to the cell concentration up to  $0.5 \times 10^5$  cells  $\text{ml}^{-1}$ . A plateau of inclusion counts was obtained at this point. However seeding concentrations greater than  $2.5 \times 10^5$   $\text{ml}^{-1}$  brought about a reduction in ifu per 100 fields. A cell concentration of  $2.0 \times 10^5$  cells  $\text{ml}^{-1}$  seeded 24 hours prior to inoculation was used throughout.

### 6.1.3 TYPE OF PIPETTE

Two available types of pipette were compared for their accuracy in dispensing a small volume of stock chlamydial suspension:-

- (i) "Gilson pipetman" with sterile disposable plastic tips adjusted to dispense 100  $\mu\text{l}$ .
- (ii) "Volac blowout" glass pipette graduated to dispense 200  $\mu\text{l}$ .

Vials were seeded with McCoy cells and incubated for 24 hours at  $35^{\circ}\text{C}$ . A stock suspension of chlamydiae was diluted in PBS (10 per cent fbs and antibiotics). 100  $\mu\text{l}$  and 200  $\mu\text{l}$  of the diluted suspension were inoculated using the Gilson and Volac pipettes respectively. 10 replicate vials were used in each case. A count of ifu per 100 fields was made.

The sample means and standard deviation for each batch of 10

vials was calculated and an F test was used to compare the variance. (Table 6.3).

Results show that the variance in inclusion counts obtained with the Volac pipette was marginally less than the counts obtained with the Gilson pipetman. The former was used for further assay procedures.

#### 6.1.4 STAINING TECHNIQUE

Three staining techniques that demonstrate cytoplasmic inclusions in infected cells were examined.

9 replicate monolayers of McCoy cells were infected with a chlamydial suspension. After inoculation by centrifugation and incubation, each coverslip was fixed and stained. Replicate cultures were stained by Giemsa, methylene blue and by iodine.

The mean ifu count per 100 microscope fields was calculated from each three replicate cultures. (Table 6.4.).

No significant difference in inclusion counts was observed using either of the three staining techniques.

Giemsa was preferred because stained inclusions were well differentiated and easily detected by dark-grown illumination.

#### 6.1.5 CENTRIFUGATION

Two centrifuges were available to isolate chlamydiae routinely:-

(i) MSE Mistral 2L

(ii) MSE GF8

Only (i) had an adjustable temperature-regulator and thermostat. (ii) operates at ambient temperatures, but had the advantage of taking four times as many specimens at (i). Two temperature settings, 4°C and approximately 37°C were selected to observe their effects on ifu per 100 fields.

A 10<sup>-3</sup> dilution of 1/382 P2M was made in PBS containing 10 per cent fbs and antibiotics. Centrifugation was carried out at 1890g on both centrifuges for 1 hour. Only one culture was tested at each temperature.

A count of ifu per 100 fields was made and a comparison was made of relative infectivity. (Table 6.5).

1890g was the maximum centrifugal force produced by the MSE GF8 centrifuge. The following equation was used to calculate correct revolutions per min. for the MSE Mistral 2L.

$$g \quad (\text{max}) = 1,118 \times 10^{-5} \times r \times n^2$$

n = revolutions per minute

r = maximum effective rotating radius of centrifuge head measured in cm from centre of the drive shaft to far end of contents of the centrifuge tube in its extended position.

r (GF8) = 17.8 cm.

r (2L) = 19.7 cm.

The temperature of rotor and tube carriers increased noticeably

using the MSE GF8 for 1 hour at 1890g. Results from Table 6.5 show that this did not adversely affect the viability of the inoculum. There was minimal variation of inclusion counts obtained with the GF8 and Mistral 2L (35-37°C). The GF8 centrifuge was used for all routine inoculations. Table 6.5 also shows that centrifugation at 35-37°C produced more than twice as many inclusions counted at 4-10°C.

## 6.2 A COMPARISON OF DIFFERENT ISOLATION TECHNIQUES FOR C. TRACHOMATIS

The following isolation techniques were compared for their relative susceptibility to chlamydial infection.

- (i) Cycloheximide treated cells;
- (ii) Cortisol treated cells;
- (iii) IUdR treated cells;
- (iv) Irradiated cells;
- (v) DEAE-dextran treated cells.

In all the isolation techniques examined (i - v), the stock chlamydial suspension (1/382 P2M) was diluted  $10^{-3}$  in PBS pH 7.2, supplemented with 10 per cent fbs and antibiotics. 0.2 ml of the inoculum was inoculated on to replicate cultures for each treatment examined. Incubation after chlamydial inoculation was for 48 hours. Infected cultures were stained by Giemsa and assayed for the number of ifu per 100 microscopic fields. The mean count and standard error was calculated and tabulated. (Table 6.6 and 6.7).

Isolation results were compared as a ratio, the numerator corresponding to inclusion counts for each treatment investigated and the denominator to the inclusion count obtained for untreated cells.

$$\text{i.e.} \quad \frac{\text{ifu per 100 fields-treated cells}}{\text{ifu per 100 fields-untreated cells}} = \frac{\text{CT}}{\text{GM}} \text{ ratio}$$

The closer this ratio approaches 1, the smaller the degree of enhanced infectivity seen in treated cells.

Results from Table 6.6. and 6.7 show that of the five cell treatments examined, cycloheximide and irradiation produced the highest CT/GM ratio. This was followed by IUdR, cortisol and finally DEAE-dextran treatments. Within each cell type (HeLa and McCoy) the difference in mean inclusion count for cycloheximide and irradiation treatments was not significant. However cycloheximide treated McCoy cells were significantly more susceptible to infection than cycloheximide treated HeLa cells (p0.001). All further assays and isolation procedures used cycloheximide treated McCoy cells.

Susceptibility to infection was measured in terms of the number of inclusion counts per unit of inoculum. Another parameter examined qualitatively was the size of inclusions produced 48 hours post-inoculation. Exceptionally large inclusions were seen in cortisol treated HeLa cells. The smallest inclusions were obtained in untreated HeLa and McCoy cells.

6.3 COMPARISON OF DIFFERENT MEDIA FOR THE MAINTENANCE  
OF CHLAMYDIAE AT DIFFERENT STORAGE TEMPERATURES

A suitable transport medium was required for sending clinical specimens to the laboratory. Four media were investigated for their ability to maintain viable chlamydiae at different temperatures. The temperatures selected for this work were those likely to be encountered during the routine transportation and long-term storage of specimens:-

- (i) Room temperature, approximately 25°C
- (ii) Domestic refrigerator, approximately 4°C
- (iii) Iced water contained in a vacuum flask
- (iv) -60°C (Revco-mechanical freezer)

The four media investigated were:-

- (a) 2SP (68.45g sucrose; 2.088g  $K_2HPO_4$ ; 1.088g  $KH_2PO_4$  in 1L deionised water pH 7.00)
- (b) Eagles MEME with 0.85g/L  $NaHCO_3$ ; supplemented with 20 per cent (w/v) sorbitol, 10 per cent fbs pH 7.3.
- (c) Phosphate buffered saline (PBS) supplemented with 10 per cent fbs pH 7.2.
- (d) Eagles MEME with 10 per cent fbs.

All media contained antibiotics.

The stock chlamydial suspension was resuscitated from storage in liquid nitrogen. The suspension was diluted  $10^{-3}$  in each of the four media. A small volume of the chlamydial suspension in each medium was distributed to separate 2 ml glass vials so that vials could be retrieved at 6 hourly intervals for a total



of 36 hours. At each interval specimens were taken from the four storage temperatures (i - iv inclusive) and assayed for the number of ifu per 100 fields.

0.2 ml of the appropriate inoculum was inoculated onto a confluent monolayer of McCoy cells. The chlamydial suspension was dispensed by 0.2 ml glass pipette. Three replicate vials were tested in each case. The rest of the inoculation procedure has been described. Cycloheximide treated McCoy cells were used. Inoculated vials were incubated for approximately 48 hours at 35 - 37°C before staining by Giemsa.

Results are shown in Tables 6.8.1 - 6.8.4 inclusive and have been plotted on graphs (Fig.6.2 - 6.5).

After 36 hours incubation three combinations of media and temperature were effective in maintaining the viability of chlamydial suspensions:-

- (i) PBS and fbs at iced water temperature 4°C (Fig.6.3)
- (ii) PBS and fbs at approximately 4°C (Fig. 6.3)
- (iii) 2SP at -60°C (Fig. 6.2)

Of these combinations (i) showed only a 20 per cent reduction in inclusion count, (ii) 50 per cent and (iii) 60 per cent.

None of the media tested protected chlamydiae at room temperature. With PBS and fbs there was a 6 hour period in which no loss in viability occurred. However from 6 - 18 hrs. there was a rapid decline in infectivity (Fig. 6.3). Other media tested at room

temperature failed to show signs of infectivity after 12 hours incubation (Fig. 6.2 - 6.5).

At  $-60^{\circ}\text{C}$  sucrose and sorbitol solutions were more protective than others. Sorbitol solutions showed an 80 per cent reduction in infectivity after 6 hours, but once this level was reached further losses were minimal. A similar pattern was seen with sucrose solution however the overall survival level was better.

The least effective medium at all temperatures examined was Eagles MEME and 10 per cent fbs. There was a substantial loss of infectivity at the beginning of each incubation suggesting that the medium itself was toxic.

For transporting clinical specimens to the laboratory and longer term storage 2SP was used at  $-60^{\circ}\text{C}$ . PBS and fbs were used for diluting chlamydial suspensions during titration procedures.

TABLE 6.1

AN ESTIMATE OF THE MINIMUM NUMBER OF FIELDS TO BE COUNTED

<u>Number of microscope fields examined</u>	<u>Mean cumulative total of ifu</u>
50	9
100	37
250	92
500	187
750	270
1200	450
1600	592
1750	641
2000	720

The minimum number of microscope-fields examined that gave a good estimate of the total number of ifu present in 2000 fields was 100.

100 fields showed 37 ifu

The predicted number of ifu at 2000 fields =  $20 \cdot 37 = 740$

True number of ifu at 2000 fields = 721

The first 50 fields were counted from each quadrant of the coverslip e.g. 12; 12; 12; 14. Fields were counted in this manner throughout.

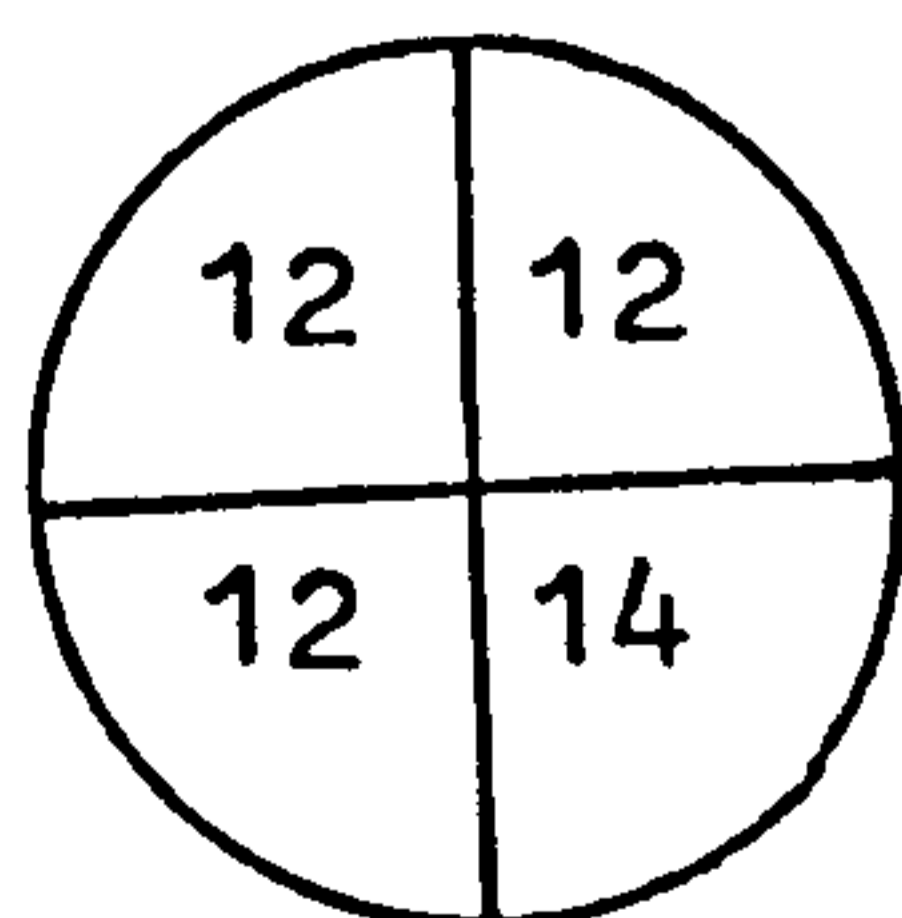


TABLE 6.2

CELL DENSITY FOR SEEDING COVERSIP CULTURES

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Cell concentration seeded ( $\times 10^5$ )	ifu per 100 microscopic fields
0.1	9 $\pm$ 1
0.25	65 $\pm$ 7
0.5	224 $\pm$ 23
1.0	221 $\pm$ 21
2.5	213 $\pm$ 16
5	27 $\pm$ 11

---

The numbers of ifu per 100 microscopic fields examined and arithmetic means  $\pm$  SD calculated in each case from 3 replicate cultures.

TABLE 6.3

COMPARISON OF VARIATION BETWEEN TWO PIPETTES

(i) 100  $\mu$ l ("Gilson pipetman")      (ii) 200  $\mu$ l ("Volac" glass pipette)

	ifu per 100 fields	ifu per 100 fields
(1)	173	351
(2)	210	370
(3)	170	448
(4)	208	457
(5)	247	487
(6)	210	364
(7)	175	438
(8)	107	410
(9)	163	422
(10)	158	431

(i) 100 $\mu$ l; 10 observations	Sample mean $\bar{N}$ =	182	
	SD(N) =	38	
(ii) 200 $\mu$ l; 10 observations	Sample mean $\bar{M}$ =	418	
	SD(M) =	44.2	

Test of hypothesis that variance of M = 4 x variance of N

$$\frac{SD^2 (M)}{4 \times SD^2 (N)} = \frac{1949}{5776} = 0.34$$

TABLE 6.4

STAINING PROCEDURES

	Giemsa	Methylene Blue	Iodine
ifu per 100 microscopic fields	282 ± 26	237 ± 28	217 ± 24

TABLE 6.5

EFFECT OF TEMPERATURE DURING CENTRIFUGATION ON THE INFECTIVITY OF CHLAMYDIAL ISOLATE 1/382 P2 at 1890 xg

Centrifuge: MSE Mistral 2L	Dilution of chlamydial stock	ifu per 100 microscopic fields
Temperature 4 - 10°C	10 <sup>-3</sup>	96
35 - 37°C	10 <sup>-3</sup>	328
<u>Centrifuge: MSE GF8</u>		
Temperature: Ambient	10 <sup>-3</sup>	319

TABLE 6.6

COMPARATIVE SUSCEPTIBILITY OF DIFFERENT CELL TREATMENTS TO CHLAMYDIAL INFECTIVITY

McCoy Cells

Dilution of chlamydial stock	ifu 100 fields <sup>-1</sup>	Cell treatment	Ratio $\frac{CT}{GM}$
10 <sup>-3</sup>	308 ± 7	None	1
10 <sup>-3</sup>	851 ± 20	Cycloheximide	2.8
10 <sup>-3</sup>	462 ± 14	IUdR	1.5
10 <sup>-3</sup>	873 ± 23	Irradiation	2.8
10 <sup>-3</sup>	483 ± 16	Cortisol	1.6
10 <sup>-3</sup>	372 ± 11	DEAE-dextran	1.2

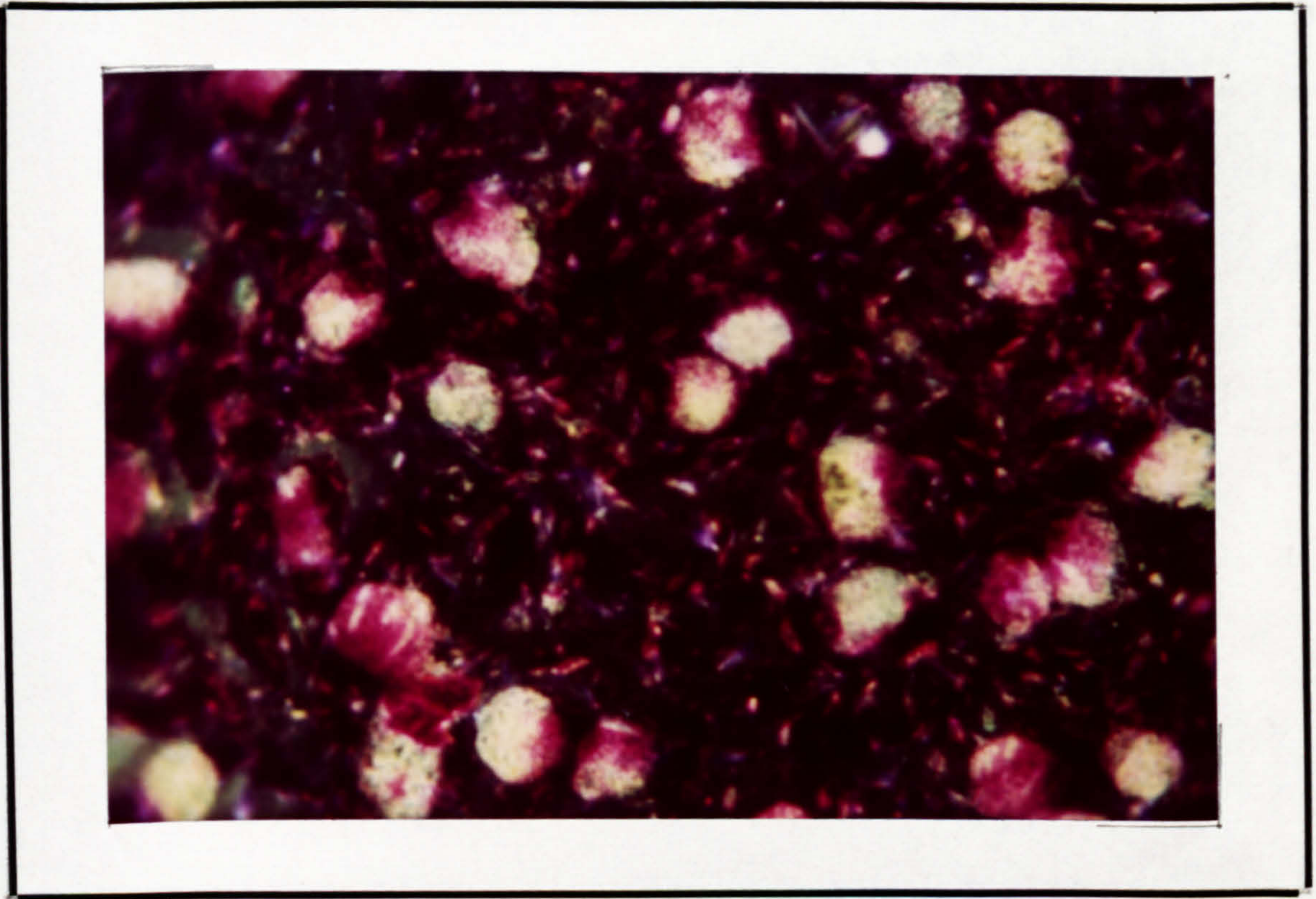
TABLE 6.7

HeLa 229 Cells

Dilution of chlamydial stock	ifu 100 fields <sup>-1</sup>	Cell treatment	Ratio $\frac{CT}{GM}$
10 <sup>-3</sup>	144 ± 2	None	1
10 <sup>-3</sup>	440 ± 27	Cycloheximide	3.0
10 <sup>-3</sup>	260 ± 9	IUdR	1.8
10 <sup>-3</sup>	502 ± 24	Irradiation	3.5
10 <sup>-3</sup>	292 ± 11	Cortisol	2.0
10 <sup>-3</sup>	187 ± 3	DEAE-dextran	1.3

$$\frac{\text{Cycloheximide treated McCoy cells}}{\text{Cycloheximide treated HeLa cells}} = \frac{851}{440} = \frac{1.9}{1}$$

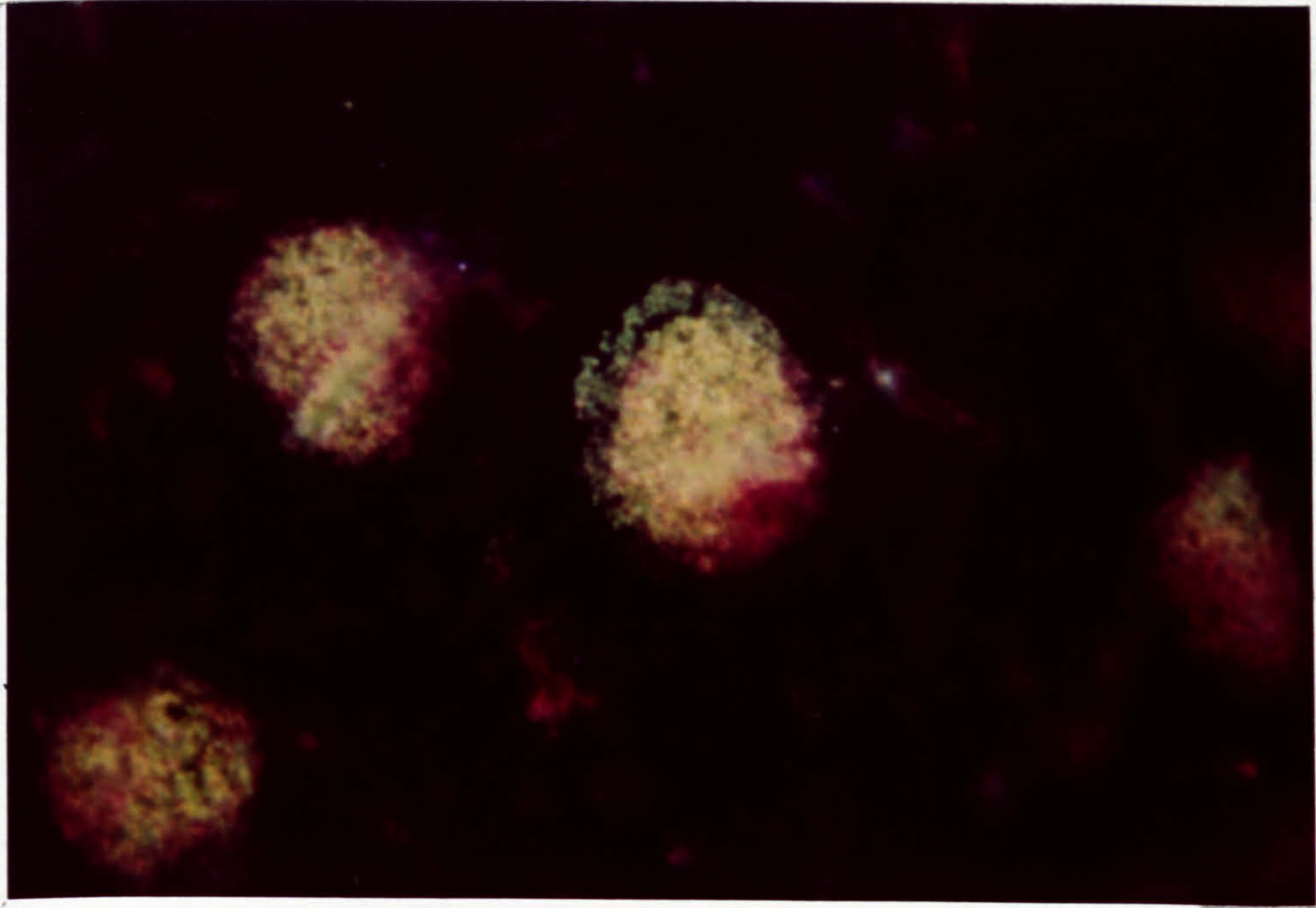
Plate 1



INFECTED MCCOY CELLS STAINED BY GIEMSA  
(Dark ground microscopy x250)

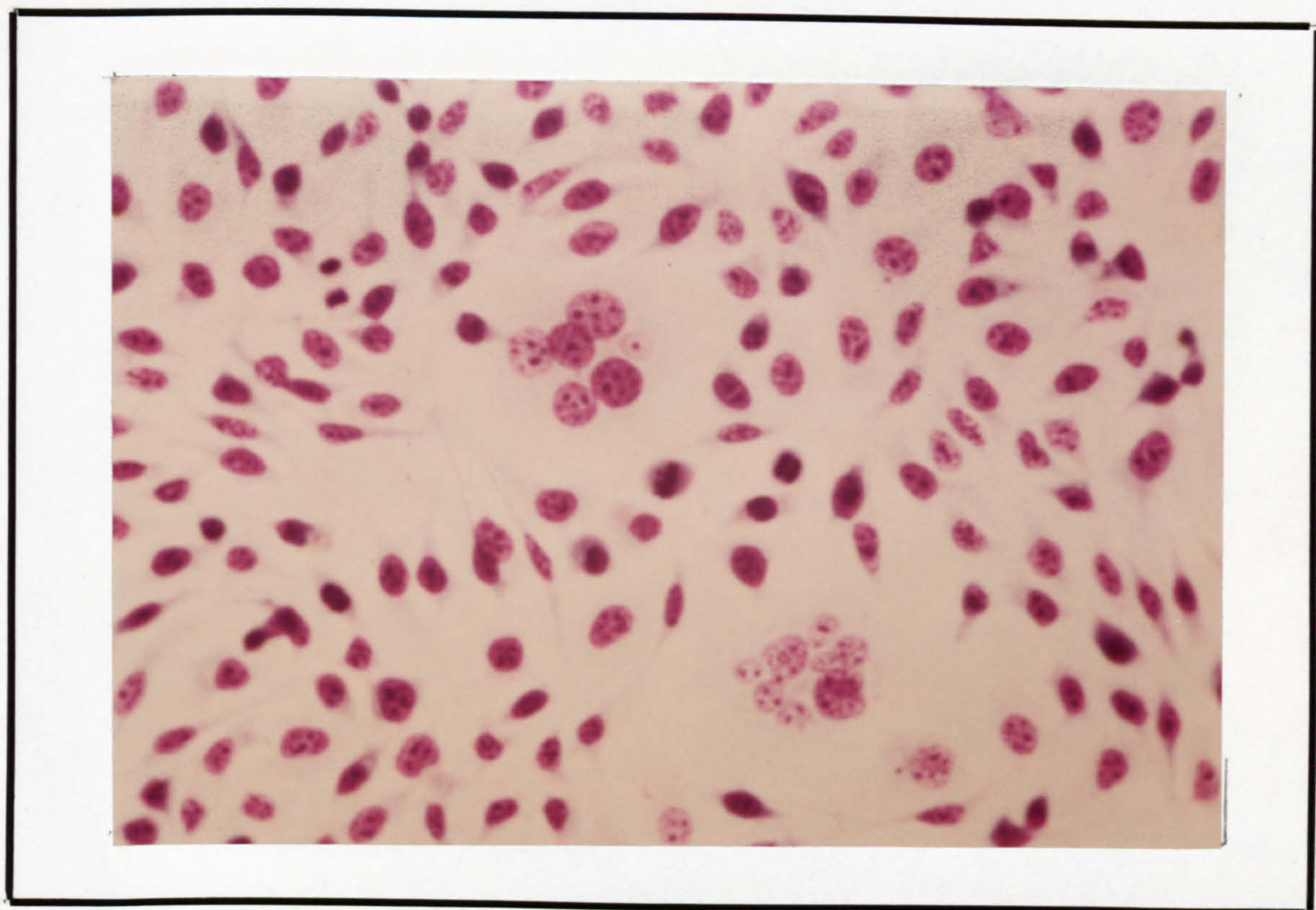


Plate 2



INFECTED MCCOY CELLS STAINED BY GIEMSA  
(Dark ground microscopy x500)

Plate 3



POST-IRRADIATION MULTI-NUCLEATED MCCOY CELLS

TABLE 6.8.1

COMPARISON OF DIFFERENT MEDIA FOR THE MAINTENANCE OF  
CHLAMYDIAE AT DIFFERENT STORAGE TEMPERATURES

(i) 2SP

INOCULUM	STORAGE TEMPERATURE	TIME OF INCUBATION (hr)						
		0	6	12	18	24	30	36
(a)	Room temperature	225	49	2	0	0	0	0
(b)	n 26°C	237	56	7	2	0	0	0
(c)		243	64	0	12	7	0	0
(a)	4 - 8°C	225	92	72	58	55	51	59
(b)		237	106	96	83	85	57	38
(c)		243	87	67	77	41	75	25
(a)	Iced water	225	40	53	30	ND	8	23
(b)		237	46	48	36	ND	17	6
(c)		243	76	34	47	ND	46	2
(a)	-60°C approx.	225	162	111	102	104	102	110
(b)		237	137	122	118	115	105	86
(c)		243	116	138	120	75	112	79

TABLE 6.8.2

(ii) Eagles MEME + 20 per cent sorbitol

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INOCULUM	STORAGE TEMPERATURE	TIME OF INCUBATION (hr)						
		0	6	12	18	24	30	36
(a)	Room temperature	189	26	0	0	0	0	0
(b)	n 26°C	176	38	0	0	0	0	0
(c)		153	41	0	0	0	0	0
(a)	4 - 8°C	189	58	32	19	16	21	15
(b)		176	63	41	27	18	16	13
(c)		153	67	37	24	19	9	ND
(a)	Iced water	189	43	37	17	6	8	3
(b)		176	37	21	21	13	4	2
(c)		153	51	35	29	7	15	8
(a)	-60°C approx.	189	72	63	68	59	67	53
(b)		176	84	67	71	63	61	49
(c)		153	61	53	48	52	72	60

---

TABLE 6.8.3

Eagles MEME + 10% fbs

INOCULUM	TEMPERATURE	TIME OF INCUBATION (hr)						
		0	6	12	18	24	30	36
i	Room temperature	63	0	0	0	12	0	0
ii		69	3	0	0	14	0	0
iii		81	6	0	0	7	0	0
i	4°C	63	27	3	2	12	0	4
ii		69	35	8	10	15	2	0
iii		81	20	15	26	21	10	9
i	Iced water	63	0	6	3	8	9	0
ii		69	30	9	17	11	23	2
iii		81	42	12	24	22	32	7
i	-60°C	63	6	0	0	17	8	0
ii		69	11	5	12	9	13	0
iii		81	24	9	21	0	0	7

TABLE 6.8.4Phosphate buffered saline + fbs

INOCULUM	TEMPERATURE	TIME OF INCUBATION (hr)						
		0	6	12	18	24	30	36
i	Room temperature	ND	255	52	66	14	6	9
ii		264	246	47	10	19	23	20
iii		240	273	72	25	32	37	34
i	4°C	ND	200	175	166	215	92	104
ii		264	186	164	162	187	173	127
iii		240	205	190	137	273	202	138
i	Iced water	ND	236	204	ND	197	192	ND
ii		264	246	211	207	208	204	ND
iii		240	252	218	219	232	210	ND
i	-60°C	ND	50	20	0	0	7	0
ii		264	22	16	11	9	8	5
iii		240	27	38	15	26	25	6

Figure 6.1

THE RELATIONSHIP BETWEEN MCCOY CELL CONCENTRATION AT SEEDING AND CHLAMYDIAL INFECTIVITY

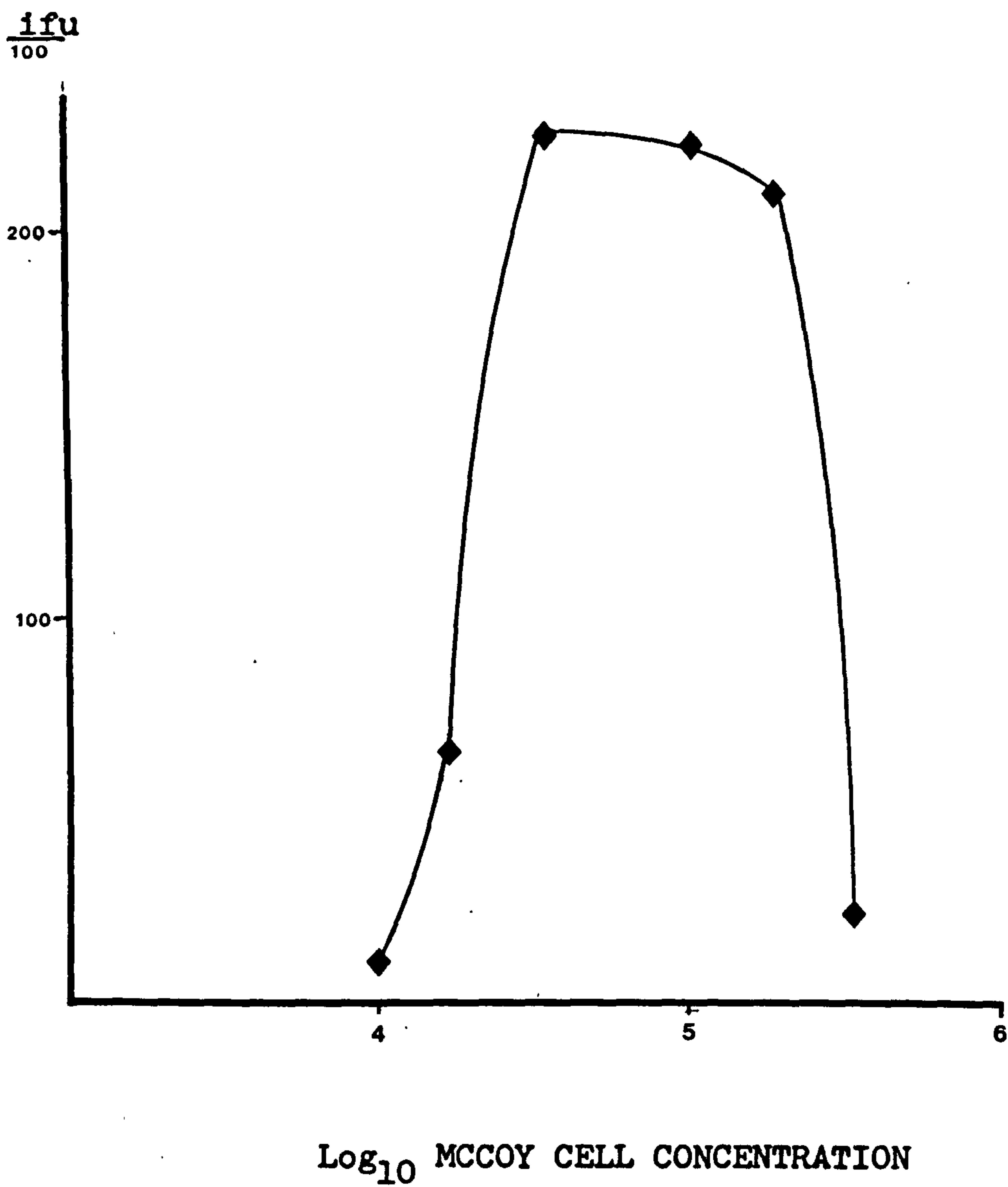
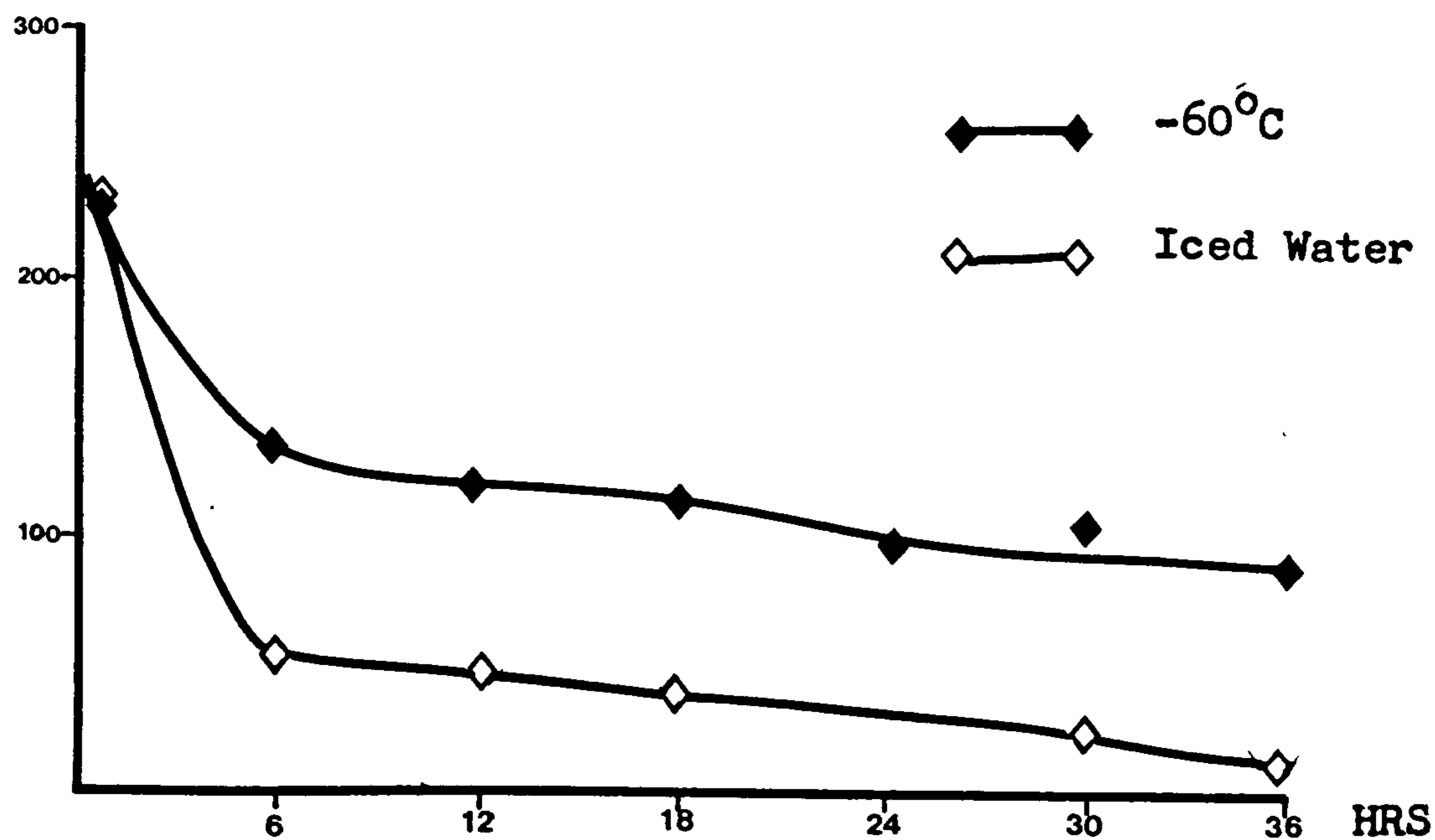
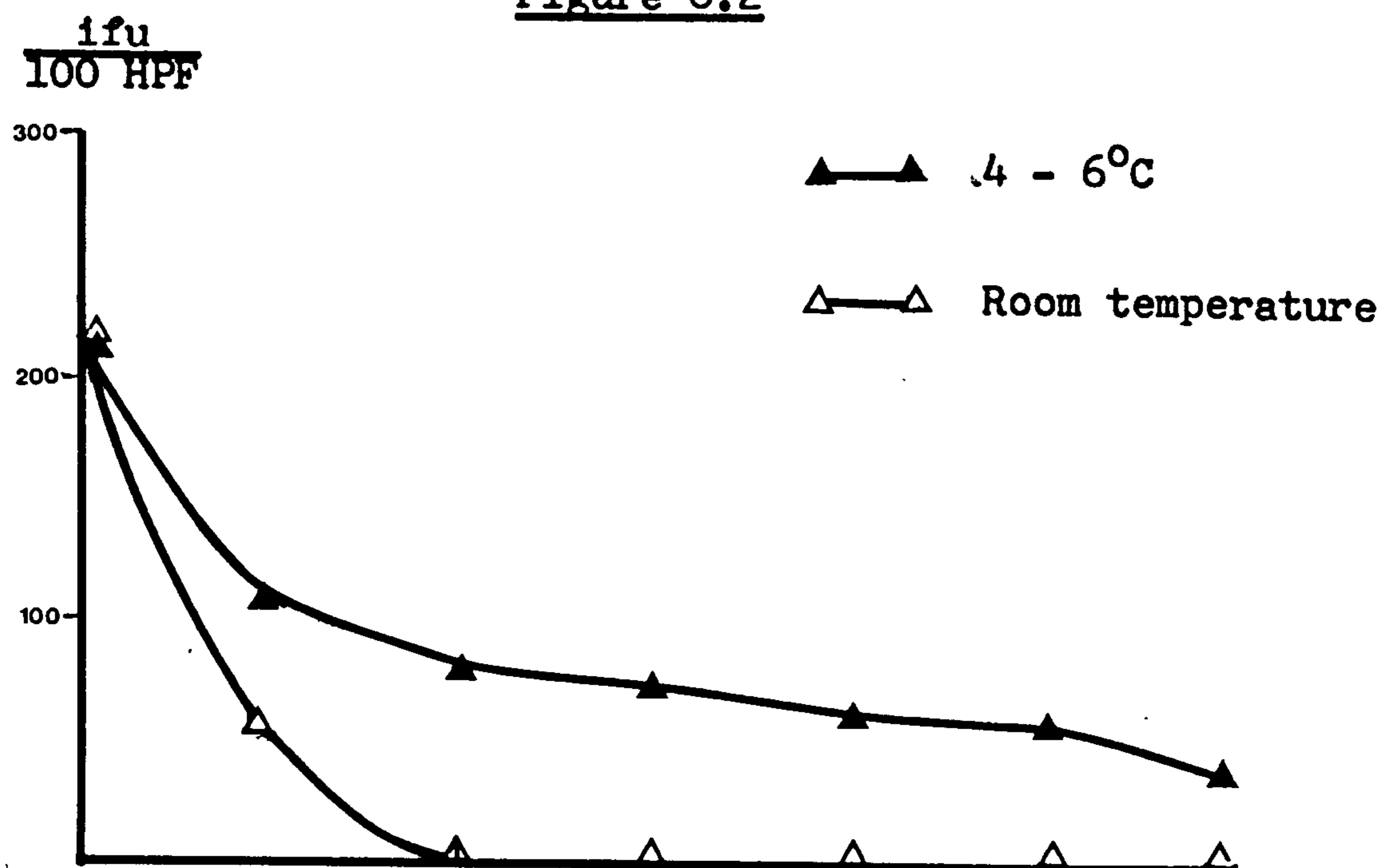


Figure 6.2

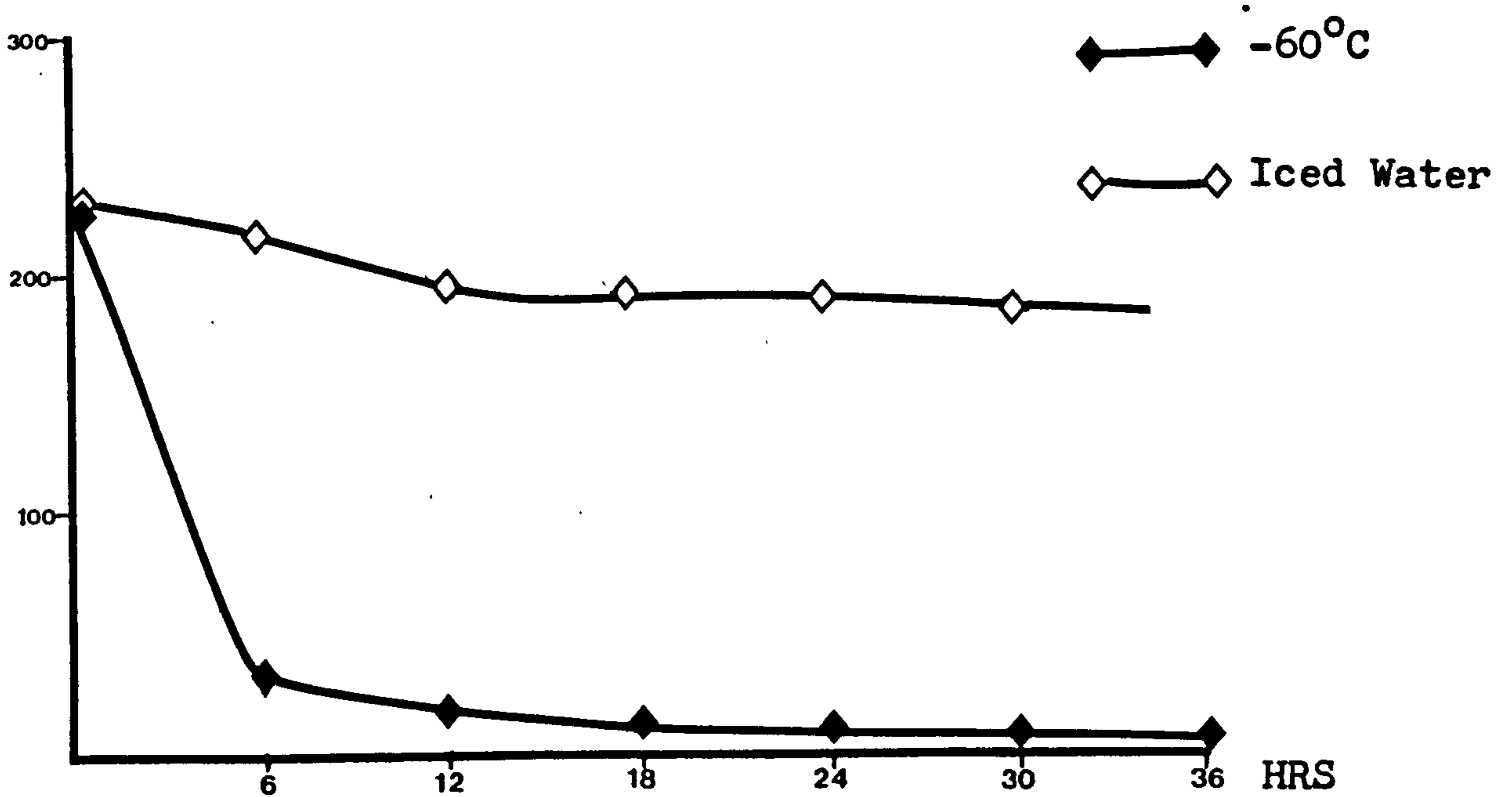
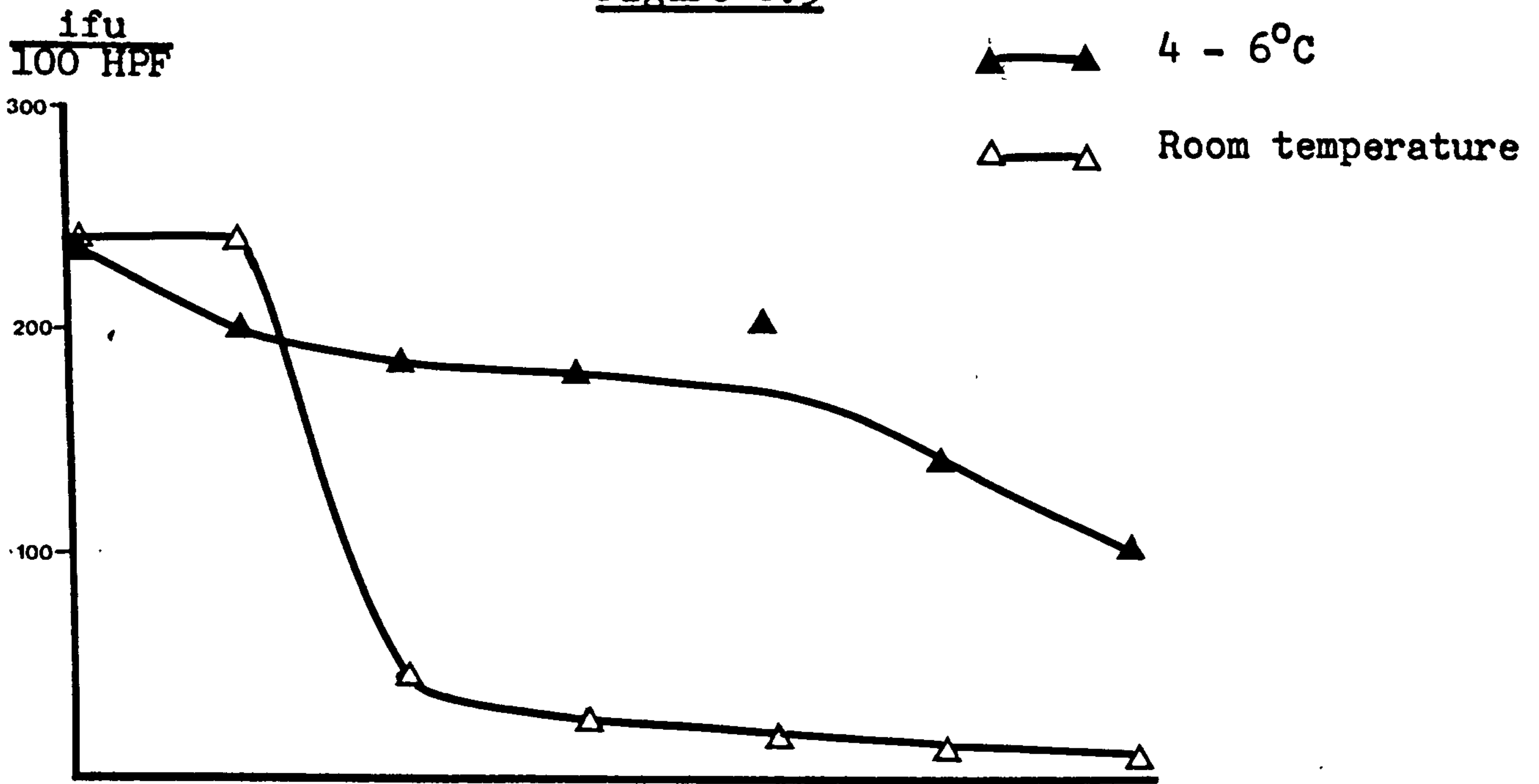


VIABILITY OF C. TRACHOMATIS IN 2SP AT FOUR  
STORAGE TEMPERATURES

Values plotted are means from three replicate cultures  
(Table 6.8.1).



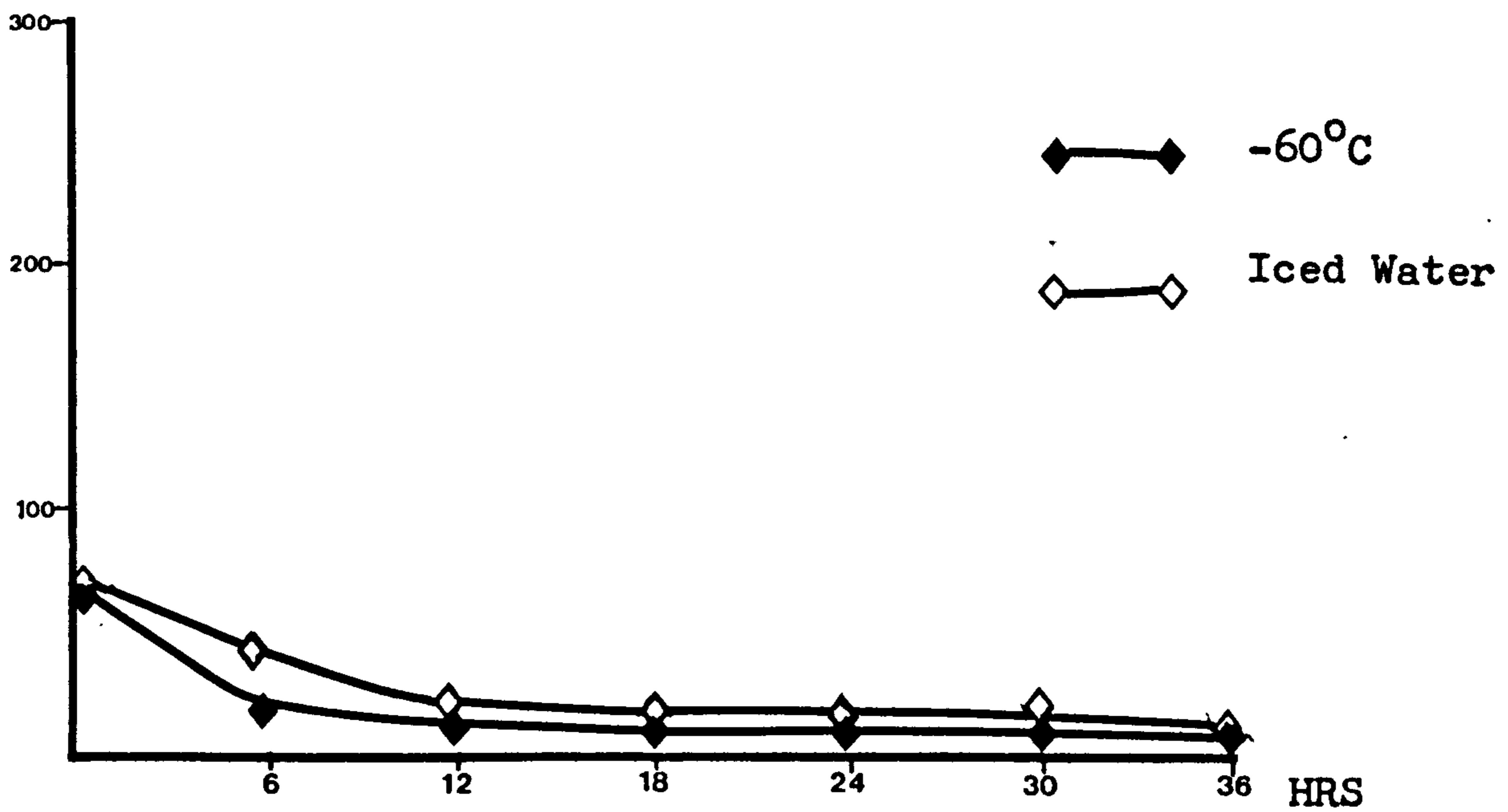
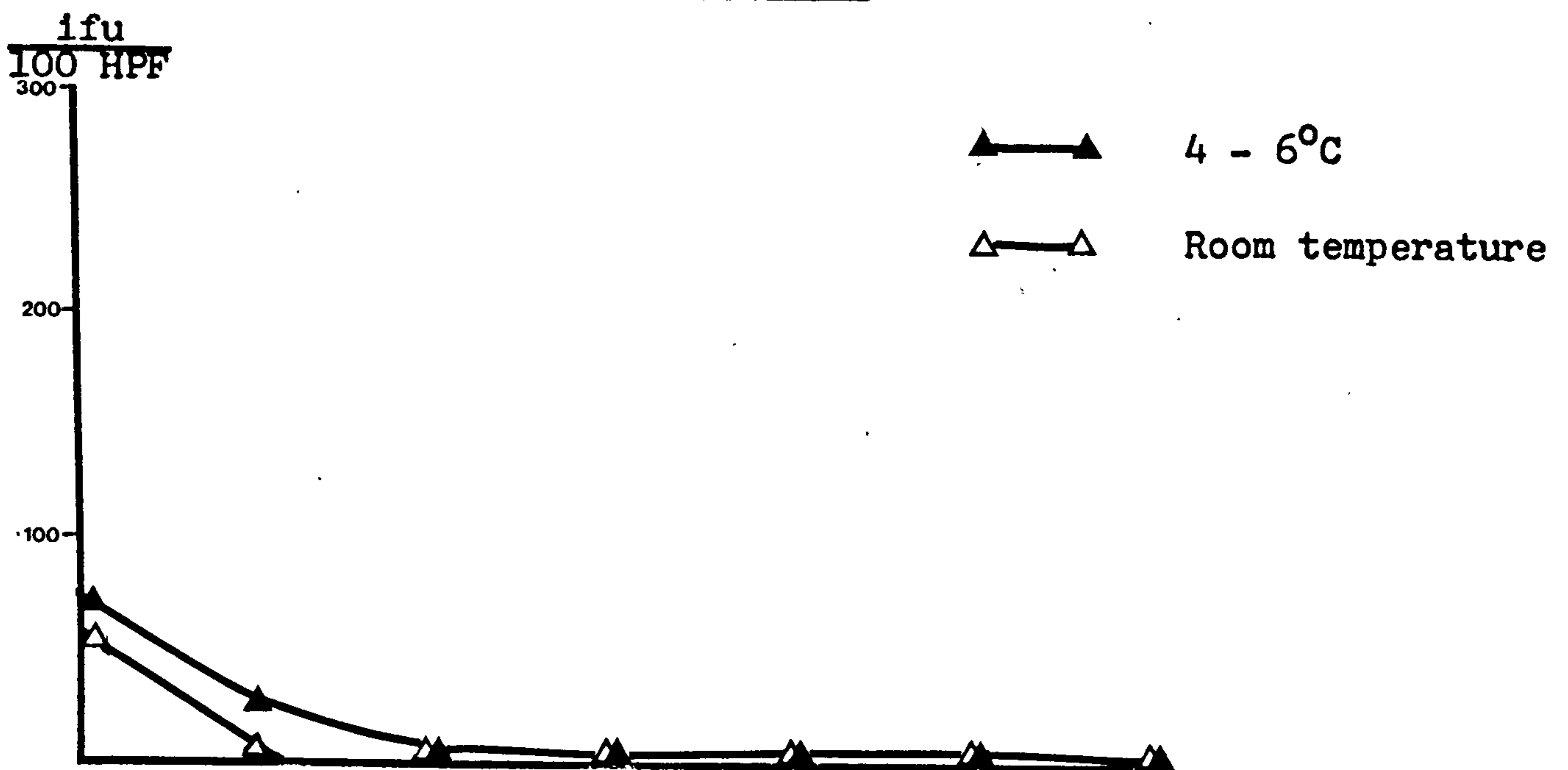
Figure 6.3



VIABILITY OF C. TRACHOMATIS IN PBS AND fbs AT  
FOUR STORAGE TEMPERATURES

Values plotted are means from three replicate cultures  
(Table 6.8.4)

Figure 6.4

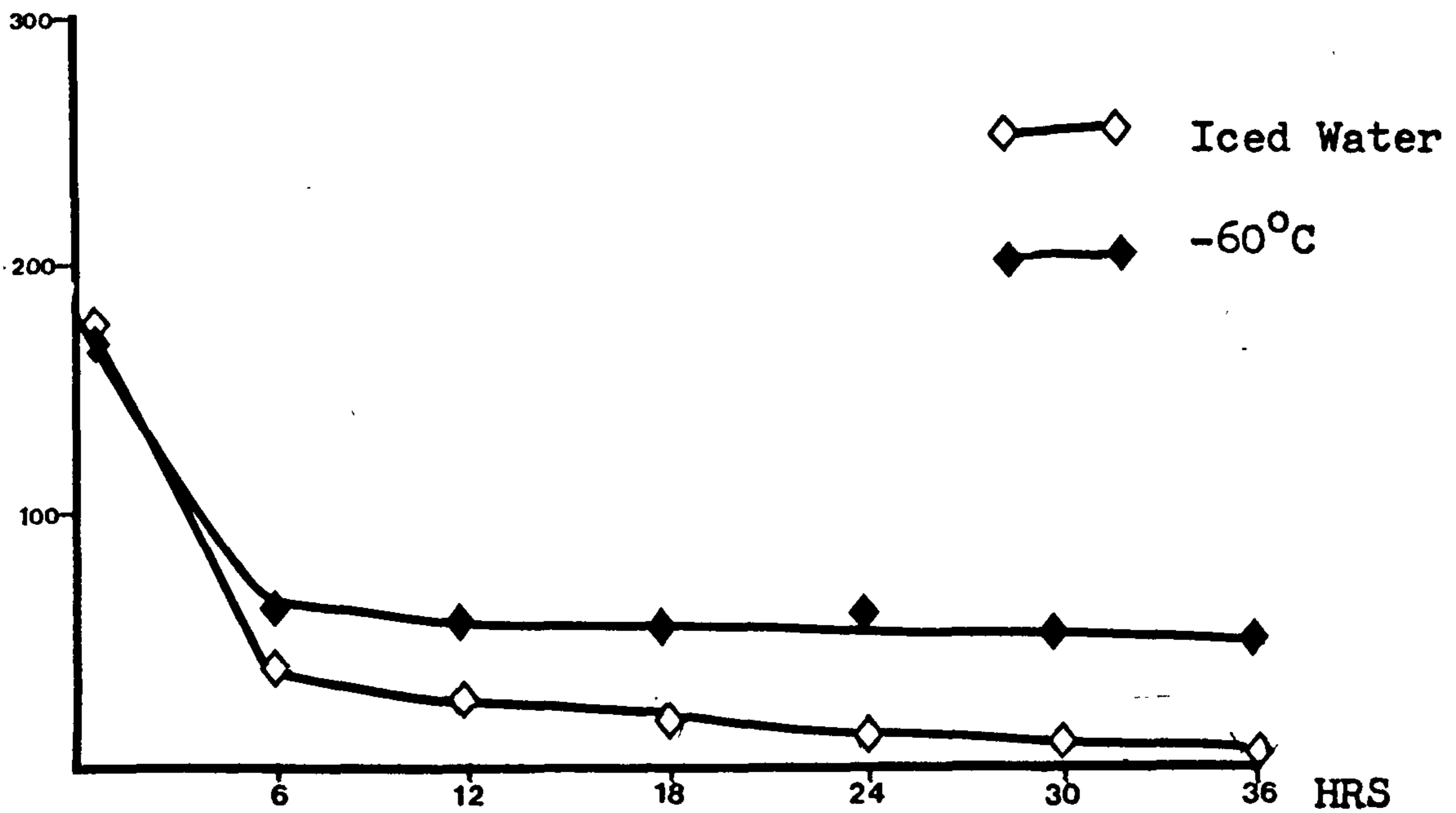
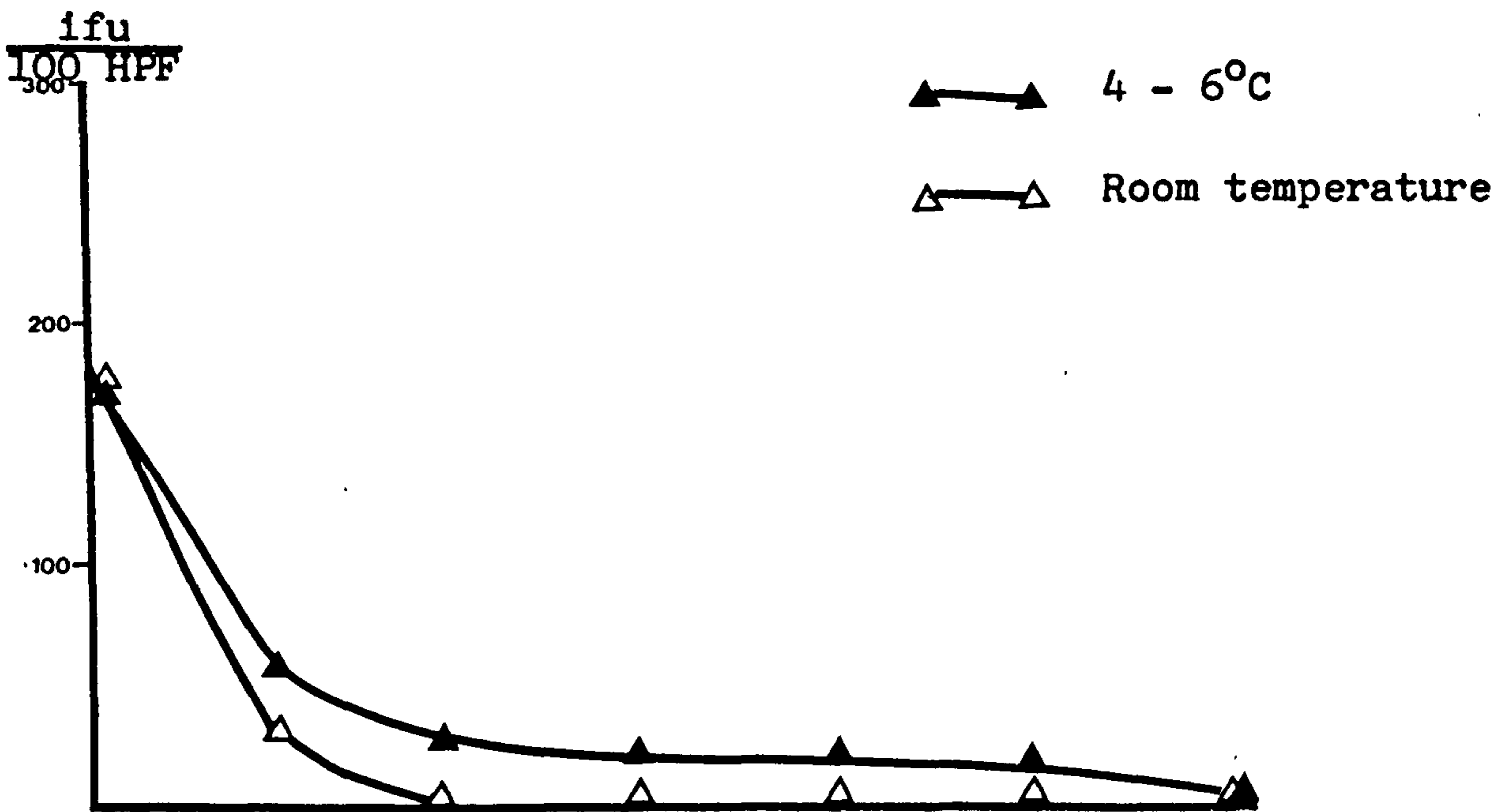


VIABILITY OF C. TRACHOMATIS IN EAGLES MEME + fbs  
AT FOUR STORAGE TEMPERATURES

Values plotted are means taken from three replicate cultures  
(Table 6.8.3)

**PAGE**  
**NUMBERING**  
**AS ORIGINAL**

Figure 6.5



VIABILITY OF C. TRACHOMATIS IN EAGLES MEME + SORBITOL  
AT DIFFERENT STORAGE TEMPERATURES

Values plotted are means from three replicate cultures  
(Table 6.8.2)

## CHAPTER 7

### MATERIALS AND METHODS II

#### 7.1 PREPARATION OF CHLAMYDIAL ANTIGENS

Initial attempts to prepare chlamydial antigen for the microimmunofluorescent antibody technique (micro-IF) were unsatisfactory. Infected yolk-sacs were homogenised, purified by differential centrifugation, placed as spots on slides, stained by Giemsa and examined by high-power dark ground microscopy. An even distribution of chlamydial particles observed by this method did not indicate whether the preparation would be satisfactory for the micro-IF technique. There was too much yolk material present which caused excessive background fluorescence and obscured chlamydial particles. Elementary bodies were visible but these were often sparsely distributed. Several purification procedures were investigated for their ability to concentrate and free chlamydial particles from contaminating yolk-sac antigen.

The bulk preparation of a chlamydial antigen in cell culture was also examined. If a suitable cell culture system was available, it would have considerable time saving advantages over yolk-sac grown chlamydiae. Even with a high titred inoculum it may take 6-12 days to obtain a heavily infected yolk-sac. A cell culture system may only take about 40 hours. Infected cells also provide a "cleaner" starting suspension for preparing antigens.

"Non-LGV" serotypes require centrifuge assisted adsorption to initiate the infectious cycle, infection of large culture flasks were therefore more difficult. A single serotype was adapted to such conditions and proved to be a useful source of antigen for the CF test and an EIA. It was again necessary to purify chlamydial particles. A suitable purification procedure was sought using small numbers of coverslip cultures; the most effective procedure was then used for 80 cm<sup>2</sup> flask cultures.

An enzyme-immunoassay (EIA) provided a measure of the purity of chlamydial suspensions from yolk-sacs and cell cultures. This technique has been shown to have a high degree of sensitivity in virology, and is highly dependent on the purity of the antigen preparation (Inglis, 1980).

The techniques and material described in the following sections were used in the serology experiments and seroepidemiology (chapters 8 and 9).

## 7.2 CHICK EMBRYO INOCULATION AND HARVESTING PROCEDURES

Serotypes of C.trachomatis were grown in the yolk-sac of 5 - 9 day old chick embryos. Each isolate was inoculated into a minimum of 10 eggs.

- I Eggs were candled and the position of the embryo head was marked.
- II The serotype to be inoculated was removed from storage in nitrogen, thawed, then homogenised by vigorous vortex mixing.

- III A dilution of the resuscitated serotype was made in 2SP containing vancomycin ( $100 \mu\text{g ml}^{-1}$ ), streptomycin ( $100 \mu\text{g ml}^{-1}$ ) and nystatin ( $100 \text{ units ml}^{-1}$ ).
- IV Tincture of merthiolate (I/1000) was used to swab the eggs over the air space and the shell was pierced at this point with an electric drill.
- V Each egg was inoculated through the hole over the air-sac with 0.2 ml of diluted infectious yolk-sac using a 21 gauge needle. The needle was inserted with the tip pointing away from the embryo. The hole was securely sealed with sellotape.
- VI Inoculated eggs were placed, upright, in a humid incubator at  $35^{\circ}\text{C}$ .

Eggs were candled daily. Embryos dying up to 72 hours post-inoculation were discarded as non-specific deaths. Thereafter, as soon as approximately 50 per cent of the surviving embryos died, the remaining living embryos were taken from the incubator and the yolk-sacs harvested.

#### HARVESTING YOLK-SACS

- I Living embryos were chilled at  $4^{\circ}\text{C}$  one hour prior to harvesting.
- II The sellotape and remaining shell covering the air-sac was removed using a pair of large sterile forceps.
- III The underlying membranes were ruptured and the whole embryo was transferred from its shell into a sterile Petri dish.

- IV A small portion of the yolk-sac, from the umbilical region, was removed and retained for making impression smears. The remainder of the yolk-sac was placed in a sterile "Universal" and stored temporarily at 4°C.
- V An impression smear of the small piece of yolk-sac was made on a clean microscope slide. Care was taken to remove excess yolk from the membrane using absorbent paper.

#### MICROSCOPIC EXAMINATION OF YOLK-SAC SMEARS

- I Impression smears were air-dried for 30 minutes and then fixed in methanol for a further 5 minutes.
- II The smears were stained with 10 per cent Giemsa for 45 - 60 minutes. Excess stain was removed by rinsing the slides carefully under tap water. Slides were air dried.
- III Each impression smear was examined using a Wild M20 microscope, fitted with a dark field condenser, at X500 magnification.

Elementary bodies (EB's) appear as bright, round yellow/green particles distributed through the yolk-sac smear. The number of EB's present in each smear was graded 1+ (minimum) to 4+ (maximum).

#### PURIFICATION OF INFECTED YOLK-SACS

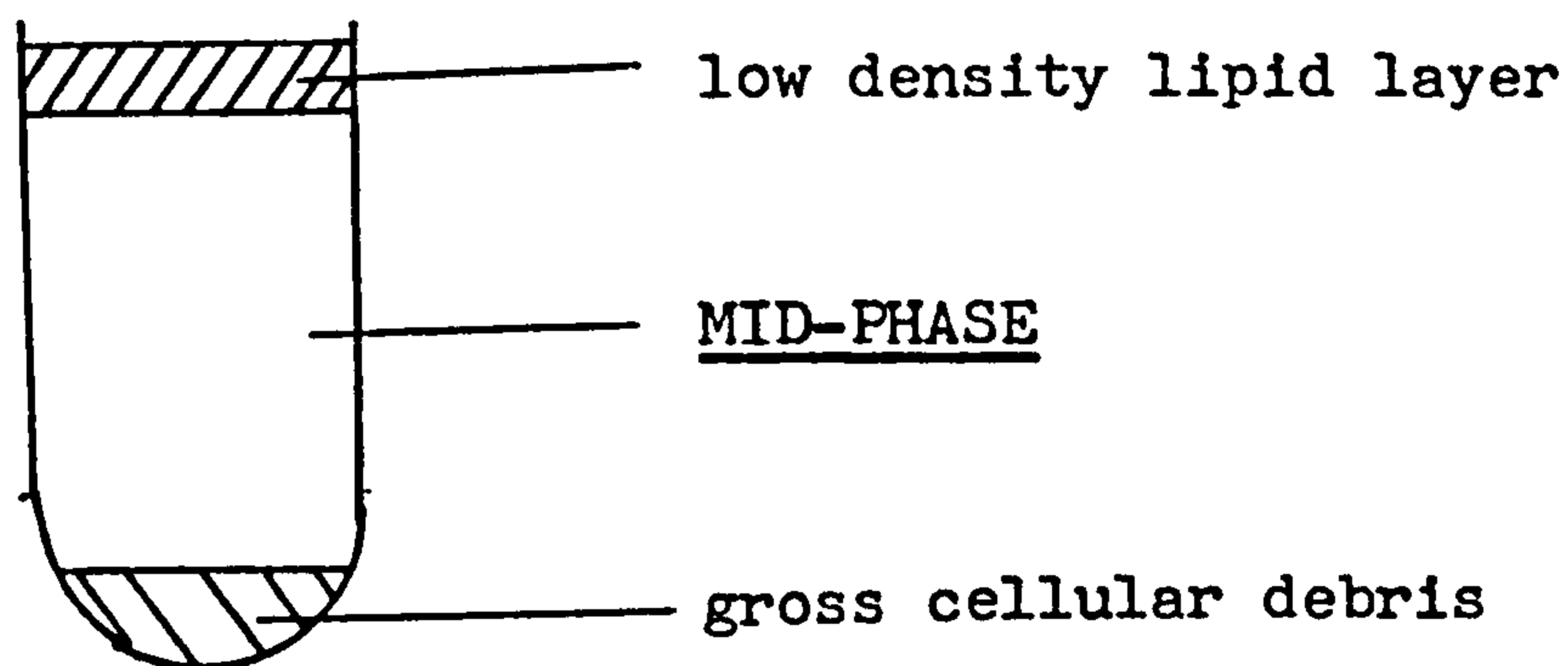
Only yolk-sacs with impression smears graded 3+ or better were used.

- I Infected yolk-sacs were weighed, pooled if necessary, and a 20 per cent (w/v) suspension was made with cold, sterile



PBS pH 7.2.

- II Sterile 3 mm diameter glass beads were added to the suspension and shaken vigorously for 5 minutes.
- III The partially homogenised suspension was removed by pipette to a sterile centrifuge tube and spun at 1,500 g for 10 minutes at 4°C.
- IV The resulting mid-phase was removed and stored, before further treatment, at 4°C.



Three layers formed after centrifuging homogenised yolk-sac (1,500 g for 10 minutes). Optimum purification procedures for the mid-phase were examined experimentally. (7.9).

#### STORAGE OF INFECTED YOLK-SAC HOMOGENATES

For each serotype passaged, a positive (3+ or better) infected yolk-sac was stored in the vapour phase of liquid nitrogen.

- I One infected yolk-sac was diluted with 2SP, containing antibiotics, to give a 50 per cent (w/v) suspension.
- II Sterile glass beads were added to the suspension and shaken vigorously (5 minutes).

III Aliquots (1.0 ml) were distributed to labelled plastic vials. (Cryotube 38 x 12.5 mm Vol 2.0 ml polypropylene Nunc Inter Med Den). Each vial was labelled:-

Serotype code

50 per cent pass number

Date of storage

SEROTYPES OF C. TRACHOMATIS PROPAGATED IN CHICK-EMBRYO  
YOLK-SACS

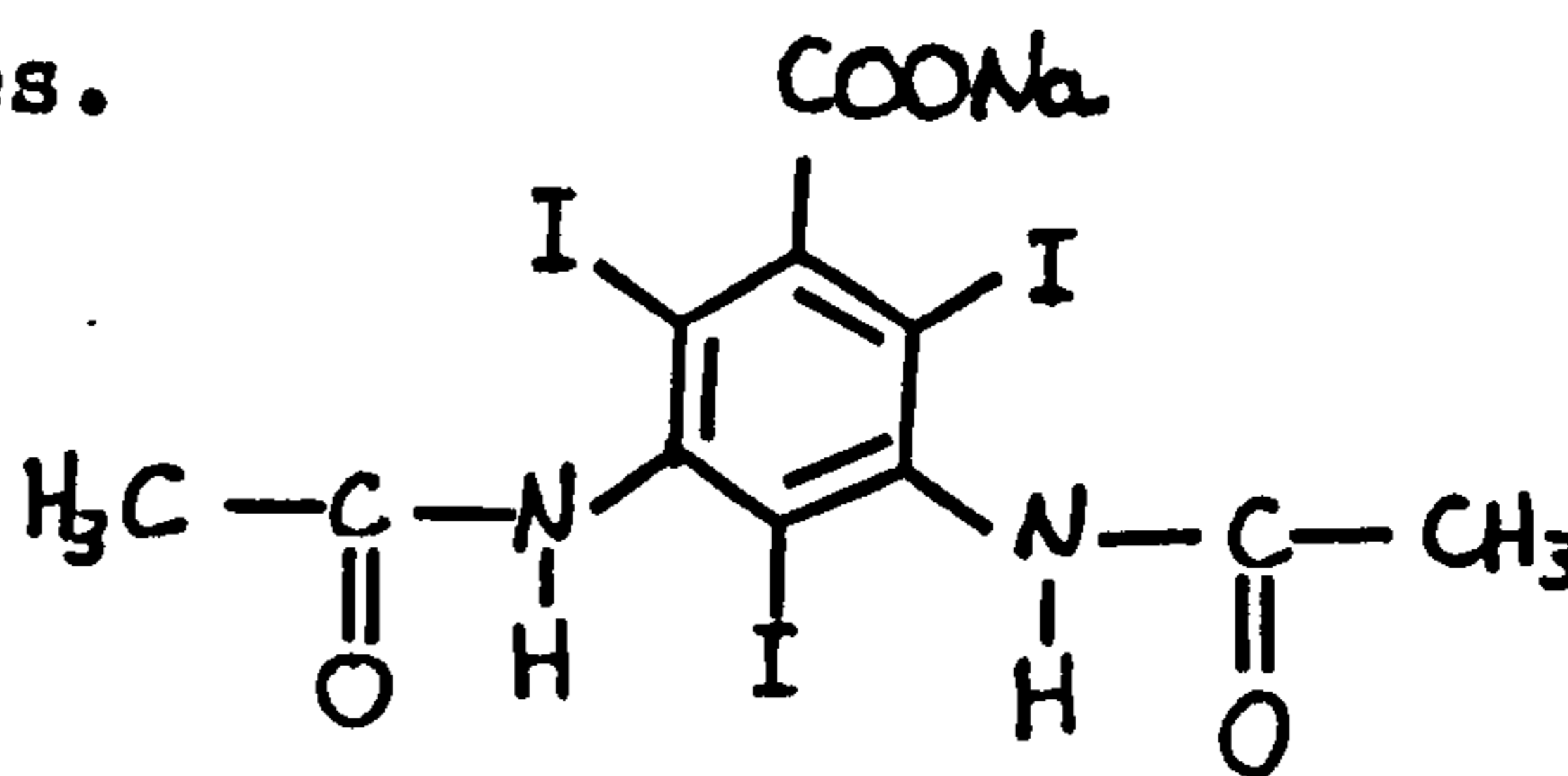
CODE	SEROTYPE	DILUTION FOR INOCULATION
SA1	A	$10^{-1}$
TW5	B	$10^{-1}$
UW1	C	$10^{-1}$
IC CAL8	D	$10^{-1}$
DK 20	E	$10^{-1}$
MRC 301	F	$10^{-1}$
IDL 238	G	$10^{-1}$
UW4	H	$10^{-2}$
UW12	I	$10^{-1}$
UW36	J	$10^{-1}$
UW31	K	$10^{-1}$
440L	LGV1	$10^{-3}$
434B	LGV2	$10^{-4}$
402L	LGV3	$10^{-3}$

### 7.3 UROGRAFIN

Urografin-76 was supplied as a sterile solution by Schering (Berlin). All diluted Urografin solutions contained PBS.

Urografin contains a mixture of the sodium and methylglucamine salts of 3, 5 - bis - acetamido - 2, 4, 6 triodobenzoic acid (sodium and meglumine diatrizoate) in the ratio 10:66 in aqueous solution. Diatrizoate salts form solutions of low viscosity and high density. The function of Urografin, employed in this work, was to provide optimal density and osmolarity necessary for the efficient removal of contaminating yolk-sac and cellular material from chlamydial elementary bodies.

SODIUM  
DIATRIZOATE



Since the compound is light sensitive Urografin was stored protected from light at 4°C.

### 7.4 ENZYME-IMMUNOASSAY (EIA) PROCEDURE

An indirect method of the EIA was used to titrate dilutions of purified chlamydial suspensions. An antichlamydial serum with known specificity and titre, by the FAT, was used at a constant dilution.

I Doubling dilutions (undiluted to 1/2048) of the chlamydial suspension in coating buffer (pH 9.6) were prepared in flat bottomed micro-EIA plates (Immulon plates; Dynatech) (200 µl).

- II Plates were stored overnight at 4°C to absorb the antigen to the wells.
- III Excess antigen was carefully removed by inverting the plate over 2 per cent sodium hypochlorite.
- IV Coated wells were washed, three times (5 minutes each), in PBS-tween pH 7.4.
- V 150 µl of diluted reference serum (1/32) was added to each well and the plate was incubated in a humid container for 90 minutes at 37°C.
- VI Plates were washed as 4.
- VII 150 µl of conjugate, (anti-human IgG, Y chain specific, alkaline phosphatase: Sigma Chemicals, London) previously titrated to determine optimal dilutions, was added to each well and incubated at 37°C for 2 hours.
- VIII Plates were washed as 4.
- IX 150 µl of enzyme substrate (p-nitrophenyl phosphate disodium: 1 mg ml<sup>-1</sup> in 10 per cent diethanolamine pH 9.8) was added and allowed to react with bound enzyme for 30 minutes at room temperature.
- X Absorbance values for each well were obtained using a Titertek Multiskan spectrophotometer fitted with a 405 nm filter.

A graph was plotted of absorbance values (ordinate axis) against antigen dilution (abscissa axis). In all the purification procedures investigated, uninfected yolk-sac and McCoy cell preparations, were prepared in an identical manner. Where possible end-point titres were recorded as the highest dilution of antigen to yield a 0.2 absorbance unit difference over the corresponding antigen dilution in the control antigen wells.

7.5 PREPARATION OF SLIDE ANTIGENS FOR THE MICRO-IF TECHNIQUE

- I The purified chlamydial suspensions were brought from storage in liquid nitrogen, thawed at 37°C and mixed thoroughly on a vortex mixer for 30 seconds. 2 SP is not a suitable diluent for plaquing antigen, so all preparations were suspended in PBS pH 7.2.
- II Using a plaquing pen nib the first antigen was carefully placed on the slide as shown in Fig 7.1. Care was taken to ensure that the remaining antigen plaques were in straight lines both horizontally and vertically.
- III Slides were left to dry at room temperature for 30 minutes and were fixed in fresh acetone for 10 minutes. Slides were stored at -70°C until required. Antigens were discarded after 4 freeze-thaw cycles.

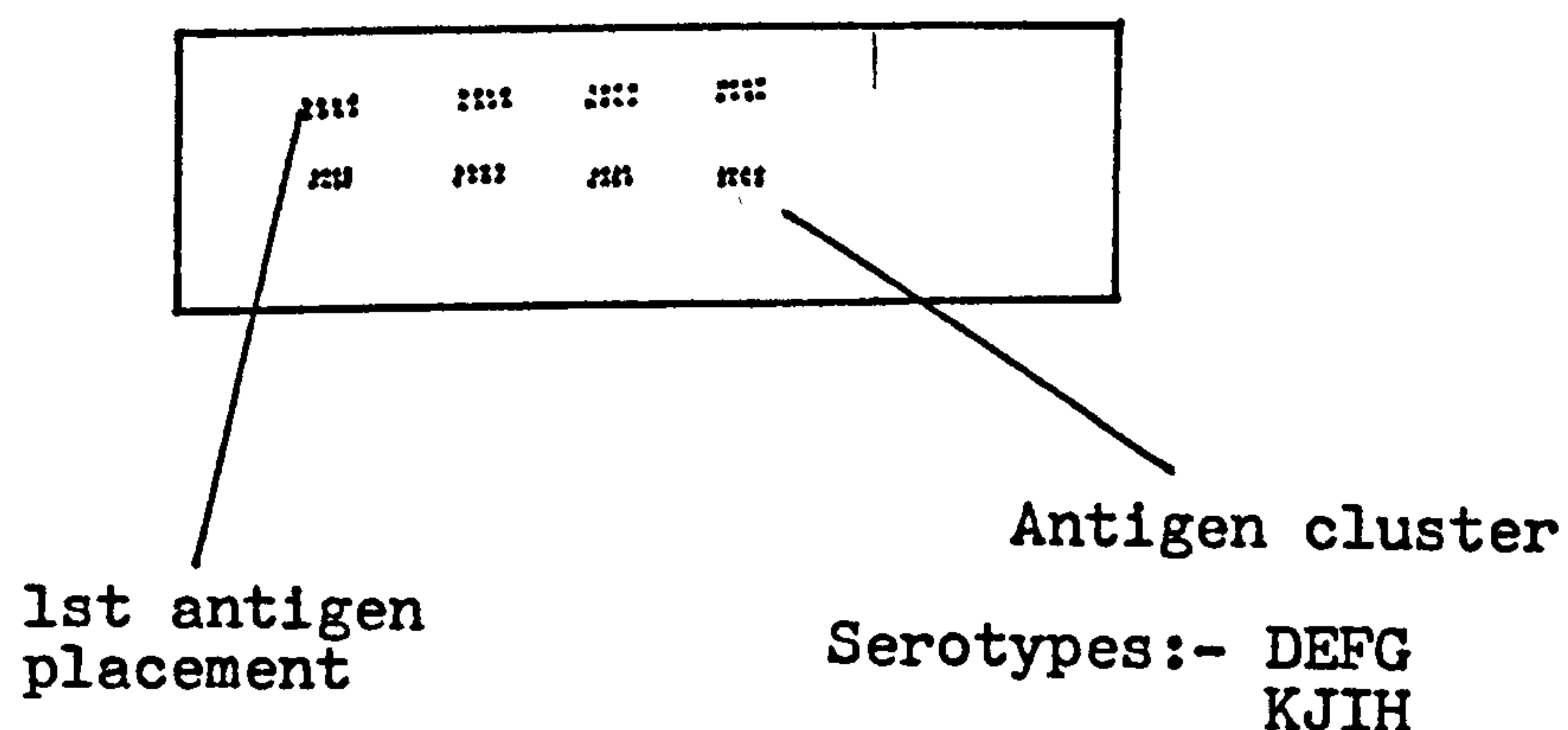


Fig. 7.1

7.6 STAINING PROCEDURE FOR THE INDIRECT IMMUNOFLUORESCENT  
TECHNIQUE (FAT)

This technique was used to determine the titre of human sera, using serotypes D - K inclusive.

- I Serum was diluted 1/16 - 1/2048 with PBS pH 7.2
- II The diluted serum was overlaid on an antigen cluster using a micropipette.
- III When each slide was completed, they were placed in a humidified slide chamber and incubated at 37°C for 30 minutes.
- IV Slides were washed twice (5 minutes each) by gentle agitation in a bath of PBS, rinsed in deionised water and air dried.
- V Lyophilized fluorescein isothiocyanate (FITC) labelled goat anti-human IgG (Kallestad, Chaska, Minn., USA) was reconstituted in sterile distilled water to the optimal working dilution. The optimal dilution for each batch of conjugate was determined by titrating it with known positive serum and homologous antigen.
- VI The diluted FITC conjugate was applied to each cluster. Slides were incubated and washed as before: 3 and 4.
- VII Coverslips were mounted over the antigen clusters using immersion oil. (Pan Scan immersion oil, Hughes and Hughes Ltd., Romford, Essex) and examined immediately at X500 magnification. The microscope used for this work was an Ortholux 2 U.V. microscope utilising Ploem illumination.

The above staining procedure was also used to determine the end-point titre of pooled sera using a suspension of infected McCoy

cells as slide antigens. Eight antigen dots were covered by each serum dilution (1/16 - 1/2048).

## 7.7 COMPLEMENT FIXATION TEST (CFT)

This test detects antibody which, on reacting with antigen, binds complement. A two stage test is normally carried out in which, firstly, antigen, complement (usually guinea-pig serum complement) and the heat inactivated serum under test are incubated together. Secondly, when this reaction has taken place, an indicator system consisting of sheep red blood cells sensitised with anti-sheep red cell antibody is added. In the presence of remaining free complement, lysis will occur. Failure of the cells to lyse indicates that the complement has been fixed in the first reaction and indicates a positive result.

### REAGENTS FOR THE CFT

Dextrose gelatin veronal buffer: This was used as diluent throughout the test. It contains calcium and magnesium ions which are necessary for full fixation of complement at higher dilutions of serum.

Sheep blood: Sheep blood in Alsever's solution was obtained weekly (50 per cent whole blood in 50 per cent Alsever's solution; Gibco, Europe). A 0.5 per cent RBC suspension, standardised photometrically, was made on the day of testing.

Haemolysin: Rabbit haemolytic serum, glycerinated, for sheep erythrocytes (Flow Laboratories; McLean Va USA).

Complement (Flow Laboratories, USA): A freeze dried preparation of preserved guinea-pig serum. One volume of reconstituted material added to seven volumes of deionised water is equivalent to a 1/10 solution of fresh guinea-pig serum in saline; further dilution was made with DGV to the strength required. After dilution, complement is unstable. It was prepared immediately before use and kept on melting ice until use in the test proper. Reconstituted complement was stored in liquid nitrogen in 0.1 ml aliquots.

Chlamydial antigens: The optimal antigen preparation was determined by experiment. ( 7.11)

Uninfected control antigens: These were made from uninfected cell cultures or egg preparations to those used to culture the chlamydial antigen.

Standardisation of complement and haemolytic serum are described in the Appendix. It is also necessary to identify the optimal dilution of the test antigen and titre of the homologous standard antiserum. Controls for non-specific reactivity were also included to confirm the specificity of the antigen-antibody reaction. This is also described in the Appendix.

#### CFT PROCEDURE

After standardising the reagents in the test the following procedure was followed to titrate antibody levels.

I Sera were inactivated (endogenous complement destroyed) in a 56°C waterbath for 30 minutes.



- II A 1/8 dilution of inactivated antiserum was prepared with DGV.
- III Using a calibrated diluter, serum was diluted from 1/8 to 1/512.
- IV 0.025 ml of the working dilution of antigen was added to each well by calibrated dropper.
- V Reconstituted complement was diluted accordingly. 0.025 ml was added to each of the test wells. Fixation of complement took place overnight at 4°C.
- VI The haemolytic system (0.05 ml) was added to the plates which were then incubated at 37°C for 45 minutes. Plates were shaken on a mechanical agitator every 20 minutes.
- VII Plates were transferred to a specially adapted centrifuge rotor and spun at 1000 r.p.m. for 5 minutes (MSE GF8 centrifuge).
- VIII The end-point titre was the highest dilution of serum giving one hundred per cent fixation of complement (all erythrocytes remaining).

## 7.8 TITRATION OF POOLED REFERENCE SERUM BY THE F.A.T.

Coverslip cultures of McCoy cells were infected with a dilution of IC CAL8 so that approximately 50 per cent of the cells were infected. The infection cycle was interrupted after 40 hours, incubation at 35°C.

- I Inoculation medium was removed from each vial and the coverslips were gently rinsed once with PBS pH 7.2. 0.2 ml trypsin/versene was added to each vial and cells were removed using a Pasteur pipette. Cells were pooled into a conical tube and pelleted by centrifugation. Taking care not to create air bubbles, cells were aspirated so that a mono-dispersed cell suspension was obtained. The density of cells was checked by placing a drop on a clean microscope slide and examined using low power microscopy. The suspension was diluted appropriately with PBS.
- II Eight drops of the infected cell suspension (approximately 5 mm diameter) were placed on a microscope slide. The drops were air dried (20 minutes) and then fixed in fresh acetone (10 minutes).
- III Dilutions of the pooled human serum (1/16 - 1/2048) were made with PBS pH 7.2 in a micro-titre plate. An end-point titre was determined by the indirect FA technique.
- IV 50 per cent reduction in cytoplasmic fluorescence occurred at a serum dilution of 1/128.

The above serum pool, stored at  $-20^{\circ}\text{C}$ , was used at a dilution of 1/32 in the EIA.

## 7.9 PURIFICATION OF C.TRACHOMATIS BY CENTRIFUGATION

Four purification procedures were examined for their ability to free chlamydial particles (EB's and RB's) from contaminating yolk-sac material.

- (a) Differential centrifugation
- (b) Centrifugation of mid-phase material through 30 per cent sucrose.
- (c) Centrifugation of mid-phase material through 25 per cent Urografin.
- (d) Centrifugation of mid-phase material through a 20-50 per cent discontinuous gradient of Urografin.

### ANTIGEN

IC CAL8 P26 (serotype D) was inoculated into ten 6-day old chick embryos at a  $10^{-1}$  dilution of stock material. After 8 days incubation at  $35^{\circ}\text{C}$ , six embryos were still alive and were harvested. Impression smears showed that four embryos had 4+ infectivity. A 20 per cent (w/v) homogenate was prepared in cold PBS pH 7.2 and the mid-phase was retained after light centrifugation (1,500 g for 10 minutes at  $4^{\circ}\text{C}$ ). 1 ml aliquots were stored at  $-70^{\circ}\text{C}$  until further use (15 x 1 ml).

## CONTROL ANTIGEN

Four x 14-day old chick embryos were used to provide uninfected yolk-sac material. The yolk-sacs were harvested, pooled, diluted (20 per cent w/v) and centrifuged in the same way as the infected yolk-sacs. 1 ml aliquots were stored at  $-70^{\circ}\text{C}$  (15 x 1 ml).

### (a) DIFFERENTIAL CENTRIFUGATION

Three ampoules of infected yolk-sac material were resuscitated from storage at  $-70^{\circ}\text{C}$ . Each 1 ml aliquot was diluted 1/5 with cold PBS pH 7.2 and vortex mixed. Three polypropylene ultracentrifuge tubes (MSE Scientific Instruments, Sussex) were filled with 5 ml of the diluted chlamydial suspension. Centrifugation was at 20,000 g for 15 minutes at  $4^{\circ}\text{C}$  (MSE Superspeed 50 preparative ultracentrifuge) using a 3 x 5 ml swing-out rotor. Each pellet was resuspended in 0.5 ml coating buffer pH 9.6 by mild sonication (Dawe Sonicleaner Type 64414) with ice and intermittent vortex mixing. This procedure was continued until no visible aggregates remained. The homogenate was finally centrifuged at 500 g for 10 minutes and the supernatant containing chlamydial particles was retained.

Uninfected mid-phase material was treated in the same way.

Doubling dilutions (200  $\mu\text{l}$ ) of antigen and control antigen were prepared in micro-EIA plates.

(b) CENTRIFUGATION OF MID-PHASE MATERIAL THROUGH  
30 PER CENT SUCROSE

1.0 ml of infectious mid-phase material was overlaid 4.0 ml of 30 per cent sucrose. Three 5 ml polypropylene ultracentrifuge tubes were prepared in this way. The material was centrifuged at 24,000 g for 90 minutes at 4°C in a 3 x 5 ml rotor.

The supernatant containing yolk-material was discarded, and the pellets were resuspended as in (a). Each 0.5 ml aliquot of the chlamydial suspension in coating buffer was pooled and centrifuged at 500 g for 10 minutes. Doubling dilutions (200 µl) of the final supernatant were made in a micro-EIA plate.

(c) CENTRIFUGATION OF MID-PHASE MATERIAL THROUGH  
25 PER CENT UROGRAFIN

1.0 ml of mid-phase material was overlaid 4.0 ml of 25 per cent Urografin. Three centrifuge tubes were prepared. Centrifugation conditions were the same as (b).

After discarding the supernatant centrifuged chlamydial particles were prepared in the same way as (b).

(d) CENTRIFUGATION OF MID-PHASE MATERIAL THROUGH A  
20 - 50 PER CENT DISCONTINUOUS GRADIENT OF UROGRAFIN

A discontinuous gradient consisting of 3.5 ml of 20 per cent Urografin on top of 0.5 ml of a 50 per cent solution was overlaid with a 1 ml sample of prepared mid-phase material. Three 5 ml centrifuge tubes were prepared in this way and centrifuged for 1 hour at 40,000 g (4°C). The band of turbidity at the 20 - 50

per cent interface was collected from each tube using a curved Pasteur pipette. The chlamydial suspensions were pooled and suspended in a total volume of 15 ml cold PBS pH 7.2. The suspension was divided between three 5 ml centrifuge tubes and chlamydiae were sedimented at 20,000 g for 15 minutes (4°C). The resulting pellets were suspended in 0.5 ml coating buffer and EIA plates were coated as before.

A band of turbidity was not visible at the 20 - 50 per cent Urografin interface in uninfected control samples. However, approximately the same volume of Urografin was collected from this position in the ultra-centrifuge tubes and then treated in the same way.

#### 7.10 PURIFICATION OF CELL CULTURE GROWN C.TRACHOMATIS

IC CAL8 (Serotype D) was sub-cultured in the yolk-sacs of chick embryos and then in McCoy cells treated with cycloheximide. A stock suspension of viable chlamydiae was prepared in 2SP which was free of gross cellular debris. The same chlamydial stock, stored in the vapour phase of liquid nitrogen, was used throughout these experiments.

Antigenic material was prepared by five different procedures:-

- (a) Mild sonication of cell monolayers and light centrifugation.
- (b) Acetone treatment of cell monolayers followed by procedure (a).
- (c) Acetone treatment followed by centrifugation through:-
  - (i) 30 per cent sucrose.
  - (ii) 25 per cent Urografin.
- (d) Cell monolayer supernatants centrifuged through 25 per cent Urografin.

In each of the procedures examined 12 coverslip cultures, seeded with  $2.0 \times 10^5$  cells, 24 hours previously, were inoculated with 0.2 ml of stock IC CAL8. The level of infectivity in McCoy cells was such that more than 80 per cent of the cells, on a Giemsa stained coverslip culture, contained cytoplasmic inclusions. Infected coverslips were processed 40 hours post inoculation.

Control antigen was prepared from 12 replicate cultures of McCoy cells inoculated with 0.2 ml 2SP alone.

The final step, before assay, involved suspending the antigen solution in 2 ml of coating buffer pH 9.6 and making doubling dilutions in EIA plates.

#### MILD SONICATION AND CENTRIFUGATION AT 1,5000 g

12 heavily infected 40 hours infected McCoy cell cultures were pooled in a glass 'Universal' container. 2 ml coating buffer pH 9.6 was added to the Universal and cells were removed from the coverslips by gently shaking with glass beads. The cell suspension was transferred to a glass centrifuge tube, subjected to mild sonication for 10 minutes (Dawe Sonicleanter Type 6441A) with ice and intermittent vortex mixing (Whirlimixer, Fisons, England). Cell debris was removed by centrifugation at 1,500 g for 10 minutes at 4°C. The supernatant was retained and used as coating antigen for EIA.

Uninfected McCoy cells were prepared in the same way.

CENTRIFUGATION OF CELL CULTURE GROWN CHLAMYDIAE  
THROUGH 25 PER CENT UROGRAFIN

12 heavily infected 40 hour McCoy cell coverslip cultures were placed in a 'Universal' container holding 3 mm diameter glass beads and 3 ml cold PBS pH 7.2. Cells were removed from coverslips, as before, followed by sonication and centrifugation to deposit cell debris. The supernatant was made up to 3 ml.

Three 5 ml ultracentrifuge tubes were filled with 4.0 ml of 25 per cent Urografin. 1 ml of the prepared supernatant was carefully overlaid the Urografin in each of the three tubes. After centrifugation at 24,000 g for 90 minutes (4°C) the three pellets were suspended and pooled in a total volume of 2 ml. coating buffer. EIA plates were prepared as before.

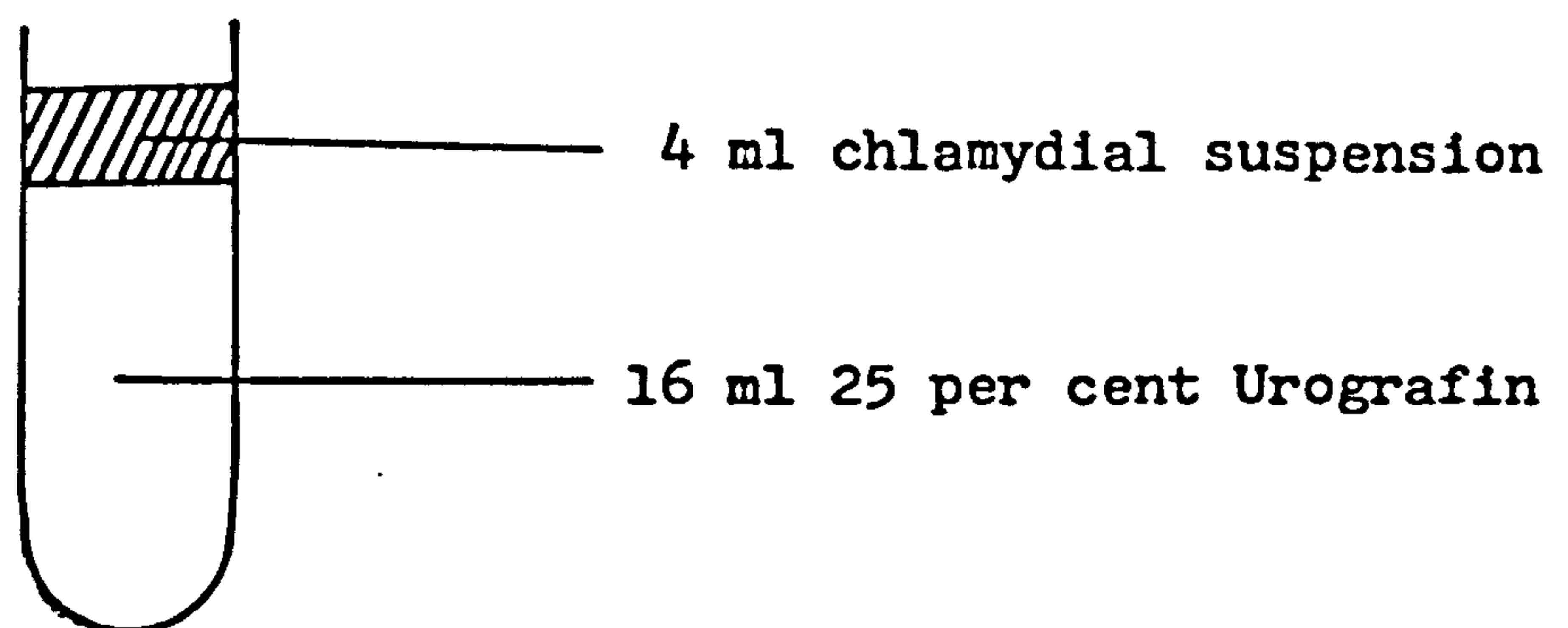
7.11 GROWTH OF C.TRACHOMATIS (IC CAL8) IN 80 cm<sup>2</sup>  
CULTURE FLASKS

The culture flasks used were sterile disposable Nunclon (R) 80 cm<sup>2</sup> bottles. A 24 hour cell monolayer was used for inoculation. Prior to inoculation, growth medium was decanted and the cell monolayer was washed twice in 5 ml of Hank's balanced salt solution containing 30 µg of DEAE-dextran ml<sup>-1</sup> (M.wt 5 x 10<sup>5</sup>; Sigma Chemical Company). A 2 ml inoculum was used. The flasks were centrifuged, on a modified rotor, at 1,000 r.p.m. for 90 minutes at room temperature (MSE GF8 centrifuge). After absorption approximately 50 ml of inoculation medium was added, and the culture was incubated at 35°C for 48 hours. The infected cell monolayer was harvested and an estimate of the degree of infectivity was made by the FAT:-



Inoculation medium was discarded and replaced with 5 ml PBS pH 7.2. Cells were carefully rolled off the flask using sterile glass beads. The suspension was gently aspirated to obtain a monodispersed suspension of infected cells. 0.5 ml of the cell suspension was aseptically removed and clean glass slides were dotted with the prepared material. Only three antigen plaques were dotted onto each slide. The rest of the procedure for the indirect FA technique has been described on (7.8).

If more than 50 per cent of the observed cells showed brightly fluorescing cytoplasmic inclusions, by U.V. microscopy, the cell suspension was purified by centrifugation through 25 per cent Urografin. The conditions for centrifugation have been described. In this case the volume of material to be centrifuged was adjusted so that three 25 ml polypropylene ultracentrifuge tubes were prepared as follows:-



Centrifugation was at 24,000 g for 90 minutes (4°C) on an 8 x 25 ml angle rotor.

Pellets were resuspended in a total volume of 7.5 ml coating buffer

and EIA plates were prepared as described (7.4).

Test antigen was also prepared for the complement fixation test. The chlamydial suspension from the flask was prepared as above. However, the pellets were resuspended in 7.5 ml DGV. The titre of the antigen was determined by two dimensional titrations using dilutions of the reference serum. The final antigen preparation was distributed in 0.2 ml amounts and stored in the vapour phase of liquid nitrogen. When this material was required for the CFT, it was sonicated (Dawe Sonicleaner), for 10 minutes in iced water, prior to dilution.

If less than 50 per cent of the stained cells showed fluorescence, the following procedure was followed:-

The 5 ml suspension of cells was sonically treated for 10 minutes and then submitted to one cycle of differential centrifugation (500 g for 10 minutes and 20,000 g for 15 minutes). The resulting pellet was resuspended in 2 ml of 2SP and sonically treated again to obtain a homogenous suspension. It was inoculated immediately into a fresh cell culture or stored in nitrogen.

## CHAPTER 8

### RESULTS OF PURIFICATION PROCEDURES FOR C.TRACHOMATIS

#### 8.1 PURIFICATION OF YOLK-SAC GROWN ANTIGEN

A chlamydial antigen preparation was required for the complement fixation test and for micro-IF. Heavily infected yolk-sacs provide a comparatively large number of chlamydial particles but have the disadvantage of excessive amounts of yolk-sac. Four centrifugation procedures were investigated for their efficiency to separate chlamydiae from yolk-sac material.

The criteria for selecting a suitable antigen preparation were, firstly, low absorbance readings by EIA for uninfected control antigen coated wells; and, secondly, high absorbance readings for infected preparations diminishing in intensity with higher dilutions giving a characteristic titration curve.

Figures 8.1 and 8.2 show absorbance readings for dilutions of purified antigen by differential centrifugation and centrifugation through 30 per cent sucrose. In both sets of results, the non-specific absorbance values for uninfected yolk-sac preparations are high relative to the chlamydia coated wells. A titration curve was obtained for test wells but the difference in these readings from control antigen was less than 0.2 absorbance units. Neither of these preparations seemed to be suitable for further studies.

Centrifugation of the chlamydial suspension through 25 per cent

Urografin and a discontinuous gradient reduced non-specific absorbance readings to acceptably low levels. It can be seen in Figures 8.3 and 8.4 that absorbance values, for IC CAL8 coated wells follow a titration curve with increasing dilutions of antigen. For the purposes of this experimental work the end-point dilution of antigen was the highest dilution giving an absorbance reading equal to or greater than 0.2 absorbance units compared with corresponding values in control antigen wells. These two procedures using 25 per cent and 20 - 50 per cent Urografin, gave titres of 4 and 5 ( $1/\log_2$ ) respectively.

Although centrifugation of the chlamydial suspension through 20 - 50 per cent Urografin gave a higher end-point dilution, this technique was time consuming and not particularly suitable for routine use. For this reason, of the four yolk-sac grown C.trachomatis antigen preparations initially investigated, the 25 per cent Urografin procedure was chosen for preparing antigen for further serology.

## 8.2 PREPARATION OF MICRO-IF CHLAMYDIAL ANTIGENS

A heavily infected mid-phase suspension of IC CAL8 was purified by centrifuging through 25 per cent Urografin. The final pellet was resuspended in PBS, pH 7.2. Doubling dilutions, 1/2 - 1/16 were prepared and dotted onto glass slides for staining by Giemsa and the F.A.T.

Giemsa stained plaques showed very little contaminating yolk-sac material and large numbers of chlamydial particles. A suitable

working dilution of the stock suspension was 1/8. However, slide antigens stained by the indirect F.A.T. appeared to have been disaggregated by the washing stages. It was possible that Urografin, still present in the chlamydial suspension, affected proper fixation.

By employing a further centrifugation step, (20,000 X g for 20 mins) to wash the chlamydial suspension, better fixation was achieved. This step was subsequently added to the 25 per cent Urografin protocol.

#### 8.3.1 PURIFICATION OF CELL CULTURE GROWN C.TRACHOMATIS

Heavily infected McCoy cell coverslip cultures were pooled and subjected to mild sonication. The supernatant formed after low speed centrifugation (1,500 X g) was collected, diluted 1/16 - 1/2048 and used to coat EIA plates.

Figure 8.5 shows that a titration curve was obtained for IC CAL8 coated wells, but corresponding control antigen wells gave high non-specific absorbance values.

#### 8.3.2 EXAMINATION OF NON-SPECIFIC ABSORBANCE READINGS RECORDED IN CONTROL ANTIGEN COATED WELLS

Results from experiment 8.3.1 showed that there was extensive cross-reaction between the pooled human reference serum used in the EIA and uninfected McCoy cell supernatant coating antigen.

Using the same serum, a coverslip of uninfected McCoy cells was stained by the indirect fluorescent antibody technique. Examination of the stained cells, by appropriately filtered UV microscopy, showed very little specific staining of cellular material. This suggested that the reference serum was reacting with a soluble cell associated antigen in the EIA which in the FA technique may have been removed by acetone fixation.

An attempt to remove non-specific absorbance readings by first treating the infected McCoy cell monolayer with fresh acetone was carried out. Infected and uninfected coverslip cultures were placed in 5 ml cold fresh acetone (10 minutes). Excess acetone was removed by pipette and the coverslip cultures were air dried at room temperature. Cells were suspended in 2 ml coating buffer. The rest of the procedure was the same as 8.3.1.

The results of this experiment can be seen on Graph 8.6. Compared with values plotted on Figure 8.5, non-specific readings have been reduced. The characteristic titration curves however have been lost. These results suggested a more stringent purification procedure was required.

### 8.3.3

The same acetone treatment of infected and uninfected coverslips was carried out as before, followed by:-

- (i) centrifugation through 30 per cent sucrose
  - (ii) centrifugation through 25 per cent Urografin
- 
- (1) After acetone treatment cells from 12 infected coverslips

were suspended in 3 ml of PBS prior to sonication and centrifugation. The 3 ml suspension was distributed in 1 ml aliquots and carefully overlaid 4 ml of 30 per cent sucrose. Centrifugation was at 24,000g for 90 minutes (4°C). The resulting pellets were resuspended in a total volume of 2 ml coating buffer, EIA plates were prepared as before.

- (ii) The chlamydial suspension was prepared as described in (i). 1 ml aliquots were overlaid 4 ml 25 per cent Urografin and centrifuged at 24,000g for 90 minutes. The plates were coated as before.

It can be seen from Figures 8.7 and 8.8 plotted absorbance values for dilutions of IC CAL8 coated wells gave a characteristic titration curve. Non-specific attachment of antichlamydial serum to control antigen wells was minimal. The titres for these preparations were 5 and 7 ( $1/\log_2$ ) for sucrose and Urografin procedures respectively.

Although acetone treatment was shown to reduce non-specific absorbance in uninfected cell culture preparations, it was unnecessary when a separation medium like Urografin was used. Figure 8.9 shows the results of chlamydial antigen prepared without acetone extraction and centrifuged through 25 per cent Urografin. The titre of this preparation was particularly high, 9, and background absorbance readings were minimal (0.2 - 0.4). The high titre obtained in this experiment can be directly compared with previous cell culture experiments because the same antigen

input was used in each case.

#### 8.4 PURIFICATION OF C.TRACHOMATIS FOR 80 cm<sup>2</sup> CULTURE FLASKS

Repeated sub-culturing in 80 cm<sup>2</sup> flasks proved to be technically more difficult than in vial cultures. The permissible centrifugal force in the former was only 210g compared with 1840g for coverslip cultures. The important factors for enhancement of the infectivity and yield of flask cultures were DEAE-dextran treatment, centrifugation and sonic disruption of the infected cell on harvesting.

The serial passage of IC CAL8 were started from infected yolk-sacs graded at 4+. All passages were carried out in culture flasks. After seven passages approximately 75 per cent of cells stained by the indirect FA technique showed cytoplasmic inclusions.

Results (Figure 8.10) of scaling up the 25 per cent Urografin method to infected flask cultures (7.9) proved to be effective. High antigen titres were obtained, 11, with low background absorbance values.

The disadvantage of this technique was difficulty in adapting a "slow growing" strain to flask cultures. In this study only one serotype was adapted to these conditions and therefore eliminated the technique for possible use in the micro-IF test.



### 8.5.1 CF ANTIGEN

Yolk-sac grown IC CAL8 purified through the 25 per cent Urografin, was titrated using human pooled serum at 1/32 to estimate a working dilution of antigen for the CFT. An end-point titre of 1/32 was obtained giving a working dilution of 1/16. There was anticomplementary activity at a 1/4 dilution of antigen.

80 cm<sup>2</sup> culture flask grown IC CAL8 was used in a two-dimensional titration using dilutions of reference serum to estimate an antigen titre for the CFT. An end-point titre of 1/128 was obtained giving a working dilution of 1/64. This chlamydial preparation was used routinely for CFT screens in preference to infected yolk-sac antigen.

### 8.6 COMPARISON OF CFT AND MICRO-IF TO DETECT ELEVATED ANTIBODY LEVELS IN CHLAMYDIA-INFECTED PATIENTS

The preceding experimental work has shown that the optimal chlamydial preparation for micro-IF used infected yolk-sac purified by centrifugation through 25 per cent Urografin. Similarly IC CAL8 antigen used in the CFT was obtained in sufficient quantities from infected flask cultures. These two tests were examined for their respective sensitivities to detect antibody in chlamydia infected patients.

72 unselected female patients attending a Department of Genito-urinary Medicine provided a serum sample for serology and cervical exudate for isolation attempts in cycloheximide treated McCoy cells. The transport medium was 2 SP.

Table 8.1 shows that of 72 patients examined 17 were culture positive. An estimate of the number of inclusions seen in 10 fields (X250) was made. The range of inclusion counts was from less than one per field to over 50 inclusions per field. The mean count was 8 ifu per field.

The micro-IF test detected elevated antibody levels in 16 of 17 culture positive patients with a reciprocal G.M.T. of 29.8. The complement fixation test showed 9 patients with titres (GMT = 1:16). There was no correlation between the number of inclusions counted in isolation specimens and the magnitude of the antibody response.

Among culture negative patients, elevated titres were detected in 29 individuals by micro-IF and 4 by CFT.

#### 8.7 CHARACTERISATION OF THE EIA COATING ANTIGEN

Chlamydial antigen (IC CAL8) was prepared from infected flask cultures, purified through Urografin and used to coat EIA plates. Hyperimmune mouse serum specific for serotype E (anti DK-20) and G (anti IOL 23B) were tested by two-dimensional titration against the coating antigen.

Results from Table 8.3 show that at a serum dilution of 1/20 and an antigen dilution of 1/80 absorbance values for the two type specific sera were indistinguishable.

A similar titration by the F.A.T. on the same sera against

a single antigen (IC CAL8) using the protocol described in Section 7.6.1 shows no type specific reaction.

If type specificity was present in both tests, anti DK-20 would be expected to cross-react with the coating antigen and the whole inclusion by the F.A.T., whereas anti IOL 23B would not. However this was not the case since both sera appeared to react equally to the same antigens. The results strongly suggest that in both assays a group rather than type specific antigen is being detected.

#### 8.8 A COMPARISON OF THE F.A.T. AND EIA FOR SCREENING HUMAN SERA

EIA plates and slides for the F.A.T. were prepared in the same way as 8.6. 64 sera from female patients attending a colposcopy clinic were titrated. For the EIA sera were screened at 1/20 and tested in duplicate.

Figure 8.11 shows that there was a high degree of correlation between the single antigen (whole-inclusion) F.A.T. and the EIA (correlation coefficient  $r = 0.91$ ). The average error between duplicate well was  $\pm 0.024$  absorbance units.

Figure 8.1 - differential centrifugation (7.9.a)

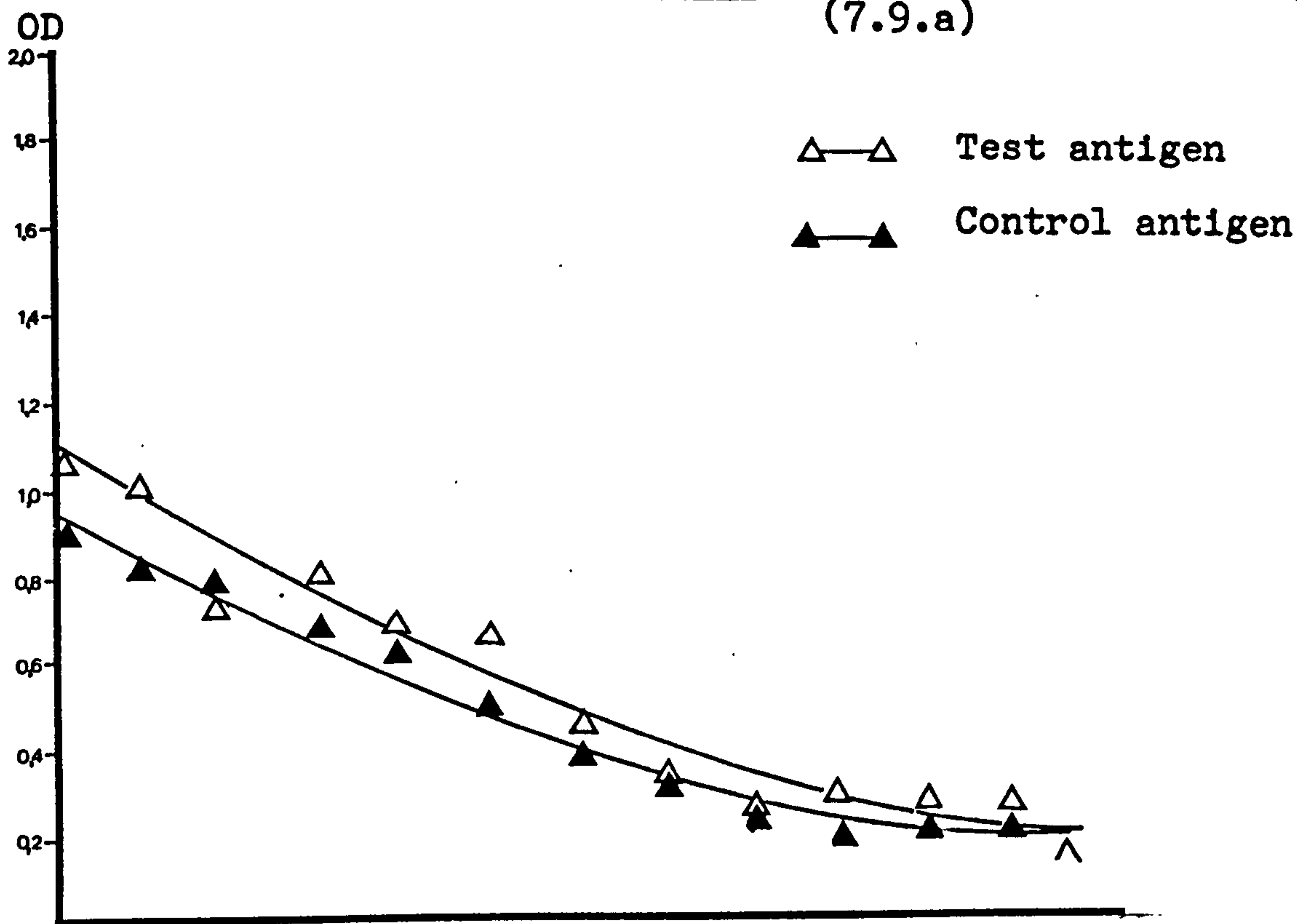
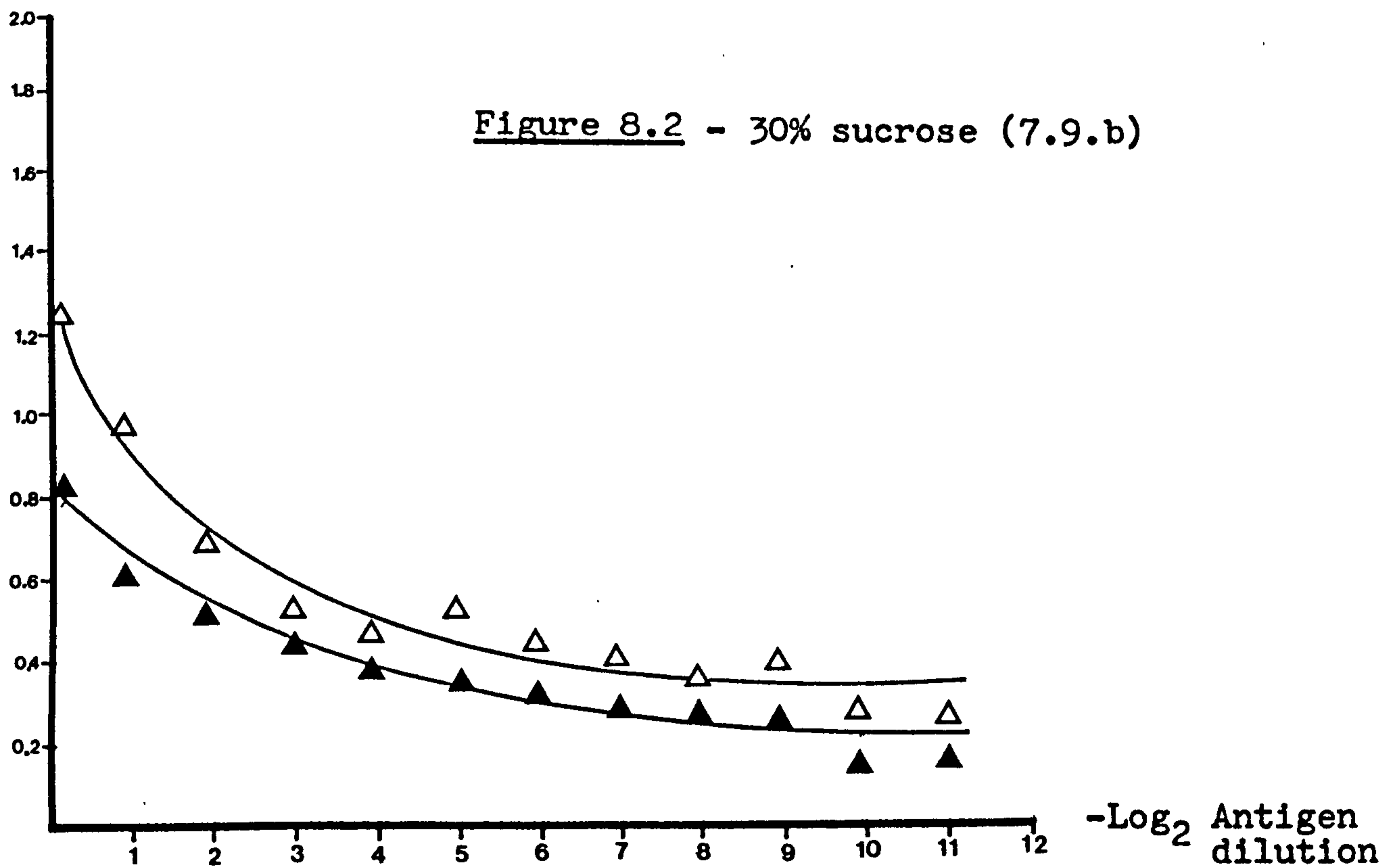


Figure 8.2 - 30% sucrose (7.9.b)



EIA VALUES FOR DILUTIONS OF YOLK-SAC GROWN  
C. TRACHOMATIS (8.1)

Figure 8.3 - 25 per cent Urografin (7.9.c)

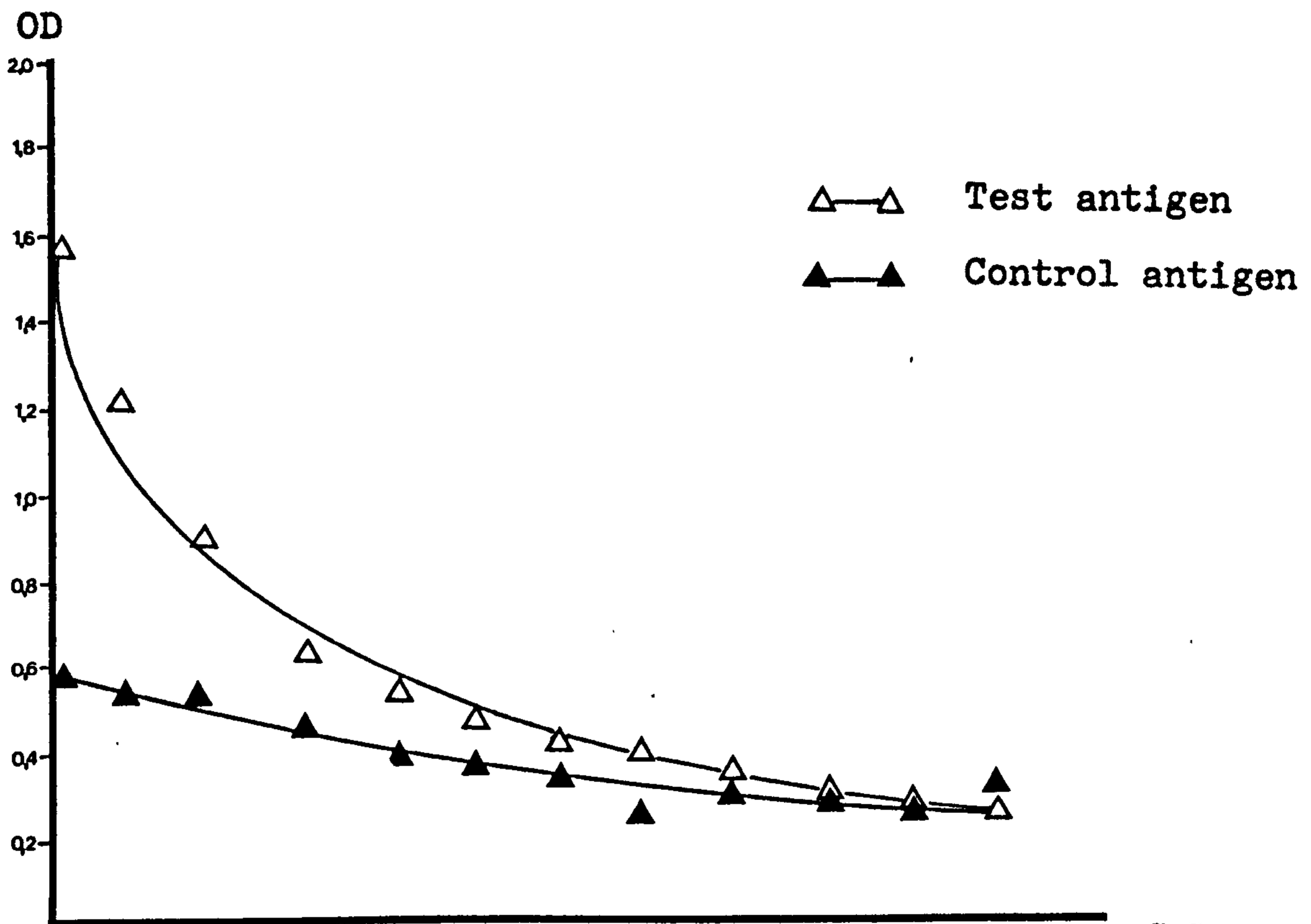
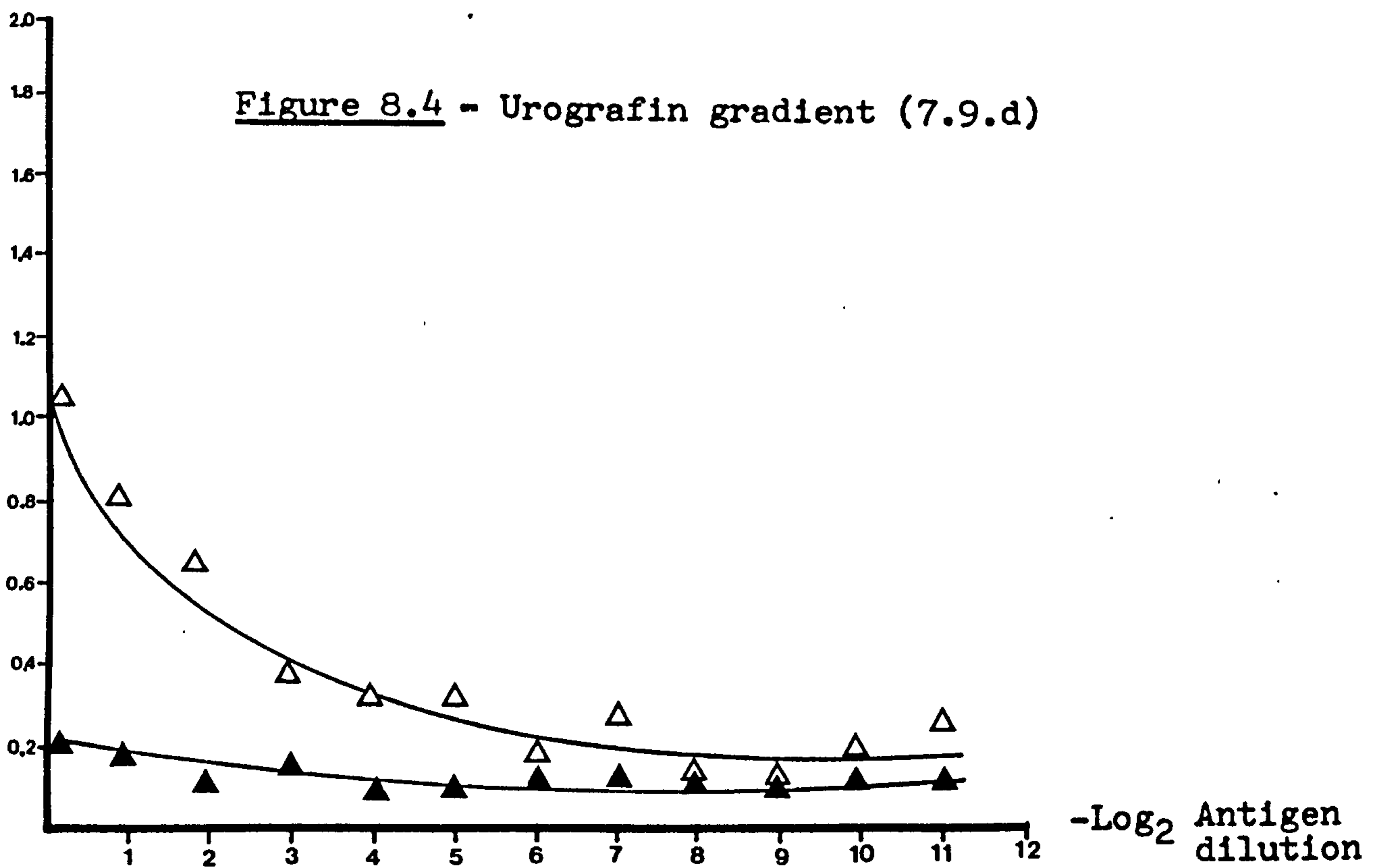


Figure 8.4 - Urografin gradient (7.9.d)



EIA VALUES FOR DILUTIONS OF YOLK-SAC GROWN  
C. TRACHOMATIS (8.1)

Figure 8.5 - low speed centrifugation (8.3.1)

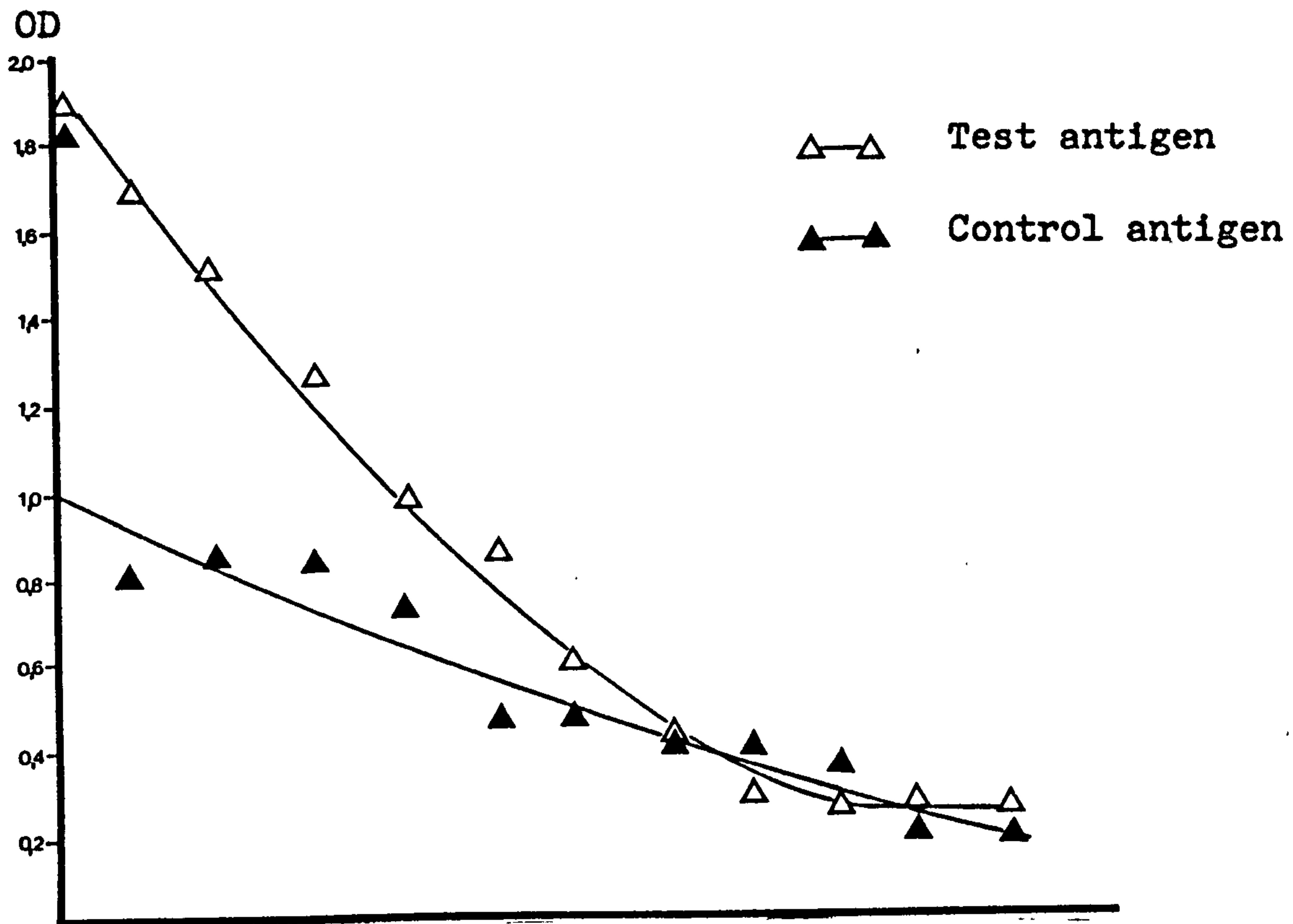
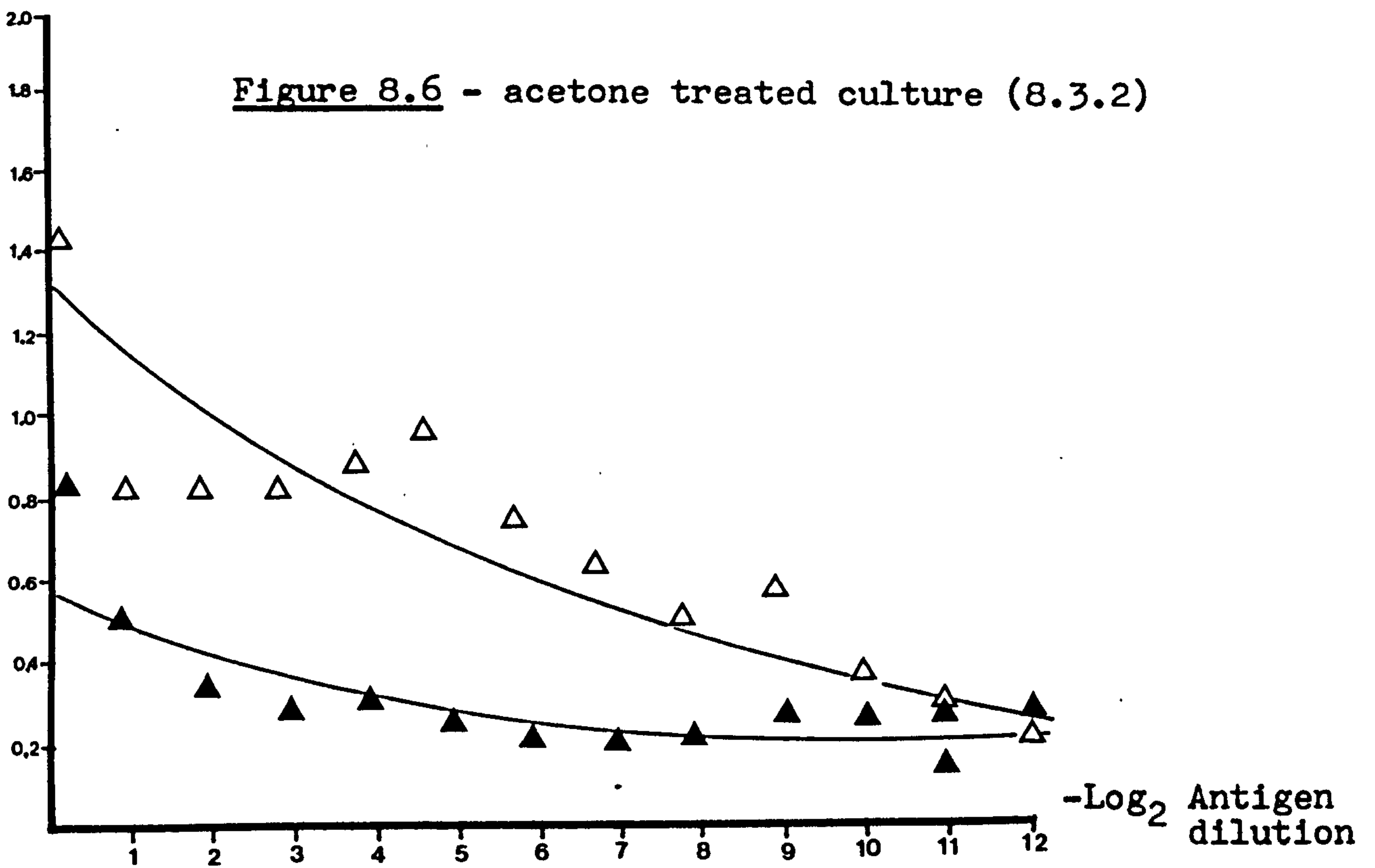


Figure 8.6 - acetone treated culture (8.3.2)



EIA VALUES FOR DILUTIONS OF C. TRACHOMATIS GROWN  
IN MCCOY CELL MONOLAYERS

Figure 8.7 - 30 per cent sucrose (8.3.3)

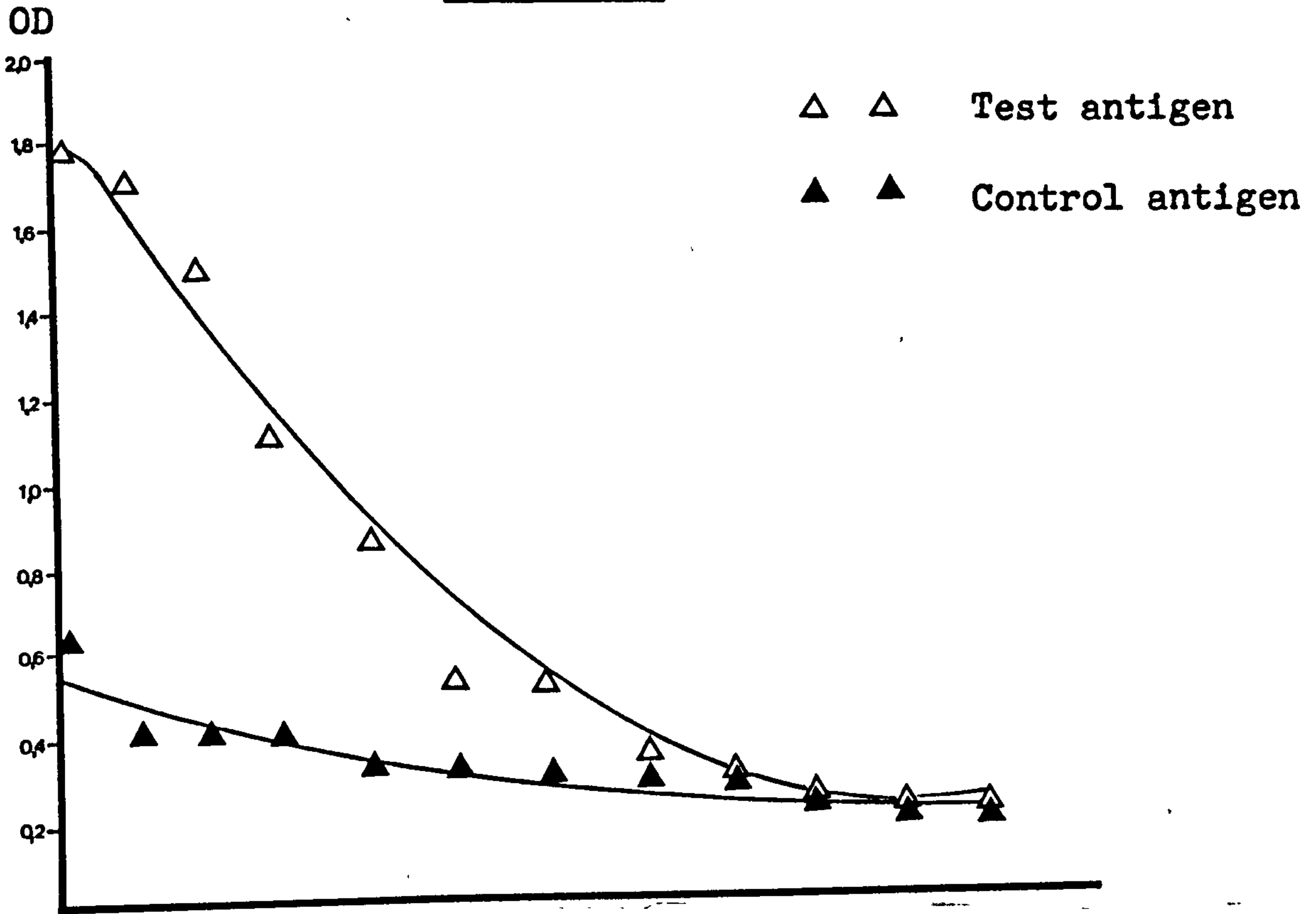
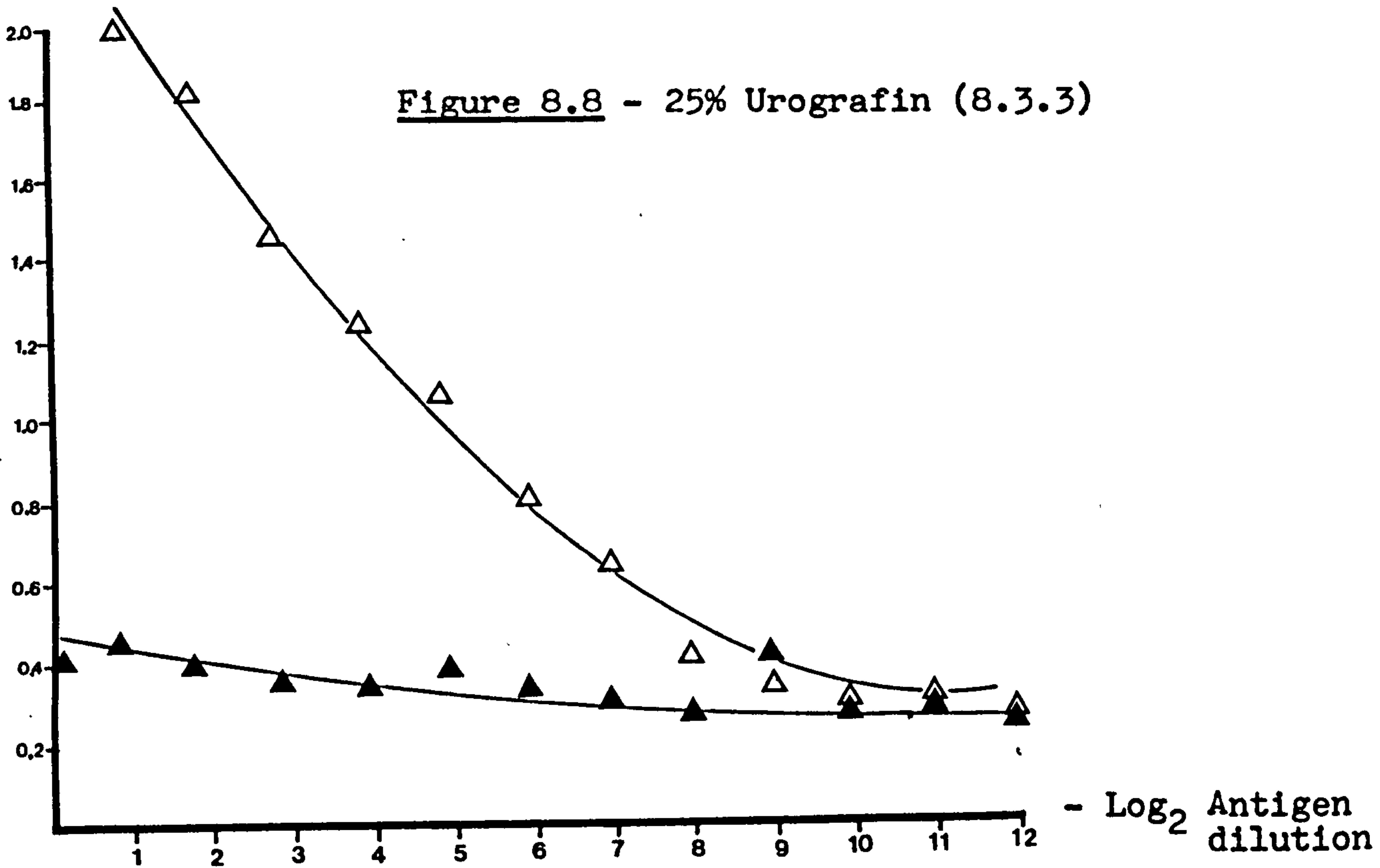


Figure 8.8 - 25% Urografin (8.3.3)



EIA VALUES FOR DILUTIONS OF C. TRACHOMATIS GROWN  
 IN McCOY CELL MONOLAYERS

Figure 8.9 - 25 per cent Urografin

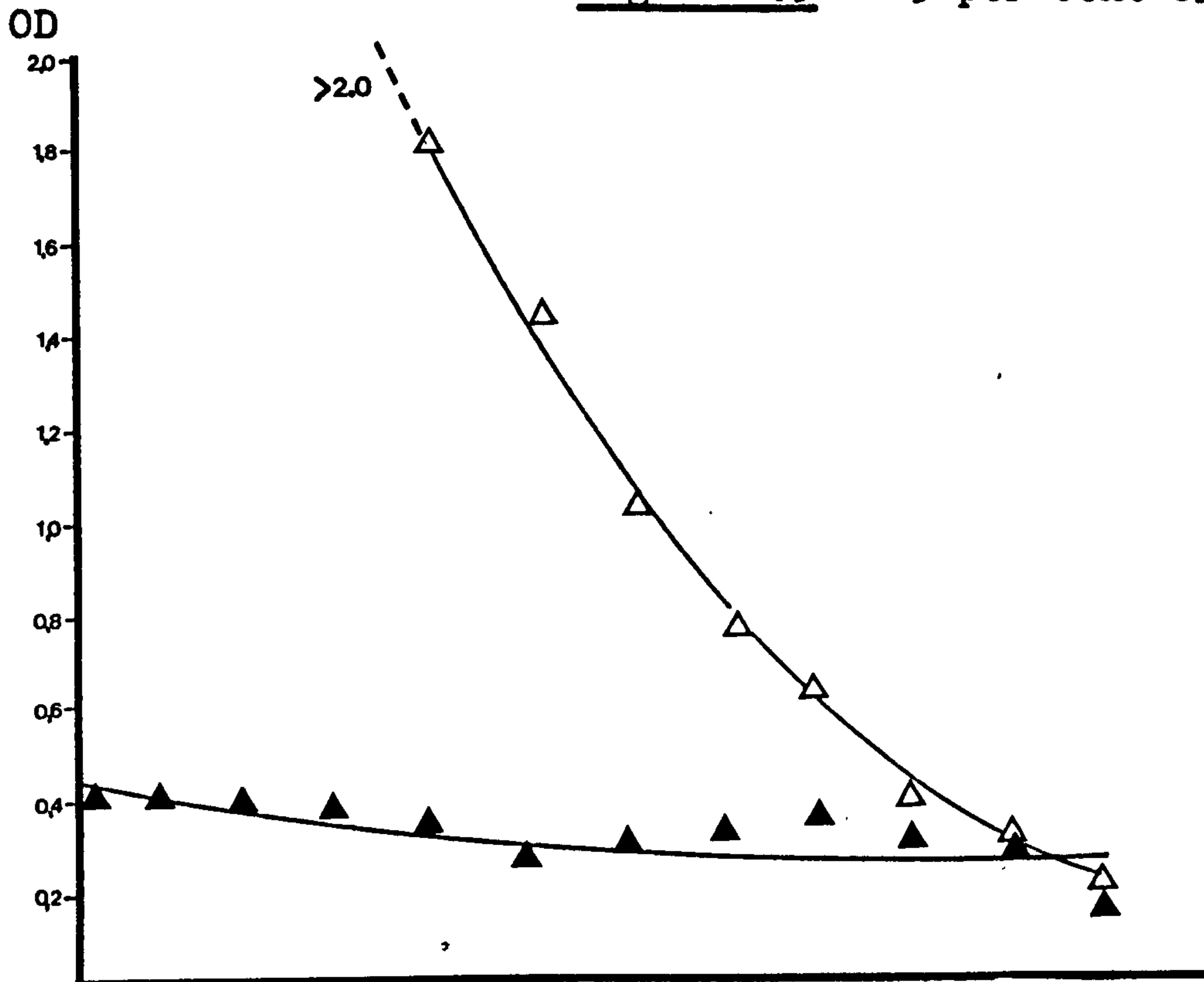
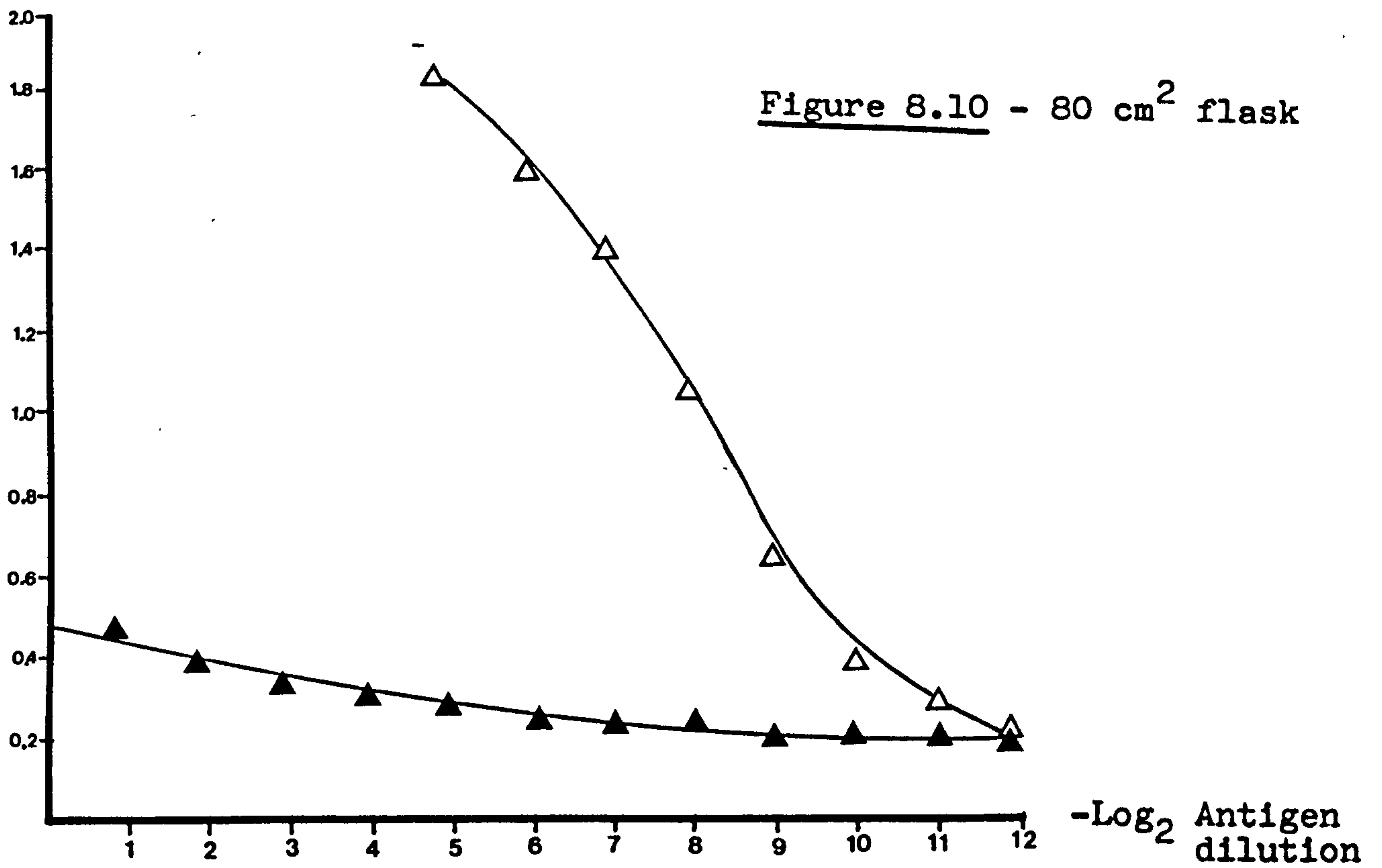


Figure 8.10 - 80 cm<sup>2</sup> flask



EIA VALUES FOR DILUTIONS OF C. TRACHOMATIS GROWN  
IN McCOY CELL MONOLAYERS



TABLE 8.1

n	Chlamydia positive cultures	Mean inclusion count per field
72	17 (23.6%)	8

TABLE 8.2.1

Elevated titres in culture positive patients:

n	Micro-IF	Reciprocal GMT	CF	Reciprocal GMT
17	16	29.8	9	16

TABLE 8.2.2

Elevated titres in culture negative patients:

n	Micro-IF	CF
55	29	4

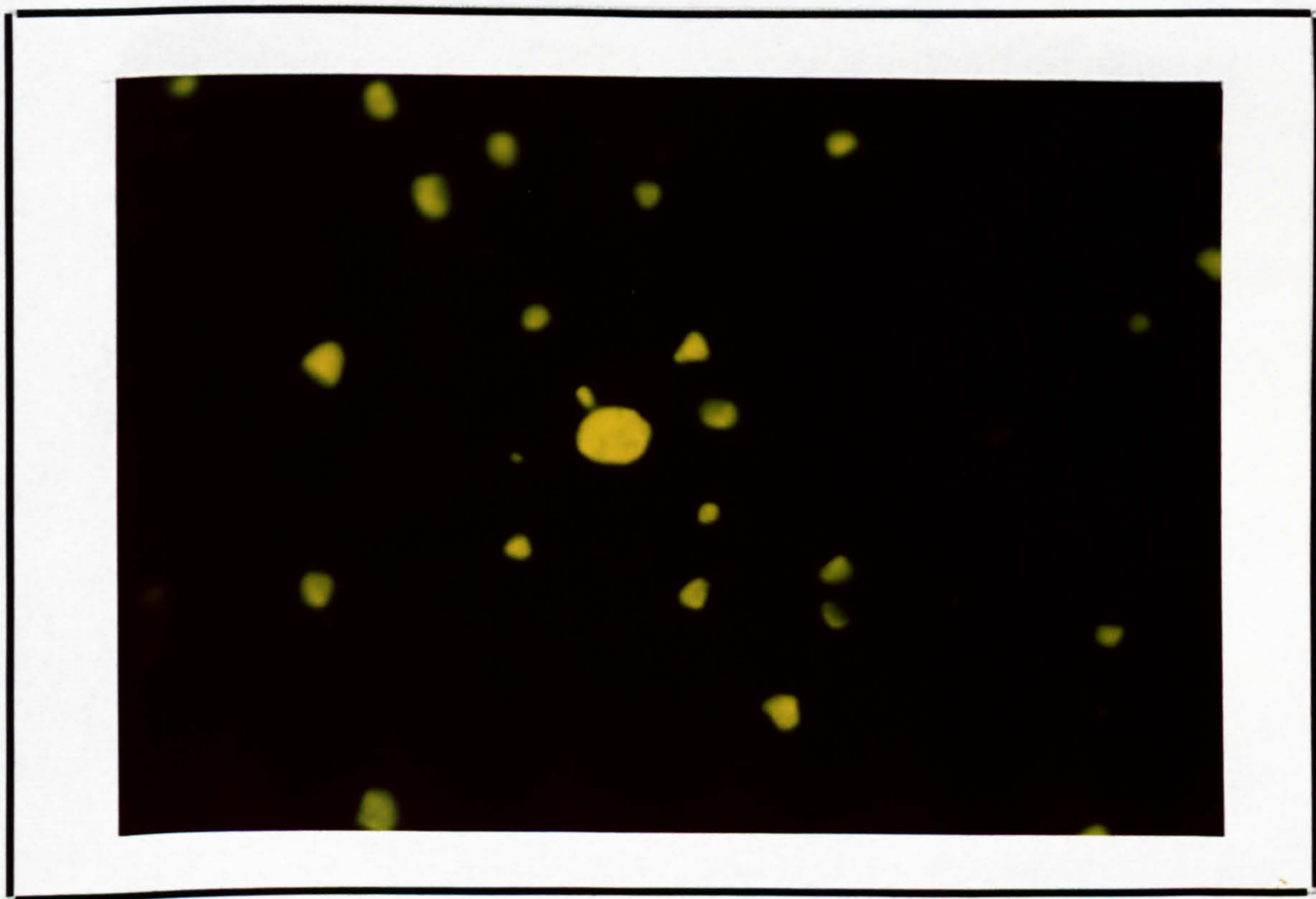
TABLE 8.3

SPECIFIC MOUSE SERUM

Serum	FA	EIA
Anti-DK-20 (E)	1/64	0.65
Anti IOL 258 (G)	1/64	0.64

Coating Antigen-IC CAL8.

Plate 4

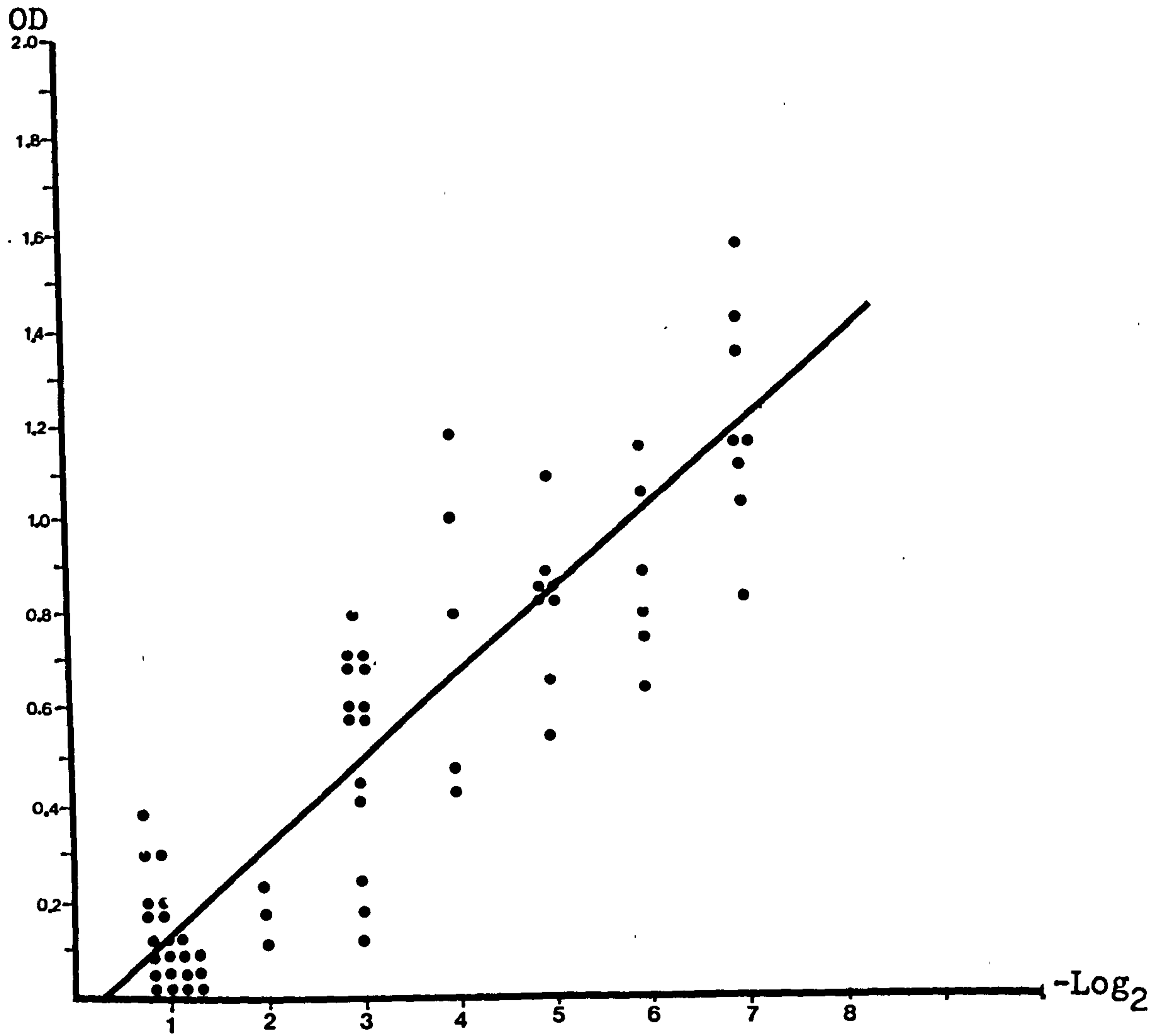


WHOLE INCLUSION FLUORESCENCE  
(Indirect FAT)

Figure 8.11

$$y = -0.08 + 0.186X$$

$$r = 0.91$$



COMPARISON OF CHLAMYDIAL EIA ANTIBODY TITRES IN SERA  
FROM 64 COLPOSCOPY PATIENTS (CD) WITH A SINGLE ANTIGEN  
FAT

## CHAPTER 9

### 9.1 AN EPIDEMIOLOGICAL INVESTIGATION OF CHLAMYDIAL INFECTION IN WEST SCOTLAND

#### INTRODUCTION

At this point I would like to describe an epidemiological survey which was carried out to investigate the prevalence of C.trachomatis infection in selected groups of patients in West Scotland. The selection criteria were based on the fact that serotypes D-K primarily infect the genito-urinary tract and are transmitted sexually. A spectrum of diseases is associated with C.trachomatis and for this reason the following groups were investigated in order to evaluate infection rates as widely as possible.

(I) FEMALE PATIENTS from the following categories:-

Ante-natal clinics (AN)

Family planning clinics (FP)

Cervical dysplasia clinics (CD)

Genito-urinary Medicine (GM)

The female groups investigated also include:-

Consorts of Reiter's Syndrome patients (cRS)

Consorts of recurrent NGU patient (crNGU)

(II) MALE PATIENTS from the following categories:-

Genito-urinary Medicine (GM)

Reiter's Syndrome (RS)

Recurrent NGU (rNGU)

Uncomplicated NGU (NGU)

Male sub-fertility clinics (MI)

Ankylosing spondylitis cases (AS)

Four main groups of female patients were investigated. Ante-natal and family planning patients represented usually healthy and sexually active females. Attenders at the Department of Genito-urinary Medicine were regarded as individuals especially exposed to infection. Patients known to have cervical dysplasia composed a smaller group and they were compared with the other groups for frequency of infection. crS and crNGU were included primarily to assess their role in relation to the disease and continuing morbidity status of their male partners.

The examination of male patients included six distinct groups as listed above. Great attention was paid to the prevalence of infection among RS patients, who were regarded as males having a complicated urethritis and a high probability of carrying the HLA-B27 antigen. Genital inflammation is an essential feature of Reiter's Syndrome which may or may not be associated with chlamydiae. The reactive arthritis which develops is associated with the localised urethral infection in generally regarded as a sterile synovitis. As part of this study patients suffering from rNGU and NGU were compared with the RS group. Patients with AS were

also included because of their common genotype with RS patients in having the HLA-B27 antigen.

To determine whether there was a greater prevalence of infection among a group of subfertile male patients, the isolation rate from these was compared with the other categories of male patients.

### POSTNATAL PATIENTS

An overall incidence rate for neonatal inclusion conjunctivitis in the Eastern District of the Greater Glasgow Health Board was established. Both the relatively short incubation period (4 - 10 days) and acute onset of symptoms of this disease allowed most cases of neonatal acute conjunctivitis, seen almost exclusively by Community Midwives on domiciliary visits to be screened for chlamydial infection.

For these epidemiological studies patients were grouped according to the clinics attended. Each disease category was defined in terms of characteristics prior to the diagnosis of chlamydial infection. The groups were observed for the frequency of isolation and/or seropositive reactors among them.

## 9.2 PATIENTS

### 9.2.1 FEMALE

#### (I) Antenatal patients (Glasgow Royal Maternity Hospital)

At their first routine obstetric examination during antenatal

care a serum sample for chlamydial serology by the CFT and micro-IF was taken from 111 patients. Between April 1979 and December 1981 all antenatal patients in two clinics were screened for current chlamydial infection by cell culture of cervical swabs.

(II) Family Planning patients (Dept. of Gynaecology, GRI)

Between January 1980 and December 1981, 1,124 patients were screened for current chlamydial infection by cell culture procedures.

(III) Cervical dysplasia patients (Colposcopy Clinic GRI)

Women with abnormal cervical cytology were referred to this clinic. Careful assessment of the disease process is made using a colposcope. This instrument enables a clinician to survey rapidly the vaginal portion of the cervix and the surrounding vaginal walls under low magnification (x20). Early signs of cervical cancer are seen as relatively gross alterations to the normal surface appearance.

176 patients were studied at the time of their presentation. Each had an endocervical swab taken for culture and a serum sample for chlamydial serology was taken from 109 of these women.

(IV) Female patients attending the Department of Genito-urinary Medicine (GRI)

Over a four year period cervical exudate from 3,321 unselected patients was examined for the presence of chlamydiae. These were mostly first attenders.



(V) Post-natal patients

During 1980 and 1981 all neonates delivered at the Glasgow Royal Maternity Hospital, and all postnatal patients cared for in the Eastern District of G.G.H.B, were observed for the onset of acute conjunctivitis. When acute conjunctivitis was seen staff at the Special Baby Care Unit and from the Department of Community Midwives obtained conjunctival swabs after removal of pus from the inner fornix. 282 neonates were screened during the two year study period.

9.2.2 MALE

(VI) Reiter's Syndrome Patients

This group was defined as those patients having two of the following symptoms: NGU, arthritis and conjunctivitis. As part of the control for this study the following groups were also included: male patients with recurrent NGU (rNGU), uncomplicated NGU (NGU) and ankylosing spondylitis (AS). (The consorts of some RS and rNGU patients were only examined to assess frequency of seropositive individuals). Urethral exudate was collected from all males for isolation studies. Serum was also collected for serology.

(VII) Male infertility patients (Department of Urology, GRI)

75 patients whose semen analyses showed either the complete absence of sperm or a relative deficiency of sperm with poor mobility and morphology were included. A sample of urethral

exudate and prostatic fluid was collected from each for chlamydial isolation, between November 1979 and March 1981.

(VIII) Male patients attending the Department of Genito-Urinary Medicine (GRI)

Between July 1981 and December 1981 urethral exudate from 145 unselected patients was examined for the presence of chlamydiae.

9.3.1 ISOLATION AND SEROLOGICAL PROCEDURE

Urethral and cervical material in 2SP was cultured for C.trachomatis in cycloheximide treated McCoy cells (5.7). Antibodies of C.trachomatis were sought by quantitative micro-IF (7.6) and CF techniques (7.7) using serotypes D-K in the former and serotype D in the latter. Serum IgG titres of 1: < 16 by micro-IF and 1: < 8 by the CFT were considered to be of doubtful significance.

#### 9.4.1 RESULTS: ISOLATION RATES FOR FEMALE PATIENTS

Cervical exudate from 916 AN patients, 1124 FP patients and 176 patients with cervical dysplasia was cultured for C. trachomatis. Chlamydiae were grown from 44 (5 per cent), 67 (6 per cent) and 13 (7.4 per cent) respectively (Table 9.1). The isolation rate is almost identical in all three groups. The monthly rates, per cent, can be compared between AN, FP and female attenders at the Department of Genito-urinary Medicine (Figure 9.1). Although there were substantial fluctuations in monthly isolation rates, the latter category had consistently higher rates. Overall figures for GM patients show that of 3,321 patients, 929 (28 per cent) were shown to be infected.

#### Chlamydial infection and primary diagnosis

Examination of patients attending the GUM clinic between January 1980 and December 1980 shows that almost one third of patients with a primary diagnosis of gonorrhoea had a simultaneous chlamydial infection (Table 9.2). Over 25 per cent of patients with other sexually transmitted diseases were also shown to be chlamydia positive. 11 of the patients who appeared to require no treatment were infected with chlamydiae.

#### Chlamydial infection and age

A comparison of C. trachomatis infection and age shows a high proportion in younger patients. One third of the patients investigated, from the GUM clinic were between 15 - 19 years. 63 per cent were under 24 years.

#### 9.4.2 ISOLATION RATES FOR MALE PATIENTS

Urethral exudate from 47 RS patients, 31 rNGU patients and 57 NGU patients was cultured for C.trachomatig. Five (11.5 per cent), 10 (32 per cent) and 14 (24.6. per cent) individuals respectively were positive (Table 9.4). Of 145 unselected GM patients 43 (30 per cent) gave positive cultures. The isolation rates for patients with rNGU and for unselected GM patients were significantly higher than for RS patients. ( $\chi^2 = 6.65, p = 0.01$ ).

Patients with Reiter's Syndrome were divided further on the basis of:-

- (I) acute, recurrent or chronic symptoms (A, R or C respectively)
- (II) presence or absence of urethral discharge at presentation (+ or -) (Table 9.5).

Patients in the A+ category had a similar prevalence of chlamydial infection to NGU patients (Table 9.5).

#### Male infertility patients

The lowest isolation rate of all categories investigated was obtained from 75 male patients with poor fertility. Only 3 positives were obtained from urethral cultures. No isolates were obtained from prostatic fluid.

#### 9.5 Perinatal Infection Rates

Conjunctival secretions were obtained from 282 patients, usually

separately from each eye. The results show the rates for unilateral and bilateral infections (Table 9.6). During 1980 and 1981, 2615 and 2660 respectively were cared for in the postnatal period by Community Midwives. The mean number of cases of inclusion conjunctivitis diagnosed per 1000 live births was 15 (Table 9.7).

## 9.6 PREVALENCE OF CHLAMYDIAL SEROREACTORS

Elevated titres of CF antibody ( $\geq 1/8$ ) and the presence of specific IgG by micro-IF ( $\geq 1/16$ ) were detected in sera from patients summarised in Tables 9.8 and 9.9 and now described here.

### 9.6.1 FEMALE PATIENTS

118 AN patients were screened for chlamydial antibodies. 11 (9.3 per cent) were positive by the CFT and 30 (25.4 per cent) by micro-IF. Similarly of 109 CD patients examined 32 (29 per cent) were positive by CFT and 58 (53 per cent) by micro-IF. The positive rate for CD patients is significantly higher than that for AN cases. (CFT:-  $\chi^2 = 16$ ,  $p < 0.001$  and micro-IF  $\chi^2 = 19.6$ ,  $p < 0.001$ ).

#### A comparison of antibody titres in AN and CD patients

32 AN and 30 CD patients were matched for age from their respective reference populations. Antibody titres obtained by micro-IF were compared. (Figure 9.2). A significantly higher median titre was found in CD patients (Mann-Whitney Test,  $V/\sigma = 9.6$ , i.e.  $> t_{0.5}$ )

Comparison of chlamydial antibody titre and grade of smear  
in CD patients

The antibody titre was compared in 63 patients from the CD clinic when grade of smear and micro-IF titre were known. (Figure 9.4). The proportion of patients in each grade did not differ significantly from the entire CD population (Figure 9.3). There was no significant difference in antibody titres between patients with grade 3 and grade 4 smears (Figure 9.4).

The higher prevalence rate of seropositives in all categories of patients by micro-IF probably reflects the increased sensitivity of this test. All seropositives detected by CF were 1-3  $\log_2$  units lower than by micro-IF

9.6.2 cRS and crNGU patients

15 of 19 (79 per cent) consorts of RS patients were shown to have titres  $\geq 16$  by micro-IF (Table 9.10), whereas all CF results indicated that only 8 patients were seropositives (42 per cent). Although the numbers were small, the cRS group had an exceptionally high proportion of seropositive patients. Consorts of rNGU patients had a lower proportion of seropositive patients at 3/12 (25 per cent) (Table 9.10).

MALE PATIENTS

34 of 43 RS patients (79 per cent), 17 of 24 (70.8 per cent) rNGU patients, 22 of 45 (49 per cent) NGU and 4 of 21 (19

per cent) AS patients had elevated antichlamydial titres by micro-IF. The reciprocal geometric mean titre in each group was 21.4, 14.2, 6.9 and 3.4 respectively (Table 9.10).

The prevalence of seropositive patients was comparable in the RS and rNGU groups. The magnitude of the IgG antibody titres in the two groups did not differ significantly. When A<sup>+</sup> patients only were included the GMT rose to 1/29.8, but this level does not represent a significant increase.

Patients with NGU had a significantly lower level of seropositive reactors ( $\chi^2 = 5.0, p < 0.05$ ) and the magnitude of the antibody response was lower compared with RS patients ( $p = \chi^2 = 2.6$ ) (Figure 9.5).

The group of patients with AS had no individuals with micro-IF titres greater than 1:16 (GMT=1:3.4).

### 9.6.3 COMPARISON OF ISOLATION AND SEROPOSITIVE (MICRO-IF) RATES (FIGURE 9.6)

The highest isolation rate was seen among rNGU patients (32 per cent). This result was reflected in the corresponding high seropositive rate (70.8 per cent). A low isolation rate was obtained from the RS group (11.3 per cent), but a high prevalence of specific IgG was detected (79 per cent).

The lower isolation rate among RS patients may be related to the variation in symptoms at presentation (Table 9.5). 4 out of 20 A<sup>+</sup> patients had C.trachomatis isolated from urethral secretions. Although the numbers of patients involved are too small for satisfactory statistical analysis, the results seem to suggest that this rate is similar to rNGU patients. A surprisingly low isolation rate was obtained from R<sup>+</sup> patients. This factor alone may account for the overall low positive rate. It is possible, on the basis of past clinical information, that these patients were treated with antibiotics. Many RS patients were referred to this survey from other departments and treatment may have taken place prior to referral. Early use of antibiotics would remove the antigen stimulus and therefore would be expected to be followed by a lower antibody level.

Female patients attending the Department of Genito-urinary Medicine were not included in the serological survey. Figure 9.6 shows that the level of current infection in CD and AN patients was similar. However, the proportion of CD patients who



were seropositive was significantly higher. This seems to suggest that some CD patients have had a significantly greater degree of exposure to chlamydial infection in the near or distant past.

TABLE 9.1

CHLAMYDIAL ISOLATION RATE IN FOUR DISTINCT FEMALE POPULATIONS

---

<u>PATIENT CATEGORY</u>	<u>n</u>	<u>POSITIVE ISOLATION</u>	<u>PER CENT</u>
AN	916	44	5
FP	1124	67	6
CD	176	13	7.4
GM	3321	929	28

---

TABLE 9.2

CHLAMYDIAL INFECTION AND DIAGNOSIS

<u>PRIMARY DIAGNOSIS</u>	<u>n</u>	<u>CHLAMYDIA POSITIVE ISOLATES</u>	<u>PER CENT</u>
Gonorrhoea	115	36	31
Other STD	268	77	28.5
No treatment required	71	11	15.5

---

TABLE 9.3

CHLAMYDIAL INFECTION AND AGE

<u>AGE</u>	<u>n</u>	<u>CHLAMYDIA POSITIVE ISOLATES</u>	<u>PER CENT</u>
15	1	-	-
15 - 19	102	34	33.3
20 - 24	155	47	30
25 - 34	139	35	25
35+	54	8	15

---

TABLE 9.4

CHLAMYDIAL ISOLATION RATE IN FOUR DISTINCT MALE POPULATIONS

---

PATIENT CATEGORY	n	POSITIVE ISOLATION	PER CENT
RS	47	5	11.5
rNGU	31	10	32
NGU	57	14	24.5
MI	75	3	4
GM	145	43	30

---

TABLE 9.5

CLASSIFICATION OF RS PATIENTS BY SYMPTOMS

<u>SYMPTOM</u>	<u>n</u>	<u>POSITIVE ISOLATION</u>	<u>ELEVATED TITRES</u>	
			<u>MICRO-IF</u>	<u>CF</u>
A <sup>+</sup>	20	4	16	9
A <sup>-</sup>	3	0	1	0
R <sup>+</sup>	13	0	11	5
R <sup>-</sup>	2	1	1	1
C <sup>+</sup>	2	0	1	0
C <sup>-</sup>	3	0	2	0
TOTAL	43	5	32	15

---

+ urethral discharge  
- no urethral discharge

A acute symptoms  
R recurrent  
C chronic

TABLE 9.6

CHLAMYDIAL ISOLATION RATE IN NEONATES WITH ACUTE CONJUNCTIVITIS

<u>n</u>	<u>POSITIVE ISOLATION</u>	<u>PER CENT</u>
282	83	29.5

TABLE 9.7

INCIDENCE RATES FOR NEONATAL INCLUSION CONJUNCTIVITIS

	<u>1980</u>	<u>1981</u>
POSTNATAL PATIENTS <u>FOR FOLLOW-UP</u>	2615	2660
<u>POSITIVE ISOLATES</u>	31	52
RATE FOR <u>1000 PATIENTS</u>	12	19

TABLE 9.8

PREVALENCE OF ELEVATED COMPLEMENT FIXING ANTIBODY TITRES  
TO CHLAMYDIA

1. FEMALE PATIENTS

<u>PATIENT CATEGORY</u>	<u>n</u>	<u>TITRE <math>\geq</math> 1: 8</u>	<u>PER CENT</u>
AN	118	11	9
CD	109	32	29
CRS	19	8	42
crNGU	12	2	16

---

TABLE 9.9

2. MALE PATIENTS

<u>PATIENT CATEGORY</u>	<u>n</u>	<u>TITRE <math>\geq</math> 1: 8</u>	<u>PER CENT</u>
RS	43	15	35
rNGU	24	5	21
AS	21	2	9.5

NOTE:

TABLE 9.10

PREVALENCE OF ELEVATED IgG C.TRACHOMATIS ANTIBODY TITRES  
BY MICRO-IF

1. FEMALE PATIENTS

<u>PATIENT CATEGORY</u>	<u>n</u>	<u>TITRE <math>\geq</math> 1: 16</u>	<u>PER CENT</u>	<u>RECIPROCAL</u> <u>G.M.T.</u>
AN	118	30	25	3.5
CD	109	58	53	9.7
cRS	19	15	79	6.4
crNGU	12	3	25	3.1

---

TABLE 9.11

2. MALE PATIENTS

<u>PATIENT CATEGORY</u>	<u>n</u>	<u>TITRE <math>\geq</math> 1: 16</u>	<u>PER CENT</u>	<u>G.M.T.</u>
RS	43	34	79	21.4
rNGU	24	17	70.5	14.2
NGU	45	22	49	6.9
AS	21	4	19	3.4

NOTE:



Table 9.12

PROPORTION OF PATIENTS CARRYING THE HLA-B27 ANTIGEN

RS Patients

rNGU patients

17/34 (51 per cent)

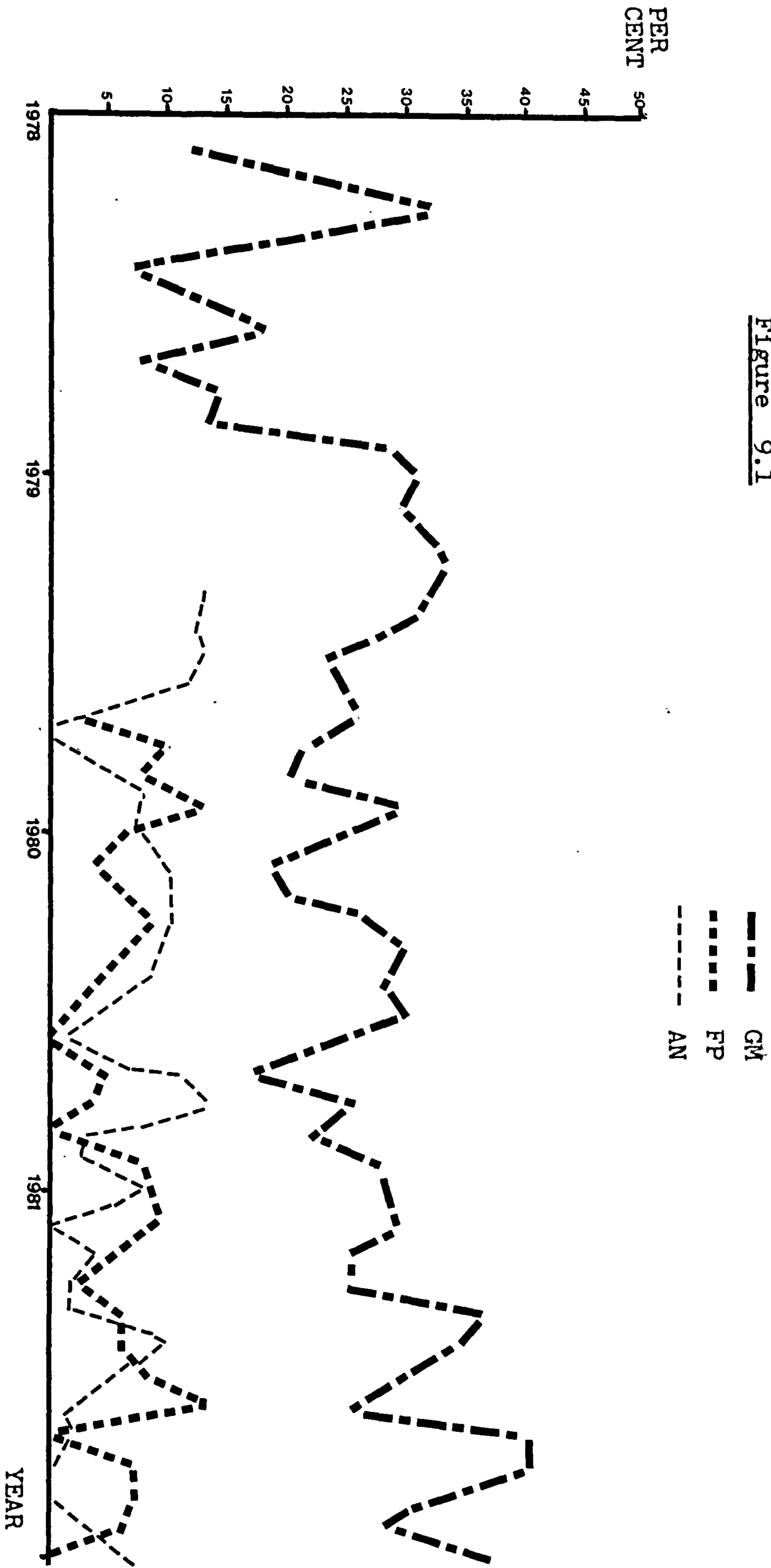
2/16 (12.5 per cent)

Table 9.13

CHLAMYDIAL INFECTION AND AGE

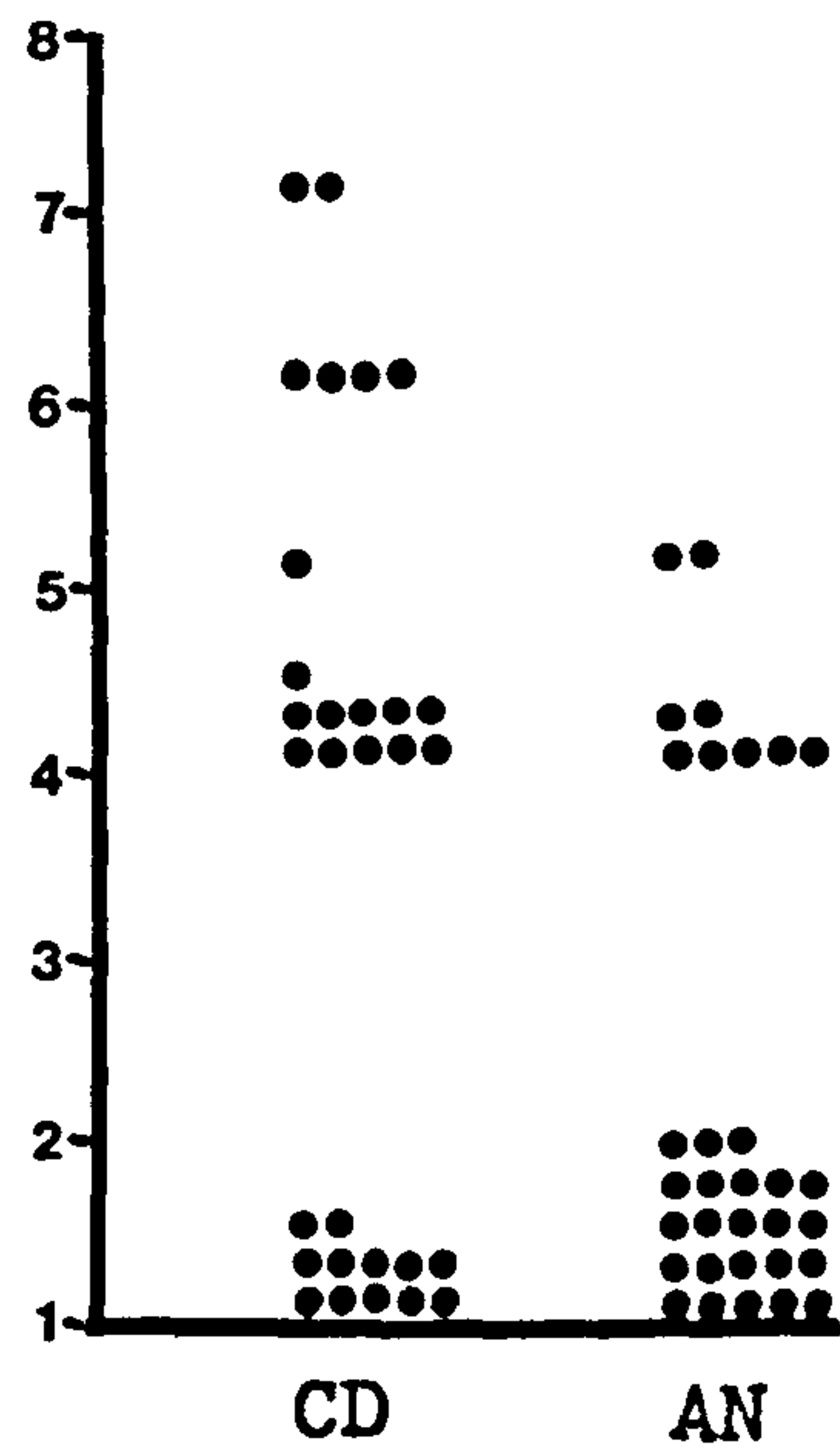
Age	n	CHLAMYDIA POSITIVE	PER CENT
15	0	0	
15-19	11	2	18
20-24	34	12	35
25-34	64	20	31
35+	28	8	28

Figure 9.1



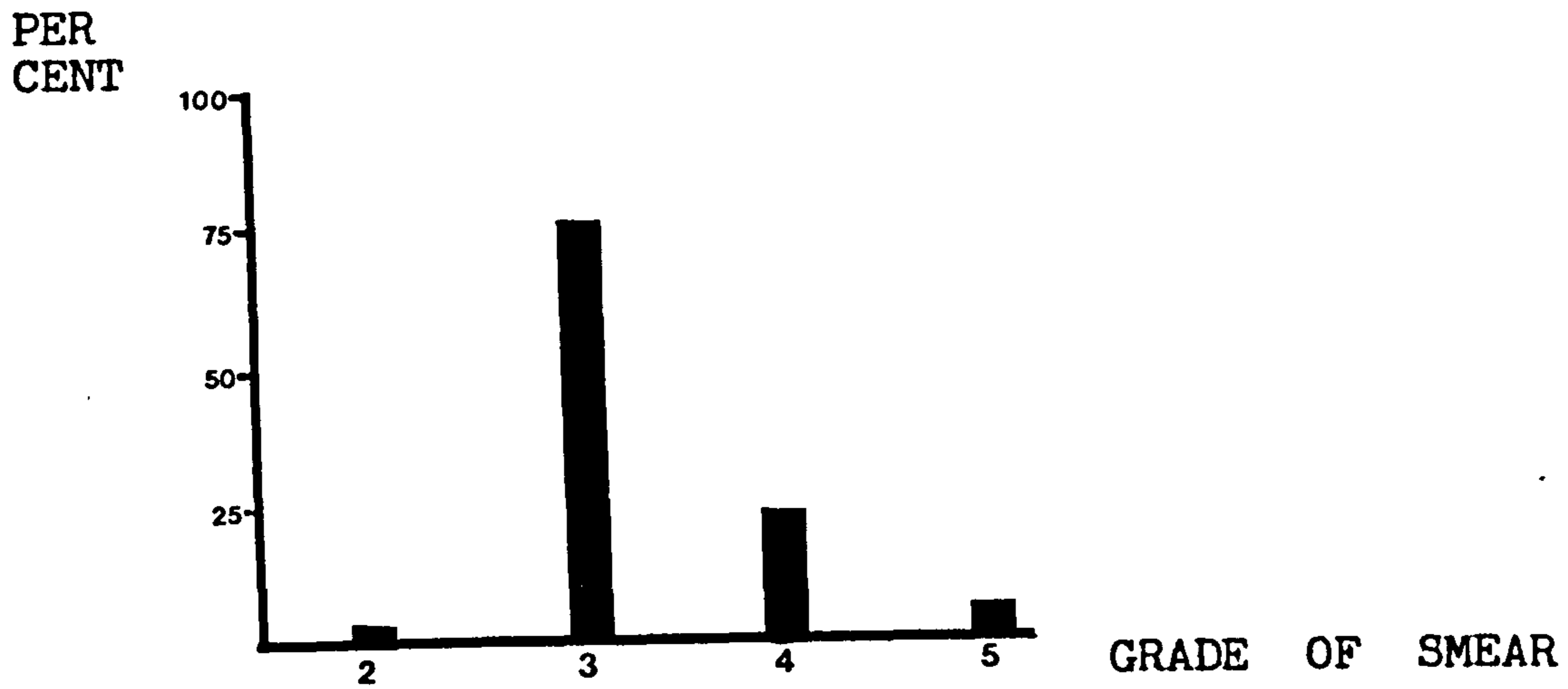
: ISOLATION RATE (per cent) FOR C. TRACHOMATIS FROM THREE FEMALE CLINICS

Figure 9.2.



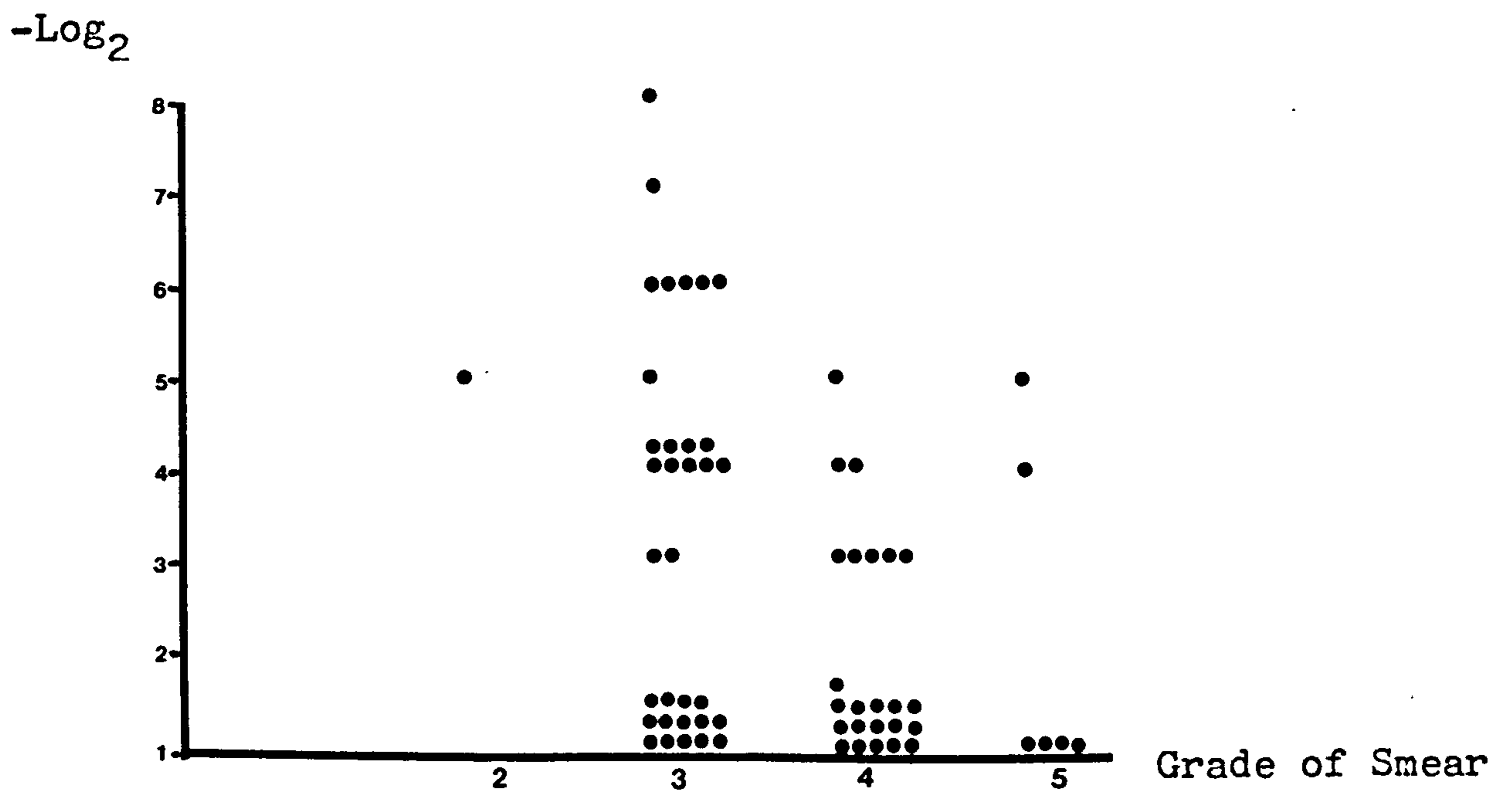
A COMPARISON BETWEEN COLPOSCOPY AND ANTE-NATAL PATIENTS FOR ANTI-CHLAMYDIAL IgG TITRES (Matched for age)

Figure 9.3



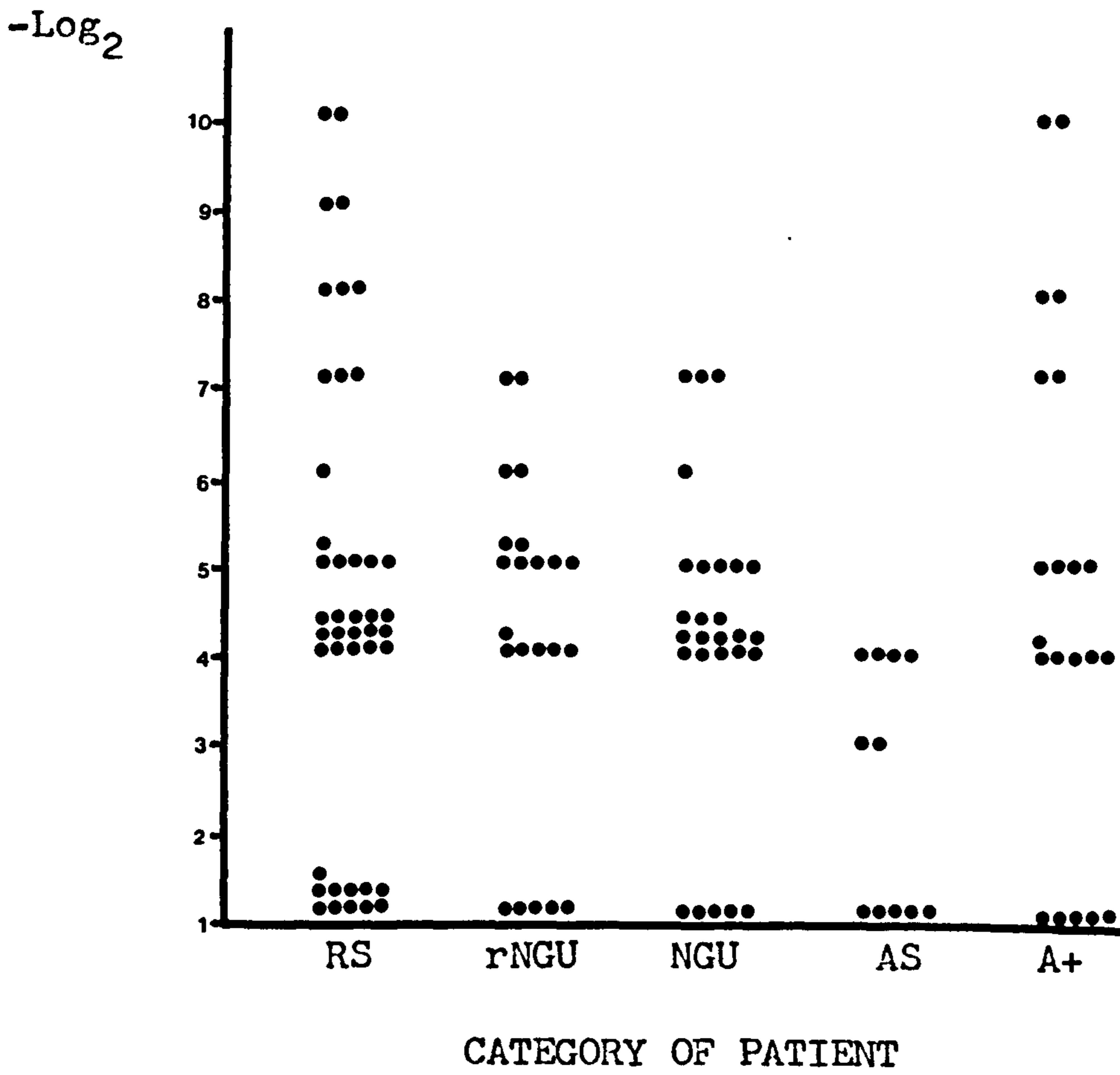
RELATIVE FREQUENCY OF GRADE OF SMEAR AT  
PRESENTATION (PER CENT)

Figure 9.4



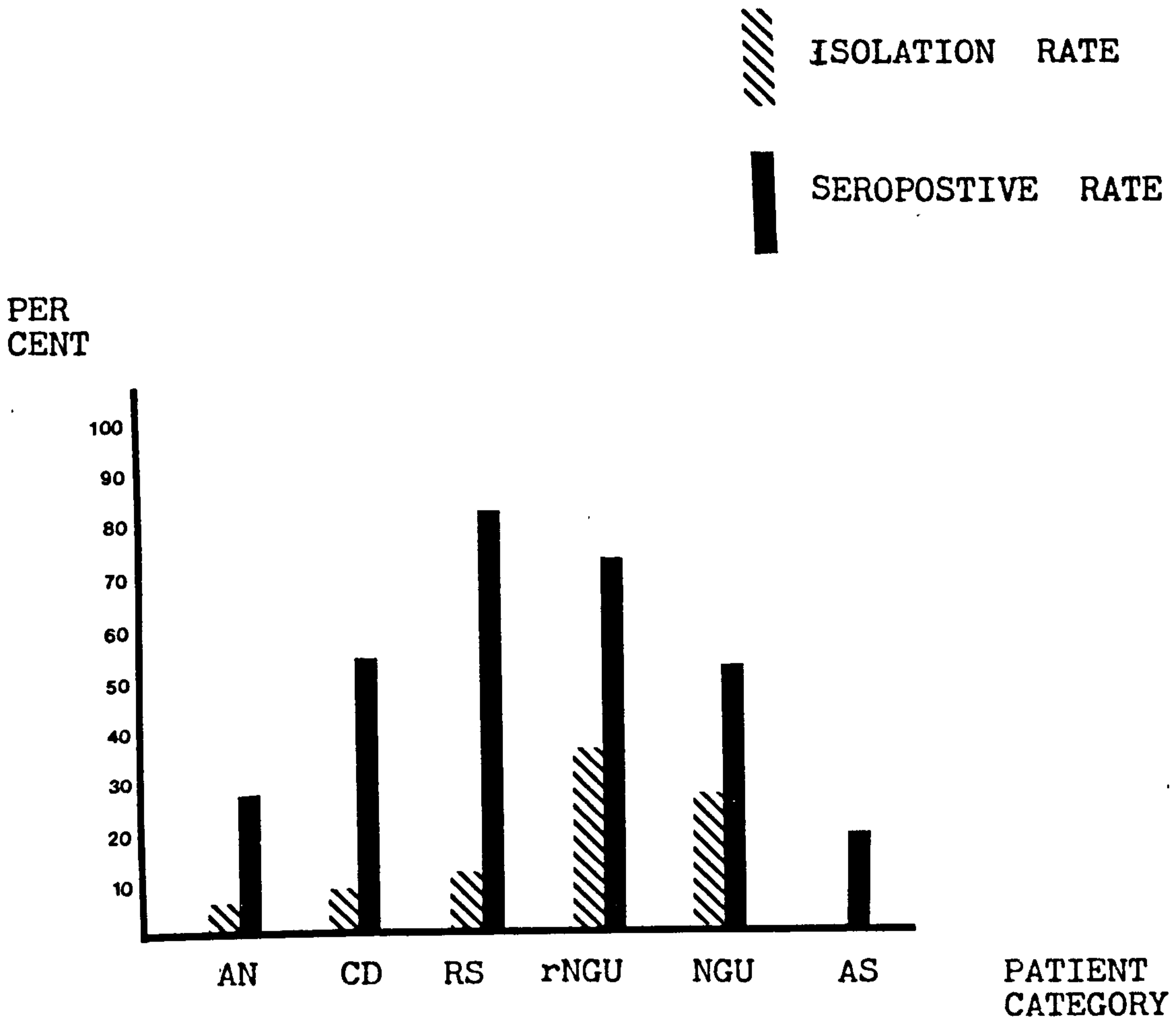
COMPARISON BETWEEN GRADE OF SMEARS AND  
ANTI-CHLAMYDIAL IgG TITRES

Figure 9.5



COMPARISON OF ANTI-CHLAMYDIAL IgG TITRES  
AMONG DIFFERENT GROUPS OF MALE PATIENTS

Figure 9.6



COMPARISON OF ISOLATION AND SEROPOSITIVE (micro-IF)  
REACTION RATE IN DIFFERENT GROUPS OF PATIENTS

**PAGE**  
**NUMBERING**  
**AS ORIGINAL**



## CHAPTER 10

### DISCUSSION

#### 10.1 INTRODUCTION

The aims of this thesis were to examine all aspects of laboratory procedures used to detect infections caused by C.trachomatis; this included both serological and isolation techniques. Where modifications in existing techniques were justified these were included. A practical EIA procedure was developed and indirectly used to measure the purity of chlamydial antigens. Finally, the more sensitive laboratory procedures were used to examine the incidence of chlamydial infections in selected groups of patients.

#### 10.2 CELL CULTURE PROCEDURES

The determination of an infectivity titre in terms of inclusion-forming units (ifu) was found to be greatly influenced by two main aspects of the assay procedure. Firstly, it was necessary to reduce variation in counts between replicate cultures by adopting a standardized counting technique. Secondly, it was obvious that even slight differences in the physiologic condition of the culture medium (including transport medium) brought about a reduction in inclusion count. In this respect conditions were sought that would maximize the infectivity titre.

At X500 magnification the area of the microscope-field was  $0.05 \text{ mm}^2$ , this represented a very small fraction of the total

infected area ( $132.7 \text{ mm}^2$ ). The count was complicated by the angle at which the coverslip lay in the culture vial and the effect this had on the distribution of seeded cells. A later experiment (6.1.2) showed that the count of ifu was directly proportional to the concentration of seeded cells. The problem of areas of high and low cell densities might have been eliminated if a high concentration of cells were seeded, which would have effectively eliminated areas of low cell density. However it was found that too high concentration had deleterious effects on inclusion counts, the reasons for which are discussed later. It was calculated that a count of 100 fields from 3 replicate cultures would give an accurate estimate of the total number inclusions on the whole infected monolayer. In practice this gave a standard error from the mean of less than 10 per cent. The only way to overcome the problem of unequal cell distribution was to count infected cells equally from different parts of the monolayer.

Results from Table 6.3 show that the type of pipette used was not a significant cause of variation. The 'Volac' glass pipette which dispensed  $200 \mu\text{l}$  produced marginally less variation. This may be accounted for by the larger number of inclusions counted, compared with the  $100 \mu\text{l}$  pipette, and therefore gave a more stable statistical result. In all the infectivity assays the dilution of stock chlamydiae was adjusted to give a convenient number of inclusions to count per field and to prevent the chance of a single host cell being infected with more than one chlamydial EB. Blyth et al (1972) showed that

BHK-21 cells infected with more than one chlamydial particle showed only one inclusion 30 hrs. after infection. Inclusions within the same infected cell tend to coalesce giving spuriously low results.

There was no significant advantage in using Giemsa, iodine or methylene blue. Rice (1936) originally demonstrated that inclusions of C.trachomatis were in a glycogen-like matrix which stained reddish-brown with iodine. However two precautions were later found to be necessary using this stain making iodine less attractive for routine use. Hanna (1977) pointed out that in cell culture the presence of a high concentration of serum with a high lipid content, may result in iodine-stained light brown masses within noninfected cells. Bernkopf (1962) studied the time course of the appearance of the carbohydrate in the inclusion bodies of the TE 55 strain grown in FL cells. It was found that the number of inclusions which were stained with iodine increased sharply during a 10 hr. period starting 20 hr. post-infection. The inclusion bodies gradually became "negative" iodine staining, indicating that the carbohydrate was degraded. Using the iodine staining technique appears to require counts made at specific times post-inoculation, which may vary between serotypes. Among the reasons for finally adopting Giemsa as a routine stain were:- infected coverslip cultures could be methanol fixed and held in this state for hours; if a pH 6.8 buffer is used for diluent, staining was found to be reproducible and uniform; the stain is available commercially; it was also possible to detect Mycoplasma

contamination of stock cell cultures.

In the review section 2.4.1 the importance of centrifugation for initiating infection of 'slow-growing' strains in cell cultures was discussed. The two important considerations that emerged were firstly centrifugation at the forces used probably only affected the overall structure of the cell membrane, secondly the process of chlamydial absorption and engulfment are temperature dependent. In this work two centrifuges were available for isolation purposes, one was controlled thermostatically but had a small capacity, the other had a much larger capacity but worked at ambient temperatures. In the latter centrifuge the temperature of the tube carriers increased noticeably during centrifugation. It was possible that this temperature rise might inactivate EB's and consequently affect isolation rates. To ensure that a loss of viability was not occurring the inclusion count obtained in the larger GF8 centrifuge at ambient temperatures was compared with the count in the thermostatically controlled (35 - 37°C) Mistral 2-L centrifuge. Results from table 6.5 show that there was no significant difference in inclusion count between the two centrifuges used. Centrifugation at 4 - 10°C brought about a dramatic decrease in infectivity emphasising the dependence of this process on temperature.

The centrifugal force used in this experimental work (1890g) was the maximum attained by the centrifuges. Although the centrifugal force used in this study was slightly lower than previous studies elsewhere, there is evidence that once a critical

centrifugal force is reached, somewhere between 1000-2000g further increases minimally enhance infectivity. Griffith et al (1976) observed that the application of increasing centrifugal force up to 1000g enhanced inclusion titres, but above this the titres were not significantly different.

The growth medium for McCoy and HeLa 229 cells was Eagle's MEM with Earl's salts supplemented with 10 per cent foetal bovine serum. Under normal cell growth conditions when cellular CO<sub>2</sub> output is high this buffered solution maintained an approximately neutral pH for 2-3 days. When infected cell cultures were exposed to the atmosphere during passage procedures the medium rapidly became alkaline, shown by a colour change of the pH indicator. Rota (1977) demonstrated that the pH of the cell culture medium is critical in maintaining the viability of EB's. A pH change from 7.0 to 7.6 reduced the inclusion count from 100 to 5, and at pH 8.0 no viable chlamydiae were detected. Experience in this work has shown that the fluctuating pH of Eagles MEME decreased the infectivity of stock chlamydiae. Care was taken therefore to maintain a neutral pH when viable particles were being manipulated. In practice this meant that cell monolayers were washed prior to inoculation or sub-culture with PBS pH 7.0 supplemented with 10 per cent foetal bovine serum (fbs).

The use of 5 to 10 per cent fbs is empirical. It may provide a source of nutrient factors such as essential amino acids, vitamins or hormones not available in commercial medium.

Alternatively the role of fbs may be physical, its macromolecular components acting for example as a protective agent or physiological buffer. Recently, (Karayiannis et al 1981) it has been demonstrated that foetal calf serum cannot be omitted from the medium of infected McCoy cells without adversely affecting the number, size and quality of C.trachomatis inclusions. The normal use of 10 per cent serum in growth medium appeared to be the optimal concentration, and decreasing the concentration gave a progressive decrease in inclusion count. When a serum filtrate containing low molecular weight compounds was used inclusion counts were again markedly affected. The physicochemical activity of the macromolecular fraction of serum could be minimised by supplementing the basic medium with a mixture of bovine serum albumin, sodium oleate and fetuin in amounts equivalent to their concentration in foetal calf serum. This mixture allowed maintenance of apparently normal McCoy cells capable of growing C.trachomatis to the inclusion counts normally reached in media with 10 per cent calf serum.

The choice of cycloheximide treated McCoy cells for routine culture attempts was based on their greater susceptibility to infection and on technical simplicity. The enhanced infectivity produced almost three times the total inclusion count obtained for untreated cells, and was significantly more sensitive than cycloheximide treated HeLa 229 cells. It is possible however that the reduced count in HeLa cells was due to a greater adaption of the chlamydial strain to McCoy cells resulting from previous passages. Allan et al (1979) found that organisms passaged through monolayers of the guinea-pig conjunctiva showed a greater capacity

to infect static monolayers than organisms grown in the chick embryo. The results strongly suggested that egg-grown organisms (EGO) and cell-grown organisms (CGO) are two homogenous populations in respect of their infectivity properties, differing in their capacity for spontaneous infection. EGO organisms purified by density-gradient centrifugation showed the same low spontaneous infectivity which suggested that the presence of yolk-sac material in EGO was not inhibitory.

This work raises the question whether animal or tissue cells endow chlamydiae with a host antigen during replication or release. Their results show instances in which the host appears to exert an effect on chlamydial properties. Although the isolate 1/382 had a low number of passages in McCoy cells, this may have been sufficient to confer phenotypic adaptation to these cells and thus a lower infectivity titre in HeLa 229 cells.

The mechanism of cycloheximide enhanced infectivity in McCoy cells still remains empirical, although recent work suggests a possible role. In a study of the competition between C. psittaci and L cells for isoleucine, Hatch (1975) suggested that chlamydiae compete inefficiently with the host cell for precursors of macromolecular synthesis. It was postulated that the addition of cycloheximide may spare essential precursors in the soluble pools of the host for the biosynthetic needs of chlamydiae. Over the last twenty years a careful assessment of the unique intra-cellular environment in which chlamydiae replicate has been made by J.W. Moulder (1969) and coworkers at the University of Chicago. Some of their observations may explain the results

in cell seeding concentration experiments (Figure 6.1).

Figure 6.1 shows that at a high seeding concentration of McCoy cells ( $5 \times 10^5$  cells  $\text{ml}^{-1}$ ) the inclusion count drops dramatically. The ecological model of the L cell - C.psittaci relationship originally proposed by Moulder and modified by Hatch (1975) suggested there are three competing species within the environment of the synthetic medium,  $S_1$  the chlamydial cell;  $S_2$  infected L cells in which  $S_1$  reside, and the L cell population surrounding infected L cells. The important results from a series of experiments carried out at the Chicago Laboratory indicated that the growth-promoting threshold of isoleucine in the culture medium was independent of the multiplicity of infection and highly dependent on the L cell density. There is strong evidence to suggest that only when the level of an essential amino-acid rises above that needed to maintain the L cell in a stationary state or when host utilization of isoleucine is blockaded by cycloheximide is C.psittaci able to sequester isoleucine for its own use. When essential macromolecular precursors are unavailable a latent phase of intracellular infection appears to be established. The reduced inclusion count seen with high seeding McCoy cell densities may be an indication of latent infection.

Pulse labelling of emetine treated infected cells demonstrated that during the initial 18 hrs post-infection it was not possible to distinguish between C.trachomatis and host protein synthesis. However the rate of chlamydial protein synthesis sharply



increased during the period 20-42 hrs. post infection. It would be at this stage in the chlamydial life-cycle that intermediary metabolites be at the growth-promoting threshold concentration. At high McCoy cell density the competition for essential amino-acids may result in their rapid decline, and at the critical 20 hr. post-infection stage, chlamydiae may be in an effectively incomplete medium Karayannis (1981) has shown that Cys, His, Leu, Ile, Phe, Tyr and Val are all essential for inclusion development.

### TRANSPORT MEDIUM

The sensitivity of EB's to slight physicochemical changes in the immediate environment poses one of the major problems in isolating these organisms from clinical specimens. This is particularly important where specimens are sent from outlying areas to a central reference laboratory. Prentice et al (1977) examined the factors influencing the survival of chlamydiae after freezing. From the data presented the preservation of laboratory-grown organisms was best achieved through the use of sucrose as a cryprotective agent. Dimethyl-sulphoxide and glycerol were found to be more toxic. It was pointed out that the period of exposure to sucrose before freezing must be kept as short as possible and be at 4°C rather than at room temperature. 2SP, a 0.25M sucrose solution in phosphate buffered saline has been widely used as a transport medium for chlamydiae and rickettsiae. Of the temperatures examined in this study 2SP was most effective at maintaining chlamydial viability at approximately -60°C. This level of freezing is generally not available at clinics and

certainly would not be achieved during transportation. At these stages in chlamydial isolation the toxic effects of sucrose may adversely affect the viability of chlamydiae (Figure 6.2).

An unexpected finding was the high level of survival of chlamydiae stored in PBS and fbs for 36 hrs. at wet ice and domestic refrigerator temperatures. This medium may be suitable for relatively short term storage, but residual enzymic activity at these temperatures may eventually lead to autolysis (Figure 6.3).

The problem of sucrose toxicity at temperatures  $> -60^{\circ}\text{C}$  may in future work may be best avoided by using phosphate buffered saline supplemented with foetal bovine serum. This might be followed by the addition of 2SP after the specimens have reached the laboratory where longer term storage at  $\leq -60^{\circ}\text{C}$  is possible. At these temperatures chlamydiae remain viable for several years if necessary with only slight losses in infectivity.

### 10.3 PREPARATION OF CHLAMYDIAL ANTIGEN FOR SEROLOGY

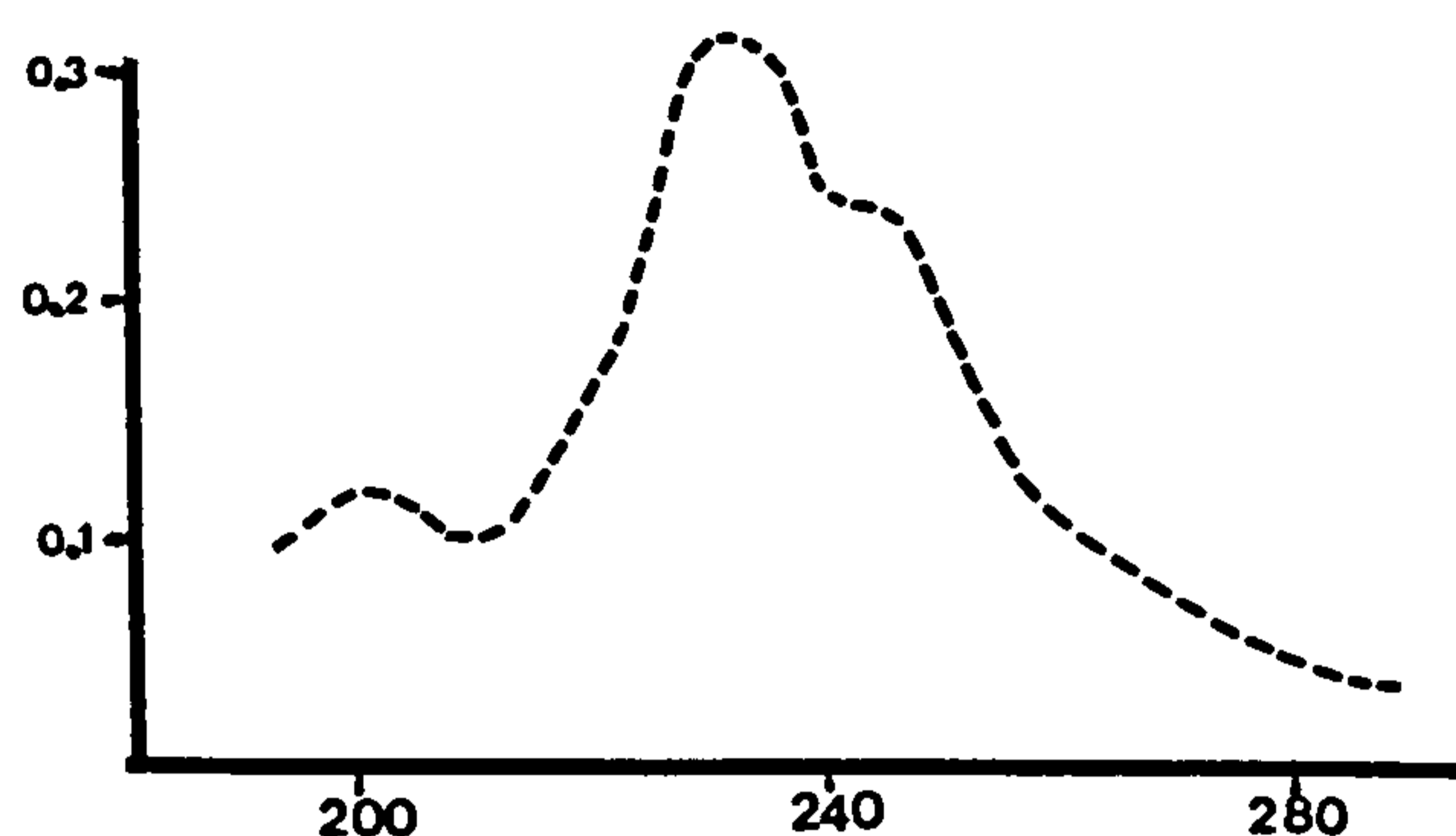
In preparing chlamydiae for serological tests either from infected yolk-sacs or from cell culture a purification technique was required which would free the organism from host material and concentrate the relevant antigens (EB's and RB's). The techniques examined in this study involved centrifuging infectious supernatant through a high density cushion allowing chlamydial particles to collect at the bottom of the centrifuge tube. The choice of solute was important:- (a) for bringing about efficient separation of host material from chlamydiae, (b) for not affecting the viability of the

organism. A loss of viability may indicate that outer membrane antigens have been denatured and consequently specificity may be lost.

The most commonly used solutes used for ultracentrifugation may be divided into the following groups:- (1) low molecular weight (mw) sugars notably sucrose (2) high mw polysaccharides eg. Ficoll (3) salts of alkali metals eg. caesium chloride. All these groups show deficiencies. Concentrated sucrose solutions tend to be lethal to living cells and may damage intracellular structures such as mitochondria. The osmotic pressure of Ficoll solutions rises exponentially with Ficoll concentration and again may adversely affect chlamydial viability. Salts of alkali metals are unsuitable for most biological separations as protein-protein and nucleic acid-protein bonds are dissociated in solutions of high ionic strengths.

A wide range of compounds have been tested as density-gradient media. Among the compounds tested the iodinated organic compounds used as x-ray contrast media appear especially suitable. A good contrast medium must be capable of forming solutions of high density and low viscosity and must be non-toxic. In many purification experiments in this present work Urografin was used as a 25 per cent (v/v) solution in PBS. Urografin contains mixtures of sodium and methylglucamine salts of diatrizoic acid.

EB's centrifuged through a 25 per cent Urografin solution maintained high levels of infectivity on reinfection in McCoy cells. The main disadvantage of these compounds was cost and the high absorbance of iodinated compounds at wavelengths less than 300 nm.



Ultraviolet absorption spectrum of solutions of Urografin

In all the EIA procedures and fluorescent microscopy work wavelengths greater than 405 nm were used. Residual amounts of Urografin present in chlamydial suspensions affected the integrity of antigen clusters in the micro-IF technique. Many 'dots' were lost during the washing procedures. Excess Urografin was subsequently removed by washing chlamydiae in PBS.

One of the first difficulties encountered in preparing infected yolk-sacs was how to initiate infection. Antibiotics were possibly being introduced to chicken feed which enters the yolk-sac and may have affected chlamydial replication. To test this hypothesis samples of inoculated yolk-sacs were introduced into wells cut in agar previously seeded with a tetracycline sensitive strain of Staph.aureus. The plates were incubated at 37°C for

24 hours and examined for zones of inhibited bacterial growth. There was no evidence of such inhibition in yolk-sac filled wells.

The diluent used in the first series of passages in yolk-sacs consisted of medium 199 supplemented with 20 per cent (w/v) sorbitol, 10 per cent foetal bovine serum and antibiotics. This was then changed to 2SP. Subsequent passages after this change showed increasing levels of infectivity. Whether this change in infectivity was due to the diluents used or coincidental is not known. Jawetz (1962) has reported a seasonal insusceptibility of embryonated eggs to C.trachomatis which lasted during the summer months. The presence of an interfering virus or of interferon was considered as a possibility. The presence of a virus was not shown. Nutritional or hormonal changes were also possibilities but there still remains no answer.

When infected yolk-sacs were finally obtained and graded 1+ (minimum) to 4+ (maximum) different purification procedures were examined. Experiment 7.7 showed that chlamydial particles could be separated from a great deal of contaminating egg materials by centrifuging through 25 per cent Urografin. Chlamydial particles observed in Giemsa-stained preparations revealed little contaminating egg material. The best preparations were obtained from heavily infected yolk-sacs. When plaquing antigens for the micro-If test prepared in this way were resuscitated from storage in nitrogen, diluted and inoculated into cell culture a high level of infectivity was seen. A  $10^{-1}$  dilution of such material would often show 90 per cent of

available cells infected with many particles seen extracellularly. The viability of chlamydiae after inoculation suggests that important surface antigens associated with virulence have not been altered.

Heavily infected cell culture grown chlamydiae purified in a similar way through Urografin provided a suitable antigenic preparation for the CFT and an EIA. Using a similar purification protocol for C.psittaci McClenaghan (personal communication) examined the final suspension by EM. There was very little contaminating material seen with numerous 300-1000 nm particles present.

#### 10.4.1 SEROLOGICAL TECHNIQUES - complement fixation

In this study two serological techniques - complement fixation and micro-IF were used in seroepidemiological studies. Although CF is regarded as an insensitive technique for chlamydial serology, four reasons suggested that modified CFT used in this work was of interest:-

(i) Zissis modification of Takatsy's original procedure allows for greater sensitivity. In the first of three trials Zissis and Clinet compared the routine method (using 2 per cent erythrocytes) with the modified technique. 100 sera from hospital patients were tested against the following viruses:- Adenovirus, Herpes Simplex, Cytomegalovirus, Influenza A, Parainfluenza I, II, III, Respiratory Syncytialvirus and Mumps virus. With the routine method they found 1-2 per cent positive results, taking a titre greater than 1/32 as indicative of a recent infection. Good correlation was observed between the routine and the sensitive method, with the latter producing titres 4-5 times higher (depending on the antigen) although only 1-2 per cent higher than 1/128 - 1/256.

Another trial involved 50 preselected sera (titres greater than 1/32 with the routine method) which were assayed by the modified CFT. In all cases the antibody level was over 1/128 - 1/256, with extreme values over 1/4000.

In the final trial an attempt was made to detect antibodies to Coxsackie B (1-5); the routine method was not satisfactory in this

case. In contrast 20 paired sera examined with Zissis modification of the CFT showed a four-fold titre or greater increase in titres, with values in excess of 1/128. Generally the following advantages made this technique attractive for screening human sera:-

1. Stable readings which could be taken at different times, 1 hr at 37°C and even 12-24 hours at 4°C.
2. Reproducibility.
3. A smaller quantity of antigen was required (25µl of 1/32 diluted stock chlamydiae per well in this work).

(ii) Commercially prepared CF antigen is normally prepared from C.psittaci (agent of enzootic abortion in ewes ) grown in the yolk-sac of embryonated hen's eggs and is heat inactivated. Results from experiment 8.5.1 showed that chlamydiae for the CFT were best prepared in cell culture. Chlamydial suspensions at lower dilutions prepared from infected yolk-sacs showed anticomplementary activity. Heat inactivation of test antigens were not carried out. Caldwell et al (1981) have isolated and identified important outer membrane protein which appear to be serogroup antigens for C.trachomatis. Commercially prepared antigens may not carry this antigen, and if they were present they may have been denatured by heat inactivation.

Using whole organisms as test antigens results in a wide range of antigenic determinates being exposed to the serum sample. Throughout this work care was taken during purification and test



procedures not to destroy labile protein antigens possibly situated on the outer membrane. However the group specific lipopolysaccharide antigen is also important and infected individuals may show an antibody response to this determinant even in the superficial uro-genital and ocular infections. Richmond et al (1981) have demonstrated that the group antigen is not restricted to the intact inclusion in cell culture. Using an indirect immunofluorescent technique antigen was seen to radiate from the host cell surface and was also seen on the single membrane vesicles probably outer membrane blebs produced by replicating chlamydiae. These results would suggest that in this work a considerable amount group antigenic material would remain in the supernatant after centrifugation through Urografin. However Dhir et al (1972) using a silver methenamine stain and EM located the group antigen at the periphery of purified organisms.

(iii) The final reason for using the CFT was based on previous seroepidemiological work carried out by Schachter et al (1977). A screen of human sera among 'normal' healthy adults showed that only 3 per cent had a positive antibody response by the CFT and 25 per cent by micro-IF. It appears that with the enhanced sensitivity of the micro-IF technique there is also a much higher 'background' seroreaction rate. The high rate by micro-IF in healthy adults is difficult to explain. Similar results were obtained in this study for example in a group of ante-natal patients, the CF reaction rate was 9 per cent whereas the micro-IF rate was 25 per cent (Table 9.8. and 9.10).

It is possible that the micro-IF test is detecting a cross-reacting antigen from another source. There has been no published evidence supporting this suggestion for type specific antigens. However Mikami (1969) has reported cross-reactivity between C.psittaci and Bacterium anitratum (Herellea). Crude biochemical tests suggested there was some similarity between cell wall antigens of the bacterium and the group antigen of C.psittaci.

Another possible explanation for the lower CF reaction rate may be related to the class or sub-class of antibody detected. The initial molecular events which take place before complement is finally 'fixed' involves an alteration of the Fc sites on the immunoglobulin. The altered Fc sites bind to the C1q fraction of the C<sub>1</sub> component of complement. Each C1q must bind to at least two Fc sites and this means that there must be several IgG molecules spatially close together on the immune complex. With IgM, several Fc sites are present on a single molecule and IgM therefore activates complement much more efficiently than IgG. On the basis of experimental animal infection IgM specific antichlamydial antibodies appear early in chlamydial infection are transient and are not recalled unless the reinfection is with a heterologous serotype. The micro-IF test may on the other hand detect low levels of IgG molecules produced by primed lymphocytes or in response to asymptomatic infection.

Only serotypes D to K inclusive were used in the micro-IF test in this work. San-pin Wang (1977) determined the serotypes of 563 isolates, 312 of which were associated with a genital epidemiology. 93 per cent of the genital isolates fell into one of the types D-K. The majority of this last group (77.5 per cent) belonged to serotype D/E or F/G. The reaction pattern of patients sera with different immunotypes was also investigated. Homologous reactions were observed among the following serotypes:- C/J, D/E, G/F; A, H, I and K appeared to have little cross-reactivity. It is interesting to note that sera from LGV patients cross-reacted with all serotypes to a greater or lesser extent.

More recently Caldwell et al (1981) isolated and identified by indirect immunofluorescence a 39.5 K dalton major outer membrane protein from serotype L<sub>2</sub> which reacted with sera to types E, D, K, L, L<sub>2</sub> and L<sub>3</sub>, but failed to react with serotypes A, B, C, F, G, H, I and J or with C.psittaci strains. It was concluded that the 39.5K dalton major outer membrane protein is a serogroup antigen of C.trachomatis.

In this study the micro-IF test failed to show single serotype reactions using patients' sera. Where more than one serotype showed fluorescence it was difficult to determine type specificity even at higher dilutions of patients' sera. With the degree of cross-reactivity described above by micro-IF

and for the 39.5K dalton antigen no attempt was made to identify serotype reactions using patients' serum. Titres were expressed as the highest dilution of serum reacting with a single or more often multiple serotype.

## 10.6

### FREQUENCY OF CHLAMYDIAL INFECTION IN DIFFERENT GROUPS OF PATIENTS

#### FEMALE PATIENTS

##### 10.6.1 ANTE-NATAL PATIENTS

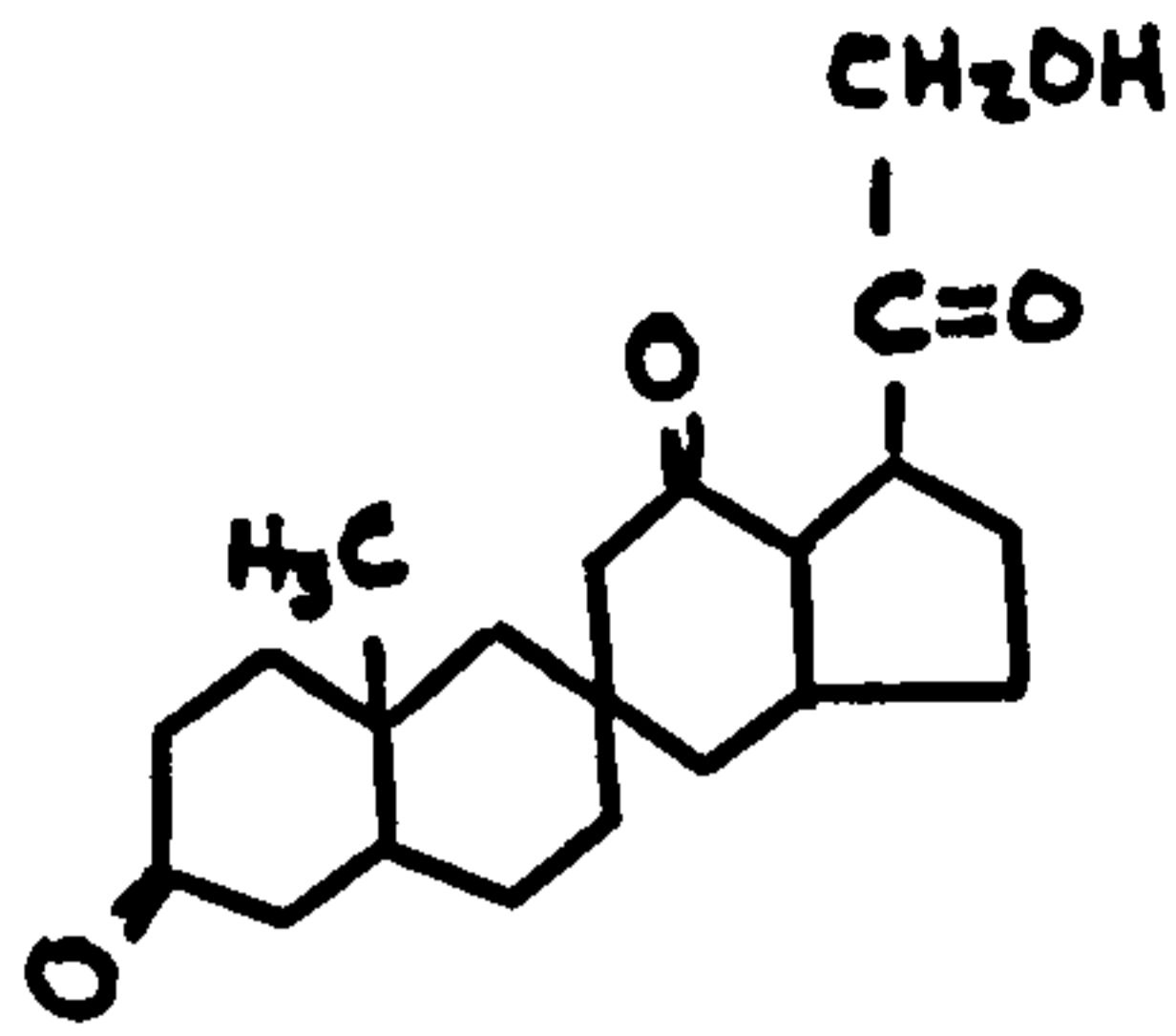
Five per cent (44/916) of women examined in this group over a 20 month period were shown to have chlamydiae infected cervixes. The complement fixation test suggested that slightly less than 10 per cent had previous exposure to C.trachomatis and the micro-IF test 25 per cent. The isolation results correlate with figures obtained by Schachter et al (1974) in a survey carried out in San Francisco. As with this study all women were tested for infection during their first trimester of pregnancy. Schachter's obstetric population was heterogeneous comprising 35 per cent white, 15 per cent black, 25 per cent Latin, 20 per cent Oriental and 5 per cent other ethnic groups. The chlamydia positive women were more likely to be black. This finding probably reflects socio-economic differences in prevalence of sexually transmitted pathogens.

Other studies in the U.S. have shown higher rates of chlamydial carriage in pregnant women. Alexander et al (1977) found 13 per cent (18/142) and Frommell (1979) 9 per cent (30/340). These studies differed from this one and Schachter's in that cervical specimens were obtained in the last trimester of pregnancy. In the U.K. Hilton et al (1974) examined 37 pregnant women who were also

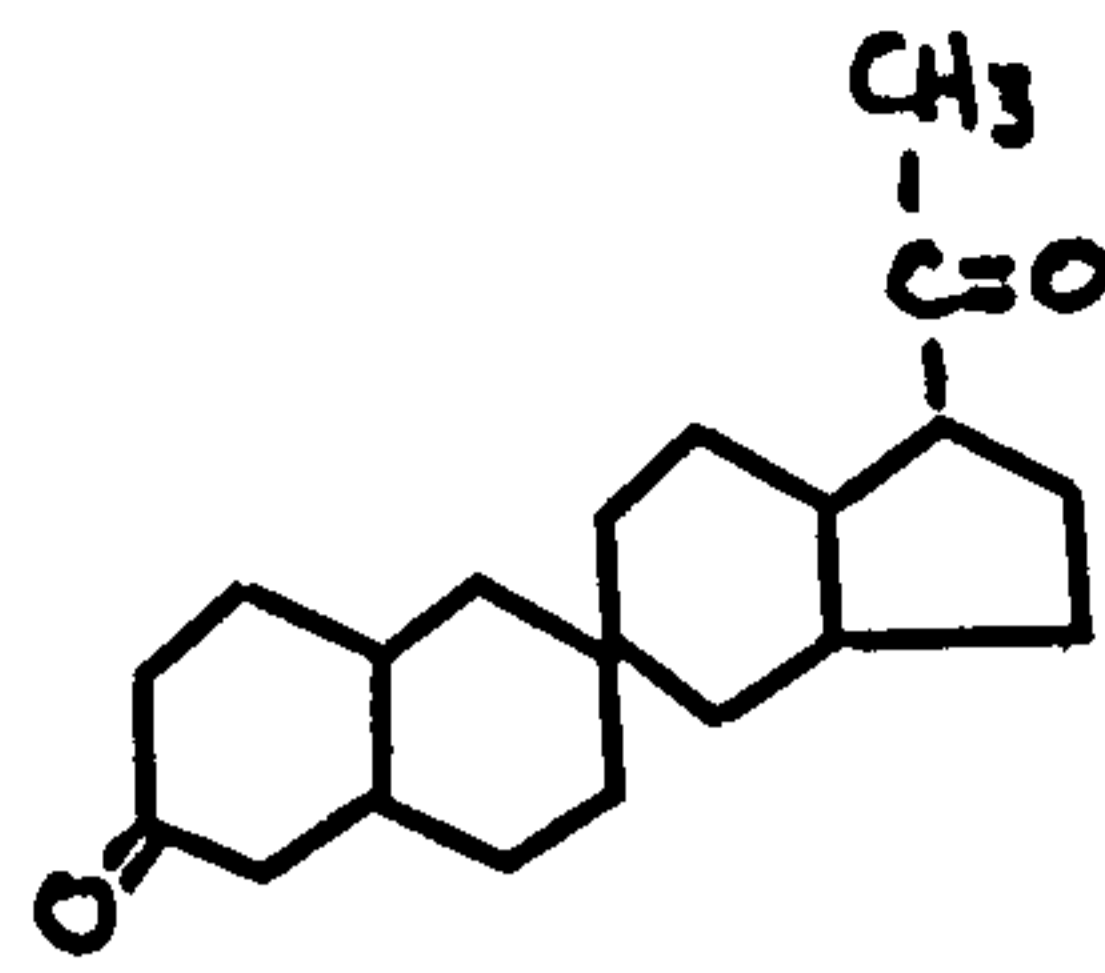
attending a G.U.M. clinic. 13 per cent (7/57) were culture positive; all were in their last trimester. This figure is considerably lower than the 38 per cent isolation rate (20/53) obtained by Goh et al (1982) in a similar group of patients.

The difference in isolation rates between the first trimester and the last is significant -  $\chi^2 = 6.5$   $p < 0.05$  (Frommell 1979 compared with this present study.) These results suggest that the variation of hormonal level during pregnancy amongst other factors may affect the outcome of isolation attempts. Studies on current chlamydial infection in women using oral contraceptives strengthen this hypothesis. Hilton et al (1974) found that almost twice as many women using oral contraceptives (45 per cent) were culture positive as non-users (24 per cent) from an unselected G.U.M. population. The isolation rate also appeared to vary according to the number of weeks that had elapsed since the first day of their last menstrual cycle.

The association between in vivo effects of orally active synthetic progestational agents, ovarian hormone levels during pregnancy and chlamydial infectivity may also be valid in vitro. Experimental results (Table 6.6) showed that treatment of McCoy cells with cortisol increased the mean ifu count from 308 in untreated cells to 483. Cortisol is biochemically related to progesterone:-



CORTISONE



PROGESTERONE

The molecular events which cause the increased infectivity in pre-treated McCoy cells are unknown. The effects of ovarian hormones on chlamydial infectivity in vitro have not been investigated. It is interesting to note that one of the physiological effects of circulating ovarian hormones (particularly oestrogen) is on the extent of cervical ectopy. The replacement of the squamous covering of the ectocervix by columnar epithelium may simply offer a larger area for colonization by chlamydiae.

An assessment of chlamydial infection rates in pregnant women is important for two reasons:- firstly the baby may become infected during birth and secondly the infected mother may have a greater risk of developing an ascending infection. Whereas inclusion conjunctivitis appears to be a self-limiting disease in the neonate the longer-term effects of an ascending infection are more serious. Rees et al (1977) carried out a prospective study of 24 mothers of babies with inclusion conjunctivitis. Seven had infected lochia or were pyrexial, five of the remaining 17 mothers developed clinical pelvic infection. Apart from the immediate morbidity, subsequent tubal

occlusion may cause infertility and there may be an increased risk of subsequent ectopic pregnancy in partially occluded tubes. The attack rate of 53 per cent (9/17) for pelvic infection seems particularly high. Recent work from Sweden (Westrom, 1982) suggests that 5 per cent of these women may have further complications. The organism may enter the peritoneal cavity and spread directly to cause periappendicitis and perihepatitis (Fitz-Hugh Curtis Syndrome). By extrapolation of these results to this present study, approximately 20 women would develop pelvic inflammatory disease and one would have abdominal complications (n = 916).

#### 10.6.2 COLPOSCOPY PATIENTS

The patients referred to the colposcopy clinic in this study had grades of smear equal to or greater than 3. The basic criteria for cytodiagnosis lie in the changes seen in the chromatin of cervical epithelial cells. Dyskaryosis of this type is well shown by the haematoxylin nuclear stain used in the Papanicolaou schedule. The most significant changes however are seen in the combined enlargement of the nucleus and the overall reduction in cell volume, so that the altered nuclear cytoplasmic ratio becomes the most distinctive feature.

Cells stained by the Papanicolaou method allow the smear to be graded as follows:-

- Grade I ; apparently normal cells
- II : inflammatory hyperplasia



- III : dysplasia: this grade includes specimens in which there were insufficient abnormal cells to assign the specimen to a more malignant grade
- IV : carcinoma in situ
- V : invasive carcinoma

As a control for colposcopy patients the antenatal (AN) group may be useful. It is assumed that the majority of AN patients had grade I and II smears. In the colposcopy group 70 per cent were grade III, 22 per cent grade IV and 8 per cent grade V (n = 176). The overall chlamydial isolation rate for the AN and CD groups were 5 per cent and 7.4 per cent respectively. This was not a significant difference ( $\chi^2 = 1.73, p > 0.05$ ). However when one examines the serological evidence of infection the results show that women with cervical abnormalities had a significantly higher probability of having antichlamydial antibody ( $\chi^2 = 19.6, p < 0.001$ ). (Table 9.10). No significant variation in antibody titre was found between women in different smear categories (Figure 9.4). There was only a two year difference in median age between AN and CD patients, the latter group being older. (Table 9.12).

The apparently high seropositive rate among CD patients indicates past exposure to C.trachomatis. The risk of cervical cancer is generally thought to be related to sexual behaviour and promiscuity. Skegg et al (1982) have suggested that the sexual behaviour of her male partner(s) is also important. Female attenders at G.U.M. clinics show a high proportion (30 per cent

between 15-19 years and 63 per cent under 24 years) who acquire chlamydial infection at a relatively young age (Table 9.3). Women in the CD group were older (Median age 29 years) by which time chlamydial infection may now be sub-clinical, latent or cured. This may explain the relatively low isolation rate seen in the CD group.

Of the several theories which try to explain the aetiology of cervical intraepithelial neoplasia (CIN) probably the most solid evidence points to a sexually transmitted virus. In situ hybridisation studies have shown that Herpes Simplex (HSV-2) specific RNA exists in the majority of samples of cervical carcinoma, whereas it can be found only in 2 per cent of samples of non-neoplastic epithelium. Also, specific herpes virus antigens are present frequently in cervical cancer cells and also in cultured cells transformed by HSV-2 DNA. (Aurrelian et al 1980).

More recently, interest has centred around the discovery that many cervical dysplasias co-exist with human wart virus even in the absence of visible epithelial changes. However it is not known whether the virus produces lesions which mimic cervical dysplasia or can be causally related to the onset of dysplasia.

Although chlamydial nucleo-proteins are not exposed to the host cellular genome to the same extent as viral DNA during replication, they are intracellular parasites and may persist in a 'latent form' for an indefinite period. Taken together

with the seroepidemiological evidence this fact strongly suggests that chlamydiae alone or concurrently with viruses have some role to play in the development of CIN.

### 10.6.3 FEMALE ATTENDERS AT A G.U.M. CLINIC

Results from Table 8.2.2. show that 52 per cent (29/55) of culture negative patients had titres  $\geq 1/16$  by micro-IF. The high seroreaction rate in these patients together with the relatively low GMT (1/29.8) in culture positive patients exclude serodiagnosis on a single specimen. The calculated GMT was lower than expected because some culture positive patients had no detectable specific IgG. The likelihood of demonstrating seroconversion using acute and convalescent serum is small. Among female patients chlamydial infections of the cervix tend to be sub-clinical for a number of weeks. Peak antibody titres may already have been reached when they attend the clinic.

Another approach is to measure serum IgM by micro-IF. The data published so far suggests that a substantial proportion of culture negative patients have detectable IgM. Schachter et al (1979) found 19 per cent (14/74) culture negative women to have IgM. Richmond and Caul (1975) have also shown that IgM persists in some culture negative patients and therefore may not be a marker for current infection.

The detection of IgM by micro-IF is complicated further by the presence of rheumatoid factor in some patients sera. (An anti-IgG antibody often of the IgM class). Also, if both IgG and IgM are present in serum IgG competes with IgM for the same antigen binding sites. IgG can be absorbed from sera using Staphylococcus aureus protein A. However IgG<sub>3</sub> which

constitutes 5 per cent of normal serum remains and 30-60 per cent of IgM present may also be lost during this procedure.

As a diagnostic criterion in chlamydial serology IgM detection is a new field which requires more detailed analysis and further work. Perhaps reported data suggesting that IgM is present in the absence of chlamydial isolation reflects the limitations of the isolation technique itself. The detection of IgM by the FAT is also subject to interpretative error. Some of the non-specific reactions recorded using immunofluorescence may not be encountered if an EIA technique is used. It has been shown that EB's readily adsorb to EIA plates if a pH 9.6 coating buffer is used. In future work this method may provide another sensitive way of detecting IgM. Although the EIA used in this present study did not have the antigenic diversity of the micro-IF technique, Richmond and Caul have presented evidence in favour of a single serotype test. Their study was based on a system which used the whole cytoplasmic inclusions found in McCoy cells infected with serotype E. It was shown that the single antigen IF test provided a simple sensitive method for screening large numbers of sera. For example 88 per cent of culture positive men suffering from NGU had chlamydial antibodies. In my study Figure 8.11 shows that there was a high degree of correlation between a modification of Richmond and Caul's single antigen FA technique and the EIA used here. The EIA had the advantage, however, of providing a measurement of antibody level on a single serum dilution, with the possible disadvantage of a complicated

calculation to work out the correlation between optical density and IF antibody titre.

Results from Table 9.3 show that almost one third of female patients with a primary diagnosis of gonorrhoea had a simultaneous chlamydial infection. This confirms previous results by Hilton et al (1974) and Ridgeway et al (1977). There has been little published information referring to concomitant infections. Evidence from trachoma endemic areas and from experimentally induced infections suggest that the presence of associated bacterial infections greatly influence the pathogenesis of these diseases in the eye.

Darougar et al (1974) have shown that C. psittaci (feline conjunctivitis agent) and Staphylococcus spp. together led to a more severe keratoconjunctivitis than either inoculum alone in experimentally infected cats. In human cases of trachoma, Haemophilus aegypti, H. influenzae and Staph. pneumoniae were commonly isolated in association with C. trachomatis. In vitro studies have also shown that chlamydial infectivity in human cervical epithelial cells was enhanced in the presence of Neisseria gonorrhoeae. Mixed infections abound making it practically impossible to consider all interactions. The initial investigative strategy might use organ cultures and might restrict investigations to other bacteria found in the vagina or urethra.

The incidence of neonatal inclusion conjunctivitis in the Eastern District of Glasgow over a two year period was 15 per 1000 live births (Table 9.7.). On the basis of the incidence of chlamydial infection in the unselected obstetric group (5%) this rate would seem to be low. A rate approaching 50 per 1000 could be predicted. However work by Alexander et al (1977) and Schachter et al (1979) in Los Angeles suggested that the attack rate of inclusion conjunctivitis in babies from predominantly black mothers known to have chlamydia infected cervixes is about 40 per cent. It is interesting to note at this point that conjunctivitis developed in those babies all of whom were treated with silver nitrate (Crédé prophylaxis). If this same attack rate is applied to the 5 per cent carriage in the AN group then 18/1000 cases would be predicted. However Schachter also found that 65 per cent (15/20) of babies born to infected mothers had laboratory evidence of chlamydial infection but only 40 per cent (7/20) had clinical disease. This means that a substantial proportion (25 per cent) of babies with chlamydial infection of the conjunctiva are not identified and may not be adequately treated.

A major development over the past five years has been the recognition of a neonatal pneumonitis associated with C. trachomatis in the U.S.A. The illness has distinctive symptoms:- typically the baby is under 3 months old and has a staccato cough with especially bad episodes occurring

once or twice a day. There appears to be no fever but a chest x-ray shows hyperexpansion with interstitial infiltrates. C. trachomatis has been isolated from nasopharyngeal secretions (NPS) and there is production of specific IgG and IgM. The initial report by Schachter was supported by Beem and Saxon (1977) and Frommell (1977). Similar findings have not been reported in the U.K., where the incidence is extremely low.

Oriel et al (1982) have suggested that the main reason for an apparently very low level or even non-existence of chlamydial pneumonitis in the U.K. is related to the type of medical care practised. Antibiotics such as erythromycin, are freely available and prescribed by general practitioners in cases of infantile neonatal respiratory infection in this country. In the U.S., hospital-based paediatricians make the diagnosis with the backing of full laboratory services. The microbiological and radiological data associated with the disease will therefore be more readily identified. Although specific surveys were not carried out in this study, clinicians were aware of the findings from the U.S. From time to time NPS's were received from cases of neonatal pneumonitis but no positive cultures were obtained. Community midwives did not report the occurrence of a pneumonia syndrome associated with inclusion conjunctivitis.



## 10.7

## MALE PATIENTS

### 10.7.1

### REITERS SYNDROME PATIENTS

The reactive arthritides have been associated in the past with enteric bacterial infections (for example: Shigella flexneri, Salmonella spp. and Campylobacter jejuni.) The proportion of patients developing sterile synovitis or enthesopathies following bacterial infections is generally in the region of 2 per cent. A similar syndrome has been described following infection with a sexually acquired bacterium. In this case genital inflammation is an essential feature. In the present study an attempt was made to determine any possible chlamydial association in this highly selected group of patients.

It was found that among RS patients, there was a higher probability of isolating chlamydiae from urethral specimens if symptoms were being experienced for the first time. Twenty per cent (4/20) A<sup>+</sup> patients had active chlamydial infections. This was not significantly different from the prevalence rate in the NGU group (Table 9.4.). However the GMT's of specific antichlamydial antibodies were significantly different between these groups; they were 1:30 in the RS group and 1:6.9 in NGU patients, respectively. Keat et al have examined a similar group of RS patients all of whom had symptoms for less than 3 weeks. It was found that there was a high level of current infection (36 per cent 11/30) and a GMT of 1:47.5. It was suggested by Keat that the exaggerated humoral immune response to

chlamydiae in some patients with reactive arthritis was a unique characteristic and may be important in the development of the disease

More than half (22/43) of the patients examined in this study were experiencing symptoms for the second (or more) time. There was a lower prevalence of active infection in these patients 4 per cent, (1/43) and the GMT was marginally lower (1:21) than A<sup>+</sup> patients. It was found that the patients with recurrent NGU (rNGU) had levels of specific antibody similar to the entire RS group. This suggests that elevated levels per se do not trigger the syndrome.

Two characteristics were seen among RS patients which were not present in the recurrent NGU group. These may be contributory factors in the disease process of the former group. In RS patients there was a higher possibility of having HLA B-27 antigen. Their consorts were found to have serological evidence of chlamydial infection.

Fifty-one per cent of RS patients possessed the HLA B-27 antigen compared with 12.5 per cent in rNGU patients. No direct association could be found between the possession of B-27 and antichlamydial antibody titre as measured by micro-IF. Keat has suggested that since high titres of antichlamydial antibody are associated with Reiter's syndrome and are also associated epidemiologically with HLA B-27 antigens, IgG and IgM levels are influenced by HLA linked immune response genes. However this present work has shown that rNGU patients generally have antibody levels similar to RS patients. This

suggests that among the known functions associated with Ir genes quantitative aspects of antibody production do not seem to be involved. For example, in the A<sup>+</sup> category, only one was B-27 out of three patients with micro-IF titres of 1:1024. Titres as high as this were not seen in any other category of patient.

One of the difficulties in interpreting these results lies in the complexity of the MHC system. Among the 1000 or so genes that make up the human MHC is genetic material coding for numerous immunological effector systems. One of the mechanisms postulated for HLA disease associations is the involvement of non-HLA genes, for example in rodents Ir genes (immune response) genes map close to the locus equivalent to HLA - D/DR. It is possible that B-27 is linked with genes involved in the immune response.

It is known for example that genetic material coding for complement components are located in the MHC. Deficiencies or functional impairment of any of the components of the complement cascade could lead either to failure or chlamydial lysis, or to the formation of circulating immune complexes. Either of these might then persist in the circulation and become deposited in the microvasculature of tissues such as synovium and blood vessel walls.

Briem et al (1980) investigated whether circulating immune complexes (IC) might contribute to the pathogenesis of reactive arthritis following enteric infections. However the techniques

for determining the presence circulating IC's are new. Although many different IC assay systems have been proposed it was clear that none is infallible. There appeared to be no true correlation between the clinical joint manifestation and the finding of circulating IC's.

The second characteristic associated with RS patients was that seventy-one per cent (15/19) of their consort had IgG titres 1/16, although none were greater than 1/32. This compares with 25 per cent in rNGU consorts. Isolation data for these patients were not available, but the results suggest that the majority of CRS have been exposed to chlamydiae and may continue to have low grade infection. If the organism were present it could be postulated that the female partner of RS patients provides a source of chlamydia antigen which on repeated exposure to their susceptible partner brings about an abnormal immune response.

At this time the mystery of HLA-linked disease susceptibility and reactive arthritis remains unsolved. The association of B-27 in patients with arthritis following enteric or chlamydial infection is well documented (Keat et al 1982), (Kosunden et al 1980) and (Brien et al 1980). It is generally agreed that there is involvement of linked non-HLA genes which have not been identified at this time.

#### 10.7.2

#### MALE G.U.M. CLINIC ATTENDERS

An overall isolation rate of 31 per cent (46/145) was obtained for this group of patients. A higher rate of 43 per cent

(19/44) was recorded amongst those who had a clinical history of of NGU. The diagnosis of NGU was usually made by one of four consultants. They may have used different criteria in their clinical assessment. These results confirm the now commonly held view that C.trachomatis is a major cause of morbidity in this category of patient. Although a control group of healthy males of comparable age was not available, patients who had no clinical history of urethral discharge had a significantly lower isolation rate (8.6 per cent, 3/39).

It was noted among female patients that 63 per cent of infected individuals were under the age of 24 years. This contrasts with the age pattern seen among infected male patients. The age range of the entire group was 15-62 years with a median of 29 years. Culture positive patients were distributed among the groups as shown in Table 9.13. Only 53 per cent of culture positive male patients were under 24 years.

The reasons for this apparent disparity of age related infection between the sexes are obscure. It may reflect a genuine difference in behaviour with females being more sexually active at a younger age. Alternatively it may be associated with the degree of difficulty in obtaining suitable material for culture from different anatomical sites (cervix and urethra). A comparatively higher level of infection may be required in the male urethra before laboratory procedures detect infection; this in turn may be either an age related phenomenon, or a difficulty in sampling.

The possibility that chlamydiae may ascend from a primary infection site in the anterior urethra to infect the epididymis has been demonstrated clearly by Berger et al (1978). Of six men aged less than 25 years suffering from epididymitis epididymal aspirates yielded chlamydiae in five. It was also shown that the majority of men with culture positive epididymitis usually had NGU.

There may be a causal association between epididymal infection and subfertility. T-mycoplasmas have been shown to occur with a higher frequency in the ejaculates and cervical secretions of couples with unexplained infertility than in fertile couples. Fowlkes et al (1971) used a scanning e.m. to examine spermatozoa. 200 nm diameter spherules attached to spermatozoa were seen in T-mycoplasma culture positive patients but were not seen in culture negative patients. It was assumed that the 200 nm spherules were sub-units of T-mycoplasma. The possibility that the spherules may in fact be chlamydiae was not discussed. It was suggested that a physical association between T-mycoplasmas and human sperm could affect reproductive capability. Three of 75 patients examined in this group had active chlamydial infection. However more detailed studies would be required to discover whether the presence of chlamydiae could be associated with each patient's sub-fertility. For example, this may be best achieved by examining spermatozoa for attached chlamydial particles by e.m. or IF.

## APPENDIX 1

### CONTRACTIONS USED IN THIS THESIS

AN	: Ante-natal patients.
Anti DK-20	: Antibody specific for serotype E (mouse).
Anti IDL-238	: Antibody specific for serotype G (mouse).
AS	: Ankylosing spondylitis patients.
BSS	: Balanced Salt Solution.
BUdR	: 5-bromo-2-deoxyuridine.
CD	: Cervical Dysplasia patients.
CFT	: Complement Fixation Test.
CGO	: Cell Grown Organisms.
COMC	: Chlamydial Outer-Membrane Complex.
crNGU	: Consorts of rNGU patients.
CRS	: Consorts of RS patients.
CT	: Treated Cells
D-K	: Serotypes D,E,F,G,H,I,J and K.
DGV	: Dextrose Gelatin Veronal Buffer.
EB	: Elementary Body.
EDTA	: Ethylene-Diamine-Tetracetic Acid.
EGO	: Egg Grown Organisms.
EIA	: Enzyme-immunoassay.
EM	: Electron Microscopy.
FAT	: Fluorescent Antibody Technique.
fbs	: Foetal bovine serum.
FP	: Family Planning patients.
g	: Gravitational force.
GM	: Genito-urinary Medicine patients.

GMT : Geometric Meant Titre.  
 GND : Gram Negative Diplococci.  
 GRI : Glasgow Royal Infirmary.  
 GRMH : Glasgow Royal Maternity Hospital.  
 GU : Gonococcal Urethritis.  
 GUM : Genito-urinary Medicine.  
 HLA : Human Leucocyte Antigen.  
 IC CAL8 : Prototype strain of C.trachomatis (D).  
 ifu : Inclusion forming unit.  
 IgG : Immunoglobulin G.  
 IgM : Immunoglobulin M.  
 IUCD : Intra-uterine Contraceptive Device.  
 IUdR : 5-iodo-2-deoxyuridine.  
 K-dalton : Kilo-dalton.  
 LGV : Lymphogranuloma Venereum.  
 Log<sub>2</sub> : Logarithm to the base 2.  
 MIC : Minimal Inhibitory Concentration.  
 micro-IF : Micro-immunofluorescence.  
 MEME : Minimal Essential Medium with Earle's Salts.  
 MI : Male sub-fertile group.  
 MLC : Minimal Lethal Concentration.  
 MTPT : Mouse Toxicity Prevention Test.  
 mw : Molecular weight.  
 NGU : Nongonococcal Urethritis.  
 NSGI : Non-specific Genital Inflammation.  
 PBS : Phosphate Buffered Saline.  
 PGU : Post-gonococcal Urethritis.  
 PID : Pelvic Inflammatory Disease.



P2M : Two passages in McCoy Cells.  
PMN : Polymorphonuclear Cells.  
r : Correlation coefficient.  
RB : Reticulate Body.  
rNGU : Recurrent Nongonococcal Urethritis.  
RS : Reiter's Syndrome Group.  
SD : Standard Deviation.  
2SP : Sucrose phosphate buffer.  
STD : Sexually Transmitted Disease.

## APPENDIX II

### STANDARDIZATION OF CFT REAGENTS

#### Haemolysin titration

This determined the concentration of haemolysin which in the presence of complement just lyses 1 unit of erythrocytes completely. An erythrocyte control was carried out simultaneously to eliminate non-specific lysis.

In Zissis' modified technique haemolysin was used at the optimal sensitising dose (OSD), the titre of haemolysin that gives most lysis with the highest dilution of complement.

The  $HD_{100}$  of complement is that dilution giving 100 per cent lysis with the highest dilution of haemolysin. In the test itself complement is used at  $2HD_{100}$ .

This two-dimensional titration determined the correct working dilution of complement and haemolysin. Different dilutions of each were simultaneously tested against each other. This titration was carried out each time a new batch of complement or haemolysin was used.

1. A 1/60 dilution of complement was prepared.
2. 1 ml of DGV was added to each of 11 bijoux.
3. 4 ml of 1/60 complement was added to the first bijoux mixed and then to further bijoux to give the following dilutions.

1	2	3	4	5	6	7	8	9
1/60	1/75	1/94	1/118	1/148	1/184	1/230	1/288	1/366

10	11
1/450	1/562

4. 0.025 ml complement from each dilution was added down the plate. The highest dilution was distributed first starting on the right hand side of the plate.
  5. 2 volumes of DGV were added to the plate to account for antigen and antiserum.
  6. The plates were covered and placed at 4°C overnight.
  7. A series of dilutions of haemolysin was prepared in 1 ml amounts.
- |       |       |       |        |        |        |
|-------|-------|-------|--------|--------|--------|
| 1     | 2     | 3     | 4      | 5      | 6      |
| 1/125 | 1/250 | 1/500 | 1/1000 | 1/2000 | 1/4000 |
8. 1 ml of 0.5 per cent sheep erythrocytes was added to each bijoux.
  9. The erythrocytes were sensitized at 37°C for 20 mins.
  10. The plate was taken for 4°C incubator and allowed to heat up to room temperature.
  11. 0.05 ml of sensitised erythrocytes was added to the appropriate wells across the plate starting with the highest dilution at the lowest row of wells.
  12. The plates were finally centrifuged at 1000 r.p.m. (GF8 centrifuge).

### CONTROLS

(a) Haemolytic serum controls:- a row of 6 wells each containing a dilution of haemolysin (1/250 - 1/8000) sensitised erythrocytes, was further filled with 3 volumes of DGV (0.025 ml each) to represent serum, complement and antigen.

(b) Complement control:- dilutions of complement (1/60 - 1/562) were aliquoted to 11 wells. Two volumes of DGV were added to each well to represent serum and antigen in the test proper. Control erythrocytes (without haemolysin) were then introduced to each well.

### Standardization of antigen and antisera.

It was necessary to obtain the optimal dilution of chlamydial antigen and the titre of the corresponding standard antiserum. Controls for nonspecific reactivity were included to confirm the specificity of the antibody reaction. DGV was used as diluent throughout.

1. Dilutions of the chlamydial suspension (antigen) from 1/2 - 1/128 were prepared in tubes (3 x ½ in. glass tubes).
2. 0.025 ml of antigen were added down the plate starting with the highest dilution on the right hand side of the plate.
3. Dilutions of inactivated reference serum (inactivated at 56°C for 30 mins) were prepared in tubes.
4. 0.025 ml of diluted antiserum were added across the plate starting at the lower end of the plate with the highest dilutions of serum.
5. 0.025 ml. of complement (2HD<sub>100</sub>) was added to all the test wells.
6. The plate was incubated at 4°C overnight.
7. 0.05 ml of sensitized cells at the OSD was added to each well.
8. The plate was incubated at 37°C for 1 hour.
9. Finally the plates were centrifuged at 1000 r.p.m. for 5 mins.

### CONTROLS

- (a) Antigen dilutions were prepared and tested against 2HD<sub>100</sub> complement with DGV replacing serum.

- (b) Antiserum dilutions were prepared and tested against  $2\text{HD}_{100}$  complement DGV replacing antigen.
- (c) Each dilution of antiserum was tested against uninfected control antigen at concentrations equal to the two highest concentrations of chlamydial antigen  $1/2$  and  $1/4$ .
- (d) Complement was prepared at 2, 1,  $\frac{1}{2}$  and 0 (cell control)  $\text{HD}_{100}$ , DGV replaced antigen and antiserum in the first three wells and 3 volumes of DGV in the cell control well to replace all three reactants.

Most antigen-antibody patterns fall into two main groups:-

- (a) a 'peak' optimal antigen dilution.
- (b) a 'plateau' of optimal antigen dilutions which may indicate anticomplementary activity at lower dilutions.

An antigen showing a peak pattern should be used at the OPD.

With an antigen showing a plateau pattern 1 unit of antigen may be taken as the highest dilution of antigen giving complete fixation of complement with the highest dilution of serum. The antigen was used at 2 units i.e. if the end-point titre was  $1/4$  it was used at  $1/2$ .

Reference antiserum was used at 4 units.

APPENDIX III

EIA REAGENTS

COATING BUFFER (pH 9.6)

Na <sub>2</sub> CO <sub>3</sub>	1.59g
NaHCO <sub>3</sub>	2.95g
NaN <sub>3</sub>	0.2g
Distilled water	1000 ml.

PBS-Tween (pH 7.4)

NaCl	8.0g
KH <sub>2</sub> PO <sub>4</sub>	0.2g
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	2.9g
KCl	0.2g
Tween 20	0.5 ml.
NaN <sub>3</sub>	0.2g
Distilled water	1000 ml.

10% Diethanolamine Buffer

Diethanolamine	97 ml.
Distilled water	800 ml.
NaN <sub>3</sub>	0.2g

Add a 1M solution of HCl and adjust to give pH 9.8. Make total volume 1000 ml with distilled water.

## REFERENCES

- Alexander, E.R., Wang, S.P. and Grayston, J.T. (1967)  
American Journal of Ophthalmology, 63, 1967-1469.
- Alexander, J.J. (1968)  
Journal of Bacteriology, 95, 327-332.
- Allan, I. and Pearle, J.H. (1979)  
Journal of General Microbiology, 112, 61-66.
- Allen, E.G., Kaneda, B., Girardi, A.J., Scott, T.F. and Siger, M.M. (1952),  
Journal of Bacteriology, 67, 718-723.
- Arita, M. and Matumoto, M. (1967)  
Japanese Journal of Microbiology, 11, 337-338.
- Aurelian, L., Gupta, P.K., Frost, I.K., Rosenheim, N.B. (1979)  
Analytical and Quantitative Cytology, Jul-Aug., 89-102.
- Barwell, C.F., (1952)  
British Journal of Experimental Pathology, 33, 268-279.
- Bedson, S.P. Sir, Western, G.T. and Levy-Simpson, S. (1930)  
Lancet, 1, 235-236
- Bedson, S.P. Sir, (1933)  
British Journal of Experimental Pathology, 14, 162-170.
- Bedson, S.P. Sir, (1936)  
British Journal of Experimental Pathology, 17, 109-121.
- Bedson, S.P. Sir, Barwell, C.F., King, E.J. and Bishop, L.W.J. (1949)  
Journal of Clinical Pathology, 2, 241-249.
- Bedson, S.P. Sir, (1959)  
Journal of the Royal Institute of Public Health and Hygiene, 22, 67-68.
- Beem, M. and Saxon, E.M. (1977)  
New England Journal of Medicine, 296, 306-310.
- Bell, S.D. and Theobald, B. (1962)  
Annals of the New York Academy of Science, 98, 337-346
- Bell, S.D. and McComb, D.E. (1967)  
Proceedings of the Society for Experimental Biology and Medicine (New York), 124, 34-38.
- Berger, R.E., Alexander, E.R., Mouda, G.D. et al (1978)  
New England Journal of Medicine, 298, 301-4.

- Bernkopf, H., Mishmi, M., Mathyr, B. and Freitelberg, I. (1959)  
Archives of Ophthalmology, 62, 33-34.
- Bernkopf, H., Mashiah, P. and Becker, Y. (1962)  
Annals of the New York Academy of Science, 98, 62-81.
- Bland, J.W. and Canti, R.G. (1935)  
Journal of Pathology and Bacteriology, 40, 231-241.
- Blyth, W.A. and Taverne, J. (1972)  
Journal of Hygiene (Cambridge), 70, 33-37.
- Bowie, W.R., Alexander, E.R., Floyd, J.F. et al (1976)  
Lancet, ii, 1276-1278.
- Breed, R.S., Murray, E.G.D. and Hitchens, P. (1948)  
Bergey's Manual of Determinative Bacteriology 6th ed.
- Brewerton, D.A., Caffrey, M. and Nichols, A. (1973)  
Lancet, ii, 996-998.
- Brewerton, D.A., Caffrey, M. and Nichols, A. (1975)  
Annals of Rheumatic Diseases, 33, 406-407.
- Briem, H., Norberg, R. and Jonsson, M. (1980)  
Journal of Infection, 2, 215-220.
- Buckley, S., Whitney, E. and Rapp, I. (1955)  
Proceedings of the Society for Experimental Biology and Medicine, 90, 226.
- Bushell, A.C. and Hobson, D. (1978)  
Infection and Immunity, 21, 946-953.
- Caldwell, H.D., Kuo, C.C. and Kenny, G.E. (1979)  
Journal of Immunology, 115, 963-975.
- Caldwell, H.G., Kromhort, J. and Schachter, J. (1981)  
Infection and Immunity, 31, 1161-1176.
- Cevenni, R., Costa, S. and Rumpianes, F. (1981)  
British Journal of Venereal Diseases, 57, 334-337.
- Chandler, J.W., Alexander, E.R. and Rheiffer, T.A. (1977)  
Transactions of the American Academy of Ophthalmology and Otolaryngology, 83, 302-308
- Chen, T.R. (1977)  
Experimental Cell Research, 104, 255-262.
- Collier, L.H. and Sowa, J. (1958)  
Lancet, i, 993-996.
- Collins, A.R. and Barrow, A.L.J. (1970)  
Journal of Infectious Diseases, 121, 1-8.



- Craighead, J.E. (1975)  
Journal of Infectious Diseases, 131, 749-754.
- Csonka, G.W. (1960)  
Arthritis and Rheumatism, 3, 164-166.
- Darouger, S., Jones, B.R., Kinnison, J.R. et al (1973)  
British Journal of Venereal Disease, 48, 416-420.
- Darouger, E., Monnickendam, M.A., El-Sheikh, H, Treharne, J.D. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D. and Holmes, K.K. pp. 186-204.  
Americal Society for Microbiology.
- Dhir, S.P. Kenny, G.E., and Grayston, J.T. (1971)  
Infections and Immunity, 4, 725-730.
- Duc-Nguyen, H. (1968)  
Journal of Virology, 2, 643-644.
- Dunlop, E.M.C., Harper, I.A., Al-Hussaidi, M.C. et al (1966)  
British Journal of Venereal Disease, 42, 77-87.
- Dunlop, E.M.C., Vaughan-Jackson, J.D. and Darongar, S. (1972)  
British Journal of Venereal Disease, 48, 425-428.
- Enders, J.F., Welles, T.H., and Robbins, F.C. (1947)  
Science, 109, 85-87.
- Ennis, H.L. and Lubin, M. (1964)  
Science, 146, 1474-1476.
- Fernandez, V.M. (1959)  
Zeitschrift Zellforschung, 50, 433-443.
- Findlay, G.M. (1933)  
Transactions of the Royal Society for Tropical Medicine and Health  
27, 35-66.
- Fowlkes, D.M., Doohar, G.M. and O'Leary, W.M. (1971)  
Fertility and Sterility, 26, 1203.
- Fraser, C.F.O. and Bermanx, D.T. (1965)  
Journal of Bacteriology, 89, 943-948.
- Friis, R.R. (1972)  
Journal of Bacteriology, 110, 706-721.
- Frommell, G.T., Bruhn, F.W. and Schwartzmann, J.D. (1977)  
New England Journal of Medicine, 296, 1150-1151.
- Gerber, G. (1972)  
Proceedings of the National Academy of Science, 69, 83-85.
- Gey, G.O., Bang, F.B. and Gey, M.K. (1952)  
Annals of the New York Academy of Science, 58, 976-999.

- Gogolak, F.M. and Ross, M.R. (1955)  
Virology, 1, 474-496.
- Goh, B.T, Morgan-Capner, P. and Limkis (1982)  
British Journal of Venereal Disease, 58, 327-329.
- Gordon, F.B., Quan, A.L. and Trimmer, R.W. (1960)  
Science, 131, 733.
- Gordon, F.B. and Quan, A.L. (1962)  
Annals of the New York Academy of Science, 98, 261-270.
- Gordon, F.B., Harper, I.A., Quan, A.L. and Treharne, J.D. (1969)  
Journal of Infectious Diseases, 120, 451-462.
- Gordon, F.B., Dressler, H.R., Quan, A.L., McQuilkin, W.T. and Thomas, J.I. (1972)  
Applied Microbiology, 23, 123-129.
- Grayston, J.T. (1967)  
Pan American Health Organization. (Scientific Publication), 147, 546-559.
- Grayston, J.T. (1975)  
Journal of Infectious Diseases, 132, 87-105.
- Griffiths, M.S. Ainsworth, S. and Reaole, J.H. (1976)  
Journal of General Microbiology, 95, 249-256.
- Hanna, L. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D. and Holmes, K.K. p. 268. Americal Society for Microbiology.
- Harnisch, J.P., Berger, R.E., Russel-Alexander, E. et al (1977)  
Lancet, 1, 819-821.
- Harper, I.A., Dwyer, R. St.C., Garland, B.R. and Jones, J.D. (1967)  
American Journal of Ophthalmology, 63, 1064-1072.
- Harrison, H.R. and Riggin, R.T. (1979)  
Journal of Infectious Diseases, 140, 968-971.
- Hayes, W. (1967)  
The Genetics of Bacteria and their Viruses.  
pp 368-375. Blackwell Scientific Publications.
- Hatch, T.P. (1975)  
Infection and Immunity, 12, 211-220.
- Hayflick, L. and Chanock, R.M. (1965)  
Bacteriology Review, 29, 185-221.
- Higashi, N., Nutake, K. and Fukudate, K. (1959)  
Annals of the Institute of Virus Research, (Kyoto University) 2, 23-56.

- Higashi, N. and Tamura, A. (196)  
Virology, 12, 578-588.
- Hilleman, M.R., Haig, D.A. and Helmholtz, R.J. (1951)  
Journal of Immunology, 66, 115-130.
- Hilton, A.L., Richmond, S.J., Milne, J.D. et al (1974)  
British Journal of Venereal Disease, 50, 1-10.
- Hobson, D., Johnson, F.W.A. and Byng, R.E. (1976)  
Lancet, Sep 18, 634.
- Hobson, D. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D and Holmes, K.K. pp 286-294.  
American Society for Microbiology.
- Hobson, D., Lee, N., Quayle, E and Beckett, E.E. (1982)  
Lancet, (ii) 872-873.
- Holmes, K.K., Handsfield, H.H. and Wang, S.P. (1975)  
New England Journal of Medicine, 292, 1199-1205.
- Inglis, G.C. (1980). The Sensitivity and Relevance of Selected Immunological Methods for the Estimation of in Serum. PhD Thesis University of Glasgow p. 288.
- Jawetz, E. (1962)  
Annals of the N.Y. Academy of Science, 98, 12.
- Jenkin, H.M., Ross, M.R. and Moulder, J.W. (1960)  
Journal of Immunology, 86, 123-127.
- Jones, B.R., Collier, L.H. and Smith, C.H. (1959)  
Lancet, i, 902-905.
- Jones, B.R., Al-Hussaini, M.K. and Dunlop, E.M.C. (1964)  
British Journal of Venereal Diseases, 40, 19-24.
- Kajima, M.N., Sharon, K. and Pollard, M. (1967)  
Journal of Ultrastructure Research, 18, 661-666.
- Karayannis, P. and Hobson, D. (1981)  
Journal of General Microbiology, 122, 47-54.
- Keat, A.C., Maini, R.M., Nkwazi, G.C, Pegrum, G.D. et al (1978)  
British Medical Journal, 1, 605-607.
- Keat, A.C., Thomas, B.G., Taylor-Robinson, D., Pegrum, G.D. et al.  
Annals of Rheumatic Diseases, 39, 431-437.
- Kosunen, T.U., Kauranen, O., Martio, J., Pitkanen, T. et al (1981)  
Scandinavian Journal of Rheumatology, 10, 77-80.
- Kruse, P.F. and Patterson, M.K. (1973) edition Tissue Culture pp. 722-734. Academic Press.

- Kuo, C.C., Wang, S.P. and Grayston, J.T. (1972)  
Journal of Infectious Diseases, 125, 313-317.
- Kuo, C.C., Wang, S.P. and Grayston, J.T. (1973)  
Infection and Immunity, 8, 74-79.
- Levine, S (1962)  
Nature, 194, 895-896.
- Loh, P.C., Hashiro, G.M. and Yau, J.T. (1978)  
Microbios, 19, 213-229.
- Macdougald, T.J., Beams, H.W. and King, R.L. (1953)  
Proceedings of the Society of Experimental Biology and Medicine, 37, 234-238.
- MacKenzie, R.D., Findaly, G.M. and McCallum, F.O. (1936)  
Transactions of the Royal Society of Tropical Medicine and Hygiene, 32, 183-188.
- Manire, G.P. and Meyer, K.F. (1950)  
Journal of Infectious Diseases, 86, 226-232.
- Matumoto, M., Arita, R. and Odam, K. (1965)  
Japanese Journal of Experimental Medicine, 35, 319-329.
- Meyer, K.F. (1967)  
American Journal of Ophthalmology, 63, 1225-1246.
- Mikami, A. (1969)  
Journal Infectious Diseases, 119, 557.
- Miyagawa, Y., Mitamura, T. and Yaoi, H. (1936)  
Japanese Journal of Experimental Medicine, 13, 331-333.
- Moll, J.M.H., Haslock, I., MacRae, I.F. and Wright, F. (1974)  
Medicine, 53, 343.
- Moulder, J.W., McCormack, B.R.S. and Itatari, M.K. (1953)  
Journal of Infectious Diseases, 93, 140-149.
- Moulder, J.W. (1964)  
The Psittacosis Group as Bacteria, pp. 79-84.  
John Wiley and Sons, In
- Moulder, J.W. (1966)  
Annal Review of Microbiology, 20, 107-130.
- Moulder, J.W. (1969)  
Bioscience, 19, 875-881.
- Murray, E.S., Bell, S.D., Hanna, A.T., Nichols, R.L. and Snyder, J.C. (1960)  
Americal Journal of Tropical Medicine and Hygiene, 9, 116-124.

- Narita, T. and Manire, G.P. (1976)  
Journal of Bacteriology, 125, 308-316.
- Nochols, R.L. and McComb, D.E. (1962)  
Journal of Immunology, 89, 545-554.
- Nichols, R.L., Bell, S.D. Jr and Murray, E.S. (1966)  
American Journal of Tropical Medicine and Hygiene, 15, 639-649.
- Nicolle, C.A., Cuenod, A. and Hanabusa, J. (1913)  
Archives of the Pasteur Institute, 3, 185-187.
- Oriel, J.D., Reeve, P., Powis, P., Miller, A and Nicol, L.S. (1972)  
British Journal of Venereal Disease, 48, 429-436.
- Oriel, J.D. (1977)  
Nongonococcal Urethritis and Related Infections,  
ed. Hobson, D. and Holmes, K.K. pp. 47-48.  
American Society of Microbiology.
- Oriel, J.D. and Ridgway, G.L. (1981)  
Genital Infection by Chlamydia trachomatis pp. 71.  
Publishers Edward Arnold.
- Paavonen, J., Saikku, P., Vesterinen, E., Meyer, B. and Vartainen, E.  
(1978)  
British Journal of Venereal Disease, 54, 257-261.
- Pagano, J.S. and Vaheri, A. (1965)  
Archiv Gesamte Virnsforschung, 17, 456-464.
- Paul, J.R. and White, C. (1975)  
Serological Epidemiology.  
pp. 1-13. Publishers Academic Press London.
- Pomerat, G.M., Kent, S.P. and Logie, L.C. (1957)  
Zeitschrift Zellforschung, 47, 158-170.
- Prentice, J.J. and Farrant, J. (1977)  
Journal of Clinical Microbiology, 6, 4-9.
- Rake, G., McKee, C.M. and Shaffer, M.F. (1940)  
Proceedings of the Society for Experimental Biology and Medicine,  
43, 332-334.
- Rees, E., Tait, I.A. Hobson, D. and Johnson, F.W.A. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D. and Holmes, K.K. pp. 67-76.  
American Society for Microbiology.
- Rice, C.E. (1935)  
Proceedings of the Society for Experimental Biology and Medicine.  
33, 317-318.
- Richmond, S.J. (1972)  
Communicable Disease Report, 72/48 Special Report.

- Richmond, S.J. and Caul, E.O. (1975)  
Journal of Clinical Microbiology, 1, 345-352.
- Richmond, S.J. and Stirling, P. (1981)  
Infection and Immunity, 34, 561-570.
- Ridgway, G.L. and Oriel, J.D. (1977)  
Journal of Clinical Pathology, 30, 933-936.
- Ripa, K.T. and Mardh, P.A. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D. and Holmes, K.K. pp. 323-327.  
American Society for Microbiology.
- Ripa, K.T., Svenson, L., Mardh, P.A. and Westrom, L. (1978)  
Obstetrics and Gynaecology, 52, 698-702.
- Robertson, D.H.H., McMillan, A. and Young, H. (1980)  
Clinical Practise in Sexually Transmitted Diseases.  
pp. 203 and 266. Publishers: Pitman Medical.
- Rose, L and Schachter, J. (1964)  
Investigative Ophthalmology, 3, 680.
- Ross, M.R. and Gogolak, F.M. (1957)  
Virology, 3, 343-365.
- Rota, T.R. and Nichols, R.L. (1971)  
Journal of Infectious Diseases, 124, 419-421.
- Rota, T.R. and Nichols, R.L. (1975)  
Applied Microbiology, 26, 560-565.
- Rota, T.R. (1977)  
Nongonococcal Urethritis and Related Infection.  
ed. Hobson, D. and Holmes, K.K. pp. 316-317.  
American Society for Microbiology.
- Sacks, D.L. and MacDonald, A.B. (1979)  
Journal of Immunology, 122, 136-139.
- Schachter, J., Barnes, M.G., Jones, J.P., Engelman, E.P.  
and Meyer, K.F. (1966).  
Proceedings of the Society for Experimental Biology. (New York),  
122, 283-285.
- Schachter, J., Banks, J., Sugg, N. et al 1974.  
Infection and Immunity, 9, 92-94.
- Schachter, J., Lum, L., Gooding, C.A. and Ostler, B. (1975)  
Journal of Paediatrics, 87, 779-80.
- Schachter, J. (1978)  
The New England Journal of Medicine (Second of Three Parts)  
298, 491-495.

Schachter, J., Grossman, M., Holt, J., Sweet, R. et al (1979)  
Lancet, (ii) 377-379.

Schachter, J., Cles, L., Ray, R. and Hines, P.A. (1979)  
Journal of Clinical Microbiology, 10, 647-9.

Silversten, S.C., Steinman, R.M. and Gohn, Z.A. (1977)  
Annual Review of Biochemistry, 46, 669-722.

Singer, S.J. and Nicolson, G.L. (1972)  
Science, 175, 720-731.

Skegg, D.C.G., Gorwin, P.A., Paul, C. and Doll, R. (1982)  
Lancet, (i), 581-583.

Smull, C.E. and Ludwig, E.H. (1962)  
Journal of Bacteriology, 84, 1035-1040.

Sowa, J. and Race, J.W. (1971)  
Journal of Hygiene, 69, 709-716.

Storz, J. and Page, L.A. (1971)  
International Journal of Systematic Bacteriology, 21, 332-334.

Storz, J. (1971)  
Chlamydia and Chlamydia induced Diseases.  
pp. 79-81. Pub. Springfield, Illinois. Charles C. Thomas, Publisher

Subak-Sharpe, J.H., Burk, R.R. and Pitts, J.D. (1966)  
Heredity, 21, 342.

Swartz, S.L. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Bogson, D. and Holmes, K.K. pp. 15-16.  
American Society for Microbiology.

Takatsy, G. (1950)  
Kiserletes Orvostudomány, 2., 393. Cited from:-

Takatsy, G. (1957)  
Archives für die Gesamte Virusforschung, 7, 344.

Tamura, A. and Manire, G.P. (1967)  
Journal of Bacteriology, 94, 1184-1188.

Tamura, A. and Manire, G.P. (1974)  
Journal of Bacteriology, 118, 144-148.

T'ang, F.F., Chang, C.L. and Huang, Y.T. (1959)  
Chinese Medical Journal, 75, 429-447.

Taylor-Robinson, D. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D. and Holmes, K.K. pp. 34-35.  
American Society for Microbiology.

- Taylor-Robinson, D. and Thomas, B.J (1980)  
Journal of Clinical Pathology, 33, 205-233.
- Thygeson, P. and Mengert, W.F. (1936)  
Archives of Ophthalmology, 15, 377-410.
- Thygeson, P. and Stone, W. Jr (1942)  
Archives of Ophthalmology, 27, 91-122.
- Thygeson, P. (1962)  
Annals of the New York Academy of Science, 98, 6-13.
- Treharne, J.D., Day, J., Yeo, C.K., Jones, B.R. and Squires, S. (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D. and Holmes, K.K. pp. 214-222.  
American Society for Microbiology.
- Treharne, J.D., Darouger, S. and Jones, B.R. (1977)  
Journal of Clinical Pathology, 30, 510-517.
- Vaughan-Jackson, J.D., Dunlop, E.M.C. and Darouger, S. (1972)  
British Journal of Venereal Disease, 48, 445-446.
- Vaughan-Jackson, J.D., Dunlop, E.M.C. and Darouger, S. et al (1977)  
British Journal of Venereal Disease, 53, 180.
- Wallis, C. and Melnick, J.L. (1968)  
Journal of Virology, 2, 267-274.
- Wang, S.P., Grayston, J.T. and Alexander, E.R. (1967)  
American Journal of Ophthalmology, 63, 1615-1630.
- Wang, S.P. and Grayston, J.T. (1970)  
American Journal of Ophthalmology, 70, 367-374.
- Wang, S.P., Kuo, C.C. and Grayston, J.T. (1975)  
Infection and Immunity, 7, 356-360.
- Wang, S.P., Grayston, J.T., Alexander, E.R. and Holmes, K.K. (1975)  
Journal of Clinical Microbiology, 1, 250-255.
- Wang, S.P., Grayston, J.T., Kuo, C.C. et al (1977)  
Nongonococcal Urethritis and Related Infections.  
ed. Hobson, D. and Holmes, K.K. pp. 237-248.  
American Society for Microbiology.
- Weiss, E. (1949)  
Journal of Infectious Diseases, 84, 125-149.
- Weiss, E. and Huang, J.G. (1954)  
Journal of Infectious Diseases, 94, 107-125.
- Weiss, E. and Dressler, H.R. (1960)  
Proceedings of the Society for Experimental Biology (N.Y.)  
103, 691-695.



Weltstein, F.O., Nall, H. and Penman, S. (1964)  
Biochimica et Biophysica Acta, 87, 525-528.

Wentworth, B.B. and Alexander, E.R. (1974)  
Applied Microbiology, 27, 912-916.

Westrom, L. (1975)  
American Journal of Obstetrics and Gynaecology, 121, 707-713.

Westrom, L., Bengtsson, L.P. and Mardh, P.A. (1976)  
Lancet, (ii), 221-224.

Westrom, L. (1982)  
Chlamydia '82.  
Symposium on Chlamydial Infections. London, Bayer Conference Service.

Zissis, G. and Clinet, G. (1974)  
Lancet, (i) 754-755.