The isolation and characterisation of temperature-sensitive mutants of R.S. virus

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Science at the University of Glasgow

by

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October, 1975

Acknowledgements

I would like to thank Professor J.H. Subak-Sharpe for provision of the facilities of the Institute and for his interest in this project. I am indebted to Dr. C.R. Pringle for his excellent supervision of the project and for valuable criticism of the manuscript of this thesis. The contribution of my mother in encouraging my interest in Virology is gratefully acknowledged. I would also like to thank Mr. Douglas Johnston and Mrs. P. McSevney for the photographic work and Mrs. A. Strachan for the excellent typing.

The work presented here was carried out with the aid of a Science Research Council Studentship for Training in Research Methods; all of the results except where specifically acknowledged were obtained by the author's own efforts.

Georgine P. Faulkner

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Summary

The RSN-2 strain of RS virus grown in BS-C-1 cells was used for the isolation and characterisation of ts mutants.

RSN-2 strain growth was examined at different temperatures and the degree of cell-association of the virus calculated. The effect of DNA inhibitors such as actinomycin D and mitomycin C as well as the effect of the concentration of phenylalanine, arginine and serum on RS virus was studied. Thin sections of RS virus infected cells were examined by electron microscopy. Infected cells were also examined under a scanning electron microscope.

Thirty-five ts mutants of the RSN-2 strain were isolated from mutagenised virus and one ts mutant from non-mutagenised virus. A few ts mutants were ts at 37°C as well as the restrictive temperature (39°C). Some ts mutants must be affected in a cytolytic function since they grow but do not induce plaque formation at 39°C. An attempt was made to characterise the ts mutants by genetic analysis. However, complementation could not be reproducibly detected in the cell system (BS-C-1 cells) used. Therefore the ts mutants were classified on the basis of their growth and immunofluorescence pattern and thermolability at 39°C. From the immunofluorescence data the ts mutants were classified into three groups. Group I ts mutants have a reduced amount of antigen and a reduced number of fluorescing cells at 39°C compared to 31°C. Group III ts mutants have a reduced amount of antigen at 39°C but the same number of fluorescing cells whereas Group II ts mutants have the same pattern at 39°C as 31°C. Group I and III ts mutants are classified by lack of

growth at 39°C into Group A. Leak occurring durin g growth of these ts mutants was less than 1%. Group II ts mutants correspond to Group B. These mutants have greater than 10% leak at 39°C.

From the temperature-shift experiments the ts mutants examined from Group I and III appeared to have a defect in an early function. However the ts mutant of Group II which was examined seemed to be required throughout the growth cycle and may be defective in a functional activity rather than synthesis. This ts mutant was heat-stable, the other ts mutants examined were heat-labile.

The thermolability of ts mutants from each group was examined at 39°C. There was no correlation between heat-sensitivity and the classification of the ts mutants as both Group A (I and III) and Group B (II) ts mutants were heat-sensitive. Ts mutants from each group were assayed for virion-associated RNA-dependent RNA-polymerase activity. Although some activity could be detected it was difficult to assess as there was a high level of background activity in uninfected cells.

Section 1

Introduction

1.1 Negative-Strand viruses

Negative-strand viruses are RNA viruses which have a single-stranded RNA genome complementary in base sequence to m-RNA. These viruses also have a virus coded RNA-dependent RNA polymerase, which allows the transcription of m-RNA from the RNA genome. The group of negative-strand viruses includes the orthomyxoviruses, paramyxoviruses, rhabdoviruses and also possibly the metamyxoviruses (Mahy and Barry, 1975; Wunner et al., 1975). The genome of the negative-strand viruses can be unsegmented (paramyxovirus, rhabdovirus) or segmented (orthomyxovirus). In viruses with a segmented genome, individual single-stranded RNA molecules of the genome are each transcribed by a virion-associated RNA-dependent RNA polymerase to complementary RNA molecules which act as m-RNA (Fenner, 1974). However, not all of the influenza virus subunits may be negative strands since the influenza virion RNA has been reported to have in vitro m-RNA activity (Siegert et al., 1973).

The genome of ~~unsegmented~~ NEGATIVE-STRAND viruses is transcribed by the virion-associated RNA-dependent RNA polymerase into m-RNA MONOCISTRONIC LENGTHS OF ~~probably by direct transcription rather than by cleavage of transcribed RNA into short lengths~~, that is genomic rather than modificative control (Subak-Sharpe and Pringle, 1975). The replication of the orthomyxoviruses (influenza virus type A, B and C) is sensitive in the early stages to the action of actinomycin D (Barry et al., 1962). However the replication of the paramyxoviruses (Newcastle disease virus (NDV) and the parainfluenza viruses) is not sensitive to actinomycin D (Barry et al., 1962). The properties of these groups are listed in Table 1 and compared with those of a proposed group, the metamyxoviruses (Melnick, 1971) which includes

human and bovine RS virus and pneumonia virus of mice (PVM). The three groups differ in the size of their nucleocapsid, that of the metamyxoviruses (11-15 nm) (Berthiaume et al., 1974) being intermediate in diameter between that of the orthomyxovirus (9 nm) and paramyxoviruses (17-18 nm). Both the ortho- and para-myxoviruses differ from the metamyxoviruses with the exception of PVM in having both haemagglutinin (Hirst, 1941) and neuraminidase (Gottschalk, 1957). Morphologically the viruses from the three groups are similar. They are pleomorphic enveloped viruses with spikes on the membrane surface and they mature by budding from the cytoplasmic membrane.

The orthomyxoviruses and paramyxoviruses differ in their genetic interactions. Both complementation and "recombination" have been shown between ts mutant of influenza virus. The ts mutant can be grouped better on the basis of 'recombination' rather than complementation data. However the recombination frequencies (5-20%) obtained were higher than would be expected due to intramolecular recombination (Simpson and Hirst, 1968). Previous hypotheses suggested that high recombination frequencies obtained were due to the possibility of reassortment of a segmented genome (Burnet, 1959 and Hirst, 1962). It has now been shown that influenza virus nucleic acid resolves into 6 or 7 single-stranded molecules (Bishop et al., 1971) and reassortment has been demonstrated between strains of influenza virus A (Kilbourne et al., 1967 and Kilbourne, 1968). Thus recombination in influenza virus is mainly due to genetic reassortment although some low frequency intramolecular recombination may occur (Staiger, 1964). The "recombination" seen between ts mutants of NDV is thought to be due to the formation of particles with

more than one genome, if ts mutant genomes are involved complementing heterozygotes can occur which produce plaques at the restrictive temperature (Dahlberg and Simon, 1969a and Dahlberg and Simon, 1969b). Complementation (Gharpure et al., 1969) and "recombination" (Wright et al., 1973) have been detected between ts mutants of RS virus (a metamyxovirus). However the "recombination" was due to complementing heterozygotes and not true intramolecular recombination.

TABLE 1

(modified from Table 5-2 in Scholtissek et al., 1969)

Property	Orthomyxovirus	Paramyxovirus	Metamyxovirus
Particle size (nm)	80-120	150-250	100-350
Filamentous forms	Common	Unusual	Common
Projections (nm)	10	12-15	12-17
Nucleocapsid (nm) (diameter)	9	17-18	11-15
Periodicity (nm)	5-6	4-6	6.5-7
Segmented nucleocapsid	Segmented	single strand	single strand
RNA sedimentation coefficient	38S	57S	ND
MW	4×10^6 daltons	6×10^6 daltons	ND
Sensitivity to actinomycin D	Sensitive	Not sensitive	Sensitive?
Eosinophilic cytoplasmic inclusions	-	+	+
RNA-RNA polymerase	+	+	+?
Haemagglutinin	+	+	[x] -
Neuraminidase	+	+	-

[x] Only PVM has haemagglutinin

1.2 Pneumonia virus of mice (PVM)

PVM as well as RS virus was proposed as a member of a new group, the metamyxoviruses (Melnick, 1971). These viruses have nucleocapsids with diameters in the range of 11-15 nm (Bachi and Howe, 1973; Berthiaume et al., 1974; Norrby et al., 1970a). It has now been proposed to extend this group to include bovine RS virus, a virus morphologically and antigenically similar to human RS virus (Ito et al., 1973). Some properties of these viruses are listed in Table 2.

The nucleocapsid of bovine RS virus is similar in diameter to that of human RS virus and PVM. The metamyxoviruses mature by budding and with the exception of PVM show no haemadsorption or haemagglutinating activity (Inaba et al., 1970a and Richman et al., 1971). PVM can agglutinate hamster and mouse erythrocytes and PVM infected cells can absorb to mouse erythrocytes (Compans et al., 1967). The centre-to-centre distance between projections of RS virus and PVM is thought to be related to their difference in haemagglutinating and haemadsorption properties. The distance between RS virus spikes is longer (10 nm) than that between PVM spikes (6 nm).

Human RS virus and PVM by complement-fixation and neutralisation tests have been found to be antigenically unrelated (Berthiaume et al., 1974) whereas human RS virus and bovine RS virus have been found to be antigenically similar (Paccaud and Jacquier, 1970).

TABLE 2

Comparison of characteristics of Human and Bovine RS virus and PVM

(modified from table 1, p48 in Berthiaume et al., 1974)

Properties	Human RS virus grown in HEp-2	Bovine RS virus grown in BK	PVM grown in Vero cells
Growth kinetics			
Latent period	6 hours	24 hours	18 hours
Peak titre	24-48 hours	72 hours	43-96 hours
% Cell-associated virus	90	ND	90
Syncytium formation	+ in some cells	+ in some cells (GBK)	-
Time for CPE to develop	3 days	3 days	> 11 days
Cytochemistry			
Acidophilic	+	+	+
Cytoplasmic inclusions			
Nuclear inclusions	-	-	-
Acridine orange staining of inclusions	yellow-green (other workers -)	-	yellow-green
Haemagglutination	-	-	+
Haemadsorption	-	-	+

TABLE 2 (contd.)

Properties	Human RS virus grown in HEp-2	Bovine RS virus grown in BK	PVM grown in Vero cells
Immunofluorescence			
Immunofluorescent AG detected (post-infection)	8 hours	ND	24 hours
cytoplasmic inclusions	+	+	+
nuclear inclusions	[x] -	-	-
Electron microscopy			
Spherical structure (diam)	100-350 nm	80-450 nm av 200 nm	100-200 nm
pleomorphoric forms	+	+	+
filamentous forms (diam)	100 nm	100-130 nm	100 nm
length of envelope projections	12 nm	13-17 nm	12 nm
projection spacing (centre to centre)	10 nm	7-9 nm	6 nm
nucleocapsid (diam)	13.5 nm	11-15 nm	13.5 nm
nucleocapsid helex pitch	6.5 nm	7 nm	6.5 nm
cytoplasmic inclusions	+	+	+
nuclear inclusions	-	-	-
budding	+	+	+

[x] Nuclear inclusions can be detected in ts RS virus infected BS-C-1 cells at 39°C

1.3 Bovine RS virus

In 1968, an inhibitor of human RS virus was found in bovine sera. The level of this RS virus inhibitor increased with the age of cattle from which it was obtained, being higher in older cattle. In some cases increased levels of inhibitors were found in cattle with respiratory disease (Doggett et al., 1968). Subsequently a virus was isolated from the nasal secretions of Swiss cattle with respiratory disease. The virus which was antigenically similar to human RS virus was shown to be the causative agent of the disease. Therefore this virus was termed ~~Bovine~~ bovine RS virus (Paccaud and Jacquier, 1970). Bovine RS virus has also been isolated from cattle with respiratory disease in Belgium (Wellemans and Leunen, 1971; Wellemans et al., 1970 and Wellemans et al., 1971), Britain (Edington and Jacobs, 1970 and Jacobs and Edington, 1971), Japan (Inaba et al., 1970a, b and Inaba et al., 1972) and America (Rosenquist, 1974; Rossi and Kiesel, 1974 and Smith et al., 1975). The symptoms of bovine RS virus disease are slight fever, anorexia, depression, pyrexia, respiratory distress, coughing lachrymation and increased nasopharyngeal secretions (Inaba et al., 1972).

The principal difference between bovine and human RS virus concerns their host range which is broader for bovine RS virus (Matumoto et al., 1974 and Paccaud and Jacquier, 1970). Bovine RS virus antibodies appear to be widespread in sheep; however the antibody level was lower (2-4-fold) than that in cattle (Smith et al., 1975). Berthiaume and his colleagues (1973) reported the prevalence of human RS virus complement-fixing (CF) antibody in sheep sera was high (81%) but low in the sera of cattle (14%) and horses (6%). Therefore it seems possible that sheep may have their own RS virus related to both human and bovine RS virus.

TABLE 3

Host range of bovine and human RS virus

(modified from table 1 in Matumoto et al., 1974)

Cell culture	Bovine RS virus		Human RS virus	
	Growth	CPE	Growth	CPE
Bovine kidney (BK)	+	+	+	+
Bovine testicle (BT)	+	+	+	+
Bovine thyroid	+	+	-	-
Bovine thymus	+	+	+	±
Bovine duodenum	+	+	+	±
Bovine rectum	+	+	-	-
Primary calf kidney (ECK)	+	+	ND	ND
Primary calf lung (ECL)	+	+	ND	ND
Madin-Darby bovine kidney (MDBK)	+	+ NS	ND	ND
Bovine kidney (GBK)	+	+	+	+
neonatal calf lung	+	+ NS	ND	ND
calf trachea	+	+ NS	ND	ND
calf synovial	+	+ NS	ND	ND
calf aorta	+	+ NS	ND	ND
calf spleen	+	+ NS	ND	ND
calf thyroid	+	+ NS	ND	ND
Swine embryo kidney (ESK)	+	+	-	-
Hamster-lung Hm Lu-1	+	-	-	-
Hamster kidney BHK21-W12	x +	x +	-	-

TABLE 3 (contd)

Cell culture	Bovine RS virus		Human RS virus	
	Growth	CPE	Growth	CPE
Primary embryonic sheep kidney	-	-	ND	ND
Chick embryo	-	-	-	-
Chick kidney	-	-	-	-
Green monkey kidney (Vero)	+	+	+	+
2° rhesus monkey kidney	-	-	+	+
Human embryo lung (HEL)	+	+	+	+
Human embryo kidney (HEK)	+	±	-	-
Human cancer (HeLa)	x +	-	+	+
Human cancer (Hep-2)	x +	-	+	+
KB	-	-	+	+
Human embryonic fibroblast	-	-	ND	ND

[x]Negative for Swiss strain of bovine RS virus

NS - non-sycytial CPE

ND - not done

1.4 Respiratory Syncytial Virus

1.4.1 Clinical and epidemiological aspects of RS virus

Information on the clinical and epidemiological aspects of RS virus is widely scattered through the literature and has never been comprehensively reviewed. The first part of the RS virus review is an attempt to remedy this deficiency, although the medical aspects of RS virus and RS virus infection are not the main theme of this thesis.

RS virus is an important respiratory pathogen in infants. It is a causative agent of upper and severe lower respiratory tract disease, especially bronchiolitis and pneumonia (Beem et al., 1962; Burton, 1964; Chanock et al., 1962; Crone et al., 1964; Gardner et al., 1973; Hilleman et al., 1962; Lewis et al., 1961; Peacock and Clarke, 1961; and Reilly et al., 1961).

Discovery and isolation of RS virus

In 1956, a virus was isolated from a chimpanzee with upper respiratory tract disease. It was termed chimpanzee coryza agent (CCA) and produced a degenerative effect in tissue culture. However no symptoms of disease developed in inoculated laboratory animals, only chimpanzees showed signs of disease. A laboratory worker in contact with the chimpanzees developed both serum neutralising and complement-fixing antibodies to CCA. Antibody to CCA was found in young adults (18 years) suggesting that they had prior infection (Morris et al., 1956). In an attempt to recover virus from children with severe respiratory disease, two similar viruses were isolated, the Long and Synder viruses. These

viruses were serologically identical to CCA and not related to any other respiratory pathogens. They formed syncytia in KB cells (human epidermoid carcinoma) and in human liver cells as did CCA (Chanock et al., 1957). The Long and Synder viruses and CCA were grouped together and termed "Respiratory Syncytial" virus (RS virus) as the virus has a syncytial cytopathic effect in some cell lines (Chanock and Finberg, 1957). RS virus association with respiratory tract disease was confirmed by Beem and his colleagues (1960) by isolation of the virus from children with lower respiratory tract disease but not from control patients.

More RS virus infections can be detected serologically than by isolation (Hornsleth, 1967 and Moss et al., 1963). The complement-fixation test is used more frequently than the neutralisation test as it is simpler to use although not as sensitive. However, neither test is ideal since both are insensitive in detecting RS virus infection in infants (<6 months old) (Beem et al., 1960; Beem et al., 1964; Chanock and Finberg, 1957; Chanock et al., 1961; Gardner et al., 1964; Parrott et al., 1961 and Wulff et al., 1964a). Immunofluorescence techniques can be used to screen clinical samples and infected cells (D'Alessio et al., 1970; Cradock-Watson et al., 1971; Gardner and McQuillin, 1968a; Hers et al., 1971; McQuillin and Gardner, 1968; MacWilliam and Herd, 1970; Nagahama et al., 1970; Urquhart and Martin, 1970; Urquhart and Walker, 1972 and Schieble et al., 1967). The detection of RS virus in cough and nasal swabs is not as efficient as in nasopharyngeal secretions (McQuillin et al., 1970). Some workers have claimed to get reliable diagnosis using smears of throat swabs (Gray et al., 1968). However Gardner and McQuillin (1968b) queried their

results since they had found examination of direct smears unsuitable due to the high amount of non-specific fluorescence detected and the low number of cells present.

Natural RS virus infection of children

Natural RS virus infection of children exhibits a number of characteristic features. RS virus is usually isolated during the winter but only when there is a RS virus epidemic in the area, the peak periods for bronchiolitis and pneumonia co-incide with the epidemic. Epidemics occur annually lasting 3-5 months. No other respiratory pathogen has this annual pattern (Chanock et al., 1961; Horn et al., 1975 and Mufson et al., 1973). Alternating long (13-16 months) and short (7-12 months) intervals have been found to occur between peaks of successive epidemics (Kim et al., 1973a). RS virus illness usually occurs after 3-5 days incubation, in children its symptoms are cough, fever, rhinitis and sometimes pharyngitis and otitis media. However, in a high percentage of cases bronchiolitis and pneumonia occur especially in children infected during the first 6 months (Beem et al., 1964; Berkovich and Taranko, 1964; Chanock and Parrott, 1965; Holzel et al., 1963; Jacobs et al., 1971 and Sandiford and Spencer, 1962).

In a recent survey the association of RS virus with bronchiolitis was further confirmed by RS virus being the only virus isolated from children (< 4 years) with the disease (Horn et al., 1975). RS virus infection can result in death, especially in infants under 1 year old. Among children in hospital with RS virus lower respiratory tract disease the death rate can be as high as 2.4% (Adams et al., 1961a; Adams et al., 1961b; Adams et al., 1963; Coates and Chanock, 1964;

Forbes et al., 1961; Holzel et al., 1963 and Lang et al., 1964). The incidence of severe respiratory disease in children decreases with age. Bronchiolitis was frequently found in children under 1 year old and rarely in older children whereas pneumonia was common up to the age of 2 years (Berglund, 1967 and Parott et al., 1973). This age-related pattern is unique to RS virus and corresponds with the decrease in maternal serum antibody which disappears by the 4th to 6th month of life. Maternal serum antibody (IgG) can be transmitted from mother to child. However its presence in infants (< 3 months) does not protect them against RS virus infection and may be related to the severity of the disease symptoms in this age group. Nevertheless, it cannot be essential since older children (4-7 months) with no serum antibody develop RS bronchiolitis (Beardmore and Hebeka, 1967; Beem et al., 1964; Hambling, 1964a; Jacobs et al., 1971; Kapikian et al., 1961; McClelland et al., 1961; Suto et al., 1965 and Wright, 1965). The severity of RS virus disease could be related to the immunological response to infection. This response is absent in children under 4 weeks old (Gardner et al., 1964) and could be the reason why these children do not develop severe symptoms on RS virus infection (Neligan et al., 1970). From 4 weeks old onwards a higher percentage of children are capable of responding immunologically to RS virus, the maximum response being reached at 5 months of age. Immunological responsiveness in infants increases with age and is related to decrease in maternal serum antibody in their sera. Also, CF antibody response is inversely related to the level of maternal serum antibody as it is impaired in infants 1 to 3 months old (Gardner et al., 1964 and Parrott et al., 1973). The percentage of children with acquired RS virus serum antibody increases from 35% of children between 3-12 months old to 95% of children of 5 years

of age (Gernez-Rieux et al., 1963; Hornsleth and Volkeit, 1964; Moss et al., 1963 and Parrot et al., 1961).

In surveys done in Scotland a higher male to female ratio was found in children (under 1 year old) with RS virus lower respiratory tract disease. This ratio was nearly 2:1 for males suffering from RS virus infection as opposed to females (Ross et al., 1971).

American workers found that although RS virus was isolated from the same proportion of males and females with respiratory disease, there were significantly more male children admitted to hospital with RS virus infections (bronchiolitis and pneumonia) than female children (Parrott et al., 1973 and Monto et al., 1974). However male children generally show a greater tendency to develop infections than female children (Winter, 1972) which could result in the apparent male-association of RS virus infection.

RS virus association with upper respiratory tract disease

RS virus infection can also produce upper respiratory tract disease (Berglund, 1967; Chanock et al., 1961; Hamparian et al., 1961 and Horn et al., 1975). However, nasopharyngeal symptoms and cough are commonly associated with most respiratory viruses and otitis media is associated with influenza B and adenovirus infections as well as RS virus infection (Horn et al., 1975). Nevertheless otitis media is frequently associated with RS virus infections of young children (Berglund, 1967 and Horn et al., 1975). Guinea-pigs experimentally infected with RS virus by inoculation of their eardrums developed otitis media. RS virus could be recovered from the eardrums at least one week after infection.

Since RS virus is very labile at the temperature of guinea-pig middle ear it is unlikely to have survived there for a week. Therefore the virus probably reproduced in guinea-pig middle ear. Although it is not known if RS virus can grow in human middle ear epithelium the virus has been isolated from the tympanic mucus of infected children (Berglund, 1967).

Children who have had severe RS virus or adenovirus infection are more prone to develop asthma or chronic obstructive lung disease than children who had no respiratory infection caused by those viruses (Strieder, 1974).

Respiratory viruses including RS virus can be isolated from cases of whooping cough. However, it is not known if these viruses can cause whooping cough (Feldman and Cradock-Watson, 1972). RS virus has only rarely been isolated from croup (Beem et al., 1960; Grauballe et al., 1974; Hamparian et al., 1961 and Horn et al., 1975). Croup , a middle respiratory tract disease, is usually associated with parainfluenza viruses (Horn et al., 1975). A case of acute complete atrioventricular heart block was noted in association with RS virus infection. However, cardiac involvement in association with RS virus is rare (Bairan et al., 1974).

Sudden infant death syndrome (SIDS)

Viruses can be associated with sudden death in infants (Aherne et al., 1970; Johnstone and Lawy, 1966). The rate of virus isolation from SIDS cases was nine times the rate from normal infants clinically free of infectious disease. Among the viruses isolated was RS virus

and other respiratory pathogens (Brandt et al., 1970). RS virus has been suggested as a possible infectious cause of SIDS since a similar age distribution has been reported to occur with both SIDS and RS virus bronchiolitis. Also RS virus antigen has been detected in the lungs of some SIDS cases giving the same distribution pattern as seen in cases of RS virus bronchiolitis (Downham et al., 1975 and Ferris et al., 1973). However, Foy and Ray (1973) found no correlation between SIDS cases and lower respiratory tract disease epidemics except that the incidence of both decreased in the summer.

Cross-infection and reinfection with RS virus

Cross-infection due to RS virus has occurred in hospitals (Chanock et al., 1957; Chanock et al., 1961; Ditchburn et al., 1971; Sterner, 1972 and Weightman et al., 1974), nurseries (Berkovich and Taranko, 1964; Kapikian et al., 1961 and Sterner et al., 1966) and families (Berglund, 1967).

Natural reinfection with RS virus is very common in children. Several workers have detected RS virus by isolation or serological evidence from the same patients in different RS virus epidemics (Beem et al., 1967; Chanock et al., 1970 and Loda et al., 1972). Reinfection with RS virus is less severe than initial infection (Beem, 1967 and Horn et al., 1975) usually asymptomatic in adults but occasionally producing mild upper respiratory tract symptoms. Experimental reinfection shows the same pattern (Hamre and Procknow, 1961; Hamre et al.,1961; Johnson et al., 1961; Johnson et al., 1962 and Kravetz et al., 1961). However, there is evidence that RS virus infection in adults over 50 years old results in more serious illness, i.e. bronchitis

and bronchopneumonia, perhaps due to a decrease in immunity to the virus (Fransen et al., 1967 and Sommerville et al., 1963). Bronchiolitis and pneumonia can also occur in children (< 4 years old) on reinfection with RS virus even if they have pre-existing serum neutralising antibody (Loda et al., 1972). This antibody does not protect against reinfection with RS virus. However RS virus nasal secretory antibody in trials, although not preventing reinfection, did modify the illness (Mills et al., 1971).

Reinfection is not caused by antigenic variation as there is no significant difference between strains of RS virus causing successive infections (Mills et al., 1971).

Antigenic Variation

Although a common complement-fixing antigen is shared by different RS virus strains (Coates and Chanock, 1962; Coates et al., 1963; Suto et al., 1965 and Wulff et al., 1964a) some antigenic variation does occur between these strains and the prototype Long strain. This difference between strains was seen in cross-neutralisation tests using post-infection animal sera (ferret, guinea-pig, monkey, rabbit). However, when human sera was used in the test in place of animal sera no antigenic variation was seen between strains (Beem, 1967; Coates and Chanock, 1962; Coates et al., 1963; Doggett and Taylor-Robinson, 1965; Suto et al., 1965 and Wulff et al., 1964a). Animals (ferrets, guinea-pigs) develop homologous but not heterologous neutralising antibody in response to primary RS virus infection. However, children undergoing primary RS virus infection probably develop both homologous and heterologous antibodies since an increase in neutralising antibody against

both the local and the prototype strain of RS virus occurs in their serum (Berglund, 1967; Wulff et al., 1964a and Ross et al., 1964).

RS virus antigenic variation does not appear to be progressive since strains which differed from the prototype Long strain did not replace it and other similar RS virus strains in the population (Coates et al., 1966).

Pathogenesis of RS virus disease

Several theories have been proposed to explain the pathogenic effect of RS virus infection in young children. RS virus disease seems to have an immunological component since children vaccinated with inactivated RS virus developed a severer response to natural infection than non-vaccinated children (Kim et al., 1969a).

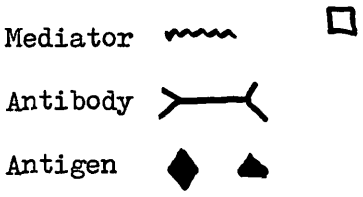
One group of workers suggested that RS virus-induced severe lower respiratory tract disease is the result of a type II or III (Gell and Coombs, 1963) immunological response (see Figure 1) (Brandt et al., 1973 and Chanock et al., 1970). However, another group proposed that RS virus bronchiolitis is the result of a type I (Gell and Coombs, 1963) reaction but RS virus pneumonia has a different mechanism (Aherne et al., 1970; Gardner et al., 1970a and Urquhart and Gibson, 1970). RS virus pneumonia was thought to result from direct virus damage to the lungs in the absence of local antibody.

Other theories have been put forward as explanations of severe RS virus illness; for example, that it results from lack of maternal antibody protection and immunological immaturity (Parrott et al., 1973 Ross et al., 1971) or that it is related to the size of airways in young

children (Simpson et al., 1974a and Simpson et al., 1974b). It has also been suggested that RS virus disease could result due to delayed hypersensity (type IV reaction) (Chanock et al., 1970). The production of interferon may be a factor in RS virus pathogenesis. Low levels of interferon have been found in patients with RS virus disease (Ray et al., 1967). However in vitro although RS virus is sensitive to interferon it is not a good inducer (Corbitt, 1971 and Gardner et al., 1970c). Moehring and Forsyth (1971) showed that the yield of interferon induced by RS virus in WI-38 cells was increased when 2% rather than 10% foetal calf serum was used in the medium and the multiplicity of infection (moi) was 0.01 or 0.01 pfu/cell rather than 1.0 pfu/cell. The non-viral inducer of interferon polyl poly C when used at low levels (1 or 10 μg/ml) which did not induce detectable levels of interferon inhibited RS virus growth (Hill et al., 1969 and Hill and Baron, 1969).

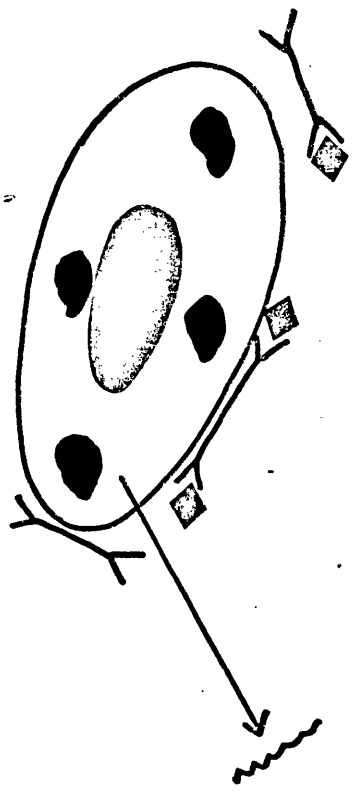
Figure 1 : Types of allergic reaction

The diagram is a modified version of figure 25.1 from Coombs and Gell, 1971.



> Specific antigen-combining receptors on membrane of specifically sensitised lymphocytes.

TYPE I



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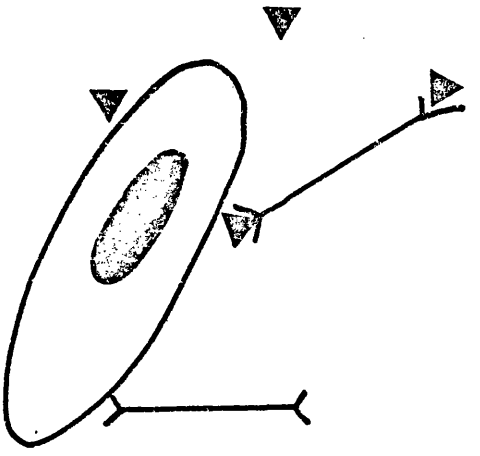
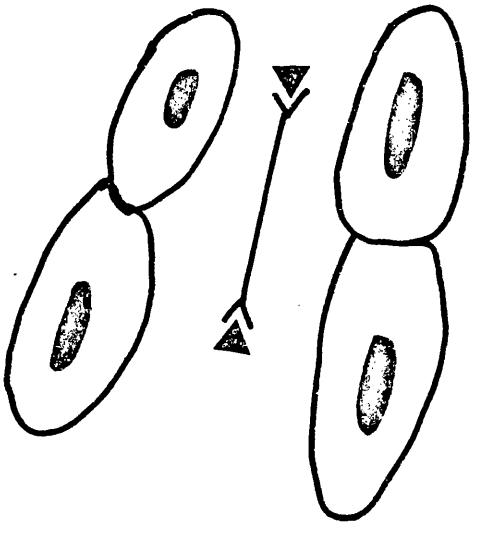


TYPE III



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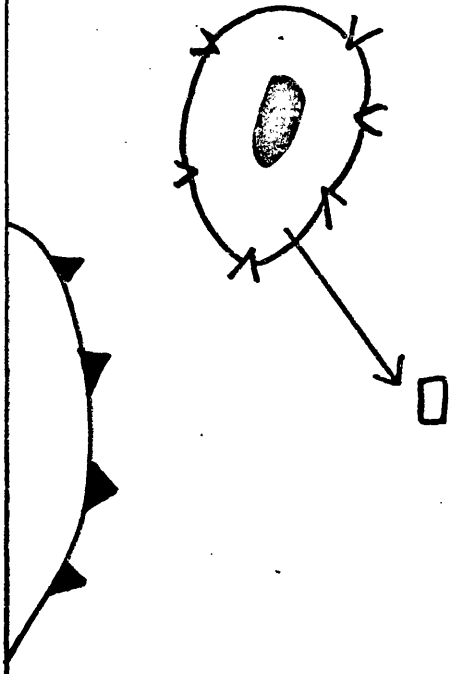


Figure 1 : Types of allergic reaction

Type I reaction (Anaphylactic, reagin-dependent)

Antibodies (IgE) are either associated with or adsorbed onto the membrane of tissue cells. A type I reaction results from the interaction of antigen with these passively sensitised cells. Histamine and other vasoactive substances are released by the sensitive cells.

Type II reaction (Cytotoxic or cell-stimulating)

The antibody (IgG or IgM) reacts by means of its combining receptors with either an antigenic component of tissue cells or antigen which is closely associated with these cells. When complement is present, interaction between the antibodies and the surface antigenic components of cells can result in cell damage.

Type III reaction (Arthus-type, antigen-antibody complexes)

Complexes are formed between antigen and antibody. When antigen is present locally in moderate excess the complexes do not precipitate but remain soluble, causing local cell damage. This is an Arthus-type reaction.

Type IV reaction (Delayed hypersensitivity)

Actively sensitised lymphocytes possibly of T (thymus derived) population respond specifically to antigen by releasing lymphokines and/or by the development of cytotoxicity.

1.4.2 Biological aspects of RS virus

RS virus infection of laboratory animals

RS virus can be recovered from the upper respiratory tract of infected ferrets (Pinto et al., 1969), minks and marmosets 24 hrs after intranasal inoculation but not 7 days after infection. Serial passage of RS virus in ferrets did not increase its virulence. RS virus CF antibody was produced in a variety of experimentally infected animals (ferrets, mink, AKR mice, chinchillas) none of which developed RS virus disease (Coates and Chanock, 1962). However, guinea-pigs inoculated with RS virus through their eardrums developed RS virus otitis media. Only a low level of neutralising antibody developed in a few guinea-pigs' sera, about 2 weeks after inoculation. When guinea-pigs were given intranasal or intracardiac injections of RS virus most of them developed both CF and neutralising antibodies but no RS virus disease was produced (Berglund, 1967). In RS virus infected guinea-pigs CF antibody decayed faster than serum neutralising antibody and was no longer detectable 8 weeks after inoculation (Berglund, 1967 and Hambling, 1966a).

RS virus in certain circumstances can be pathogenic for mice. It has been adapted to grow in the brain of suckling mice causing death between 6 to 14 days after inoculation (Cavallaro et al., 1966). Encephalitis of infected suckling mice was associated with widespread necrosis and liquefaction of the brain. The adapted strain of RS virus is neuropathic; it only produces lesions in the nervous system of intracerebrally inoculated suckling mice.

Initially, it was found that this neuropathic strain could not be passaged intracerebrally in adult mice, 3-4 weeks old. However it has now been shown to grow in the brain of 7 to 9 day old mice (Cavallaro et al., 1967). RS virus is not pathogenic for mice when intraperitoneal, intranasal or intrathoracic injections are given. No RS virus was recovered from the lungs of inoculated animals (Cavallaro et al., 1966).

RS virus growth in tissue culture

Cells

RS virus cytopathic effect (C.P.E.) in tissue culture is dependent on the type of cell and composition of medium used. The characteristic CPE of RS virus infected cells is the formation of syncytia. However, not all RS virus infection of cells results in the production of syncytia, for example RS virus infected DMB cells round up rather than form syncytia. Syncytia are pronounced in RS virus infected HEp-2 cells but minimal in KB and HeLa cells.

When maintenance medium with Scherer's solution was used CPE was delayed and the development of syncytia was restricted. However, the yield of infectious virus was not altered by the type of CPE produced (Jordan, 1962). The sensitivity of cells to RS virus infection varies, human diploid cells such as WI-38, HeL-1 (human embryonic lung) (Anderson and Beem, 1966), L-4, L-49 (Dreizin et al., 1967), human embryonic lung fibroblasts (HLDC-1,-4,-6), human embryonic brain cells (HBC-1,-4) (Larionov and Soloneva, 1968) and HL cells (human heteroploid cells (Cavallaro and Monto, 1972) were more sensitive to RS virus than

HEp-2 cells. A continuous human amnion cell line (U) has also been reported to be more sensitive than HEp-2 cells, especially for initial isolation of RS virus (Young and Matthews, 1969).

However, HEp-2 cells seem to be more sensitive for RS virus than HeLa, monkey kidney, human amnion and human kidney cell lines (Bennet and Hamre, 1962). One group of workers found unlike other groups (Bennet and Hamre, 1962; Chanock et al., 1962 and Lewis et al., 1961) that monkey kidney cells were much more sensitive to RS virus than HEp-2 cells (Wulff et al., 1964b). Bovine testis cells appeared to be more sensitive to RS virus than HeLa cells (Caul et al., 1974).

RS virus can grow well in suspensions of HEp-2 and MA-160 cells (human heteroploid cell line derived from benign prostate cells). Suspension cultures would be a good method to use in the production of large quantities of RS virus (Richman and Tauraso, 1971). Using the RSN-2 strain of RS virus I found that it grew in HeLa, HEp-2, WI-38, Vero and CV-1 cells but not in BHK/C_{13} or L cells (Wunner et al., 1975).

RS virus plaque assay

In 1963, a plaque assay using HEp-2 cells was described for RS virus (Long strain). This method was found to be both reliable and sensitive. Infected cells were incubated at 36°C and plaques first appeared 9 days after infection. These plaques reached their maximum size (2 mm in diameter) 11 to 12 days after infection (Kisch and Johnson, 1963). Another plaque assay also using HEp-2 cells but a different RS virus strain (Randall strain) was developed about the same time. This assay differed from the above assay in the time required for the development of RS virus CPE; it was much shorter, 3 days at 37°C,

as compared with 9 days at 36°C. However, this method had a longer adsorption period, 12 hours at 37°C as opposed to 2 hours at 36°C in the above assay and used liquid instead of agar overlay. A linear relationship existed between concentration of virus and the number of syncytia formed. Secondary plaques were not thought to develop before 76 hr after inoculation as the distribution of syncytia was Poissonian until that time (Taylor-Robinson and Doggett, 1963).

Although RS virus antiserum added to the overlay medium prevented the formation of secondary syncytia it also reduced the size and number of primary syncytia produced. This suggested that syncytium formation was not solely due to intercellular spread of virus (Taylor-Robinson and Dogett, 1963).

Long strain of RS virus grown in HeLa cells forms macroscopic plaques under agar 5 - 6 days after infection (Levine and Hamilton, 1969).

Overlay for RS virus infected cells

DEAE Dextran (Diethylaminoethyl dextran)

DEAE dextran added to either the virus inoculum or the maintenance medium has been reported to increase RS virus infectivity by various amounts. Some workers found that the addition of 15 µg/ml DEAE dextran to the maintenance medium of RS infected HEp-2 or HeLa cells increased infectivity of RS virus 100 fold (Nisevich et al., 1972) whereas another worker only found a 2-5 fold increase in RS virus infectivity when 30 µg/ml and 10 µg/ml of DEAE dextran was added to RS virus inoculum used in HEp-2 and AGMK (African green monkey kidney cells) cells respectively. DEAE dextran incubated with RS virus (30 µg/ml)

at 36°C for 2 hours before inoculation increased virus infectivity 2-fold (Normura, 1968).

Protamine sulphate

Protamine sulphate at low concentrations (5 μg/ml) in the virus inoculum marginally increased RS virus infectivity; however higher concentrations (60 μg/ml) can cause as much as 50% inhibition of infectivity. Protamine sulphate incubated with RS virus for 2 hours at 36°C before inoculation increased RS virus infectivity as much as DEAE dextran (2-fold) (Normura, 1968).

Since protamine sulphate has a different effect to DEAE dextran when added to RS virus inoculum, ionic charge is not the main factor involved in the action of polycations in increasing RS virus infectivity. Probably DEAE dextran complexed with RS virus particles and increased their efficiency of attachment by binding to cell membrane. Protamine sulphate could in process of binding to the cell membrane block cellular receptors thus restricting the attachment of RS virus particles.

However, both DEAE dextran and protamine sulphate incubated with the virus before inoculation increase its infectivity. This could be due to these polycations having a stabilising effect on RS virus particle (Normura, 1968).

Dextran sulphate and Heparin

Dextran sulphate and heparin in low concentrations (5 μg/ml) in the virus inoculum or incubated with the virus before inoculation for 2 hours at 36°C have an inhibitory effect on RS virus growth in HEp-2 cells (Normura, 1968).

RS virus can be grown in the absence of CO_2 if L-15 medium overlay is used. There was no marked difference in the type of plaques formed or total virus yield compared with virus grown in the presence of CO_2 using ordinary overlay medium (Potash and Gilbertson, 1967).

RS virus infected HEp-2 cells required Eagle's MEM overlay medium to have at least 18 μg/ml glutamine otherwise syncytium formation was totally inhibited. When sufficient glutamine was added to infected cultures in glutamine-free medium RS virus CPE occurred after 2 days irrespective of the time infected cultures were in deficient medium. When the normal concentration of glutamine (290 μg/ml) in Eagles MEM medium was reduced to 72 μg/ml RS virus CPE was delayed although no reduction in virus titre occurred (Marquez and Hsuing, 1967). These findings are in direct conflict with previous experiments, where the absence or presence of glutamine had no effect on the production of syncytia in RS virus infected HeLa, KB and HEp-2 cells (Jordan, 1962).

Haemagglutination test

No haemagglutinins have been detected in RS virus infected tissue culture fluids when human type O, guinea-pig and chicken erythrocytes were used (Bennet and Hamre, 1962 and Chanock et al., 1957 and Peacock and Clarke, 1961). Other workers using the supernatant of RS virus (Randall Str) infected HEp-2 culture fluid found no haemagglutination of rooster, chicken and guinea-pig erythrocytes (Beem et al., 1960).

A thorough attempt to demonstrate RS virus haemagglutinin was carried out using RS virus (Burnett Str) grown in MA-104 (embryonic rhesus

monkey kidney), MA-160 (adult human prostate), WI-38 and HEp-2 cells. The haemagglutination test was performed at various temperatures (4°, 23° and 37°C) and at a pH range of 5.8 to 7.4 using wide range of red blood cells: human type O, rhesus monkey, African green monkey, Hartley guinea-pig, NIH albino rabbit, golden ~~Syrian~~ SYRIAN hamster, Swiss NIH random bred mouse strain, Sprague-Dawley albino rats, White Rock cockerel, White Leghorn chickens (1 day old), White Embden gander, Hampshire sheep, and Hampshire swine. The test was carried out for 30 to 90 mins depending upon temperature till control cells had sedimented. No haemagglutinin was detected in any of the experiments (Richman et al., 1971).

Haemadsorption test

Guinea pig, chicken and human type O erythrocytes did not adsorb to RS virus infected monkey kidney cells (Beem and Hamre, 1962) and rooster, chick and guinea-pig erythrocytes did not adsorb to RS virus infected HEp-2 cells (Beem et al., 1960). More recently an extensive search for haemadsorption has been carried out using RS virus infected MA-104 cells when CPE was well developed (25% to 50% of cell sheet involved). Red blood cells (RBC) from more than one animal of the same species were tested separately to avoid variation in sensitivity to RS virus infected cells which might occur between animals. The RBC's used were from rhesus monkey, African green monkey, chimpanzee, gelada, guinea-pig, rat, hamster, Swiss NIH inbred strain mouse, NIH-CFW white strain mouse, Pekin duck, goose, White King pigeon, cockerel and one-day old chickens (pooled). However haemadsorption could not be detected in any of the tests (Richman et al., 1971).

Neuraminidase

It has been reported that RS virus has no neuraminidase; however no experimental results in support of this claim have been presented (Waterson and Hobson, 1962).

Cell Fusion

Using various intravital stains such as janus green (for chondriome), neutral red (for vacuoles) and trypan blue (for dead cells) as well as phase contrast microscopy alterations in RS virus infected cell chondriome and vacuoles were followed.

Cell chondriome alteration was suggested to be induced by virus penetration of the cell. During the first 20 hours after infection cell chondriome became swollen, and later towards the end of the RS virus replication cycle disappeared as RS virus syncytia were formed. Syncytia can be seen as early as 16 hours after infection although most develop later. RS virus infection of cells does not destroy the nuclei (Wilczynski, 1971). The nuclei stay on the periphery of RS virus syncytia (Kisch et al., 1962 and Wilczynski, 1971) whereas in measles virus syncytia the nucleus stay in the centre. (Klone et al., 1966). However the alterations in cell chondriome and vacuoles were similar to those occurring during myxovirus infection (Wilczynski, 1971).

It has been reported that the formation of RS virus syncytia resulted in total inhibition of alkaline phosphatase activity in the cytoplasmic membrane and the complete loss of nuclear histones. An accumulation in the amount of phospholipids and a concentration of lysosomes in the middle of syncytia were also reported (Bonissol, 1966).

RS virus infection of cells can induce cell fusion. Polykaryocytes developed in HEp-2 cells 16-18 hours after RS virus infection. This was followed by lysosomal activation which preceeded cell fusion. Pretreatment of cells with hydrocortisone hemisuccinate, a lysosome stabilising agent, reduced the amount of RS virus induced cell fusion. Polykaryocytes were still produced but they were smaller and had fewer nuclei (Greenham and Poste, 1971; Poste, 1971a and Poste, 1971b).

Physical properties of RS virus

Density of RS virus particle and complement-fixing antigen

Initially it was shown that RS virus infectious particles could be separated from soluble complement-fixing (CF) antigen by differential centrifugation (30,000 rpm/45 min). The CF antigen was recovered from the supernatant fluid while the pellet contained 90% of the recoverable infectivity (Chanock et al., 1957). Further experiments were performed to separate and identify components of RS virus soluble CF antigen.

The tissue culture fluid from RS virus (Long and 18537 strains) infected cells (HEp-2, BEK) was concentrated by vacuum dialysis and used in the experiments. Most of the infectious RS virus (90%) from concentrated RS virus tissue culture fluid was recovered at a density of 1.22-1.24 g/ml after CsCl equilibrium centrifugation, whereas using unconcentrated RS virus fluid the infectivity was recovered at a higher density (1.25-1.27 g/ml). There was no difference in the density of virus particles between different strains of RS virus using this method. The complement-fixing (CF) antigen activity was detected in density range

1.23-1.37 g/ml in CsCl gradient (Coates et al., 1966).

Other workers have also reported similar results using concentrated RS virus material in CsCl equilibrium density gradients. Infectious particles had a density 1.21-1.23 g/ml and the CF antigen a density range 1.25-1.34 g/ml. Two populations of particles were found when RS virus concentrate of material was examined by discontinuous CsCl gradient. The rapidly sedimenting particles had most of the virus infectivity as well as 10% of the total CF activity associated with them, whereas the remaining CF antigen activity was associated with the slower sedimenting particles (Bloth and Norrby, 1966).

Soluble CF antigenic material from RS virus infected tissue culture fluid can be separated by G200 Sephadex column chromatography into two fractions (A and B). Specific antibodies to A or B were produced in guinea-pigs inoculated with A or B respectively. Antigen A was found to have a density of 1.28-1.32 g/ml and Antigen B a density of 1.34-1.37 g/ml in a CsCl equilibrium density gradient. Guinea-pigs immunised with antigen A developed CF antibody to antigen A and neutralising antibody, whereas those inoculated with antigen B only developed CF antibody to antigen B. There was no difference in density between antigen A and B when RS virus culture fluid was centrifuged to equilibrium through preformed sucrose gradient. Therefore the difference in density seen in CsCl gradients could be due to the difference in reaction to ~~calcium~~ CAESIUM ions rather than actual difference in density.

The density of antigen A in CsCl (1.28-1.32 g/ml) indicates that it could probably be the virion coat antigen. Antigen B is probably a component of the virion since it can be released from intact

particles by ether-Tween treatment. However, it is unlikely to be associated with the surface of the virion as it does not induce neutralising antibody. Human serum reacts equally well with Sephadex-purified antigens A and B; however the main distribution of CF antigen activity reacting with human serum was found at a lower density (1.23 g/ml) which suggests the presence of a third antigen (Coates et al., 1966 and Forsyth et al., 1966). Tween 80-ether treatment of RS virus destroyed all virus infectivity but not complement-fixing activity (Bloth and Norrby, 1966). Antigen A was not sensitive to Tween-ether treatment whereas antigen B was very sensitive (Forsyth et al., 1966).

Identification of RS virus antigens by agar gel diffusion and electrophoresis

Sephadex-purified antigens A and B each produced only one precipitin line when examined by agar gel diffusion. The serum of guinea-pigs inoculated with either antigen A or B only produced on precipitin line when tested. Concentrated (200 - fold) RS virus infected tissue culture fluid gave only two precipitin lines on agar gel diffusion (Forsyth, 1970). However other workers on examination of serum from RS virus infected animals and RS virus material have detected three precipitin lines by agar gel diffusion (Berglund, 1967; Chanock and Parrott, 1965 and Manyjarvi et al., 1967). Precipitin lines (A and B) produced by sera with antibodies to A and B have different electrophoretic mobilities. The precipitin line corresponding to antigen A moves towards the cathode whereas B remains close to the point of application. When human RS virus convalescent serum was examined by electrophoresis, only two antigens were detected, no third antigen was found (Forsyth, 1970).

Factors affecting RS virus stability

Thermolability

RS virus is very thermolabile. Hambling (1964b) reported that it loses infectivity rapidly at 55°C, only 10% of the virus survived for 5 minutes. Rechsteiner (1969a) using partially purified RS virus in distilled water showed a linear relationship between the logarithm of the inactivation rate constant and the reciprocal of the absolute temperature from 50°C to 37°C below this temperature there was no linear relationship. In earlier experiments with crude virus preparations (Beem et al., 1960; Bennet and Hamre, 1962 and Hambling, 1964b) the inactivation rates were lower probably due to the presence of stabilising agents in the tissue culture fluid used as a source of infectious virus (Rechsteiner, 1969a).

RS virus stored at -70°C or below is stable (Jordan, 1962; Rechsteiner, 1969a and Rowe and Michaels, 1960). However RS virus stored at -20°C is inactivated more rapidly than at room temperature (Wulff et al., 1964b).

Sensitivity to freezing and thawing

It has been general experience that RS virus can more easily be isolated from fresh rather than frozen material as it is sensitive to freezing and thawing especially if frozen slowly (Beem et al., 1960; Chanock et al., 1961; Hambling, 1964b and Holzel et al., 1963). However if RS virus is frozen rapidly and stored at -70°C or below, a single cycle of freeze-thawing may even enhance RS virus infectivity (Jordan, 1962 and Rechsteiner, 1969a).

The effect of serum concentration on the stability of RS virus

Hambling (1964b) found that increasing the concentration of heat-inactivated rabbit serum from 5% to 45% in RS virus suspensions did not protect RS virus against loss of infectivity at 4°C or -65°C. However, 45% serum showed a slight protective effect at -30°C.

The effect of pH

Jordan (1962) reported that RS virus growth was better in alkaline than acidic medium. Other experiments confirmed this finding as RS virus was found to be most stable at pH 7.5. If the pH deviated from this value RS virus lability increased especially if pH was lowered (Hambling, 1964b).

The effect of salt concentration

The concentration of different salts in RS virus suspensions affected the stability of the virus.

Partially purified RS virus in distilled water was protected against heat inactivation at 30°C, 20°C and 10°C by the addition of molar amounts of NaCl, $MgSO_4$, Na_2SO_4 and sodium phosphate. Protection decreased with decreasing molarity and no protection was given to RS virus by 0.1M solutions of these salts (Rechsteiner, 1969a).

Other experiments using RS virus in 0.1M tris buffer (pH 8) also showed that the addition of 1M NaCl protected RS virus against inactivation. The stabilising effect obtained by addition of 2M NaCl, 2M $MgSO_4$ or 2M Na_2SO_4 to RS virus suspension was similar to that obtained by addition of 1M NaCl (Yamamoto, 1969). However, RS virus in distilled

water was not protected by addition of 1M $MgCl_2$ (Rechsteiner, 1969a) whereas some protection was given to RS virus in 0.1M tris buffer by addition of $MgCl_2$ (Yamamoto, 1969).

The effect of glucose and sucrose

Glucose (1M) had a similar effect to 1M NaCl in the protection of RS virus against heat inactivation except that its effectiveness was not reduced by decreasing the temperature to 0°C (Rechsteiner, 1969a).

Sucrose (44.5%) also had the same stabilising effect on RS virus suspensions as 1M NaCl (Yamamoto, 1969). It preserved RS virus infectivity for at least 2 years when the virus was stored at -70°C or below (Law and Hull, 1968).

The effect of lecithin

Lecithin enhanced RS virus inactivation at 10°C and 20°C as the virus in lecithin suspensions was inactivated more rapidly than the controls (Rechsteiner, 1969a).

The effect of skim milk

Bennet and Hamre (1962) reported that the addition of skim milk (20%) to RS virus culture fluids preserved the virus infectivity for at least 30 days at -40°C and longer at -60°C.

The survival of RS virus in aerosols

The inactivation of RS virus in aerosols has been studied at different relative humidities. Two inactivation mechanisms are possibly involved since two peak rates of inactivation occurred.

However, oxidation is not the cause of the inactivation since the same experiments in an atmosphere of pure N_2 gave similar results (Rechsteiner and Winkler, 1969 and Rechsteiner, 1969b).

Cytochemical studies

Eosinophilic intracytoplasmic inclusions are more frequently found in syncytia than in single cells (Adams et al., 1961a; Bennet and Hamre, 1962 and Kisch et al., 1962).

Acridine Orange

In one experiment acridine orange stained perinuclear RS virus inclusions red (Levine et al., 1971), in other experiments acridine orange stained RS virus inclusions green (Berthiaume et al., 1974; Bonissol, 1966 and Corbitt and Beswick, 1972) whereas in yet other experiments no staining of inclusions occurred (~~Homsleth~~ HORNSLETH et al., 1969; Kisch et al., 1962 and Paccaud and Jacquier, 1971). If single-stranded RNA was present in the inclusions acridine orange would stain them a bright orange-red colour. Green fluorescence with acridine orange staining indicates that the inclusions contain denaturated nucleoprotein (Bonissol, 1966), DNA or double-stranded RNA (Corbitt and Beswick, 1972). Enzyme digestion (RNase and DNase) did not remove the green coloration from the RS virus inclusions, neither did hot ~~trioarboxylic~~ TRICHLORACETIC acid (TCA) treatment. Therefore, the green fluorescence is probably due to acridine orange binding to protein (Corbitt and Beswick, 1972).

Methyl green-pyronin

When RS virus infected cells were treated with methyl green-

pyronin the inclusions stained deep pink. This suggested the presence of RNA especially since pretreatment of a fixed cell preparation with RNase before staining prevented coloration of the inclusions. DNase had no effect on the staining of the inclusions but hot TCA removed the stain. This indicated that RNA if present was mostly in the single-stranded form (Corbitt and Beswick, 1972). However, Bonissol (1966) using the methyl green-pyronin staining technique did not find RS virus inclusions stained, whereas parainfluenza types 2 and 3 inclusions stained red.

Immunofluorescence

Levine and his colleagues (1971) detected RS virus specific immunofluorescence in infected HEp-2 cells from adsorption onwards; only 8-10 hours after infection could specific fluorescence be detected in the perinuclear inclusions. However, other workers did not detect RS virus specific antigen in HEp-2 cells to between 8 and 12 hours after infection (Bennet and Hamre, 1962; Berthiaume et al., 1974 and Kisch et al., 1962 and Nagahama et al., 1974).

Cytoplasmic inclusions which can be stained by haematoxylin and eosin have been reported to be both different from (Nagahama et al., 1970 and Paccaud and Jacquier, 1970) and similar to (Bennet and Hamre, 1962 and Levine et al., 1971) the inclusions detected by immunofluorescence.

Electron microscopic studies of RS virus

RS virus is very pleomorphic, both filamentous and spherical particles can be detected. The difference in size of particles seen is

outlined in Table 4. The variation in size of the RS virus virion may be due to the different techniques used as the virion could be distorted as a result of osmotic pressure or centrifugation (Bloth et al., 1963). Incomplete virus particles, i.e. without a nucleocapsid, were predominant when concentrated RS virus culture fluid and thin sections of infected Vero cells were examined (Bachi and Howe, 1973).

The RS virus nucleocapsid is fragile and has a tendency to unwind. Its diameter has been reported to be various sizes ranging from 12 nm (Bloth and Norrby, 1965) to 17 nm (Bloth and Norrby, 1967) (see Table 4). However, some workers found that by avoiding harsh treatments such as concentration of RS virus by centrifugation and dialysis, they could obtain reproducible results for the diameter of the RS virus nucleocapsid, 13.5 nm (Berthiaume et al., 1974; Joncas et al., 1969 and Zakstelskaya et al., 1967).

RS virus envelope projections have been reported to be of varying lengths, ranging from 10 nm (Bachi and Howe, 1973) to 17 nm (Bloth et al., 1963). Bloth and Norrby (1965) considered these projections to be similar in size and shape to the haemagglutinin spike of NDV. When RS virus is grown in the presence of 10 mM 2-deoxy-D-glucose (2dG) virions are produced in fewer numbers and without projections. This seems to indicate that 2dG inhibits the formation of RS virus projections (glycoproteins) (Hodes et al., 1975).

No nuclear inclusions have been seen in thin sections of RS virus infected cells (Kalica et al., 1973; Kisch et al., 1963 and Norrby et al., 1970) with the exception of one report of nuclear inclusions in RS virus infected HeLa cells (Armstrong et al., 1962). If the nucleus

is involved in RS virus infection, its role cannot be essential since RS virus grows in enucleate BSC-1 cells (Follett et al., 1975). Two types of cytoplasmic inclusion have been observed; the paranuclear fibrillar inclusions and denser, more granular inclusions (Armstrong et al., 1962; Berthiaume et al., 1974; Kalica et al., 1973 and Norrby et al., 1970). RS virus buds from the cytoplasmic and vesicular membranes about 15 hours after infection. The RS virus particles usually contain 4-5 granules per particle. Inclusions are usually detected at the same time or a few hours earlier than budding (Bachi and Howe, 1973; Berthiaume et al., 1970; Norrby et al., 1970).

Some workers have reported that free RS virus nucleocapsids have a herring-bone pattern similar to that seen in the nucleocapsids of paramyxoviruses (Bachi and Howe, 1973 and Bloth and Norrby, 1967). However, Berthiaume and his colleagues (1974) have reported that RS virus nucleocapsids were arranged in straight parallel fibres in the virion. This is different from the superhelical arrangement seen for the nucleocapsids of the ortho- and para- myxoviruses.

TABLE 4

Electron microscopic studies of RS virus

Method of Preparation	Virion Diameter (nm)	Nucleocapsid Diameter (nm)	Projection Length (nm)	Reference
Sedimentation on sucrose. Particle diameter estimated from formula. Long strain used.	90-130	ND	ND	Chanock et al., 1957
Thin section RS virus (Long) infected HeLa cells.	Spherical particle 65 nm Filamentous particle 60-70 nm	ND	ND	Armstrong et al., 1962
Negative Staining 2% PTA (1) Clarification virus material (3,000 rpm for 30 mins). (2) Concentration by forced dialysis (3) Centrifugation on 30% sucrose or centrifugation at 20,000 rpm for 1 hour. Long strain used.	Spherical and filamentous particles. 90-860 Average 340	ND	Club-shaped 13-17. width 4-7 centre to centre distance 7-9	Bloth et al., 1963
Negative Staining PTA (1) PEG concentration (2) Centrifugation 20,000 rpm/60 min on CsCl density gradient (3) Material used or treated Tween 80 and Ether Long strain was used	Tween 80 and ether treated virus - Rosette-like particle 50-70 average 60	Free nucleocapsid from disrupted particles 10-12 pitch 7	12-16 resembled HA spikes of NDV and Sendai virus	Bloth and Norrby, 1965

TABLE 4 (contd)

Method of Preparation	Virion Diameter (nm)	Nucleocapsid Diameter (nm)	Projection Length (nm)	Reference
Negative Staining 2% PTA (1) Virus material frozen and thawed or homogenised (2) Low speed centrifugation (3) Concentration by ultra-centrifugation 41, 190 x g for 30 min Long strain used	spherical particles 150 filamentous particles	13-18 nucleocapsid material from disrupted particles	ND	Eckert et al., 1965
Negative Staining STS and PTA (1) concentration by forced dialysis (2) clarification low speed centrifugation (3) centrifugation in discontinuous CsCl gradient Long strain used	ND	16-18 estimated diameter of 20 intact nucleocapsids Herring-bone like structure Pitch 7	ND	Bloth and Norrby, 1967
Negative Staining (1) low speed centrifugation (3,000 rpm/5 min) (2) cells kept and suspended in distilled water and allowed to disrupt. Stain 3% PTA. Long strain of RS virus was used.	80-100 spherical and filamentous forms. Rosettes also seen	Helix herring-bone arrangement just seen - 13.5	15 resemble projections of avian infectious bronchitis virus	Zakstelskaya et al., 1967

TABLE 4 (contd)

Method of Preparation	Virion Diameter (nm)	Nucleocapsid Diameter (nm)	Projection Length (nm)	Reference
Negative Staining (1) Centrifugation at low speed (2) Sediment freeze-thawed or given hypotonic shock. Stain 3% PTA Supernate of high speed centrifugation 30,000 rpm/1 hr also used. Three newly isolated strains of RS virus were used	Spherical, filamentous and pleomorphic particles. 80-500 spherical 100-350	Nucleocapsid more delicate flexible and narrower than that of Parainfluenza type 2 13.5 range 12.5-15	15	Joncas et al., 1969
Thin Section RS virus (Long) infected Vero cells	90-130 Filamentous forms 100-130 length 2 nm	11-15	12-15	Norrby et al., 1970
Thin Section RS virus (Long) infected Vero cells Negative Staining (1) infected culture fluid centrifuged 120,400g/60 min at 4°C onto cushion of 55% sucrose (2) sucrose removed by overnight dialysis Stain 2% PTA	Spherical particles Av. 150-250 range 150-500 Filamentous particles 90-100 length 1-4 mμ some 10 mμ seen	15 herring-bone pattern	10	Bachi and Howe, 1973

TABLE 4 (contd)

Method of Preparation	Virion Diameter (nm)	Nucleocapsid Diameter (nm)	Projection Length (nm)	Reference
THIN SECTION RS virus (A_2) infected HeLa cells	Spherical and filamentous particles 80-140 length of filaments 2 mμ	11-15	11-120	Kalica *et al.*, 1973
THIN SECTION RS virus (Long) infected HEp-2 cells	Negative Staining Spherical particles 100-350	13.5 pitch 6.5 centre to centre length 10	12 club-shaped	Berthiaume *et al.*, 1974
Negative Staining Infected cells pelleted 450 g for 10 mins	Filamentous particles 60-110 Length 5 mμ Pleomorphic particles 80-500 Thin Section spherical or filamentous particles 100 nm in diameter	Thin section 12	Thin Section 12	

1.4.3 RS virus nucleic acid

Intracellular RNA

Although on indirect evidence (e.g. ether sensitivity, resistance to mitomycin C, growth in enucleate cells) RS virus appears to be an RNA virus, the virion RNA has not yet been isolated.

In uninfected HeLa cells labelled with ^{3}H-uridine the main species of RNA detected were 28S, 18S and 4S, whereas in RS virus infected cells cellular RNA synthesis was depressed and the major species detected was 4S RNA.

In experiments using actinomycin D (5 μg/ml) the incorporation of ^{3}H-uridine into RS virus infected cells was poor. However, reproducible peaks of radioactivity could be detected. In RS virus infected HeLa cells, 24 hours after infection, a peak was detected at 10S; 36 hours after infection when maximum incorporation of label occurred two peaks were seen, one at 10S and one at 28S respectively. No heavier RNA species was detected although the characteristic size of paramyxovirus virion RNA's is 50S. The nucleic acid detected in RS virus infected cells was mainly single-stranded RNA since most of the radioactivity of the sample disappeared on alkaline hydrolysis or *N. Crassa* nuclease treatment (Hodes *et al.*, 1974b).

The RNA in RS virus infected BS-C-1 cells labelled with ^{3}H-uridine in presence of actinomycin D (2.5 μg/ml) was heterogeneous, with the major species sedimenting at 16S and a minor species sedimenting at approximately 50S. The main RNA species detected in ^{3}H-uridine labelled partially purified RS virus were at 16S and 4S. When RS virus infected

cells labelled in presence of actinomycin D with ^{3}H-uridine were co-extracted with ^{14}C-uridine labelled uninfected cells using SDS-phenol method, the main species found in infected cells was 4S RNA with small amounts of 13-16S, 25-28S and approximately 50S RNA (Wunner et al., 1975).

Inhibition of viral RNA synthesis in RS virus infected cells

Indirect methods have been used to detect the period in the RS virus growth cycle when synthesis of viral RNA occurred. The uridine analogues, 6-azauridine (6-AU) and 2-thiouridine (2TU) prevent viral RNA synthesis. The effects of these inhibitors on RS virus growth was studied by their addition at different times after adsorption to RS virus infected cells. When 6-AU (10 μg/ml) or 2TU (50 μg/ml) were added 2 hours after adsorption no RS virus was produced. However if they were added any time between 5 and 18 hours post-infection some RS virus production occurred. Therefore, RS virus RNA must have accumulated in the cells during this period since it was available for virus production after the addition of inhibitors of RNA synthesis. The curves for virus production and viral RNA synthesis were parallel, the curve for viral RNA synthesis being 5 hours ahead (Levine and Hamilton, 1969 and Levine et al., 1971).

The data indicated that synthesis of RS virus RNA started 5 hours after infection (Levine and Hamilton, 1969). This was supported by a fluorescent photometry experiment which reported an increase in RNA-content of RS virus infected cells 4 hours after RS virus infection. An increase in the DNA-content of RS virus infected cells 10 hours after infection was also noted (Augusten, 1974). In phenylalanine deficient

medium RNA synthesis in RS virus infected cells occurred at the same rate as in normal medium (Levine et al., 1971)

Inhibitors of DNA-dependent RNA synthesis

Actinomycin D

Hornsleth (1969) found that low concentrations of actinomycin D (0.04 μg/ml) did not inhibit RS virus replication when added together with the virus or 1 hour before, 3 hours after or just after adsorption of the virus. Pretreatment of the HEp-2 cells with actinomycin D (0.04 μg/ml) for 24 hours reduced the RS virus yield at 44 hours post infection but it also reduced the number of cells present by about 50%. Therefore, the effect of actinomycin D on the cells as well as the virus must be considered. Other workers found that actinomycin D (0.05 - 0.5 μg/ml) when added 1 hour before infection and present until the virus harvest reduced RS virus yield at 72 hours but enhanced it at 24 hours after infection (De Jong and Harmsen, 1973). Formica and his colleagues (1969) found that RS virus replication was inhibited in the presence of 0.1 to 2.0 μg of actinomycin D/ml. Therefore the effect of actinomycin D on RS virus growth appears to be variable.

Proflavine

Proflavine has been found to be more effective in inhibiting DNA polymerase activity than DNA-dependent RNA polymerase activity, unlike actinomycin D which has been found more effective in inhibiting DNA-dependent RNA synthesis (Hurwitz et al., 1962). Hornsleth (1966a) reported that proflavine (0.05 - 2 μg/ml) reduced the RS virus growth cycle in HEp-2 cells. The acceleration of virus growth on the addition

of proflavine was suggested to be the result of the inhibition of cellular RNA and protein synthesis by proflavine. Riboflavine (0.5 or 5 μg/ml) can reverse the effect of proflavine on the RS virus growth cycle.

1.4.4 RS virus proteins

Viral polypeptides

RS virus grown in HEp-2 cells and labelled with ^{3}H-amino acid was purified and analysed by SDS-polyacrylamide gel electrophoresis (PAGE). Seven polypeptides ranging in molecular weight from 26,500 to 90,000 were reported.

HEp-2 intracellular proteins from infected and uninfected cells were examined at 36 hours post infection. The number of newly synthesised proteins associated with the mitochondrial and microsomal subcellular fractions was markedly reduced in infected cells compared to uninfected cells. However most of the newly synthesised proteins produced by the infected cells which were associated with these fractions had similar electrophoretic mobilities to the virion polypeptides. The profile of proteins associated with the soluble fraction of infected and uninfected cells was similar and there was no relationship between these proteins and viral synthesis (Treuhaft and Beem, 1972).

When RS virus grown in BS-C-1 cells and labelled with ^{35}S-methionine was analysed by PAGE nine to ten polypeptides, six consistently, could be detected. The same number and size of polypeptides could also be detected using a discontinuous ~~SDS~~-PAGE system (Wunner and Pringle, in preparation). In previous experiments using partially purified RS virus (high speed centrifugation and isopycnic banding in sucrose gradient) labelled in the presence of actinomycin D, five major and several minor polypeptides were detected. The proteins extracted from cells simultaneously labelled with ^{14}C-leucine and ^{3}H-arginine had a high ratio of

arginine to leucine incorporation. This could explain the requirement of arginine for RS virus production (Wunner et al., 1975). In these experiments the molecular weights of the viral polypeptides were calculated on the basis of comparison with vesicular stomatitis virus (VSV) structural protein markers. The molecular weights of the RS virus polypeptides ranged from 48,000 to 2,5000 daltons (see Table 5).

Some of the polypeptides have been tentatively assigned functions, for example polypeptide VP48 (48,000 daltons) has been suggested as the glycosylated membrane protein. Nucleocapsids can be obtained by treatment of RS virus concentrated material with Triton X-100 followed by centrifugation of this treated material in a metrizamide density gradient (15-45%). The nucleoprotein peak contained polypeptide VP_{41} and polypeptide VP_{38} and some small amounts of higher molecular weight polypeptides. The nucleocapsids banded in CsCl density gradient at 1.3 g/ml which is similar to the density of Pneumonia virus of mice (PVM) (Compans et al., 1967) and paramyxovirus nucleocapsids (Wunner and Pringle, in preparation).

Other workers using polyethylene glycol (PEG) concentrated RS virus have reported that on treatment of this material with SDS (0.5%) followed by centrifugation on a sucrose gradient (30-70%) two bands are detected. These bands had densities of 1.27 g/ml and 1.19 g/ml in sucrose. ^{3}H-uridine was incorporated into the heavier of these bands which was suggested to be composed of nucleoprotein, whereas ^{3}H-lysine was incorporated into the band which was suggested to be composed of protein (Senterfit and Balridge, 1974).

TABLE 5

Summary of data abstracted from Wunner and Pringle, in preparation

Polypeptide	Molecular weight (daltons)	Glycosylation	Function
VP_{48}	48,000	+	Major polypeptide probably spike protein (G)
VP_{41}	41,000	-	Major polypeptide probably nucleocapsid protein (N), bands 1.22-1.38 g/ml in CsCl gradient
VP_{38}	38,000	-	Minor polypeptide associated with nucleocapsid polypeptide
VP_{31}	31,000	-	Major polypeptide function not known
VP_{27}	27,000	-	Probably matrix polypeptide (M)
VP_{25}	25,000	-	Minor polypeptide function not known
P_1P_2 P_3P_4	> 48,000	?	High molecular weight polypeptides present in small amounts - could be cellular

The effect of inhibitors of glycoprotein synthesis

The glucose analogue, 2-deoxy-D-glucose (2-dG) has been shown to have an inhibitory effect on the growth of some enveloped viruses; for example influenza A (fowl plague), Sindbis, Semlike Forest, and herpes simplex virus. Experiments have indicated that 2-dG appears to specifically inhibit the maturation of surface glycoproteins (Courtney et al., 1973; Kaluza et al., 1973 and Klenk et al., 1972).

The replication and cytopathic effect (CPE) of RS virus as well as parainfluenza virus type 3 (para 3) and measles virus was inhibited in the presence of 2-dG (1 mM or higher concentration). However the effect of 2-dG was reversible. The inhibitory effect of 2-dG occurred from 12 hours post-infection onwards. Although the presence of 2-dG in virus cultures caused a reduction in the amount of complement-fixing antigen produced (4-fold) some antigens, for example the immunofluorescent antigen, were not inhibited. When 2-dG was removed from RS virus cultures in the middle of the virus cycle (17.5 hours), exponential growth of the virus occurred after 4 hours. This short latent period indicated that early virus products probably accumulated during the 2-dG block since mature virions were released more quickly than normal when the block was removed.

It is unlikely that 2-dG directly affects the cell surface since pretreatment of cells with 2-dG and treatment during the virus adsorption period did not inhibit virus multiplication or CPE. Electron microscopic studies with RS virus ts-2, showed that virions produced in the presence of 2-dG had no spike-like projections. This indicates that 2-dG appears to act on RS virus by the inhibition of formation of surface

glycoproteins. Other workers have found this effect of 2-dG with different viruses, for example influenza A (fowl plague) (Klenk et al., 1972 and Scholtissek, 1974). However, not all enveloped viruses are inhibited by 2-dG, for example vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) replication were not affected. It has been suggested that this could be due to poor incorporation of 2-dG into VSV or NDV infected cells or that defects in the processing of viral glycoproteins in these viruses do not prevent the formation of infectious virus particles (Scholtissek, 1974).

The effect of inhibitors of energy generating systems on RS virus growth

Oxidative phosphorylation appears to be involved in RS virus replication as dinitrophenol (DNP), an uncoupling agent, causes marked inhibition of RS virus growth. However, the normal functioning of the TCA cycle does not seem to be essential for RS virus replication since sodium fluoroacetate and sodium malonate had no inhibitory effect. Sodium fluoroacetate inhibits aconitase, an enzyme which catalyses the oxidation of citric acid and sodium malonate inhibits succinic dehydrogenase, an enzyme which catalyses the oxidation of succinic acid to fumaric acid in TCA cycle.

RS virus replication could involve glycolytic pathways since sodium iodoacetate inhibited RS virus production. However, since RS virus did not grow in HEp-2 cells in an atmosphere of 5% carbon dioxide and nitrogen, aerobic glycolysis rather than anaerobic glycolysis would seem to be involved (Hornsleth, 1966b).

1.4.5 Genetics of RS virus

Isolation of ts mutants of RS virus (Gharpure et al., 1969)

Four conditional-lethal ts mutants (ts A1, ts A2, ts A3, ts A4) of RS virus (A_2 strain) on bovine kidney (BK) cells from plaque purified virus grown in the presence of 5-Flurouridine (10^{-4}M). The frequency of isolation of these ts mutants was low (0.8%) and their restrictive temperature was 39°C. Some of the ts mutants did not produce infective virus at 39°C (ts A1, ts A4) whereas other ts mutants (ts A2, ts A3) grew to a limited extent. The progeny obtained at 39°C did not produce plaques at that temperature and therefore represented "leakage". Ts A2 was more "leaky" than ts A3 since its growth was only reduced 16-fold at 39°C in contrast to 100-fold for ts A3. None of the ts mutants produced plaques at 38°C and ts A2 and ts A4 did not produce plaques at 37°C. Ts A1 and ts A3 did produce plaques at 37°C although there was a 100-fold decrease of their plaquing efficiency at that temperature. The plaques formed at 37°C were smaller, half the size, than plaques formed at 32°C.

It was suggested by Gharpure and her colleagues (1969) that spontaneous ts mutants of RS virus were rare, since no ts mutants were isolated from the survivors (249 plaques) of Nitrosoguanidine (NTG) treatment. Plaque purified RS virus was treated with NTG (100 μg/ml) for 15 minutes at room temperature and NTG was then removed by dialysis.

Other ts mutants of RS virus were isolated from different treatments. Ts A5 and ts A6 were isolated from the progeny of virus grown in the presence of 5-Flurouracil ($10^{-2.5}$M) whereas ts A7 was

isolated from virus exposed to ethylmethanesulphonate (EMS). Two of the new mutants (ts A6, ts A7) resembled ts A3 in that they grew to a limited extent at 39°C whereas the other mutant, ts A7 did not grow at 39°C. One of the seven ts mutants (ts A2) did not produce characteristic syncytial plaques on BK and HeLa cells. This mutant produced non-syncytial plaques which consisted of an area of pyknotic cells. BK cell line was used for the isolation and initial growth of the ts mutants since it was a suitable cell line for RS virus growth for vaccine production. However, RS virus produced very small irregular plaques on BK cells from which it was difficult to recover virus. Therefore, HeLa cells were used for characterisation of the ts mutants since they gave better CPE and grew to higher titre in these cells (Wright et al., 1973).

Whether the RS virus ts mutants isolated were the result of single or double step mutation is not known. The low frequency of isolation of the ts mutants (0.8%) coupled with the low concentration of 5-Fluorouridine used would suggest that single-step mutation was likely. However, the in vitro stability, i.e. low reversion frequency 10^{-5} to 10^{-7}, of the ts mutant might indicate that they have more than a single change in the nucleotide sequence.

The method used for isolation of the ts mutants would favour the more stable mutants since virus from each plaque was grown in BK cells before screening for plaquing efficiency at permissive (32°C) and restrictive (39°C) temperatures. Therefore, if any single step mutants with high reversion frequencies occurred they would not be detected by this method since they would appear to be wild type on screening (Gharpure et al., 1969).

Complementation between ts mutant of RS virus

Yield complementation tests for RS virus ts mutants were performed in HeLa cells infected at 39°C with mixtures of RS virus ts mutants at a multiplicity of infection (moi) of 5-10 pfu/cell for each mutant. The seven ts mutants examined were found to form three mutually exclusive complementation groups -

Definite complementation occurred between group A (ts A1, ts A3, ts A4) ts mutants and the group C (ts A7) ts mutant. Ts A7 complemented each of the other ts mutants to give an increase (11 to 210-fold) in yield at 39°C.

In mixed infections between group B (ts A2) and other ts mutants, a significant increase (83-fold) in the yield of syncytial virus occurred. However, because ts A2 was so "leaky" it was difficult to know if true complementation was occurring. If ts A2 had the same genetic lesion as either or both of the other complementation group mutants, it could be "leaky" enough to allow the ts mutants of these groups to grow at the restrictive temperature (39°C). Therefore a "plate complementation" test using HEp-2 cells was used, based on the principle that single infections of ts mutants at 39°C produced no plaques, whereas if complementation occurred between two mutants at 39°C plaques would be produced. Using this system ts A2 was found to complement with ts mutants of group A and group C. Complementation also occurred between group A and group C, but not between the ts mutants included in each group.

Although the seven ts mutants formed three mutually exclusive complementation groups it was suggested that this does not necessarily

mean that these ts mutants contained single genetic lesions since their stability would indicate the opposite to be the case (Wright et al., 1973).

Absence of recombination between ts mutants of RS virus

Recombination tests were performed in HeLa cells infected with single ts mutants and crosses of ts mutants and incubated for 20 hours at 32°C. The progeny virus was then plated on HeLa cells and incubated at 39°C and 32°C. "Recombination" was noted between ts A2 and ts A1, ts A3 and ts A4. However the frequency of recombination was found to be dependent on the temperature control of the plaque assay. Recombination frequency was higher (10.5 - 7.5%) when a hot air incubator was used and lower (0.1 - 3.0%) when a controlled temperature water-bath (39°C ± 0.05°C) was used for the plaque assay. No "recombination" was seen between ts mutants of the same complementation groups.

Apparent recombination in animal viruses can occur as a result of several different mechanisms. For example, reassortment of genome segments can result in high frequency of "recombination" in reovirus and influenza A virus (Shatkin, 1971). Complementing heterozygotes can produce what appears to be "recombination" in Newcastle disease virus (Dahlberg and Simon, 1969a and b). However, in poliovirus, it has been suggested that the low levels of recombination detected could be due to true intra-molecular recombination (Cooper, 1969).

Examination of the progeny of mixed infections of RS virus ts mutants was performed by picking clones at 32°C and determining their phenotype. The clones were picked at the permissive temperature to reduce selective pressures. No true wild type recombinant plaques were found

among the progeny tested (68 plaques). Some of the clones (24%) yielded progeny which were a mixture of ts A1 and ts A2 (i.e. some ts A1 plaques and some ts A2 plaques). Ts A1 clones were syncytial, ts A2 clones were non-syncytial. The clones which yielded a mixture of ts A1 and ts A2 were probably formed by virus particles which contained both ts genomes. Therefore, this data indicates that "recombination" detected between RS virus ts mutants was due to complementing heterozygotes. It is unlikely that the apparent recombination was due to complementation occurring in virus aggregates, since sonication of the virus prior to plaque assay did not alter the "recombination" frequency. Also the frequency of "recombinant" plaques followed one hit kinetics during dilution. Mixtures of ts A1 and ts A2 which were grown separately before mixing produce fewer (100-fold) "recombinant" plaques than the progeny of mixed infection.

The possibility that mixed progeny produced by some clones was due to cross-contamination was unlikely. Clones were picked from plates containing no more than 3 plaques and "clones" picked from areas where no virus CPE was seen did not yield virus (Wright et al., 1973).

Electron Microscopic study of RS virus ts mutants

Thin sections of HeLa cells infected at permissive (32°C) and non-permissive (39°C) temperature with wild type RS virus and ts mutants of RS virus, were examined by electron microscopy. Growth of the ts mutants and wild type RS virus was similar at 32°C. Dense cytoplasmic inclusions but no nuclear inclusions were seen in infected cells. Maturation of the virions occurred by budding from the cytoplasmic or vesicular membranes. Various types of RS virus particle were seen,

spherical forms with diameters 80-140 nm and filamentous forms measuring 2 μm in length. The RS virus envelope projections measured 11 to 20 nm and the internal components (nucleocapsid) had diameters of 11 to 15 nm. Usually 6 to 12 of these internal components could be seen symmetrically arranged inside the virus particles. The same virus production was seen in cells infected with wild type RS virus at 39°C and 32°C.

At the restrictive temperature (39°C), Group A ts mutants (ts A1, ts A4, ts A5) showed a marked decrease in the number of buds and filaments formed. However, there was no reduction in the number of cytoplasmic inclusions seen at this temperature, except in one ts mutant, ts A5. Only cytoplasmic inclusions could be seen in cells infected with ts A4 at 39°C, and only a reduced number of these in cells infected with ts A5. The ts mutants of Group A appear to have a block in the process of transformation of the cytoplasmic membrane as this was limited at 39°C. It was suggested that ts A5 may have another defect as well since it shows a reduction in the number of inclusions produced at 39°C and initially grows poorly in bovine kidney cells at the permissive temperature.

Group B ts mutant (ts A2) showed virus production (buds, filaments, cytoplasmic inclusions) similar to that seen for wild type virus at the restrictive temperature. This was expected since this mutant is so "leaky" and immunofluorescence studies gave similar results. However, Group C ts mutant (ts A7) infected cells showed no evidence of virus growth, only more microvilli were seen in the infected cells compared with the uninfected cells. Therefore, it was suggested that

ts A7 has probably a defect which blocks nucleocapsid formation and aggregation. Membrane transformation would seem to be dependent on the aggregation of nucleocapsids in the cytoplasm near the membrane since no transformation occurred in their absence (Kalica et al., 1973).

1.4.6 RS virus vaccine

Inactivated RS virus vaccine

As RS virus is a major cause of severe lower respiratory tract disease in infancy (Berglund, 1967; Chanock et al., 1961 and Gardner et al., 1973) a method of protecting infants against this virus was needed. One feasible method was the production of an RS virus vaccine.

Initially a mixed vaccine containing inactivated RS virus as well as other respiratory pathogens was tested but this induced a very poor immunological response (Woodhour et al., 1966). Trials using inactivated RS virus vaccines which were highly antigenic were not successful. Although children vaccinated with these vaccines developed high levels of serum neutralising and CF antibodies they were not protected by the vaccine; developing severer symptoms than non-vaccinated children on natural infection with the virus (Chin et al., 1969; Fulginiti, 1969; Kapikian et al., 1961; Kapikian et al., 1969 and Kim et al., 1969b).

Low temperature-adapted RS virus vaccine

In other myxoviruses local respiratory tract antibody had been found to be associated with protection against infection (Chanock et al., 1970; Smith et al., 1966 and Waldman et al., 1968). Therefore since young children developed neutralising response to RS virus in their nasal secretions (Kim et al., 1969b; Scott and Gardner, 1970 and Scott and Gardner, 1974) a vaccine stimulating local respiratory tract antibody

was proposed (Kim et al., 1971). An attenuated (low (26°C)-temperature-adapted) strain of RS virus was developed for use as a vaccine. The trial of this vaccine in adult volunteers was successful since they developed an immunological response to RS virus but no RS virus associated illness (Friedwald et al., 1968). Therefore the vaccine was then tested in children. However, it was not suitable since vaccinated children who had not had RS virus infection developed mild respiratory tract disease (Kim et al., 1971). As an alternative to a low-temperature-adapted RS virus vaccine a temperature-sensitive (ts) mutant vaccine was developed. Trials of the low (26°C)-temperature-adapted RS virus vaccine and the ts virus vaccine were undertaken in asthmatic children (McIntosh et al., 1974). The response of asthmatic children to these vaccines was similar to that of normal nonatopic children. No excess wheezing occurred in any of the vaccinated children which was in marked contrast to the response of asthmatic children to natural wild type RS virus infection (McIntosh et al., 1973).

Ts mutant RS virus vaccine

Ts mutants were isolated for use as a live vaccine based on the theory that they would grow at the temperature of the nasopharynx (32 - 34°C) and induce nasal secretory antibody, but they would not grow at the temperature of the lower respiratory tract (37°C) (Wright et al., 1970).

Four ts mutants of RS virus (A_2 strain) were tested in vivo in hamsters. Hamsters are not ideal for evaluation of RS virus ts mutants because they do not develop symptoms of disease on RS virus infection. A non-specific inhibitor has been found in the lungs and nasal turbinates of hamsters. However, despite the lack of suitability some valuable

conclusions could be drawn from the RS virus ts mutant tests as the temperatures of the upper and lower respiratory tracts in hamsters are similar to those in man. Ts Al was found to be the best mutant for use as a live vaccine since it replicated in hamster nasal turbinate but not in lungs (Wright et al., 1970). Therefore ts Al was tested in adult volunteers (Wright et al., 1971). The mutant appeared to be attenuated in comparison to wild type as it produced less extensive infection and no illness. No revertants were isolated from the infected adults which agreed with the results of ts Al infection of hamsters (Wright et al., 1970). Ts Al did not induce a marked immunological response in adults since only one of the nine infected adults developed a rise in serum and nasal secretory antibody. Nevertheless, infection with the ts Al vaccine induced resistance to challenge by wild type virus, 45 days after the initial inoculation. In this study the local secretory tract antibody did not seem to be the factor involved in the induction of resistance to RS virus. It was suggested that cell mediated immunity or interferon might be involved in the resistance to challenge by wild type virus.

Therefore, following the successful trial of the ts Al vaccine in adults (Wright et al., 1971) trials were undertaken of this vaccine on children (Kim et al., 1973b; McIntosh et al., 1974 and Parrott et al., 1972). However, these trials were not successful since children who had no previous RS virus infection developed mild respiratory symptoms as a result of vaccination and otitis media developed in one of these patients. Ts Al appears to be communicable since it was isolated from people in contact with the vaccinated children (Kim et al., 1973b and McIntosh et al., 1974). No challenge of ts Al vaccinated children was reported to occur during the trials, therefore it was not possible to

assess the effectiveness of the ts A1 vaccine. Some revertants were 'recovered' from the nasopharyngeal secretions of vaccinated children who shed virus during infection. However no correlation occurred between the age of the vaccinated children, their serum neutralising titre and the shedding of revertants. The revertants were less than 0.1% of the total virus isolated from the respiratory tract at the peak of virus replication. It was not known whether this genetic change occurred in vivo in the infected children or in vitro during the growth of ts A1 in HEp-2 cells at permissive temperature (Hodes et al., 1974). Therefore to resolve this question ts A1 was passaged twice in HEp-2 cells (20 separate tubes); none of the progeny (20 populations) of the second passage had a different ts pattern from ts A1 virus.

It was then suggested that the genetic change probably occurred in vivo in the infected children rather than through passage in vitro in HEp-2 cells. The revertant virus isolated from children was cloned. These clones showed various patterns of temperature sensitivity intermediate between ts A1 and wild type RS virus, some of the clones had completely reverted to wild type.

Three possible explanations for this reversion pattern were proposed. Firstly, that ts A1 could have undergone a change at more than one nucleotide site therefore reversion (back-mutation) occurring in one or more of these altered sites could result in a reduction in temperature sensitivity. This mechanism was thought unlikely since clones showing complete reversion to wild type were isolated quite frequently. If the suggested mechanism was correct, complete revertants would be rare as complete reversion would require simultaneous reversion

at several mutation sites.

Another possible explanation could be that a second mutation occurring at the same site as the first mutation might produce a less temperature sensitive gene product. However, this theory was also thought to be unlikely as different types of mutation would have to occur at the same site in order to explain the different intermediate patterns of temperature sensitivity. This might be possible if a locus with a very high tendency towards mutation existed in the RS virus genome (Hodes et al., 1974).

A third explanation for the intermediate reversion pattern seen, is that a second (suppressor) mutation distinct from the original mutation could occur (Hodes et al., 1974 and Kim et al., 1973). This suppressor mutation could affect the original mutation by producing a gene product which could be used as a substitute for the original mutation product or it could open alternative pathways for the synthesis of viral products. A suppressor mutation could change the conditions in the host cell so that the original mutation's defective product could be by-passed.

Intracistronic suppression could occur, that is, a second change in the same product, which would enable it to function almost normally. Another possibility which was suggested was that a change in the way genetic information is transformed into structural proteins could result in the intermediate pattern of reversion.

At present, which of these possible mechanisms occurs in RS virus is not known (Hodes et al., 1974).

Section 2

Materials and Methods

Section 2 - Materials and Methods

2.1 Cell culture

2.1.1 African Green monkey kidney cells (BS-C-1)

BS-C-1 cells are epithelial African Green monkey kidney cells. (Hopps et al., 1963). They were maintained in continuous culture in rotating Winchester bottles (80 oz). The cells were grown in Eagles minimum essential medium (Glasgow modification) supplemented with 5 or 10% foetal calf serum (FCS).

Foetal calf serum was used in preference to ordinary calf serum as the latter contains antibodies to bovine respiratory syncytial virus (bovine RS virus) (Doggett et al., 1968) which is antigenically similar to human respiratory syncytial virus (RS virus) (Paccaud and Jacquier, 1970). Antibodies if present in growth medium may become attached to the cells, therefore FCS was used in growth medium as well as in maintenance and overlay medium to avoid inhibition of RS virus by antibodies to bovine RS virus.

The BS-C-1 cells used were obtained at passage 75 from Dr. D. Bell, Biochemistry Department, University of Glasgow.

Production of BS-C-1 cell monolayers for growth and assay of RS virus

BS-C-1 cells (12×10^6 cells/bottle) were seeded in medium (180 ml) in a Winchester bottle (80 oz) and gassed using 100% CO_2 (100 ml). The bottles were rotated at 37°C for 3-4 days until confluent monolayers formed. Then, the cells were removed from the glass by washing twice with versene, once with trypsin, leaving 2 ml of trypsin in each bottle

and returning it to the rotating rack at 37°C for 5 to 10 minutes. The cells were centrifuged (1,000 rpm/5-10 mins), resuspended in growth medium (5 ml) and dispensed into Winchester bottles (80 oz) or used for plates or bottles (1 oz).

BS-C-1 cells were passed every 3 to 4 days using the above procedure and could be propagated for 4 to 5 months (≈ 102 passages) before losing their sensitivity to RS virus or overgrowing on assay plates making it difficult to detect RS virus cytopathic effect. Low passage number (P76) BS-C-1 cells were revived routinely every three months or when required.

BS-C-1 cells, standard plating densities for confluence in 24 hours and 48 hours

Container	No. of cells (24 hrs)	(48 hrs)
Plate (35 mm)	7×10^5	3.5×10^5
Plate (50 mm)	1×10^6	5×10^5
Linbro well (6 mm)	1×10^5	5×10^4
Vial (12 mm)	2×10^5	1×10^5
Bottle (1 oz)	1×10^6	5×10^5

The BS-C-1 cells were tested for Mycoplasma, however no mycoplasmal contamination could be detected.

2.1.2 African Green monkey kidney cells (CV-1)

CV-1 cells are adult African Green monkey kidney cells which unlike BS-C-1 cells are fibroblastic not epithelial in appearance. These cells were obtained from Dr. J. Williams of this department, who had earlier obtained them from the Cold Spring Harbour Laboratories.

The cells were propagated by the method used for BS-C-1 cells, and seeded at the same densities used for BS-C-1 cells.

2.1.3 African Green monkey kidney cells (Vero)

Vero cells are also an African Green monkey kidney cell line which like CV-1 cells are fibroblastic rather than epithelial. They were grown using the method detailed for BS-C-1 and seeded at the same densities. The Vero cells used were obtained from Flow Laboratories Ltd.

2.1.4 Human diploid lung cells (WI-38)

WI-38 cells (Hayflick, 1965) are fibroblastic human diploid lung cells. They were grown using the method used for BS-C-1 cells. Plates (50 mm) were seeded at 2×10^6 cells/plate for confluence in 24 hours. The cells were obtained from Dr. F. Perkins, National Institute for Biological Standards, Hawstead.

The WI-38 cells were tested for Mycoplasma and found to have M. arginini.

2.1.5 Human cervical carcinoma cells (HeLa)

HeLa cells (Gey et al., 1952) are epithelial cells derived from a human cervical carcinoma. The cells were propagated in a similar manner to BS-C-1 cells. Plates (35 mm) seeded with 1.7×10^6 cells were confluent in 24 hours. The HeLa cells were obtained from Dr. J. Williams of this department, who originally obtained them from Flow Laboratories Ltd.

2.1.6 Human carcinoma cells (HEp-2)

HEp-2 cells are epidermoid cells derived from carcinoma of the larynx (Toolan, 1954). The cells were grown in the same way as BS-C-1 cells. Plates (35 mm) were seeded with 1.6×10^6 cells to form confluent monolayers in 24 hours. The HEp-2 cells were obtained from Flow Laboratories Ltd.

2.1.7 L cells (NCTC clone 929)

L cells (Earle, 1943) were derived from normal subcutaneous areolar and adipose mouse tissue. These cells are fibroblastic in appearance and were grown in the same way as BS-C-1 cells. Plates (50 mm) seeded with 2×10^6 cells were confluent in 24 hours. The L cells were obtained from Bio-cult Laboratories Ltd., Glasgow.

2.1.8 BHK_{21} clone 13

BHK_{21} C_{13} cell line was derived from hamster kidney fibroblast cells (Macpherson and Stoker, 1962). The cells were grown in a similar manner to BS-C-1 cells except that the growth and maintenance medium used contains tryptose phosphate broth (10%) as well as FCS.

2.2 Storage of cells

The storage medium consisted of 4 parts of Eagles MEM (Glasgow modification), 4 parts of FCS and 2 parts of glycerol (non-toxic). The cells to be stored were removed from the Winchester bottle using trypsin as detailed earlier, then pelleted at 1,000 rpm for 5-10 min. Then, the cells were resuspended in 10 ml of Eagles MEM, and 1 ml of the cell suspension mixed with 1 ml of the storage medium and transferred to vials (5-6 x 10^6 cells/vial).

The cells were stored at -195^{o}C and revived by thawing quickly and then transferring the suspension to a bottle (1 oz) with maintenance medium (5 ml).

2.3 Virus

2.3.1 Respiratory Syncytral virus (R.S. virus)

Long strain

The Long strain of RS virus (Chanock et al., 1957) was obtained from the American Type Culture Collection, Rockville, Maryland, USA.

Before receipt the Long strain had been passaged six times in KB cells (Human epidermoid carcinoma of mouth) twice in Chang L cells (non-malignant human liver epithelial cells) and twice in HEp-2 cells. For experiments detailed in this thesis the Long strain was grown and assayed on BS-C-1 cells.

RSN-2 strain

The RSN-2 strain of RS virus was isolated from a five month old child with bronchitis by Dr. P.S. Gardner, Royal Victoria Infirmary, Newcastle, in 1972. It was received from Dr. P.S. Gardner as an infected human diploid culture (MRC - 5 cells) and then passaged twice in BS-C-1 cells, before being cloned four times to ensure a genetically uniform stock. RSN-2 strain was grown and assayed on BS-C-1 cells.

A_2 strain temperature sensitive (ts) mutants

The ts mutants (tsA1, A2, A7) were kindly supplied by Dr. R.M. Chanock, National Institute of Health, Bethesda. Before receipt the ts mutants had been passaged four times in HeLa cells and once in HEp cells. On receipt the mutants were passaged twice in BS-C-1 cells before being used in Complementation experiments.

2.3.2 Vesicular Stomatitis Virus (VSV)

The strain of VSV used was a triple cloned wild type stock of the Indiana - C strain obtained from Dr. C.R. Pringle of this department. This strain belongs to the Indiana serotype of VSV and was obtained originally from A.V.R.I. Pirbright by kind permission of the Director Dr. J.B. Brookesby. It had been attenuated by serial egg passage and was no longer virulent for cattle. VSV is an RNA virus which causes an animal disease of economic importance. It was used with the consent of the Animal Health Division of the Department of Agriculture, Scotland.

2.3.3 Pseudorabies virus (PSRV)

A cloned wild type stock of PSRV was obtained from Dr. C.R. Pringle. This wild type stock was a derivative of the Kaplan strain of PSRV and its characteristics have been described by Pringle, Howard and Hay (1973).

2.4 Virus stocks

2.4.1 Preparation of Virus stock

A confluent monolayer of BS-C-1 cells in a bottle (1 oz) was inoculated with virus (0.2 ml) after the removal of growth medium. The multiplicity of infection (moi) was less than 1 plaque forming unit per cell (pfu/cell). Adsorption was allowed for 1 hour at 31°C or room temperature then the maintenance medium (5 ml) was added.

The infected cells were incubated stationary at 31°C until the C.P.E. was well developed. Then, they were harvested by freeze-thawing in an alcohol-dry ice bath or by scraping using a Pasteur pipette bent at the tip. The virus stock (infected cells + medium) was stored either in a liquid nitrogen cylinder (-195°C) or in a low temperature storage cabinet (-70°C).

2.4.2 The concentration of virus using polyethylene glycol (PEG)

A Winchester bottle (80 oz) containing a confluent monolayer of BS-C-1 cells ($60-70 \times 10^6$ cells) was inoculated with virus (1-2 ml) in maintenance medium (8-9 ml). The moi was less than one. The bottle was rotated at 31°C for one hour to allow the virus to adsorb. Then, maintenance medium (60 ml) was added to the bottle and it was returned to 31°C until the CPE was just visible. The maintenance medium was removed and replaced by fresh maintenance medium (20 ml). After which the bottle was returned to 31°C and rotated until CPE was well developed. Then, the infected cells were harvested into the medium by scraping with a piece of silicone rubber on the end of a metal rod. The virus (infected cells + medium) was spun at 2,000 rpm for 20 minutes in a

refrigerated centrifuge (4°C). The cell pellet was discarded and the supernatant retained. PEG was added to the supernatant to give a final concentration of 6% PEG and the mixture was then placed at 4°C for 2 hours. During this time virus and proteins were precipitated. Then the mixture was centrifuged (2,000 rpm/20 mins at 4°C) the supernatant discarded and the pellet retained (precipitate). The centrifuge used was an MSE Mistral 6L. The pellet was resuspended in a small volume of 20 mM Tris HCl (0.1 ml) pH 8.4. Usually, the virus pellets obtained from PEG treated virus suspensions from 5 Winchester bottles (80 oz) were pooled.

2.4.3 Virus for Immunofluorescence experiments

Bottles (20 oz) of confluent BS-C-1 cells were inoculated with 3-5 ml of virus, the moi was less than one. Adsorption was allowed for 1 hour at 31°C and then maintenance medium (20 ml) was added. The infected cells were incubated stationary at 31°C and harvested when the CPE was well developed by scraping infected cells into the medium using a piece of silicone rubber on the end of a glass rod. Then, the virus (infected cells + medium) was clarified by centrifugation (2,000 rpm/20 mins at 4°C) and the supernatant retained and used as the virus inoculum.

2.5 RS virus assay and growth

2.5.1 RS virus plaque assay

BS-C-1 cell monolayers on plates (50 mm) were used for the RS virus plaque assay. These cells were used in preference to the other susceptible cell lines studied (HeLa, WI-38, HEp-2) because RS virus produced a clear distinctive cytopathic effect (CPE) in these cells. RS virus formed foci of darker staining cells on BS-C-1 cell monolayers (plates 1, 2) whereas in WI-38 cells RS virus produced irregular syncytia (plate 3).

Dilutions of RS virus (0.2 ml) in PBS/2.5% FCS were inoculated onto BS-C-1 cell monolayers. Then adsorption was allowed for one hour at 31°C or room temperature. After this time the agar overlay (5 ml) was added and the plates incubated for 6-7 days at 31°C or 5 days at 39°C. The plates were fixed using formal saline (2 ml/plate), then the agar was removed and the cell sheet stained with Giemsa Stain (2 ml) for 5-10 minutes. Plaques were counted using a Watson dissecting microscope at magnification times 8.75.

2.5.2 Growth curves

Method (a) - Total virus

Bottles (1 oz) containing confluent monolayers of BS-C-1 cells were inoculated with virus (0.2 ml). The moi was less than one. Adsorption was allowed for one hour at 31°C, after which the monolayers were washed twice with maintenance medium and a final volume of 5 ml added. Then, the infected cells were incubated at the appropriate

temperatures in waterbaths for various lengths of time. Samples were taken by removing duplicate bottles from the waterbaths and harvesting the infected cells into the medium by scraping or freeze-thawing. The virus sample (infected cells + medium) was stored at -70°C or below (-195°C) and titrated when BS-C-1 cells were available.

Method (b) - Total virus

This method was used for ts mutant growth curves. The smaller number of cells used enabled an increase in moi.

Confluent monolayers of BS-C-1 cells on plates (35 mm) were inoculated with virus (0.2 ml). The moi varied according to the ts mutant used. Adsorption was allowed for one hour at 31°C or room temperature. Then, the plates were washed twice with maintenance medium and a final volume of 2 ml added. The plates were incubated in CO_2-gassed incubators at 31°C and 39°C. Samples were taken by removing duplicate plates from the incubators at various times after adsorption. The infected cells were scraped into the medium using a piece of silicone rubber on the end of a glass rod. The virus sample (infected cells + medium) was stored at -70°C or -195°C and titrated on BS-C-1 cells at 31°C. The 39°C samples were also assayed at 39°C as well as 31°C to determine the amount of reversion as opposed to leak occurring at this temperature.

For temperature-shift experiments this method was modified. As well as harvesting plates at various times after adsorption, plates were also transferred from 31°C to 39°C (upshift) and from 39°C to 31°C (downshift) at 12, 24, 36 and 48 hours after adsorption.

Method (c) - Cell-released and cell-associated virus

Monolayers of BS-C-1 cells on bottles (1 oz) were inoculated with RS virus (0.2 ml). Adsorption was allowed for one hour at room temperature. Then the RS virus infected cell monolayers were washed twice with maintenance medium and a final volume of 5 ml added. The infected cells were incubated in a waterbath at 37°C. Samples were taken at various times from 0 hours to 48 hrs after adsorption by removing duplicate bottles from the waterbath. Then, the maintenance medium was removed from the infected cells and stored at -70°C before being assayed for RS virus (cell-released virus).

Fresh maintenance medium (2 ml) was added to the bottles (1 oz) of infected cells. Then the cells were sonicated for 1 minute and after this scraped into the medium using a Pasteur pipette. The sample (infected cells + medium) was stored at -70°C and then titrated (cell-associated virus).

The titres of the samples were corrected for the difference in final volume between the cell-released (5 ml) and the cell-associated (2 ml) samples.

Plate 1 : RSN-2 strain plaque on BS-C-1 cells

The cells were stained with giemsa.

RS VIRUS FOCI OF DARK STAINING CELLS.

Plate 2 : Enlarged view of a RSN-2 plaque on BS-C-1 cells

The cells were stained with giemsa.

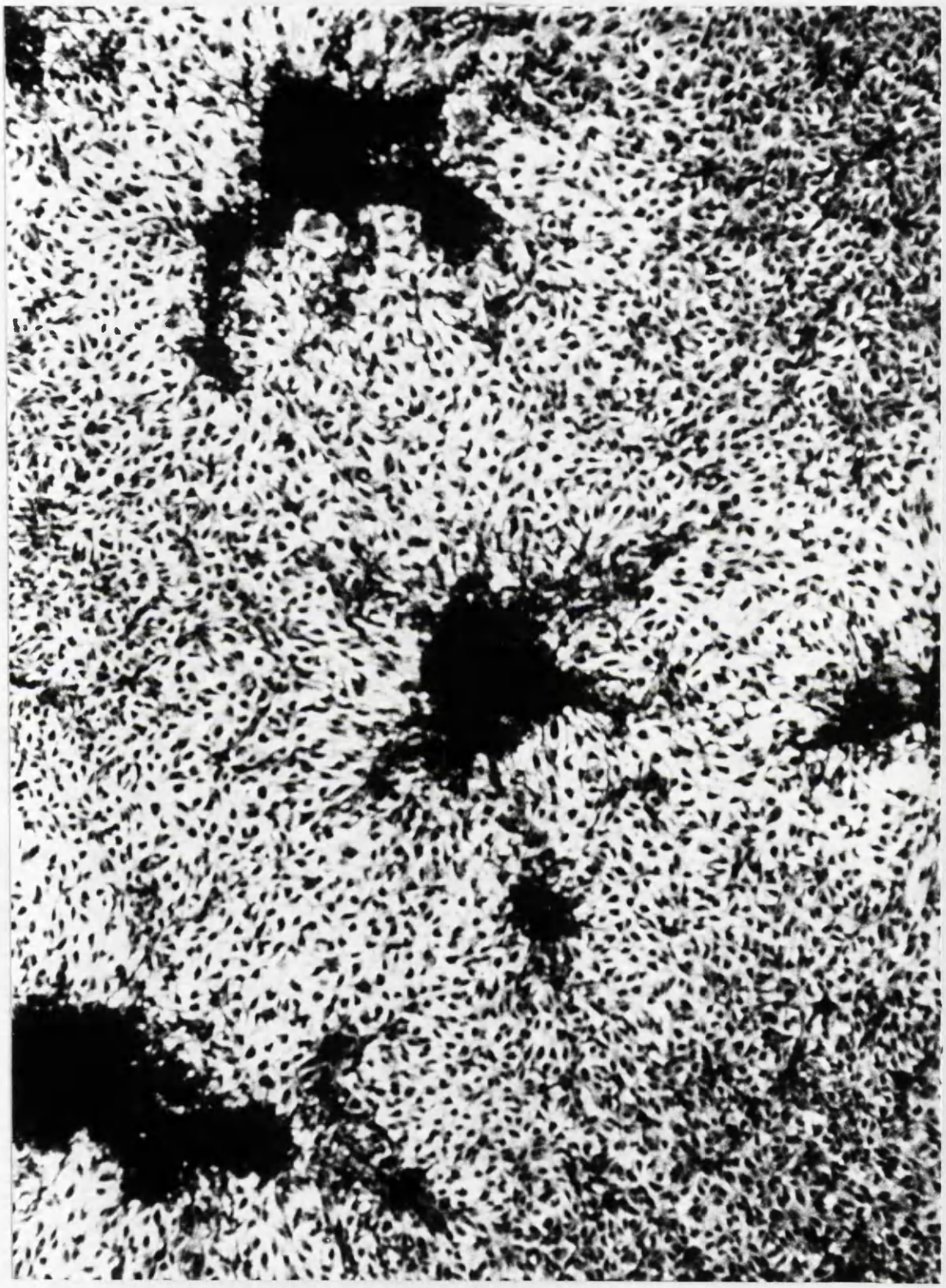


PLATE 1

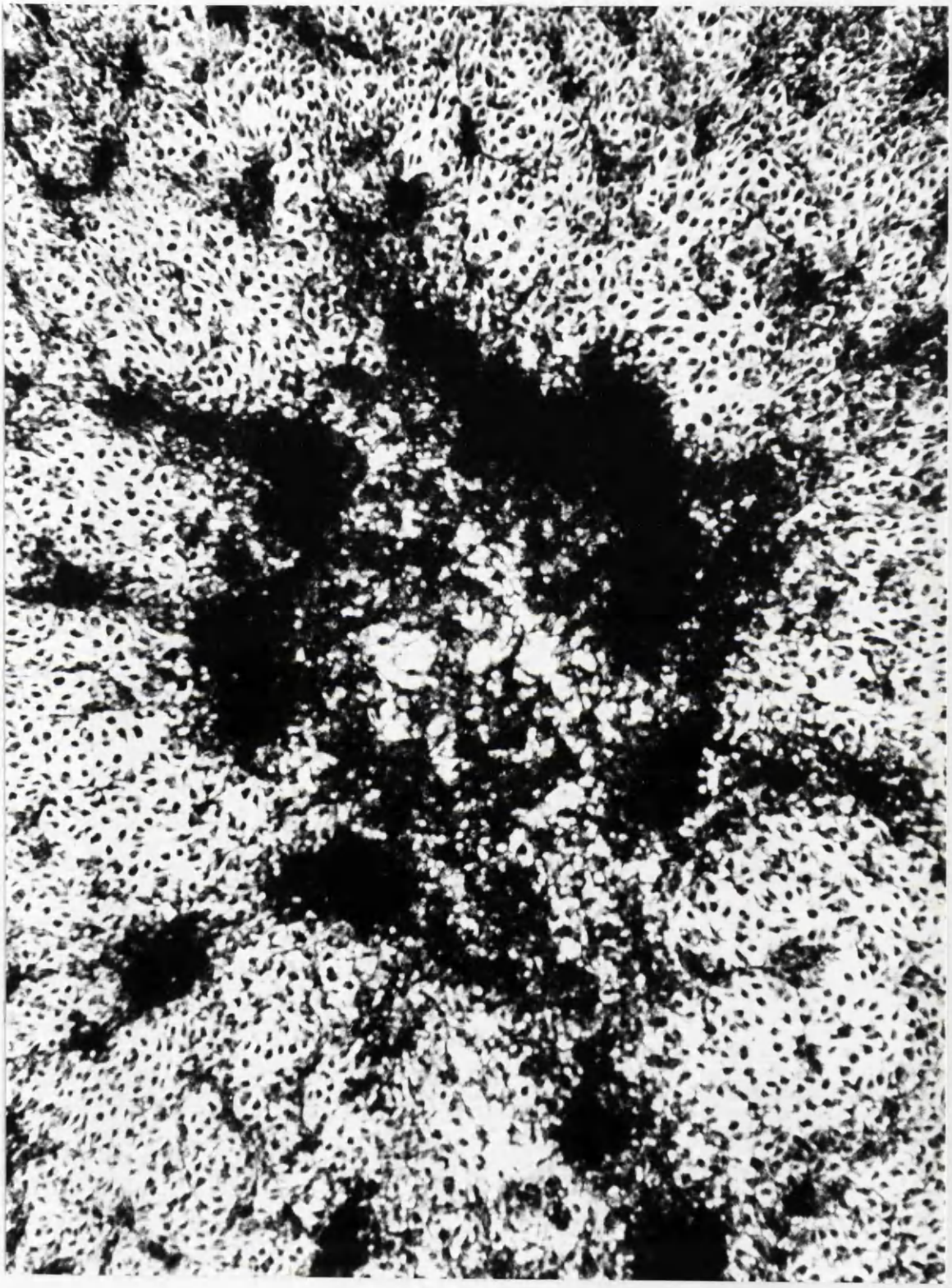


PLATE 2

Plate 3 : RSN-2 Strain plaque on WI-38 cells

The cells were stained with giemsa.

AN IRREGULAR SYNCTIUM PRODUCED BY RS. VIRUS ON WI-38 CELLS.

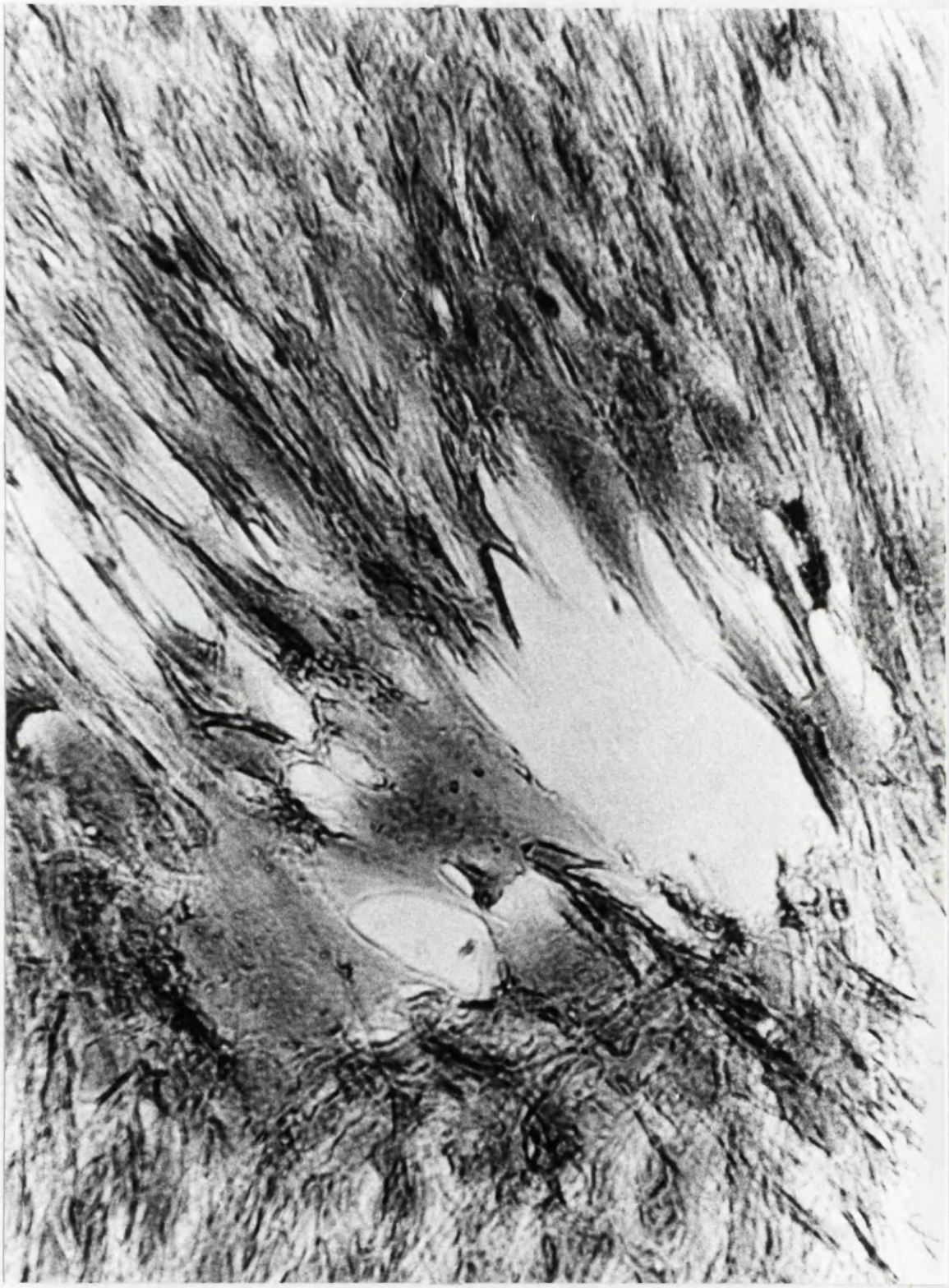


PLATE 3

2.6 Chemical agents

2.6.1 Mutagens

5-Flurouracil (5-FU)

This chemical was obtained from Koch-Light Laboratories Ltd., Buckinghamshire, England. Stock solutions were made by dissolving the chemical in sterile distilled water.

5-Azacytidine (5-ACR)

This chemical was obtained from Sigma Chemical Co., St. Louis, Mo., USA. Stock solutions were made by dissolving the chemical in dissolved water.

Ethyl methanesulphonate (EMS)

EMS was obtained as a liquid from Sigma Chemical Co., St. Louis, Mo., USA.

N-Methyl-N'-nitro-N-nitrosoguanidine (NTG)

NTG was obtained from Research Chemical, Ralph N. Emanual Ltd., Wembly, England.

2.6.2. Inhibitors

Actinomycin D

Actinomycin D was obtained as D-actinomycin MSD under the trade name Lyvac Cosmegen from Merk, Sharpe and Dohme Ltd., Hoddesdon, Hertfordshire. Each vial contained 0.5 mg of actinomycin D and 20 mg

of mannitol (inactive). Solutions of actinomycin D were made up with distilled water and used immediately.

Mitomycin C

Mitomycin C was obtained from Sigma Chemical Co., St. Louis, Mo., USA. Solutions of mitomycin C were made by the addition of distilled water to the chemical.

2.6.3 Amino acids

Arginine

Arginine was obtained from Koch Light Ltd. Stock solutions were prepared using sterile distilled water.

2.7 Complementation and Recombination

2.7.1 Yield Complementation Method

Mixtures of ts mutants for use in complementation experiments were prepared and then inoculated onto BS-C-1 monolayers. Single-infection contained equal volumes of ts mutant and diluent (PBS) whereas mixed infection contained equal volumes of both ts mutants.

Virus (0.2 ml) inoculated onto BS-C-1 cell monolayers was allowed to adsorb for one hour at 4°C or 31°C. Then, the monolayers were washed twice with cold maintenance medium and a final volume of medium added. The monolayers were incubated at 39°C and then harvested and stored at -70°C. When BS-C-1 cells were available the samples were assayed at 31°C and 39°C.

The above method was used when HeLa and HEp-2 cells replaced BS-C-1 cells in some complementation experiments.

A complementation index (CI) of 10 or above was taken as significant. This was a purely arbitrary figure.

$$CI = \frac{(A + B)^{31^\circ} - (A + B)^{39^\circ}}{A^{31^\circ} + B^{31^\circ}}$$

A - ts mutant A

B - ts mutant B

The different yield complementation methods used are detailed below.

Method - 1

In this method the BS-C-1 cell monolayers used in the complementation experiments were in bottles (1 oz). The incubation temperature was accurately controlled as an exact 39°C waterbath was used. Infected cells were harvested by freeze-thawing or scraping.

Method - 2

Blocks of Linbro wells (6 mm) obtained from Linbro Chemical Co. Ltd., were used in this method. BS-C-1 monolayers in these wells were inoculated with virus (20 λ) and after adsorption overlaid with maintenance medium (150 λ).

The blocks were incubated in a CO_2 incubator at 39°C. Infected cells were harvested 4 days after adsorption by freeze-thawing.

Method - 3

In this method BS-C-1 monolayers were grown on the bottom of vials (12 mm in diameter). The virus inoculum was 0.1 ml and after adsorption the cells were overlaid with maintenance medium (1 ml). Then the infected cells were incubated in an accurate waterbath at 39°C for 3 days before being harvested by freeze-thawing.

Method - 4

This method was the most efficient since BS-C-1 cell monolayers on plates (35 mm) can be easily harvested by scraping using a piece of silicone rubber on a glass rod. The incubation temperature was also accurate as an exact 39°C waterbath was used; the plates were placed in

a CO_2-gassed airtight jar which was immersed in the water.

The virus inoculum was 0.2 ml and after adsorption the infected cells were covered with maintenance medium (2 ml).

2.7.2 Recombination

Mixtures of ts mutants were prepared and then inoculated onto BS-C-1 monolayers on plates (35 mm). The virus inoculum (0.2 ml) was allowed to adsorb for one hour at 31°C. Then, the monolayers were washed twice with maintenance medium and a final volume of 2 ml added. The monolayers were incubated at 31°C for 4 days and then harvested by scraping the cells into the medium. Then the virus samples (infected cells + medium) were stored at -70°C until BS-C-1 cells were available for the assay. The virus samples were titrated at 31°C and 39°C and the recombination frequency was calculated using the formula given below.

% Recombination frequency

$$= \frac{\text{Virus titre at } 39^\circ \text{ (AB)}}{\text{Virus titre at } 31^\circ \text{ (AB)}} - \frac{\text{Virus titre } 39^\circ \text{ AA}}{\text{Virus titre } 31^\circ \text{ AA}} \times \frac{\text{Virus titre } 39^\circ \text{ BB}}{\text{Virus titre } 31^\circ \text{ BB}}$$

$$\times 100$$

A - ts mutant A

B - ts mutant B

AA - self cross ts A

BB - self cross ts B

2.8 Thin sectioning technique

The following method was used to obtain sections of RS virus infected cells for examination by an electron microscope (Siemens, 101).

2.8.1 Preparation of RS virus infected cells

BS-C-1 cell monolayers on plates (50 mm) were inoculated with virus (0.2 ml). The virus was allowed to adsorb for one hour at 31°C. Then, maintenance medium (5 ml) was added to the plates and they were incubated at 31°C and 39°C in CO_2-gassed incubators.

When RS virus CPE was just visible, 4-5 days after adsorption, the plates were removed from the incubators and the cell sheets fixed by the addition of 2.5% glutaraldehyde (4 ml/plate) for 30 minutes. Then, the glutaraldehyde was removed and the cell sheet washed twice with PBS before the addition of 1% osmium tetroxide (10 drops/plate) for 15 minutes to stain the cells. After this the cell sheet was washed twice with PBS and a final volume of 2 ml of PBS was added to the plates. The infected cells were scraped into the PBS using a piece of silicone rubber on the end of a glass rod. Then the infected cell suspensions were placed in conical bottomed centrifuge tubes and centrifuged (4,000-5,000 rpm/10 mins) using a MSE bench centrifuge. The supernatant was discarded and the pellet of infected cells retained. The cells were dehydrated in alcohol; the following percentages of alcohol 30, 50, 70, 90 were each successively added to the infected cells for 5 minutes and then removed. If required the infected cells can be left overnight in 70% alcohol.

After 90% alcohol treatment for 5 mins, the cells were given two 15 minute treatments with 100% alcohol. Then the alcohol was removed and replaced with a 1:1 mixture of alcohol and propylene oxide (PO) for 15 minutes. The mixture was removed and PO added for 30 minutes, followed by the addition of a 1:1 mixture of PO and Epon. At this stage the cells can be left overnight if required.

The mixture of PO and epon was removed and the cells were then immersed in epon for 1 hour. If the pellet was not compact, it was centrifuged before being layered on top of a little epon in a BEEM capsule. After the cells were placed in the capsules, the capsules were filled nearly to the top with epon. Then they were placed in an oven at 60°C for 48 hours to allow the epon to polymerise. When they were removed from the oven the embedded cells were ready for sectioning. The LKB Ultromicrotome fitted with a glass knife was used to cut sections of the cells embedded in epon.

2.8.2 Staining thin sections for examination by an electron microscope

Thin sections cut using an LKB ultromicrotome were picked up on uncoated grids and left on the grids for 24 hours before staining. Then, the grids were placed, section side down, on a drop of a saturated solution of uranyl acetate in methanol for 5 minutes. The grid (section side up) was then washed with distilled water and dried on a piece of filter paper. A drop of sodium hydroxide (0.1N) was placed on the grid (section side up) before it was placed on a drop of lead citrate for 5 minutes. Then the grid was washed thoroughly with distilled water and dried on filter paper. The grid was examined using a Siemens, 101 electron microscope at magnifications between 4,000 and 8,000 and photographs taken.

2.9 In vitro assay for RNA polymerase of RS virus

The reaction mixtures (0.2 ml) were prepared in test tubes immersed in ice water. The same amount (0.1 ml) of sample (ts mutant, wild type RS virus or uninfected BS-C-1 cells) was added to each test tube. Then these solutions were added in the following order, 20 mM Tris HCl, pH 8, (0.04 ml), 70 mM dithiothreitol (0.01 ml) and reagent mixture (0.05 ml). The reagent mixture (1 ml) contained 400 mM Tris HCl buffer, (pH 8), 2.56 mM each of ATP, CTP and GTP, 256 μM of cold UTP and 0.24 mCi/mole ^{3}H -UTP. Therefore the final concentration of reagents in the reaction mixture (0.2 ml) was 104 mM Tris HCl buffer (pH 8.0), 3.5 mM dithiothreitol, 640 μM each of ATP, CTP and GTP, 64 μM of cold UTP and 12 μCi ^{3}H -UTP. The 5,6 ^{3}H -uridine 5'-triphosphate was obtained from the Radiochemical Centre, Amersham and had a specific activity of 49 Ci/mmole in 50% ethanol. This ethanol was evaporated before the triphosphate was added to the reagent mixture.

The reaction mixtures were mixed using a Whirlimixer and zero time samples were taken. Then the mixtures were warmed to the incubation temperatures (31°C and 39°C) for 1 minute and then the reaction was started by the addition of 220 mM $MgCl_2$ (0.005 ml) with mixing. Incubation was continued and samples (0.02 ml) were taken at the following times 10, 20, 30, 60 and 90 minutes. The samples (0.02 ml) were placed on Whatman No. 1 filter paper discs and allowed to dry. Then the discs were washed 7 times with 5% trichloracetic acid containing 0.04M sodium pyrophosphate, 3 times in ethanol and once in diethyl ether. After drying the discs were placed in scintillation vials and a toluene based scintillant was added. The radioactivity was counted using an Inter-technique SL30 liquid scintillation spectrometer.

2.10 Indirect Immunofluorescence Method of detecting RS antigen in infected cells

This method involved staining coverslips of infected BS-C-1 cells with specific absorbed antiserum (Bovine anti-bovine RS) and after washing with PBS (0.01M), staining the coverslips with rabbit anti-bovine antiserum conjugated with fluorescein isothyocyanate.

Only the internal and/or nuclear antigens could be detected as the infected cells were fixed by acetone. Surface antigens can be detected only if live cells are used. The indirect immunofluorescence method is more sensitive than the direct method but it can produce problems due to non-specific fluorescence.

2.10.1 Antisera

Bovine anti-bovine RS virus serum

Bovine anti-bovine RS virus serum was provided by Dr. J. Stott, Animal Disease Research Institute, Compton. The antiserum was absorbed with acetone treated uninfected BS-C-1 cells and its titre was estimated using the indirect immunofluorescent technique. It was used at a dilution of 1 in 50.

Human anti-RS virus serum

Two convalescent sera from children with RS virus infection were provided by Dr. J.H. Connolly, Virus Reference Laboratory, Department of Microbiology and Immunobiology, Royal Victoria Hospital, Belfast. The sera were absorbed with acetone treated uninfected BS-C-1 cells and used at a dilution of 1 in 10.

Rabbit anti-herpes serum

The rabbit anti-herpes simplex virus (ts 99) serum was kindly provided by Dr. Morag Timbury of this department. This antiserum was absorbed with acetone treated rat embryo cells and BS-C-1 cells. Using the indirect immunofluorescence technique the antibody titre in the serum was estimated. The titre of the ~~anti-serum~~ antiserum was 320. It was used at a dilution of 1 in 30.

Monkey anti-measles virus serum

The monkey anti-measles virus serum was provided by Dr. P.V. Shirodaria, Department of Microbiology and Immunobiology, The Queen's University of Belfast, Belfast. This antiserum was absorbed with acetone treated HEp-2 cells, Vero cells and BS-C-1 cells. Its titre was estimated by indirect immunofluorescence and it was used at a dilution of 1 in 50.

Rabbit anti-measles virus serum

The rabbit anti-measles virus serum was provided by Dr. P.V. Shirodaria, Department of Microbiology and Immunobiology, The Queen's University of Belfast, Belfast. It was absorbed with rat liver powder, rat embryo cells and BS-C-1 cells. This antiserum was used at a dilution of 1 in 20.

2.10.2 Fluorescein Conjugates

Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-bovine globulin

This conjugate was a commercial preparation obtained from Nordic Pharmaceuticals and Diagnostics, London. The titre of the conjugate was 48. It was absorbed with acetone-treated BS-C-1 cells and used at a dilution of 1 in 15.

FITC-Conjugated sheep anti-human IgG.

Sheep anti-human IgG was obtained from Wellcome Reagents Ltd. It was conjugated by Dr. P.V. Shirodaria with fluorescein isothiocyanate (FITC) obtained from Sigma Chemical Company. The conjugate was absorbed with acetone-treated mouse liver powder, HEp-2 cells and BS-C-1 cells. Its titre was 1 in 160 and it was used at a dilution of 1 in 90.

FITC-conjugated rabbit anti-monkey globulin

The conjugate was a commercial preparation obtained from Nordic Diagnostics and Pharmaceuticals, London. Its titre was estimated by the indirect immunofluorescent technique. The conjugate was absorbed with acetone treated mouse liver powder, HEp-2 cells, Vero cells and BS-C-1 cells and it was used at a dilution of 1 in 15.

2.10.3 Absorption of antisera

Preparation of cells used to absorb antiserum

BS-C-1 cells in bottles (80 oz) were harvested into the medium by scraping using a piece of silicone rubber attached to the end of a metal rod. Then the cell suspension was centrifuged at 1,000 rpm for 15 minutes and the cell pellet retained. The cells were resuspended in PBS (0.01M) and pelleted as before; this procedure was repeated three times. After, this treatment the cells were resuspended in acetone (10 times the volume of the cell pellet) for 5 minutes and then pelleted (2,000 rpm/15 mins). The acetone was discarded and the cells resuspended in PBS (0.01M) and then pelleted (2,000 rpm/15 mins). The cells were resuspended in PBS (0.01M) and pelleted five times or until no trace of acetone remained. Then the cells suspended in PBS (0.01M) were stored at -20°C until required.

Other cell lines used for absorption were treated in the same way as BS-C-1 cells.

Absorption of antiserum or conjugate with uninfected BS-C-1 cells

The cells were treated with acetone as described earlier. Before use the cells were washed in PBS (0.01M) and pelleted (2,000 rpm/15 mins).

Antiserum or conjugate was mixed with a third of its volume of acetone treated cells. The mixture was rotated for 48 hours at 4°C and then for a further 1 hour at 37°C. It was then centrifuged in a refrigerated centrifuge (10,000 rpm for 30-60 minutes) and the supernatant retained. The supernatant contained the absorbed antiserum which was clarified by filtration using a HA millipore filter (0.45 μ). This procedure was repeated between each adsorption when adsorptions with more than one type of cell were required.

2.10.4 Fixation and Fluorescent Staining

Preparation of virus infected cells

BS-C-1 cell monolayers on coverslips (13 mm) in plates (30 mm, 50 mm) were inoculated with clarified virus suspensions (0.5 ml, 1 ml). Before inoculation the cell monolayers were washed with medium (Eagles MEM/1% FCS) containing a lower percentage of FCS than maintenance medium. The virus was allowed to adsorb for 1 hour at 31°C and then the cell monolayers were washed with low serum supplemented Eagles MEM (1% FCS) and a final volume of this medium was added to the plates (2 ml, 5 ml). Then the infected cells were incubated in a CO_2-gassed incubator at 31°C and at 39°C in either a CO_2-gassed incubator or a CO_2-gassed airtight jar immersed in water in a 39°C waterbath. The 39°C waterbath was used to ensure greater temperature control. When the plates were incubated in the airtight jar in the waterbath the percentage of FCS had to be increased to 2.5% in order to prevent the cells from detaching from the coverslips.

Fixation of infected and uninfected cells

Maintenance medium was removed from the infected and uninfected cells and they were washed twice with serum free Eagle's medium. The coverslips were then washed twice in PBS (0.01M) and once in cold acetone (4°C) before being placed in a metal rack in a cold acetone bath (4°C) for 5 minutes. After treatment the coverslips were immediately removed from the acetone bath and allowed to dry in air (30-60 minutes). Then the coverslips were stored in plates (50 mm) with silica gel at -20°C.

Indirect immunofluorescence staining method

The coverslips to be stained were washed in PBS (0.01M) and arranged on rods in the staining box. Then the coverslips were covered without overflow with appropriate dilutions of non-conjugated antiserum (2-3 drops). The antiserum was allowed to react for 1 hour at 37°C. Each coverslip was removed and washed in PBS (0.01M) and then placed in the same order in a bath of PBS (0.01M) containing a magnetic stirrer. The washing was continued for 30 minutes. Then the coverslips were removed and their undersurfaces dried before they were placed on rods in the staining box. The appropriate conjugated globulin (anti-species globulin) was placed on each coverslip (2 drops) covering the whole surface without overflow. Staining was continued for 45 minutes at 37°C. After this, the coverslips were washed with PBS (0.01M) as described above. Then the undersurfaces of the coverslips were dried and the coverslips mounted on slides (0.8 mm thick) in buffered glycerol-saline pH 8.3.

Specificity of staining

The specificity of bovine anti-bovine RS virus serum was established by the absence of staining with foetal bovine calf serum, rabbit anti-measles virus serum, rabbit anti-herpes simplex virus serum and monkey anti-measles virus serum. After adsorption of immune serum with RS virus infected BS-C-1 cells no staining was detected; this shows the specificity of RS virus staining.

2.10.5 Equipment

Microscopes used for fluorescence observations

Fluorescence observations were made using a Reichert fluropan fluorescent microscope under dark-field illumination with a Tungsten halogen lamp (G.E.C. 12 volts, 100 watts). A primary Baltzer filter and barrier filter 3 were used when necessary. Specimens were photographed in a Zeiss standard R.A. fluorescence photomicroscope using a U or I exciter filter and barrier filter 41.

Preparation of coverslips for tissue culture

Glass coverslips (Chance no. 1, 13 mm diameter) were cleaned prior to use by boiling in distilled water for 10 to 15 minutes. Then, the coverslips were allowed to cool (5 mins) and the procedure repeated three times. After washing, the coverslips were rinsed twice in absolute alcohol, and dried in air at 37°C. Then, each coverslip was polished with lens tissue. The coverslips were placed in glass Petri dishes (8 coverslips per dish) and sterilised in a hot air oven at 180°C for 1½ hours.

The coverslips were obtained from Chance propper Ltd., Smethwick, Warley, England.

Section 3

Results

Section 3 - Results

3.1 General properties of RS virus

3.1.1 Growth of the RSN-2 strain in various cell lines

Several cell lines were examined in order to find a suitable cell system for assay and growth of the RSN-2 strain of RS virus.

The RSN-2 strain did not grow in BHK/C_{13} or L cells. However, it did grow in HeLa, HEp-2, WI-38, CV-1, Vero and BS-C-1 cells. As noted in section 2.5.1 BS-C-1 cells were chosen as the assay system for the RSN-2 strain because of the clear viral CPE produced in these cells (plates 1, 2). No clear plaques were seen when the RSN-2 strain was titrated on HeLa cells whereas irregular syncytia were formed in WI-38 cells (plate 3).

Stocks of the RSN-2 strain cloned and grown in WI-38 cells and cloned and grown in BS-C-1 cells were titrated on both cell lines (Table 6). The plating efficiency of the RSN-2 strain on WI-38 cells was lower than on BS-C-1 cells. However, the RSN-2 strain which had been cloned and passed in WI-38 cells had a higher efficiency of plating on WI-38 cells than virus which had been grown in BS-C-1 cells.

Therefore the RSN-2 strain grows better and plates more efficiently on BS-C-1 cells than on WI-38 cells. However, the virus titre and plating efficiency can be improved if the RSN-2 strain is adapted by passage in the cell line used.

TABLE 6

Comparison of RS virus (RSN-2 strain) growth and plating efficiency in BS-C-1 and WI-38 cells

RS virus cloned 3 times in:-	RS virus grown in:-	Assayed on:-	Titre (log_{10}/ml) after 6 days at 31°C	(%) Efficiency of plating of RS virus compared to RS virus grown and assayed on BS-C-1 cells
BS-C-1	BS-C-1	BS-C-1	7.23	/
	BS-C-1	WI-38	4.78	0.35
WI-38	WI-38	WI-38	5.04	0.65
	WI-38	BS-C-1	6.98	56.23

3.1.2 Growth of RS virus (RSN-2 and Long strains) in BS-C-1 cells

The rate of growth of RS virus at different temperatures was examined to determine whether the rate of growth was dependent on temperature.

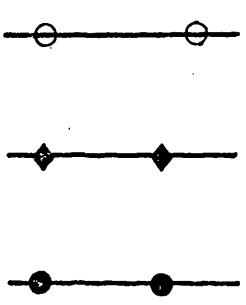
The Long strain of RS virus was grown in BS-C-1 cells at three different temperatures (31°, 37°, 39°C). Stock of this strain was used as it was available at a higher titre than that of the RSN-2 strain. The growth cycle of the Long strain at these temperatures was different, the best yield and highest growth rate occurring at 39°C (Figure 2).

To determine whether the low titre of both strains of RS virus was due to RS virus remaining cell-associated, the levels of cell-associated and cell-released virus were measured throughout the growth cycles.

At 36 hours post adsorption 16 % of Long strain (Figure 3) was released whereas only 1% of RSN-2 (Figure 4) was released. Therefore both strains of RS virus are very cell-associated, however the Long strain is less cell-associated than the RSN-2 strain. This difference in cell association could account for the higher yield of Long strain compared with RSN-2 strain.

In these experiments the latent period of RS virus (RSN-2 and Long strains) in BS-C-1 was 10-12 hours and the exponential phase was 20 hours or more. The entire RS virus growth cycle takes 36 to 48 hours in BS-C-1 cells.

Figure 2 : The growth of the Long strain of RS virus in BS-C-1 cells at 31°C, 37°C and 39°C



Long strain growth at 31°C

Long strain growth at 37°C

Long strain growth at 39°C

MOI : 0.05 PFU/CELL

TIME : HOURS POST-ADSORPTION

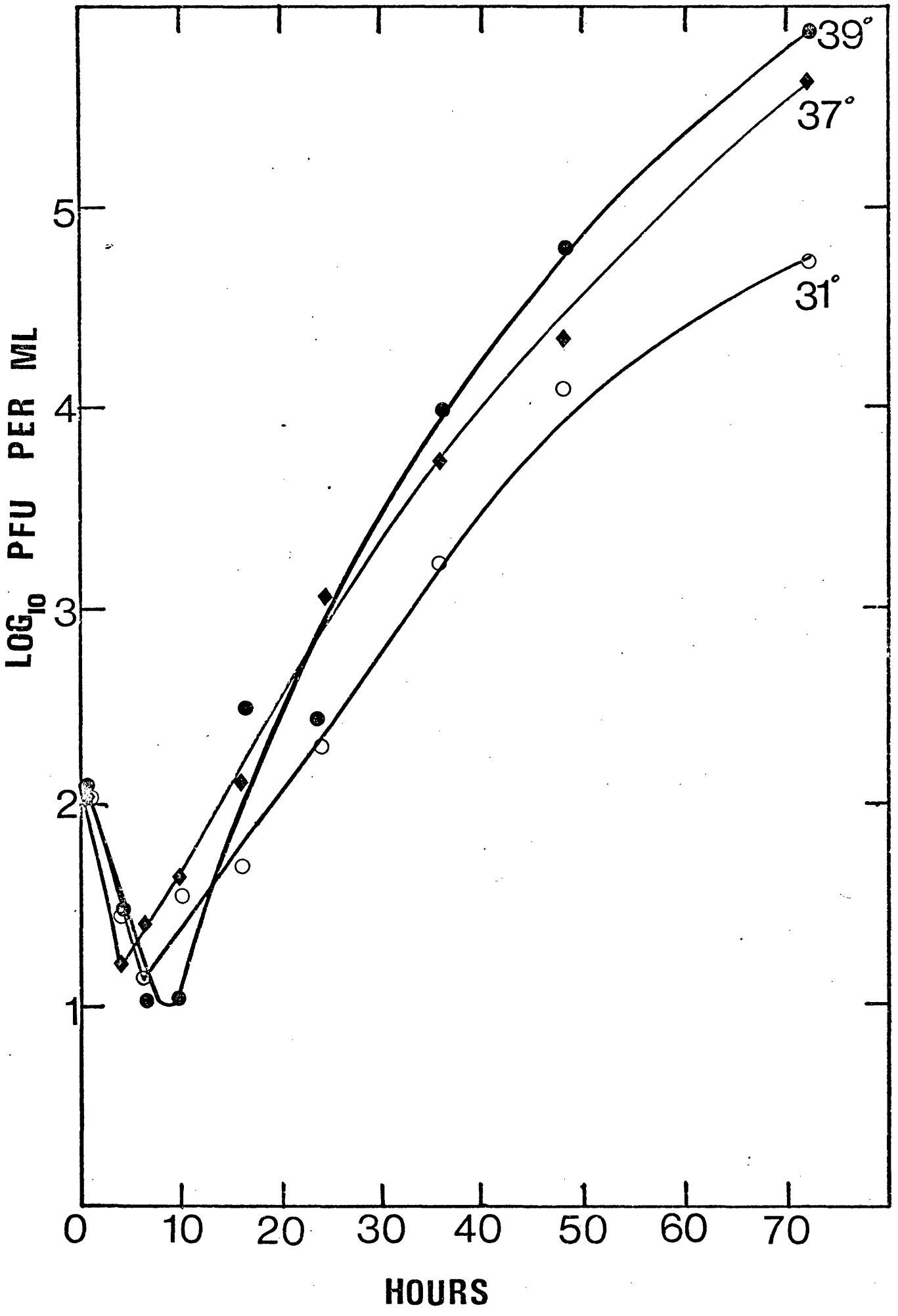

39°
37°
31°
LOG10 PFU PER ML
1
2
3
4
5
0
10
20
30
40
50
60
70
HOURS

Figure 3 : Growth of the Long strain of RS virus at 37°C


C.A.V. - Cell-associated virus


S.V. - Supernatant virus (cell-released virus).

MOI : 0.18 PFU/CEL

TIME : HOURS POST-ADSORPTION

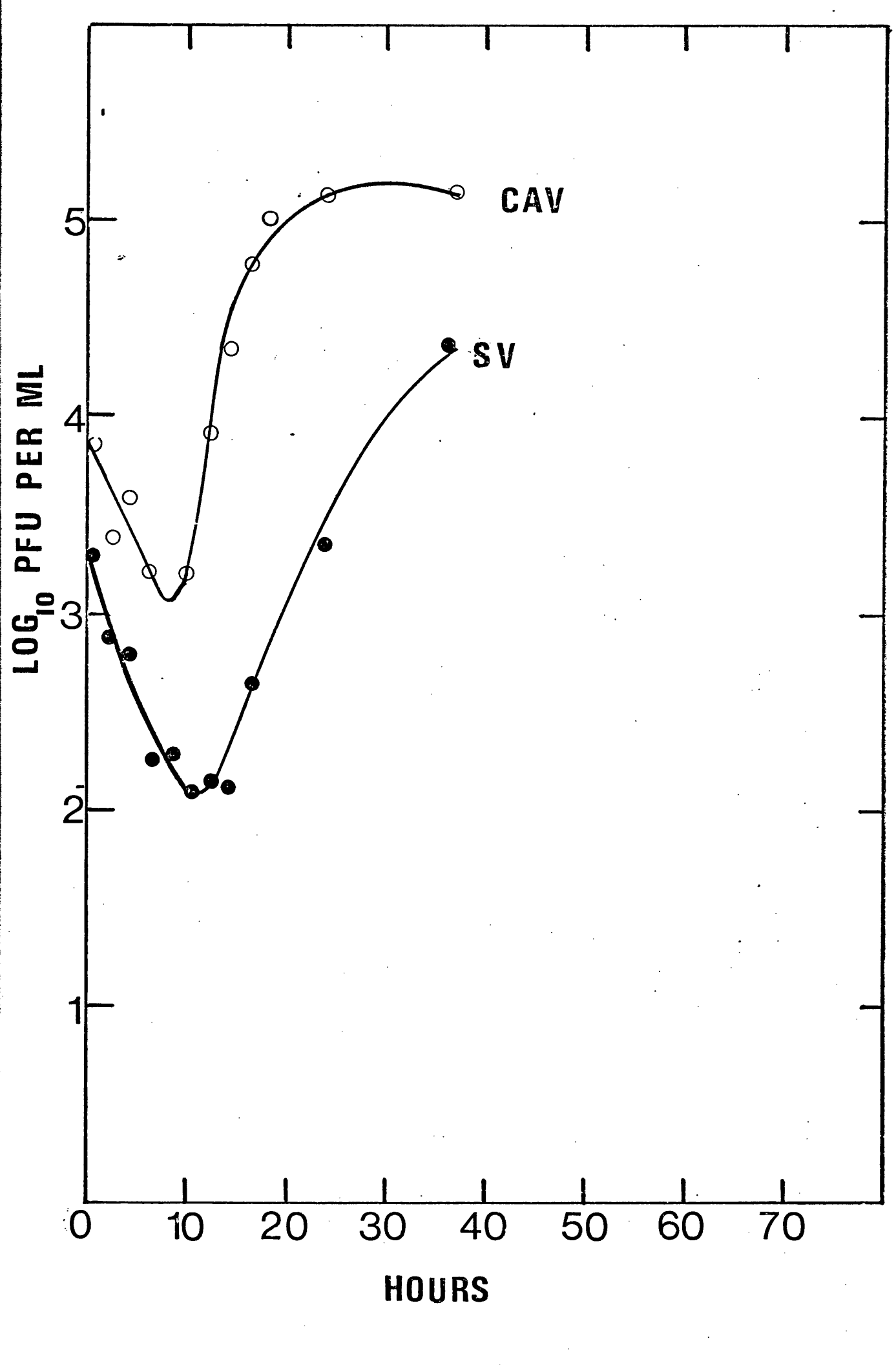
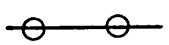
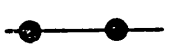

CAV
SV
LOG10 PFU PER ML
1
2
3
4
5
0
10
20
30
40
50
60
70
HOURS

Figure 4 : Growth of the RSN-2 strain of RS virus at 37°C



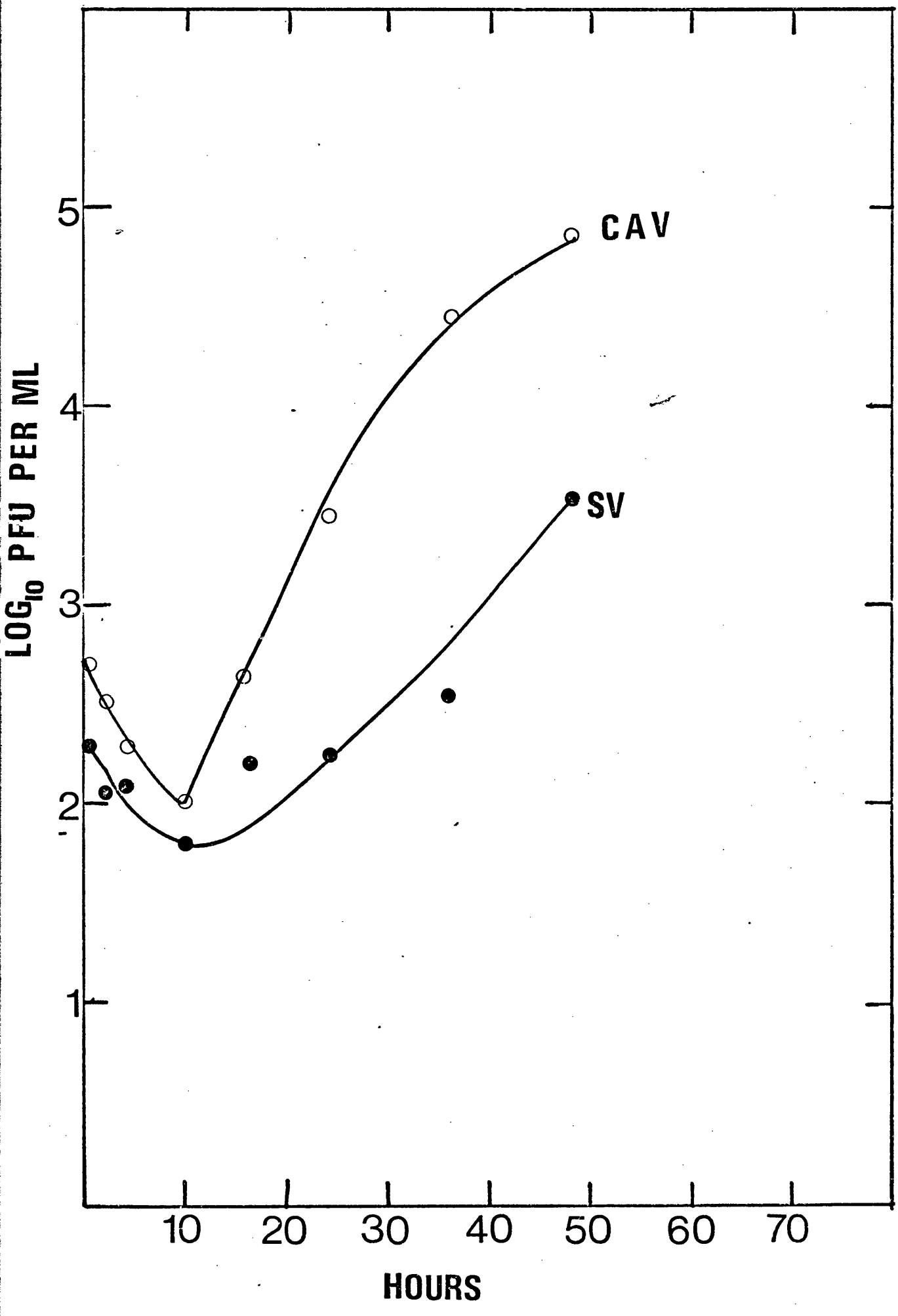
C.A.V. - Cell-associated virus



S.V. - Supernatant virus (cell-released virus).

MOI : 0.03 PFU/CELL

TIME : HOURS POST-ADSORPTION


CAV
SV
5
4
3
2
1
10
20
30
40
50
60
70
HOURS
LOG10 PFU PER ML

3.1.3 Adsorption of the RSN-2 strain of RS virus to BS-C-1 cells

The efficiency of adsorption of the RSN-2 strain of RS virus to BS-C-1 cells was examined to determine whether poor adsorption of virus contributed to the low virus yield.

The percentage of RSN-2 strain adsorbed during 1 hour at room temperature (25°C) was 90%. This figure was the average of three separate experiments (24 samples) in which both wild type and ts mutant virus was used. The percentage of ts mutant virus adsorbed to BS-C-1 cells is shown in Table 7. There was no difference in adsorption between wild type RSN-2 virus and ts mutants (ts2, ts3, ts5, ts15, ts19) of this strain. However, loss of virus due to thermal inactivation during the adsorption period was not taken into account. The thermal inactivation rate of RSN-2 at 25°C was considered to be low based on the results of experiments by other workers using the Long strain of RS virus. A suspension of partially purified Long strain virus in distilled water had an inactivation rate of 0.2 log pfu/hour (Rechsteiner, 1969a), whereas a suspension of the Long strain virus in culture medium, pH 7.8, was not inactivated during the first hour at 25°C (Hambling, 1964b).

In the above experiments the RSN-2 virus was suspended in tissue culture medium. If no or very little inactivation occurs at 25°C adsorption of the RSN-2 strain is very efficient. Thermal inactivation of ts mutant virus may occur more readily at 25°C than that of wild type virus. Therefore infectivity lost due to thermal inactivation could result in an apparent increase in efficiency of adsorption.

TABLE 7

Percentage of RS virus (RSN-2 strain) adsorbed to cells during 1 hour at room temperature

Sample Number	Inoculum	Inoculum recovered	Amount Adsorbed	Percentage of virus adsorbed
1	3.5×10^6	1.2×10^5	3.4×10^6	97
2	7.0×10^5	7.7×10^4	6.2×10^5	89
3	2.7×10^6	7.6×10^4	2.6×10^6	96
4	1.3×10^6	2.2×10^5	1.1×10^6	85
5	4.2×10^6	1.7×10^5	4.0×10^6	95
6	6.2×10^6	2.0×10^5	6.0×10^6	97
7	4.8×10^6	1.3×10^5	4.7×10^6	98
8	3.4×10^6	3.7×10^4	3.4×10^6	99
9	2.0×10^6	1.2×10^5	1.9×10^6	95
10	4.0×10^6	9.8×10^4	3.9×10^6	98

Average 95%

3.1.4 Treatment of RS virus infected BS-C-1 cells to release cell-associated virus

Since RSN-2 strain of RS virus is very cell-associated, methods of releasing cell-associated virus were used to try to increase virus yield (Table 8).

An initial experiment using sonication as a means of releasing cell-associated virus seemed promising. However, on further examination no correlation was found between time of sonication and increase in virus yield.

Another method used in an attempt to release cell-associated virus was trypsin-versene treatment. A solution of 5 ml of trypsin (0.25% w/v) and 20 ml versene was used. The RSN-2 virus infected BS-C-1 cells were pelleted and resuspended in the trypsin-versene solution (2 ml) and then left in suspension for various lengths of time (5-30 minutes). The action of the trypsin-versene solution was stopped by the addition of foetal calf serum (20%). Then the suspension of cells in trypsin-versene was centrifuged and the supernatant retained. The supernatant was assayed for RS virus infectivity.

There was no increase in virus yield due to trypsin-versene treatment. Therefore cell-associated virus was either not released by this treatment or if released, the virus must have been inactivated probably by the trypsin in the solution.

Other methods of increasing the titre of RSN-2 strain of RS virus were tried such as arcton and polyethylene glycol (PEG) treatment as well as the addition of the polycation polybrene (4 μg/ml) to the PBS

solution used to dilute the virus. PEG precipitation did increase the virus titre but arcton treatment drastically reduced it and polybrene had no effect.

TABLE 8

Treatment to release cell-associated RS virus (RSN-2 strain)

Method	Titre (log_{10}/ml)	Increase in yield (Log_{10} treatment - Log_{10} no treatment)
Experiment I - SONICATION		
No treatment	6.78	/
Sonication (1 min)	7.58	0.80
Frozen and thawed then sonication (1 min)	7.62	0.84
Experiment II - SONICATION		
No treatment	5.15	/
Sonication (30-30 sec) interval of 1 minute between sonication	4.78	$\bar{1}$.63
Sonication (minutes)		
0.5	5.40	0.25
1.0	5.26	0.11
1.5	5.15	0
2.0	4.78	$\bar{1}$.63
2.5	5.26	0.11
3.0	5.48	0.33
3.5	5.15	0
4.0	5.04	$\bar{1}$.89
Trypsin-versene treatment		
Time (minutes)		
0	5.66	/
5	5.66	0
10	5.61	$\bar{1}$.95
20	5.59	$\bar{1}$.93
30	5.31	$\bar{1}$.65
Experiment I - Arcton		
No treatment	6.78	/
Arcton (10 ml)	6.00	$\bar{1}$.22

TABLE 8 continued

Method	Titre (log_{10}/ml)	Increase in yield (Log_{10} treatment - Log_{10} no treatment)
Experiment II - Arcton		
No treatment	5.15	/
Arcton (5 ml)	3.48	$\overline{2}$.33
Arcton (10 ml)	3.30	2.15
Sonication (1 min) + Arcton (10 min)	3.00	$\overline{3}$.85
Experiment - Polyethylene glycol (8%) treatment at 4°C for 24 hours		
No treatment	5.15	/
PEG	5.56	0.41
PEG	5.34	0.19
Experiment - Polybrene (4 µg/ml)		
No treatment	5.15	/
Polybrene in diluent	5.18	0.03

3.2 Electron microscopic studies on the RSN-2 strain of RS virus infected BS-C-1 cells

3.2.1 Thin sectioning of RS virus infected BS-C-1 cells

RSN-2 virus-infected BS-C-1 cells were treated as described in the Material and Methods section. Thin sections of infected and uninfected cells (plates 4, 5, 6) which had been grown at 31°C and 39°C were examined under the electron microscope.

The RSN-2 virus matures by budding from the cell membrane. Budding virus in various stages of development can be seen in Plates 7, 8, 9, 10. In RSN-2 virus-infected cells typical dense cytoplasmic inclusions and marked margination of the chromatin could be seen which were not present in uninfected cells. Also in RSN-2 virus-infected cells there was a marked increase in the number and length of membrane processes (Plates 11, 12) compared with those in uninfected cells (Plates 4, 5, 6).

Thin sections of ts mutant infected cells (ts 15, ts 16, ts 23) were also examined but because of the number of particles seen at 31° in the sections, the absence of virus particles at 39°C could not be evaluated.

Plate 4 : Thin section of an uninfected BS-C-1 cell

The magnification of the photograph is 20,000 X.

N : NUCLEOLUS

NO PROCESSES ON THE CELL MEMBRANE.

Plate 5 : Thin section of an uninfected BS-C-1 cell

The magnification of the photograph is 20,000 X.

NO MARGINATION OF THE CHROMATIN IN THE NUCLEUS

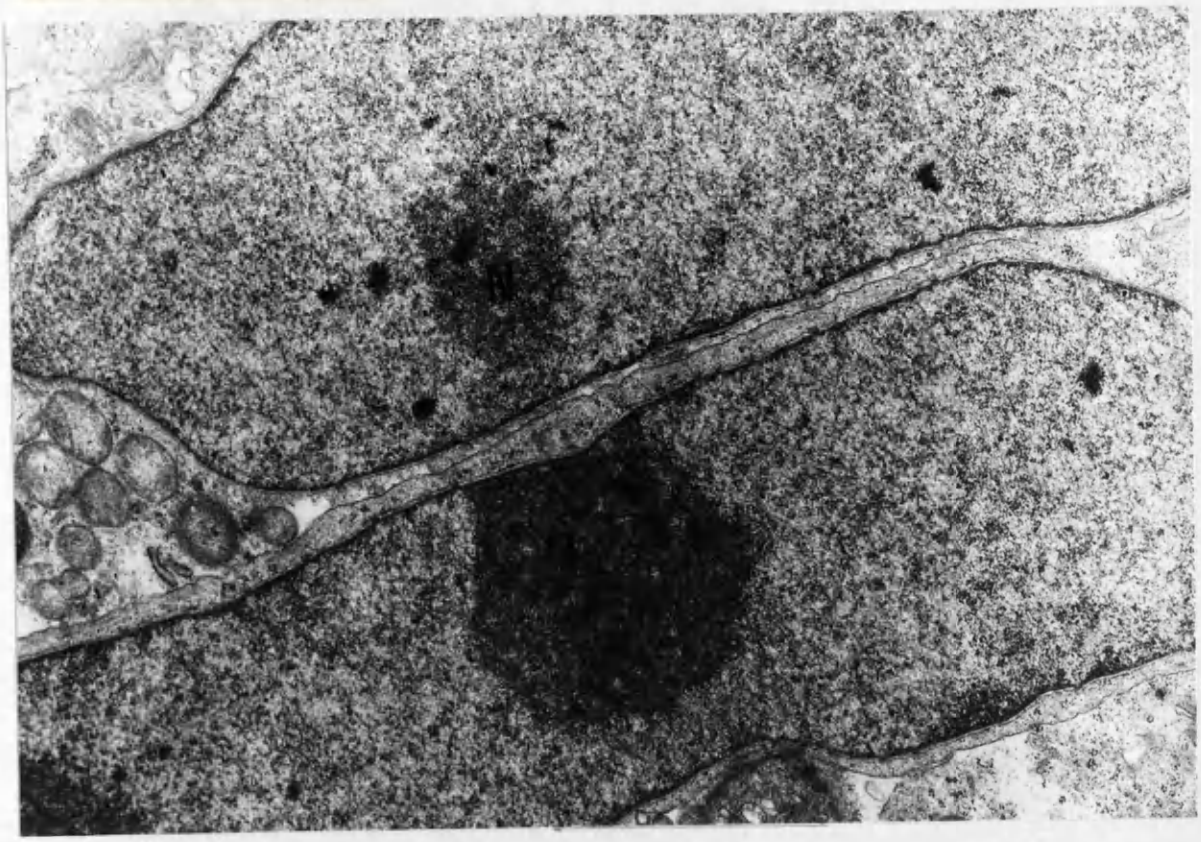


PLATE 5

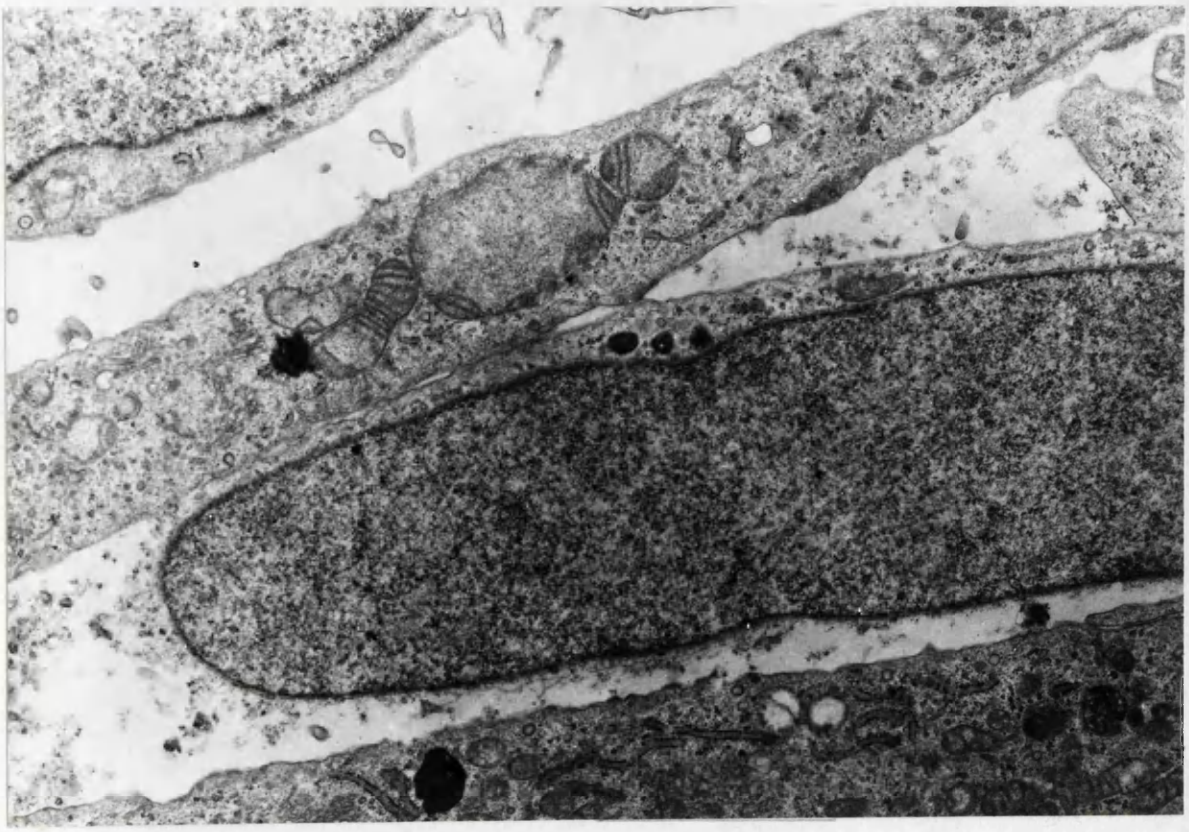


Plate 6 : Thin section of an uninfected BS-C-1 cell

The magnification of the photograph is 20,000 X.

NO CELL PROCESSES.

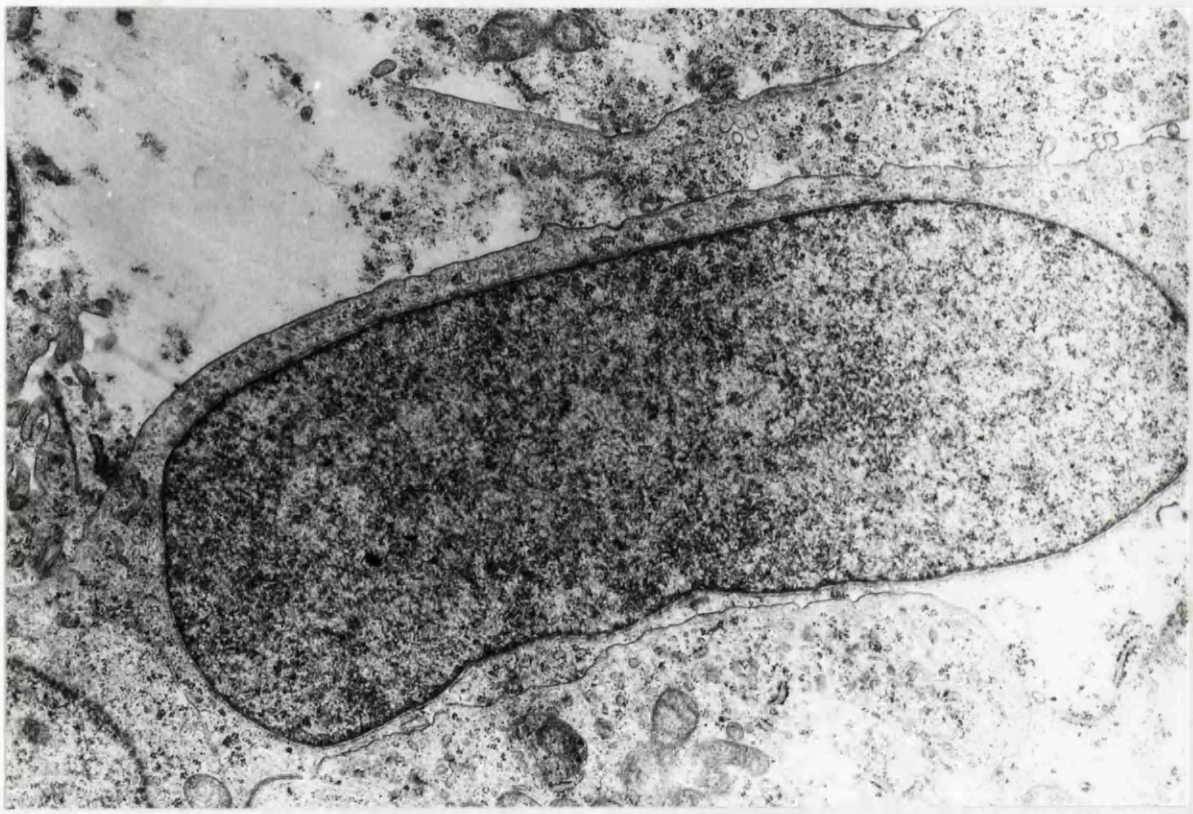


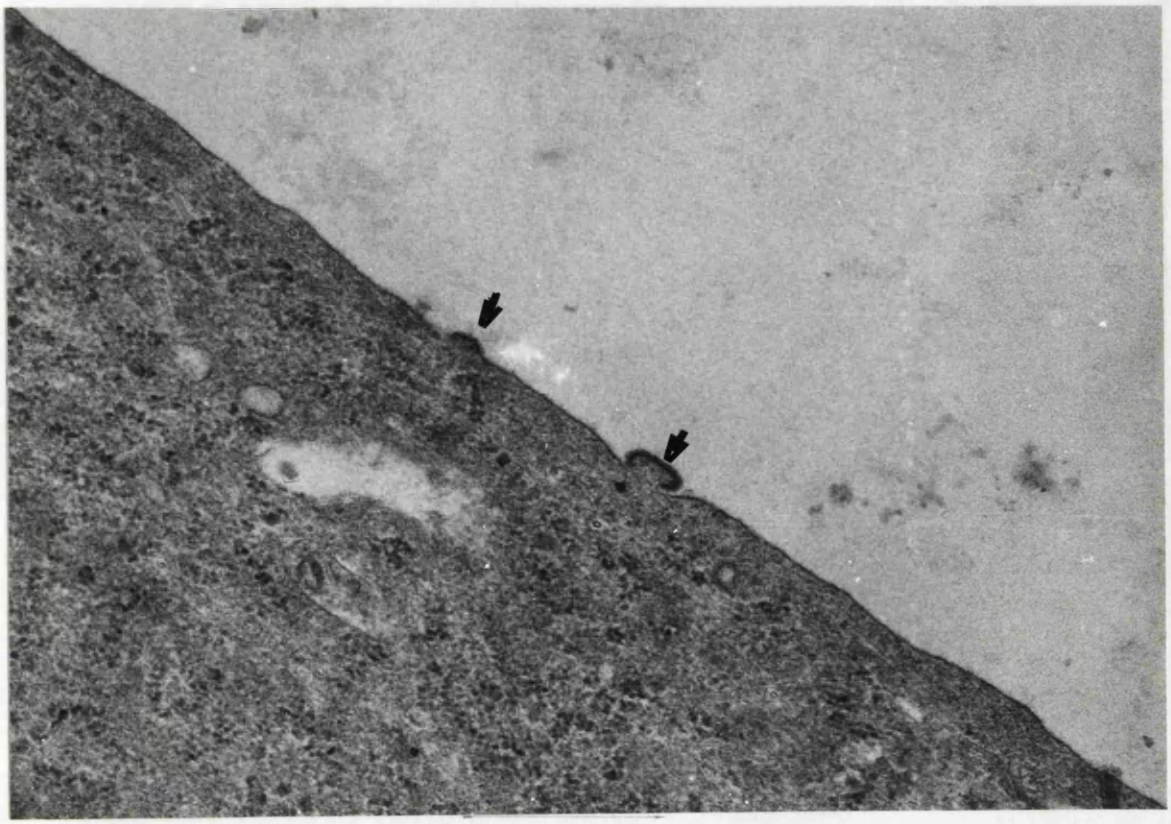
PLATE 6

Plates 7 and 8 : Thin sections of RSN-2 strain infected BS-C-1 cell

RS virus particles can be seen in the first stages of budding from the cell membrane.

The magnification of the photographs is 20,000 X (Plate 7) and 16,250 X (Plate 8).

PLATE 8

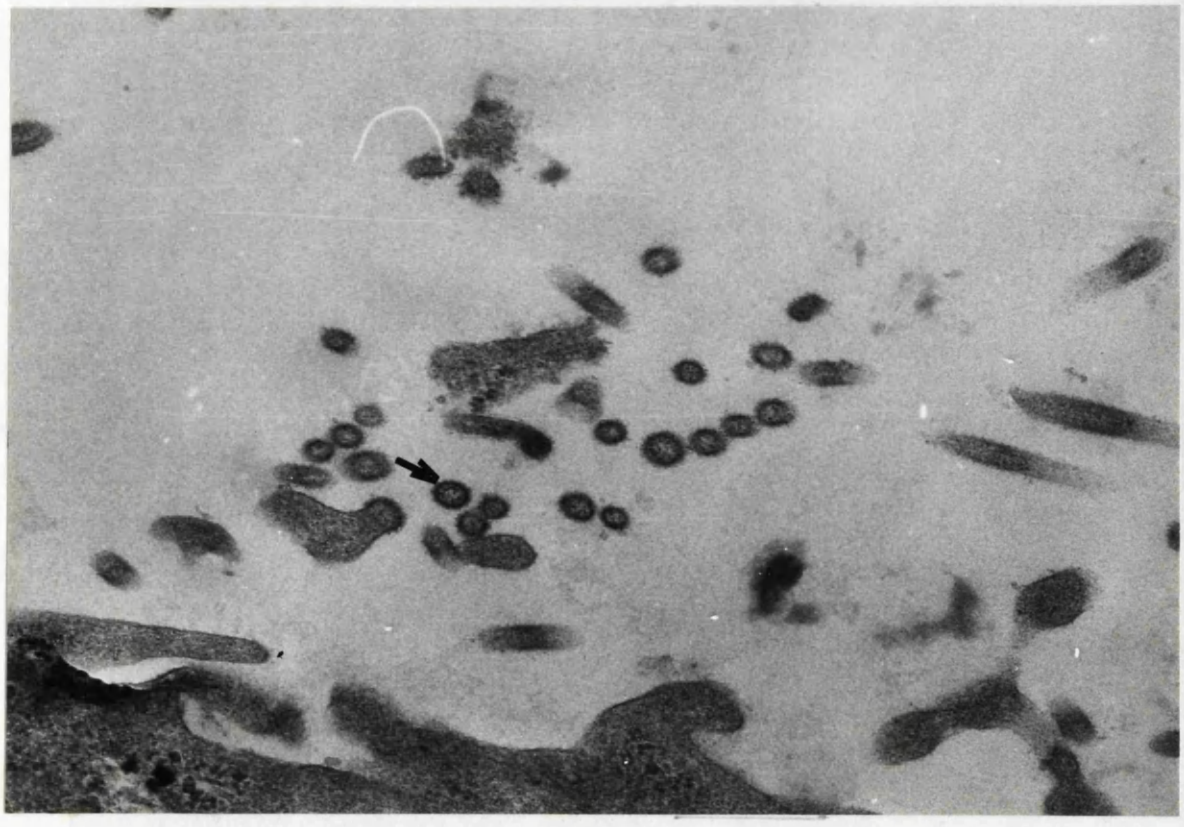


Plates 9 and 10 : Thin sections of RSN-2 strain infected BS-C-1 cells

RS virus particles can be seen in late stages of budding from the cell membrane and free in the cytoplasm.

The magnification of the photographs is 20,000 X (Plate 9) and 16,250 X (Plate 10)

PLATE 10



Plates 11 and 12 : Thin sections of RSN-2 strain infected BS-C-1 cells

RS virus processes can be seen protruding from the cell membrane.

The magnification of the photographs is 16,250 X.

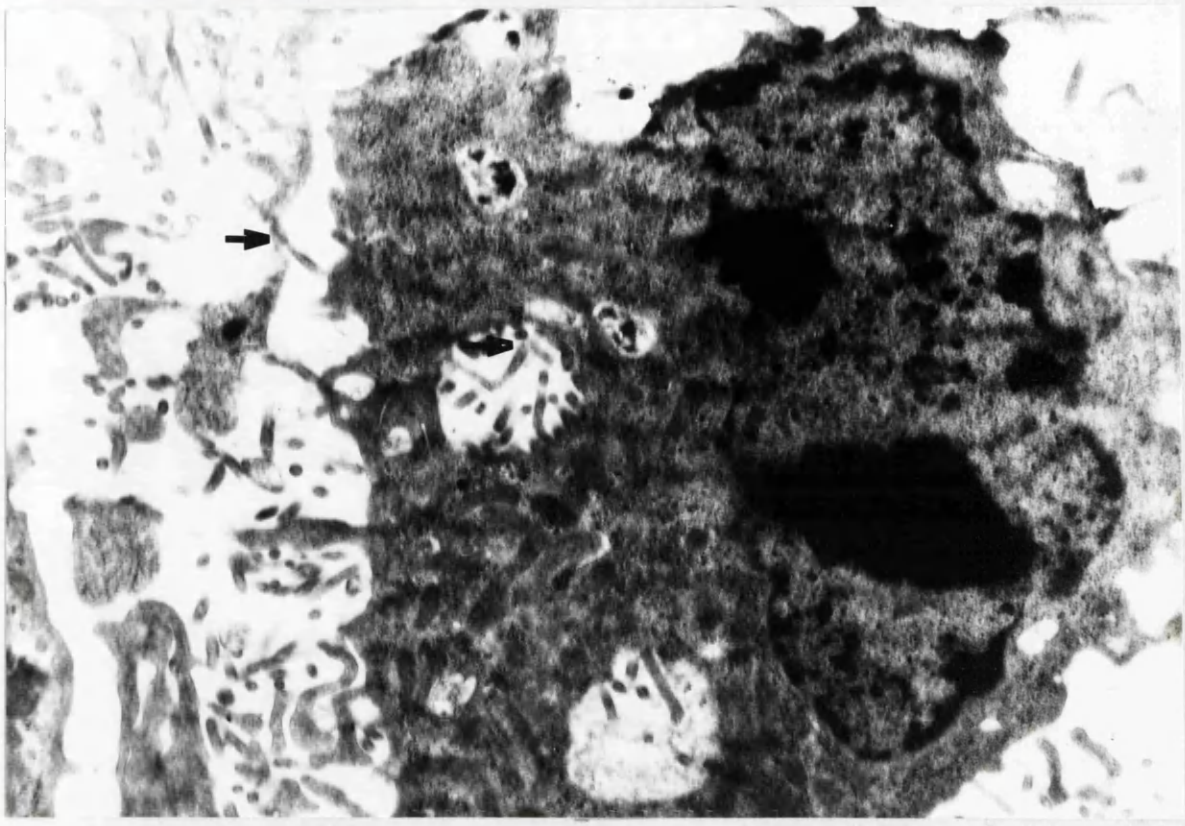
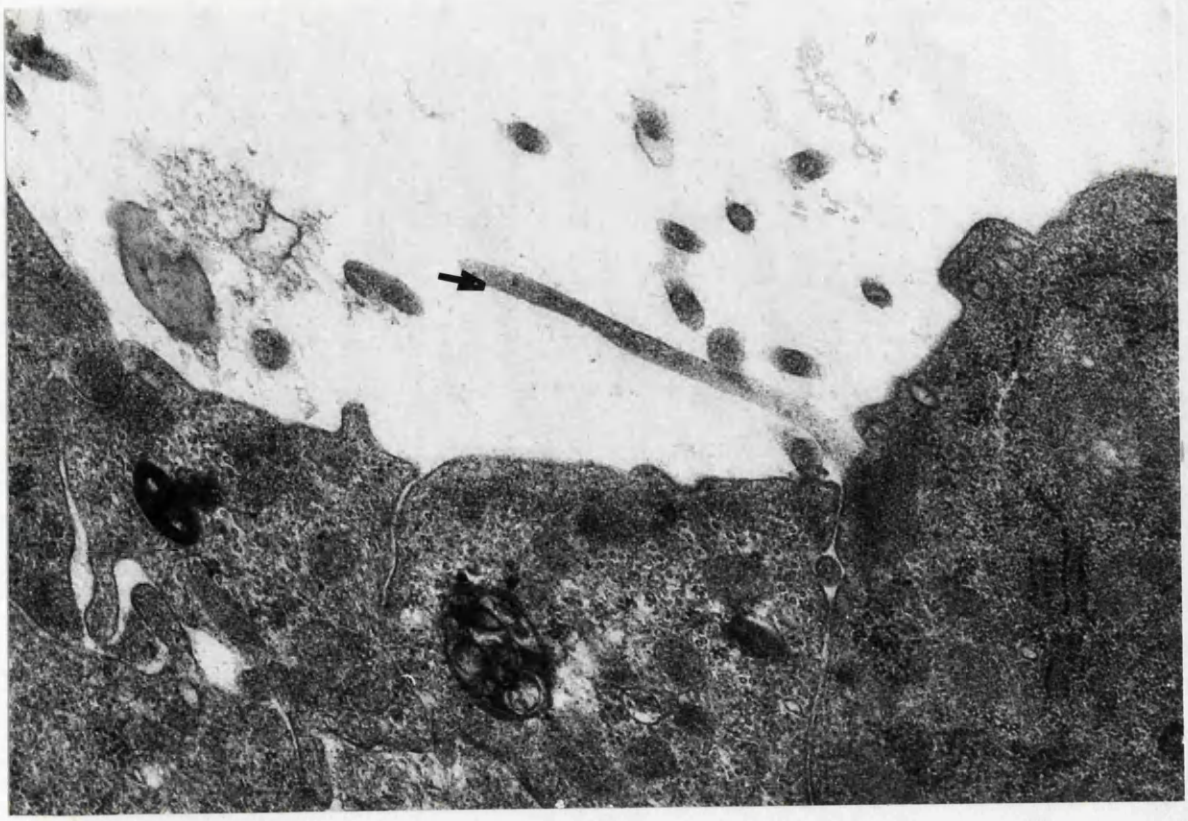


PLATE 12



3.2.2 Scanning electron microscope (EM) study of virus-infected cells

Virus-infected and uninfected BS-C-1 cells grown on coverslips at 31°C were prepared for the Scanning EM as described in Appendix 2.

The microvilli present on the surface of uninfected BS-C-1 cells are seen in plate 13. When the cells were infected with the RSN-2 strain the microvilli became longer and more numerous (Plates 14, 15, 16, 17). Studies using fluorescent antibodies by Dr. P. Shirodaria indicate that these processes carry virus antigen (personal communication).

Mrs. P. McSevney prepared the samples for the Scanning EM and Dr. E. Follett took the photographs.

Plate 13 : uninfected BS-C-1 cells

The magnification of the photograph is 2,000 X.

IN THE CENTRE OF THE FIELD MICROVILLI CAN BE SEEN ON THE EDGE OF TWO CELLS

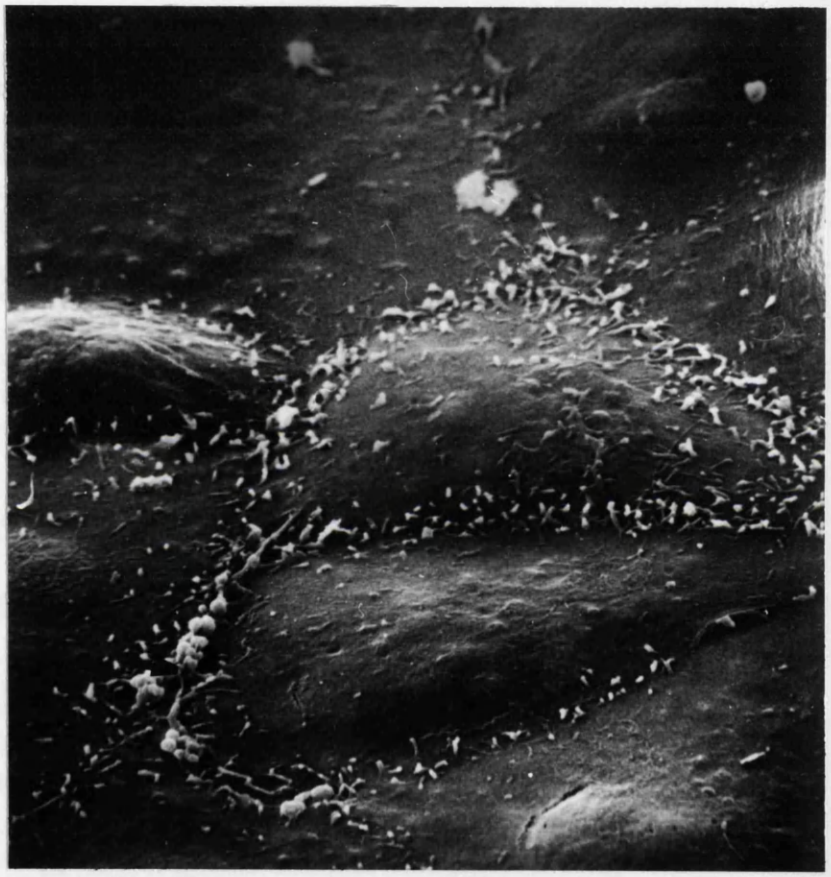


PLATE 13

Plates 14 and 15 : RSN-2 strain infected BS-C-1 cells

The magnification of the photographs is 1,500 X (Plate 14) and 10,000 X (Plate 15).

INFECTED CELLS INCUBATED AT 31°C FOR 72 HOURS. THE NUMBER AND LENGTH OF CELL PROCESSES INCREASES WHEN CELLS INFECTED WITH RS. VIRUS

PLATE 15
AN ENLARGED VIEW OF THESE PROCESSES.

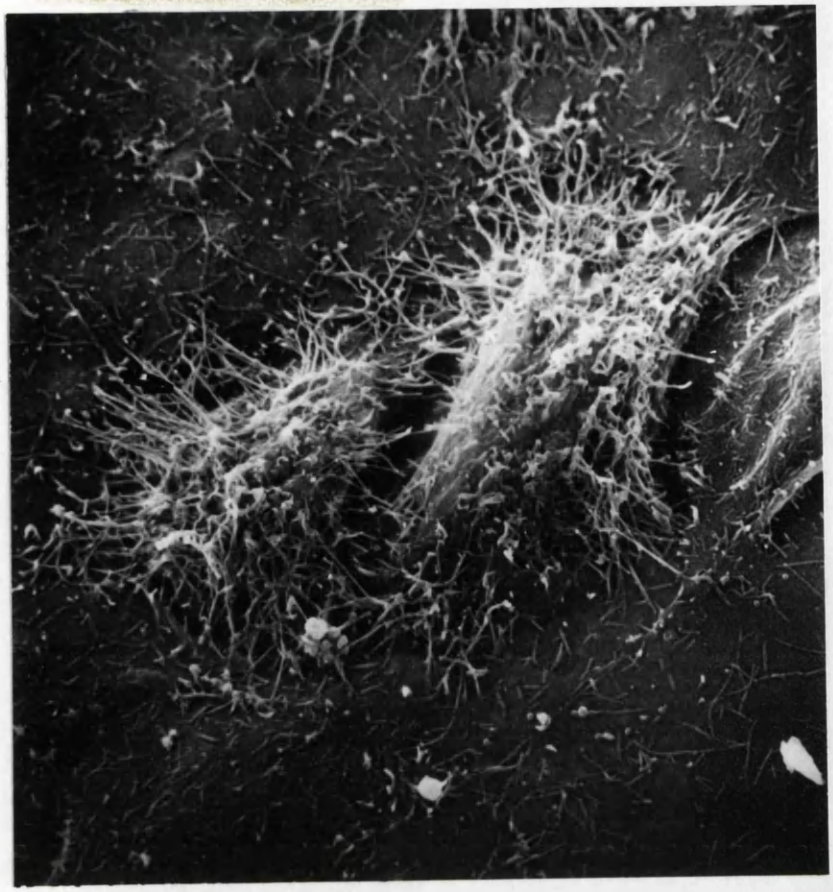
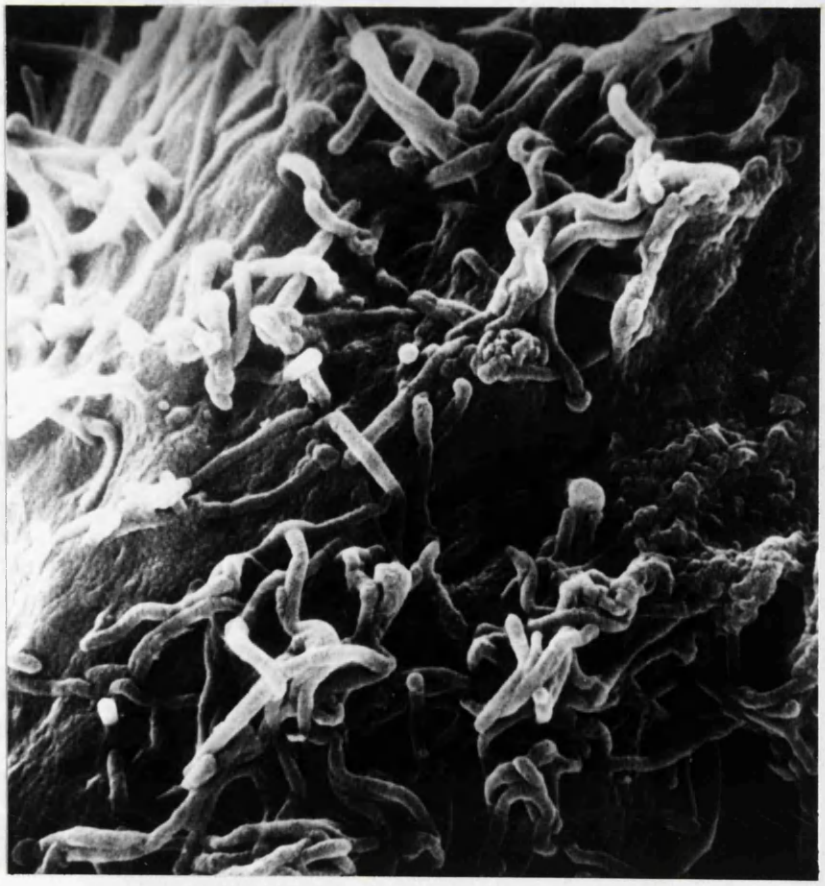


PLATE 15



Plates 16 and 17 : RSN-2 strain infected BS-C-1 cells

The magnification of the photographs is 1,500 X (Plate 16) and 4,800 X (Plate 17).

INFECTED CELLS INCUBATED AT 31°C FOR 72 HOURS. THE NUMBER AND LENGTH OF PROCESSES INCREASES WHEN CELLS INFECTED WITH RS VIRUS.

PLATE 17

AN ENLARGED VIEW OF THESE PROCESSES

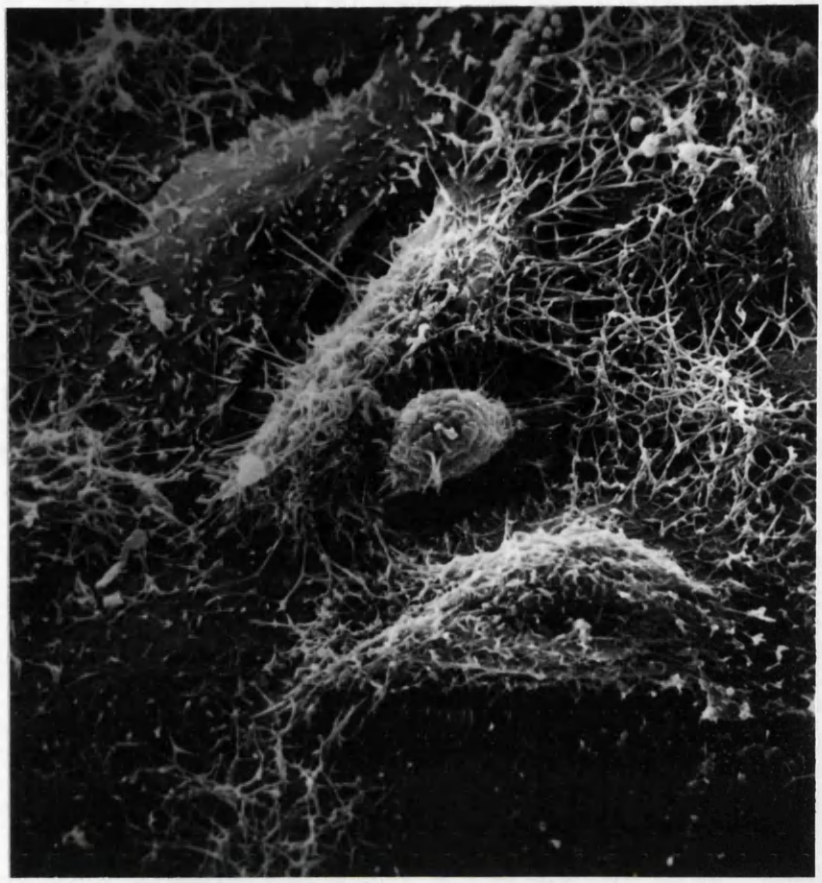


PLATE 16

PLATE 17

3.3 The effect of serum and amino acid concentration in medium on RS virus growth

3.3.1 The effect of serum concentration on the growth of the RSN-2 strain of RS virus

The concentration of serum in Eagle's medium was examined to determine the minimum percentage of foetal calf serum (FCS) required for the optimum growth of RS virus over 48 hours at 31°C.

RS virus growth occurred in the absence of FCS in the medium. However, RS virus yield was increased almost ~~two~~ FOUR-fold by the addition of 1% FCS to the maintenance medium (Table 9).

The virus yield was not further increased until the serum concentration reached 10%.

TABLE 9

The effect of serum concentration on the growth of RS virus

Time (hours)	Percentage of foetal calf serum	Titre (log_{10}/ml)	Mean Titre (log_{10}/ml)	Yield Log_{10} at 48 hrs - Log_{10} at 0 hr
0	10	3.64	3.71	/
0	10	3.78		
48	0	4.46	4.46	0.75
48	1	5.00	5.09	1.38
48	1	5.18		
48	2	5.32	5.03	1.32
48	2	4.74		
48	5	4.93	4.93	1.22
48	10	5.20	5.26	1.55
48	10	5.32		

3.3.2 The effect of arginine concentration on RS virus growth

The effect of arginine concentration in Eagle's medium on the growth of two strains of RS virus, Long and RSN-2, in BS-C-1 cells was examined. The results for both strains were similar. Long strain growth was markedly reduced when no arginine was present in the maintenance medium (Figure 5). However, the addition of arginine (42 μg/ml) to arginine free medium 18 hours after adsorption reversed this effect. Therefore RS virus requires arginine for optimal growth. This confirms earlier results of other workers using the Long strain of RS virus grown in HeLa cells (Levine et al., 1971).

Since RS virus required arginine for growth further experiments were undertaken to study the effect of increasing the arginine concentration in Eagle's medium. It was hoped that an increase in arginine concentration would result in higher RS virus yield. The addition of extra arginine (42 μg/ml) to the maintenance medium of RSN-2 virus infected BS-C-1 cells at 12 hours and at 12 and 24 hours post adsorption only slightly increased the yield of RSN-2 virus at 48 hours.

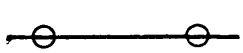
Whereas increasing the concentration of arginine in the maintenance medium by 42 μg/ml immediately after adsorption resulted in an increase in RSN-2 virus yield ($\frac{1}{2}$ log unit). However increasing the total concentration of arginine beyond this level (84 μg/ml) did not result in any further increase in RSN-2 virus yield (Figure 6).

Figure 5 : The effect of arginine concentration on the Long strain of RS virus

Temperature : 37°C

Long strain grown in medium containing arginine.

Long strain grown in arginine free medium for 18 hours, then supplemented with arginine (42 µg/ml).



Long strain grown in arginine-free medium.

MOI : 0·05 PFU/CELL

TIME : HOURS POST-ADSORPTION

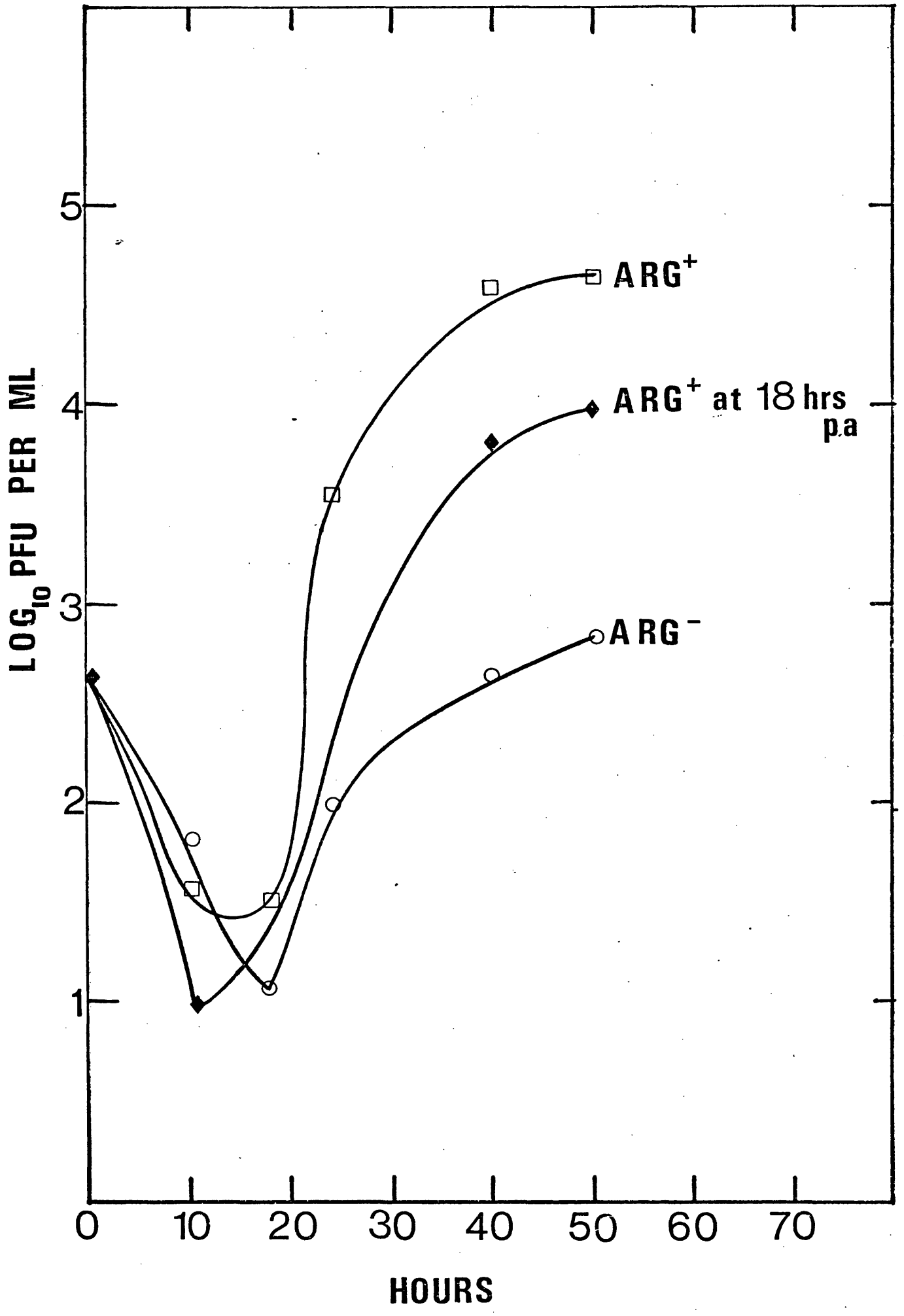
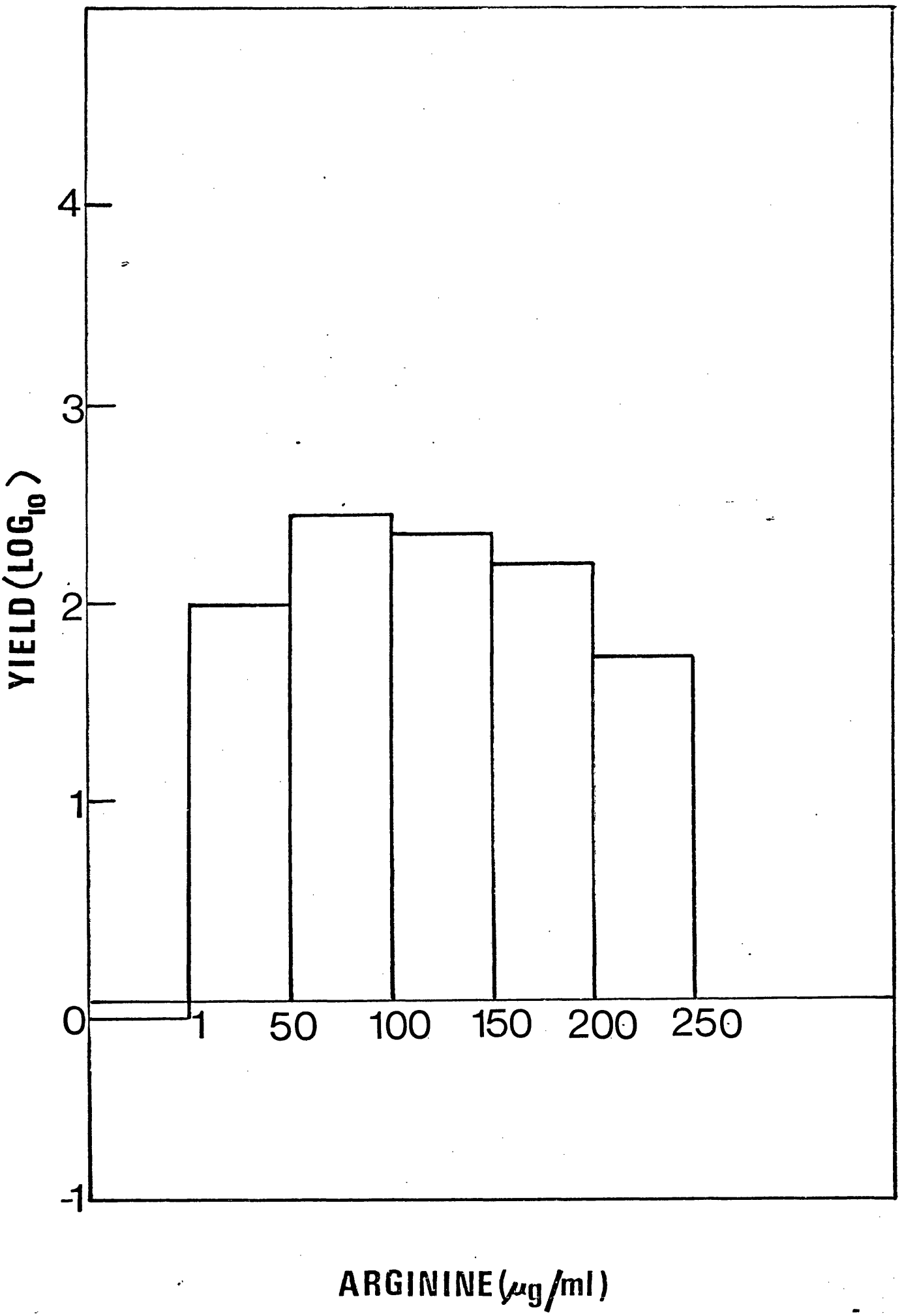

5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS
ARG+
ARG+ at 18 hrs p.a
ARG−

Figure 6 : The effect of arginine concentration in medium on RS (RSN-2 strain) virus yield

Yield $\log_{10}$ = $\log_{10}$ Yield at 72 hrs - $\log_{10}$ yield at 0 hrs.

Infectivity is measured as $\log_{10}$/ml.


4
3
2
1
0
-1
YIELD (LOG10)
1
50
100
150
200
250
ARGININE (µg/ml)

3.3.3 The effect of methionine concentration on growth of the RSN-2 strain of RS virus

If RS virus could be grown and labelled in methionine free medium more ^{35}S-methionine label would be incorporated into viral polypeptides as ^{35}S-methionine would be the only source of methionine in the medium apart from serum.

The effect of methionine concentration on RS virus growth was examined because ^{35}S-methionine is used as a radioactive label for virus polypeptides. RS virus was grown in Eagle's medium containing various concentrations of methionine (15 μg/ml, 7.5 μg/ml, 0 μg/ml). Eagle's medium (Glasgow modification) contains 15 μg/ml of methionine. There was no reduction in RS virus yield when the virus was grown in Eagle's medium without methionine (Table 10). Therefore methionine-free medium can be used when RS virus polypeptides are labelled using ^{35}S-methionine.

TABLE 10

The effect of methionine concentration on RS virus growth

Time (hours)	Methionine µg/ml	Titre (log_{10}/ml)	Mean Titre	[x]Yield
0	0	3.60	3.50	
0	0	3.40		/
48	0	4.84	5.10	1.60
48	0	5.36		
48	7.5	4.90	5.08	1.58
48	7.5	5.26		
48	15	5.30	4.92	1.42
48	15	4.54		

[x]Yield is taken as the difference in value between titre at 48 hours (log_{10}/ml) and 0 hr (log_{10}/ml).

3.4 The effect of inhibitors of nucleic acid and protein synthesis

3.4.1 The effect of actinomycin D on the growth of RS virus (RSN-2 strain)

Actinomycin D is a polypeptide antibiotic which at the level of 1-5 μg/ml almost completely inhibits (95%) DNA-dependent RNA synthesis. However, RNA synthesis which does not require cellular or viral DNA is not affected by these levels. Actinomycin D also inhibits the transport of RNA from the nucleus to the cytoplasm (Reich and Goldberg, 1964). *In vivo* and *in vitro* actinomycin acts by binding to the guanine residues of helical deoxypolynucleotides containing guanine (Reich, 1966). The replication of DNA and some RNA viruses (fowl plague, Rous sarcoma virus, reovirus) is inhibited by actinomycin D.

The effect of actinomycin D on RS virus was examined to determine whether RS virus replication was inhibited like that of the orthomyxoviruses or unaffected like that of the paramyxoviruses by the presence of actinomycin D.

Initially the yield of RS virus in the presence of 5 μg actinomycin/ml and 10 μg of actinomycin D/ml was examined (Table 11). At 24 hours and 48 hours there was no decrease in RS virus titre in the presence of 5 μg of actinomycin D/ml. However, in the presence of 10 μg of actinomycin D/ml at these there was a decrease in RS virus titre ($1\frac{1}{2}$ log units at 48 hours). Since high concentrations of actinomycin D are toxic to cells the effect of 10 μg/ml could be non-specific.

To confirm these findings RS virus and vesicular stomatitis virus (V.S.V.) were grown in the presence of 5 μg/ml of actinomycin D.

TABLE 11

The effect of actinomycin D on RS virus growth

Experiment	Time (hours)	ACTINOMYCIN D		
		NONE	5 µg/ml	10 µg/ml
1	0	3.94	3.87	3.87
	5	3.49	3.56	3.09
	10	3.39	2.45	2.91
	24	4.34	4.43	4.15
	48	5.15	5.16	3.78
2	0	3.83	3.83	ND
	72	5.90	4.77	ND

ND - not done

Titre of virus given as $\log_{10}$/ml

In this experiment RS virus yield in the presence of 5 μg of actinomycin D/ml was reduced; $1\frac{1}{2}$ and $2\frac{1}{2}$ log units at 37 and 48 hours respectively (Figure 7). This result was in contrast to the previous finding that there was no decrease in RS virus titre at 48 hours. VSV also showed a reduction in yield (1 log unit at 14 hours) but not at 37 hours. The effect of actinomycin D on VSV was presumed to be non-specific.

Since these experiments with RS virus gave contradictory results further experiments to examine the effect of actinomycin D concentration on RS virus yield were performed. In two similar experiments, one using Pseudorabies virus, a DNA virus, for comparison with RS virus there was no correlation between concentration of actinomycin D and the degree of reduction in virus yield (Figure 8).

The cells were pretreated for 2 hours before infection with the concentration of actinomycin D which was added after adsorption. Pseudorabies virus was completely inhibited by even the lowest concentration of actinomycin D (0.1 μg/ml) and by pretreatment of cells with actinomycin D (10 μg/ml). The cells were washed twice with medium without actinomycin D before infection and no further actinomycin D was added to the samples to show the effect of pretreatment alone.

RS virus yield was reduced by pretreatment of the cells with actinomycin D but not as much as by the addition of various concentrations of actinomycin D after adsorption. In the presence of actinomycin D (5 μg/ml) RS virus yield was reduced (about 1 log unit). This reduction was smaller than that found in a previous experiment (Figure 7) with no pretreatment of cells. The results were calculated as percentage

resistance to actinomycin D (Figure 8). RS virus yield was reduced 80-90% in the presence of actinomycin D whereas Pseudorabies virus was reduced 99%.

The effect of actinomycin D on RS virus yield can be variable, since RS virus yield has been reduced (Figures 7, 8) as well as not affected (Table 11) by the presence of 5 μg/ml of actinomycin D.

A variability in response to actinomycin D has also been reported for poliovirus. Several factors were reported to affect poliovirus resistance to actinomycin D; the strain of virus used and the age of the cells, but the main factor seemed to be the presence of a substance in serum which induced an actinomycin-resistant state. The substance was nondialysable and heat stable and was considered to be a hormone. Experiments showed that the hormone insulin even in low concentration (0.004 μg/ml) had a similar effect to the substance present in serum. Pretreatment of cells with insulin had no effect on poliovirus growth in the absence of actinomycin D. However, when actinomycin D was present insulin pretreatment of cells produced an increase in resistance to actinomycin D. This effect was not seen when insulin was added after adsorption to cells which had not been pretreated (Cooper, 1966).

Therefore an experiment based on the poliovirus findings was undertaken. The effect of pretreatment of cells with Eagle's medium alone or supplemented with either FCS (5%), bovine serum albumin, BSA (fraction V) 1 mg/ml, insulin (4 μg/ml) or combinations of insulin and FCS or BSA before infection was examined (Table 12). Cells which had been pretreated for 25 hours with any one of the media described above

Figure 7 : The effect of actinomycin D (5 µg/ml) on the growth of RS virus (RSN-2 strain) and VSV

Temperature : 31°C

VSV grown in the presence of actinomycin D (5 µg/ml).

VSV grown in the absence of actinomycin D.

RSN-2 strain grown in the presence of actinomycin D (5 µg/ml).

RSN-2 strain grown in the absence of actinomycin D.

MOI

VSV : 7.4 PFU/CELL

RS.VIRUS : 0.1 PFU/CELL

TIME : HOURS POST-ADSORPTION.

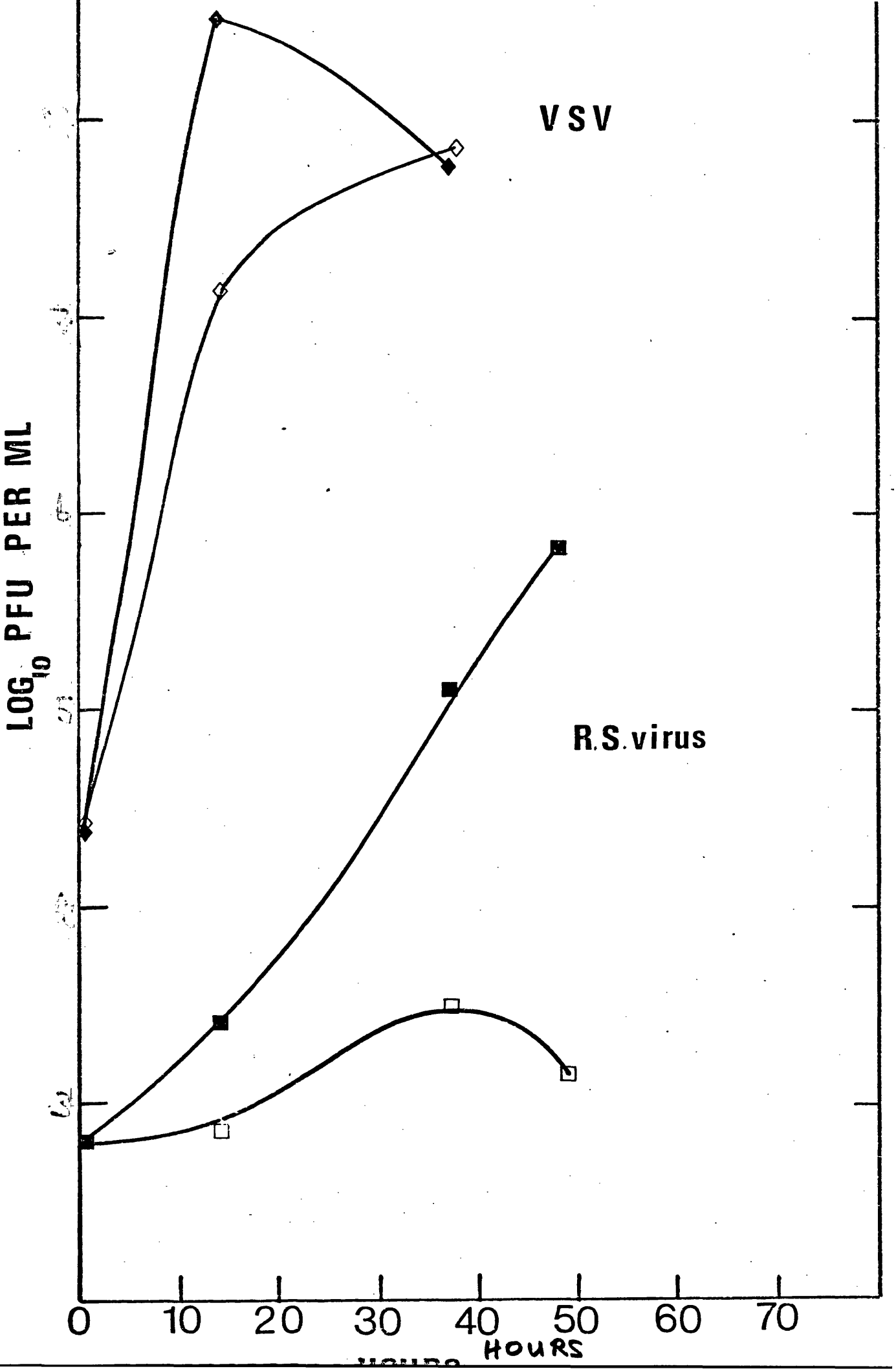
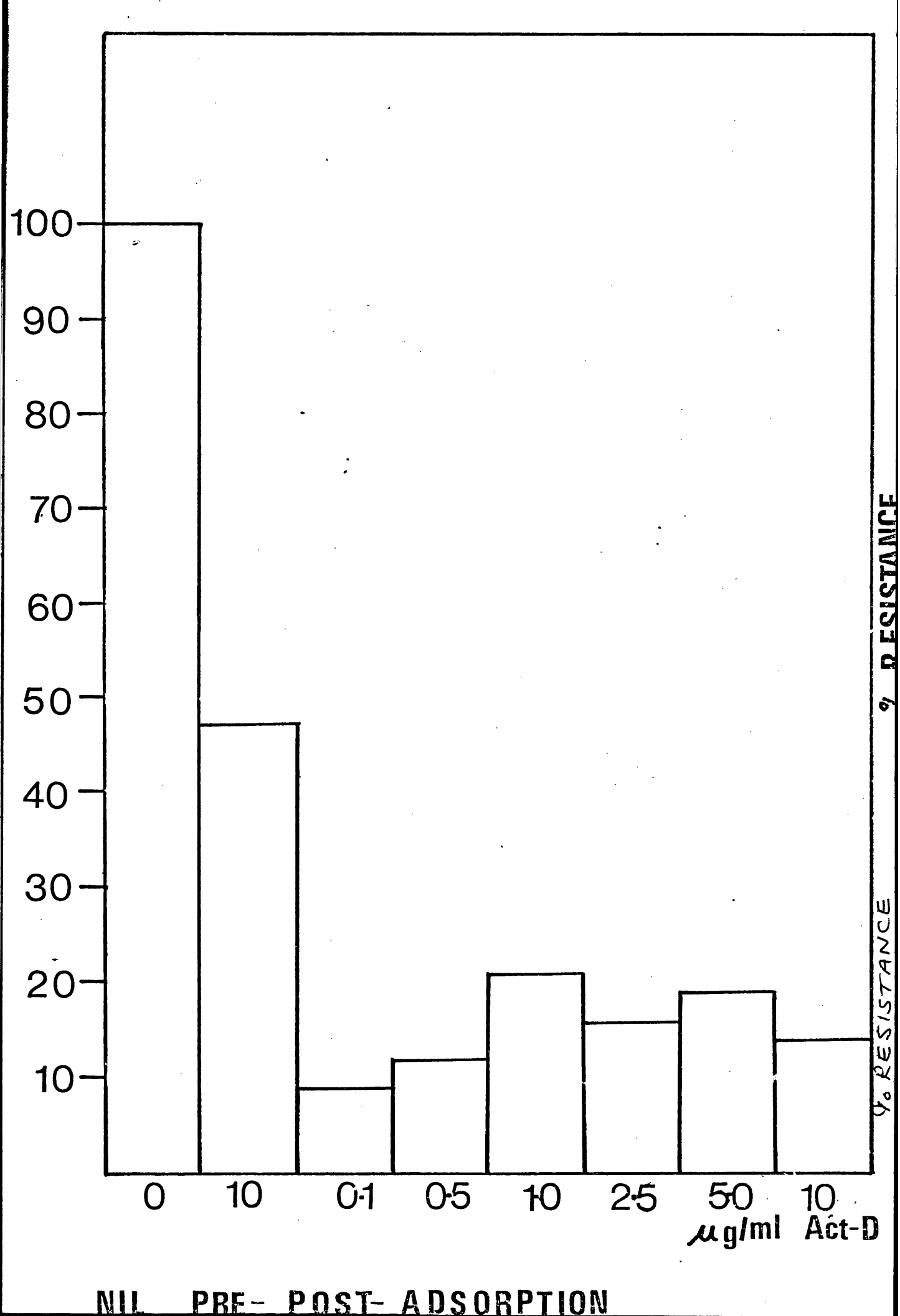

VSV
R.S.virus
LOG10 PFU PER ML
0
10
20
30
40
50
60
70
HOURS

Figure 8 : % Resistance of RS virus (RSN-2 strain) to actinomycin D

Temperature : 31°C



NIL PRE- POST- ADSORPTION

TABLE 12

The influence of medium on the action of actinomycin D on RS virus replication

Time (hours)	Medium	no Actinomycin D		Actinomycin D 5 µg/ml		** % Yield
		Titre log_{10}/ml	*Yield (log_{10}/ml)	Titre log_{10}/ml	*Yield (log_{10}/ml)	
0	FCS batch 1	3.22	/	/	/	/
48	no FCS	5.95	2.73	4.16	0.94	1.6
48	FCS batch 1	6.03	2.81	4.61	1.39	3.8
48	FCS batch 2	5.91	2.69	4.85	1.63	8.7
48	FCS batch 3	5.77	2.55	5.04	1.82	18.6
48	FCS batch 4	6.31	3.09	5.22	2.00	8.1
48	BSA (1 mg/ml)	6.52	3.30	4.95	1.73	2.7
48	Insulin (4 µg/ml)	5.80	2.58	4.80	1.58	10.0
48	Insulin + BSA	6.66	3.44	4.65	1.43	0.98
48	Insulin + FCS_4	5.92	2.70	4.95	1.73	10.7

Cells were not pretreated with actinomycin D but they were pretreated with different media for 25 hrs before infection.

* Yield is the difference between titre at 48 hr (log_{10}/ml) and 0 hr (log_{10}/ml).

** % Yield is yield in the presence of actinomycin D expressed as a percentage of yield in the absence of actinomycin D.

FCS - 5% foetal calf serum.

were infected with RS virus. After adsorption the same type of medium was used as maintenance medium and in one set of duplicate cultures it was supplemented with 5 μg of actinomycin D/ml. Cultures pretreated with medium containing insulin (4 μg/ml) were slightly less sensitive to actinomycin D than those pretreated with medium containing no serum, FCS and BSA (fraction V). The combination of insulin with BSA resulted in an increase in sensitivity to actinomycin D. This must be due to the action of BSA since insulin in combination with FCS did not show the same effect. The combination of insulin and BSA in medium resulted in the best RS virus yield in the absence of actinomycin D. Therefore since the percentage yield was calculated on the difference between virus yield in the absence and presence of actinomycin D, the sensitivity of the virus to actinomycin D would appear to be higher than in the other examples. The results show that insulin did not markedly influence RS virus resistance to actinomycin D. An interesting feature was that insulin (4 μg/ml) and BSA (1 mg/ml) or BSA (1 mg/ml) alone could substitute for FCS in Eagle's medium. RS virus grown in medium supplemented with these factors gave the best yields.

Following a personal communication from Dr. Meera Gharpure that RS virus like influenza virus was only sensitive to actinomycin D if added during the early part of the growth cycle, an experiment was undertaken to study the effect of addition of actinomycin D at various times after adsorption (Table 13).

Actinomycin D (1 μg/ml) was added to maintenance medium at various times after adsorption. The effect of actinomycin D was most marked when it was added directly after adsorption virus yield being

TABLE 13

The effect of addition of actinomycin D to RS virus maintenance medium at various times after adsorption

Harvested .(hours)	Actinomycin D (1 µg/ml) added	Titre (log_{10}/ml)	Mean Titre	*Yield (log_{10}/ml)	**Percentage Yield
0	None	3.78	3.37	/	/
0	None	2.95			
72	None	5.81	5.76	2.39	/
72	None	5.70			
72	Pretreatment 2 hours	4.85	4.98	1.61	16.6
72	Pretreatment 2 hours	5.11			
72	0 hr pa	3.46	3.46	0.09	0.5
72	0 hr pa	3.46			
72	2 hr pa	4.57	4.21	0.84	2.8
72	2 hr pa	3.85			
72	4 hr pa	4.40	4.35	0.98	3.9
72	4 hr pa	4.30			
72	6 hr pa	4.64	4.57	1.20	6.5
72	6 hr pa	4.49			
72	8 hr pa	5.08	5.14	1.77	24.6
72	8 hr pa	5.20			

Pretreatment - cells pretreated with 1 µg/ml actinomycin D for 2 hours before infection, then actinomycin D removed.

* Yield is the difference between titre (log_{10}/ml) at 72 hour and titre (log_{10}/ml) at 0 hr.

** % Yield in the presence of actinomycin D expressed as percentage yield in absence of actinomycin D.

reduced 99.5%. However, as the actinomycin D was added later in the growth cycle its effect decreased progressively.

The addition of actinomycin D to medium 8 hours post adsorption resulted in only a 76% reduction in RS virus yield. Therefore, actinomycin D appears to affect RS virus growth at an early stage in the growth cycle.

3.4.2 The effect of mitomycin C on RS virus (RSN-2 strain) growth

High concentrations of mitomycin inhibit cellular but not viral RNA synthesis and DNA synthesis by combining with the cellular DNA and inactivating it. Actinomycin inhibits DNA dependent RNA synthesis but does not destroy the genes controlling it since cellular DNA synthesis is not inhibited. The effect of mitomycin C (10 μg/ml, 5 μg/ml) on RS virus growth was examined.

The presence of 10 μg of mitomycin C/ml reduced RS virus yield at 48 hours by 41% and completely inhibited the growth of PSRV, a DNA virus (Table 14). Therefore RS virus did not appear to require cellular DNA or DNA synthesis for replication. The reduction in RS virus yield was probably due to the non-specific effect of mitomycin C on the cells.

TABLE 14

The effect of mitomycin C on RS virus growth

Experiment	Virus	Time	Mitomycin C			*Percentage yield
			None	5 µg/ml	10 µg/ml	
1	RSN-2	0	3.94		3.80	
		5	3.49	/	3.46	
		10	3.39		3.06	/
		24	4.34		3.53	15
		48	5.12		4.89	59
2	RSN-2	0	3.83	/	/	/
		72	5.90	4.80	4.90	10
1	Pseudorabies virus	0	0		0	/
		24	0	/	0	/
		48	4.37		0	<0.004
3	Pseudorabies virus	0	4.08		4.78	/
		10	4.11		4.08	/
		24	7.20	/	3.30	0.013
		36	7.32		3.00	0.005
		48	7.11		2.18	0.001

*Yield in the presence of mitomycin C (10 µg/ml) expressed as a percentage of yield in absence of mitomycin C.

3.5 The isolation and growth properties of RS virus ts mutants

3.5.1 Mutagenisation of the RSN-2 strain of RS virus

RS virus was cloned four times to obtain a homogeneous stock before mutagenisation. Then RS virus was mutagenised using various concentrations of different mutagens. The virus was grown in the presence of 5-flurouracil (5-FU), N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or 5-azacytidine (ACR) to induce mutations during replication. It was also exposed to ethyl methane sulphonate (EMS) a mutagen which acts by causing direct chemical modification of the viral nucleic acid.

Clonally related mutants may occur when the virus is mutagenised during replication. To avoid isolating a large number of clonally related mutants, mutants were isolated from FIVE independently treated clones of virus.

5-Flurouracil (5-FU)

5-FU is an analogue of uracil and acts by replacing uracil in RNA during replication. Miscoding occurs in RNA viruses grown in the presence of 5-FU because halogenated uracils are more likely than uracil to undergo tautomeric change. When this happens uracil resembles cytosine in pairing with its complementary base guanine during nucleic acid replication. 5-FU present during replication is a good mutagen for RNA viruses, for example poliovirus (Cooper, 1964).

RS virus was grown in the presence of concentrations of 5-FU from 5 to 200 μg/ml at 31°C (Experiment 1). The infectivity of virus grown in the presence of 200 μg/ml of 5-FU was reduced by 2½ log units

(Figure 9). PLAQUES ~~Clones~~ were picked from RS virus treated with 5, 10, 25, 50, 75, 100 and 200 μg/ml of 5-FU.

In another experiment (Experiment 2) RS virus was grown in the presence of concentrations of 5-FU from 5 to 100 μg/ml at 31°C and 39°C. The infectivity of RS virus grown in the presence of 100 μg/ml of 5 -FU was reduced by 3½ log units (Figure 10). PLAQUES ~~Clones~~ were picked from RS virus grown in the presence of 50 μg/ml of 5-FU as the greatest difference in plating efficiency between virus grown in the presence of 5-FU at 31°C and 39°C occurred at this concentration. The smallest difference in plating efficiency occurred when virus was grown in the presence of 100 μg/ml of 5-FU; PLAQUES ~~clones~~ were also picked from this virus. Clones of RS virus were grown in the presence of 50 μg/ml of 5-FU and PLAQUES ~~clones~~ were picked from this virus (Experiments 3 and 4).

N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

NTG is an effective mutagen for RNA viruses *in vitro*. It is also a mutagen for DNA viruses *in vitro* and *in vivo* (Fenner, 1974).

In earlier experiments other workers had found that treatment of RS virus with NTG *in vitro*, exposure to 100 μg/ml NTG for 15 minutes, was not an effective means of inducing *ts* mutants (Gharpure *et al.*, 1969). Therefore in this experiment RS virus growth in presence of varying concentrations of NTG (0-10 μg/ml) was examined. The infectivity of RS virus grown in the presence of 10 μg/ml of NTG was reduced by 4 log units (Figure 11).

PLAQUES ~~Clones~~ were picked from RS virus grown in the presence of 0.1, 0.25, 0.5, 2.5 and 10 μg/ml of NTG.

The reduction in titre of RS virus in the presence of NTG was probably due to the effect of NTG on the cells rather than NTG affecting RNA virus replication.

Ethylmethane sulphonate (EMS)

EMS has been used in vitro as a mutagen for both RNA and DNA viruses. It appears to affect the guanine bases in DNA and RNA.

In this experiment the concentration of EMS was constant, only the time of exposure of the virus to EMS varied. RS virus was treated in vitro with EMS (4 ml virus + 0.04 ml EMS) for various lengths of time. When required samples were taken and transferred into vials containing a solution of PBS and 2% thiosulphate which neutralised the EMS in the sample.

When RS virus was treated with EMS for 30 minutes 71% of the virus infectivity was lost due to inactivation (Figure 12). PLAQUES ~~Clones~~ were picked from RS virus treated with EMS for 10, 15 and 20 minutes.

5-azacytidine (5-ACR)

A clone of RS virus was grown FOR 7 DAYS in the presence of 50 µg/ml of 5-ACR at 31°C. PLAQUES ~~Clones~~ were picked from the progeny virus. ONE TS MUTANT (ts 35) WAS ISOLATED FROM THIS CLONE.

Figure 9 : The effect of 5-Flurouracil (5-FU) on RS virus (RSN-2 strain) growth

Temperature : 31°C

—⊖——⊖— The growth of RS virus FOR 6 DAYS in the presence of various concentrations of 5-FU.

MOI : 0.07 PFU/CELL

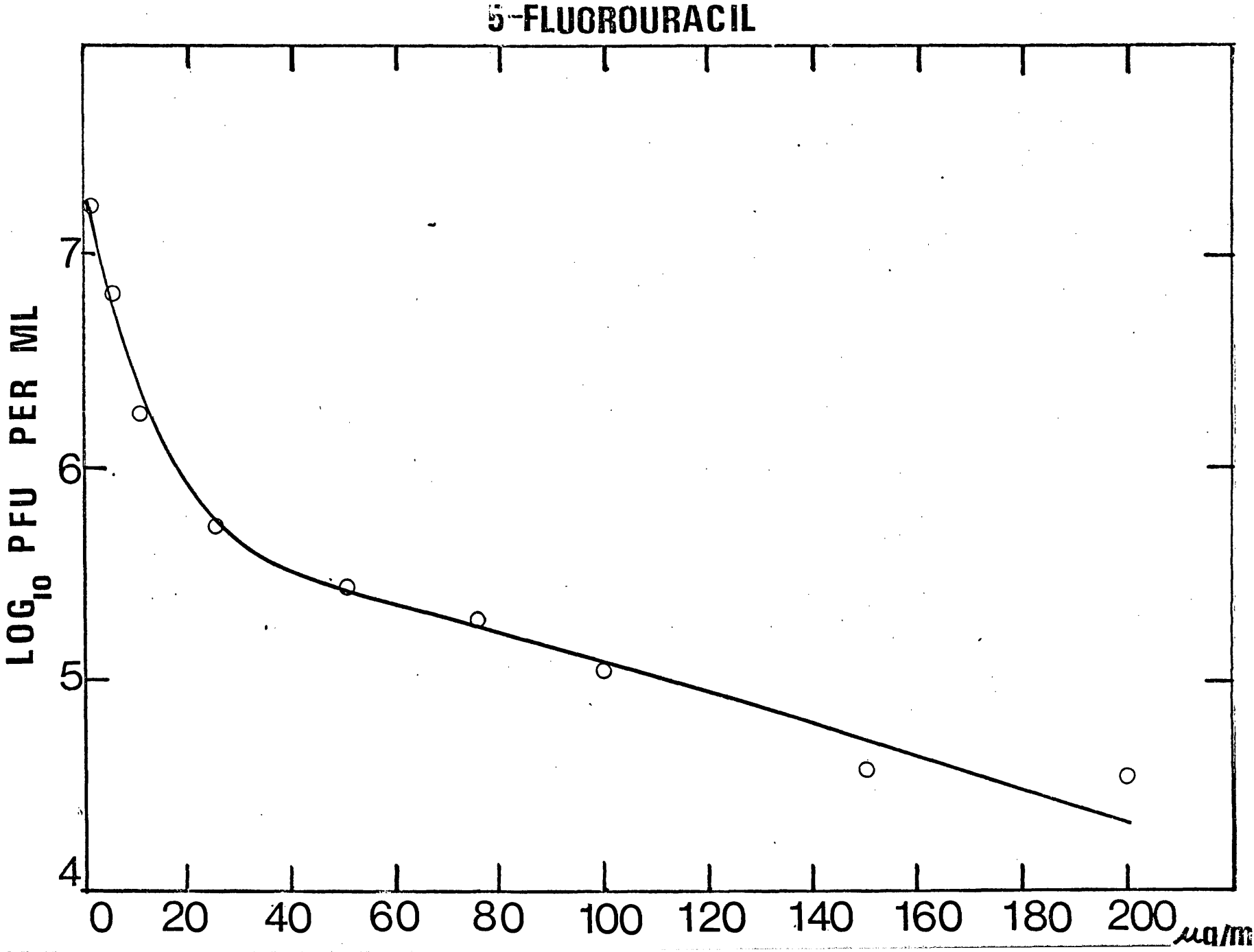

5-FLUOROURACIL
LOG10 PFU PER ML
7
6
5
4
0
20
40
60
80
100
120
140
160
180
200
μg/m

Figure 10 : The effect of 5-Flurouracil on RS virus (RSN-2 strain) growth

Growth of RS virus at 31°C in the presence of 5-FU. FOR 5 DAYS

Growth of RS virus at 39°C in the presence of 5-FU. FOR 5 DAYS

MOI : 0.07 PFU/CELL

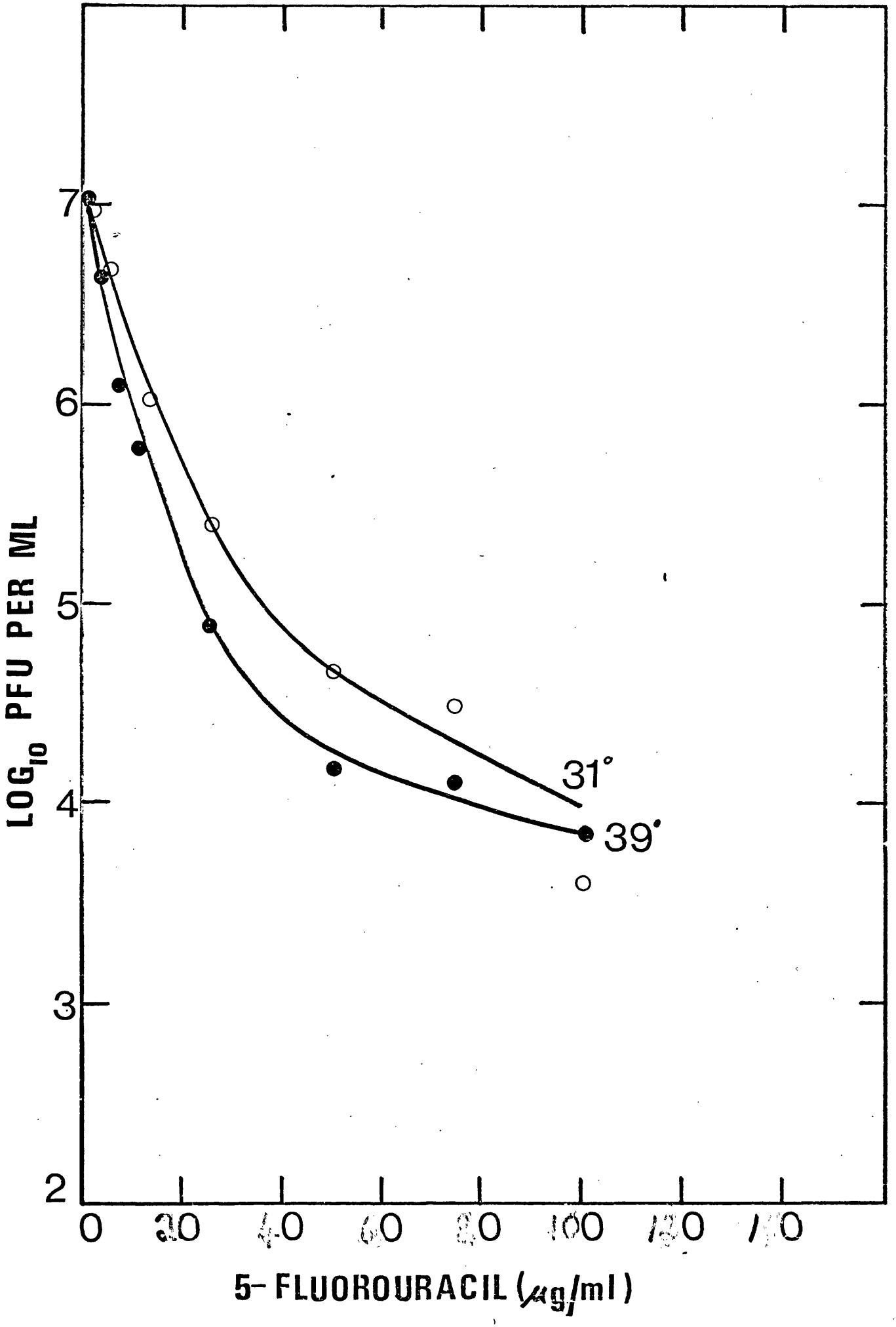

7
6
5
4
3
2
LOG10 PFU PER ML
31°
39°
0
20
40
60
80
100
120
140
5-FLUOROURACIL (μg/ml)

Figure 11 : The effect of NTG on RS virus (RSN-2 strain) growth

Temperature : 31°C

Growth of RS virus for 6 DAYS in the presence of NTG.

MOI : 0.07 PFU/CELL

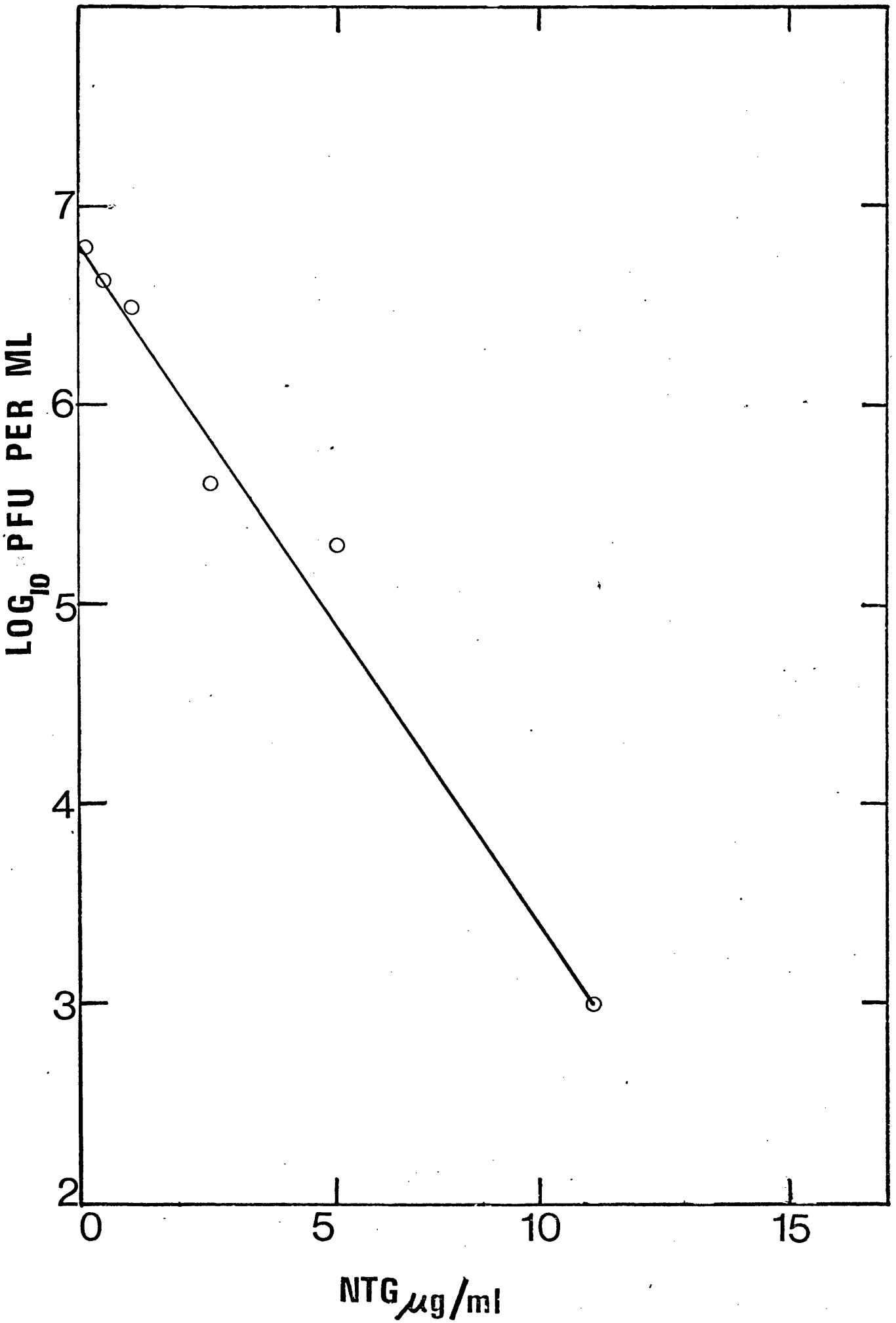
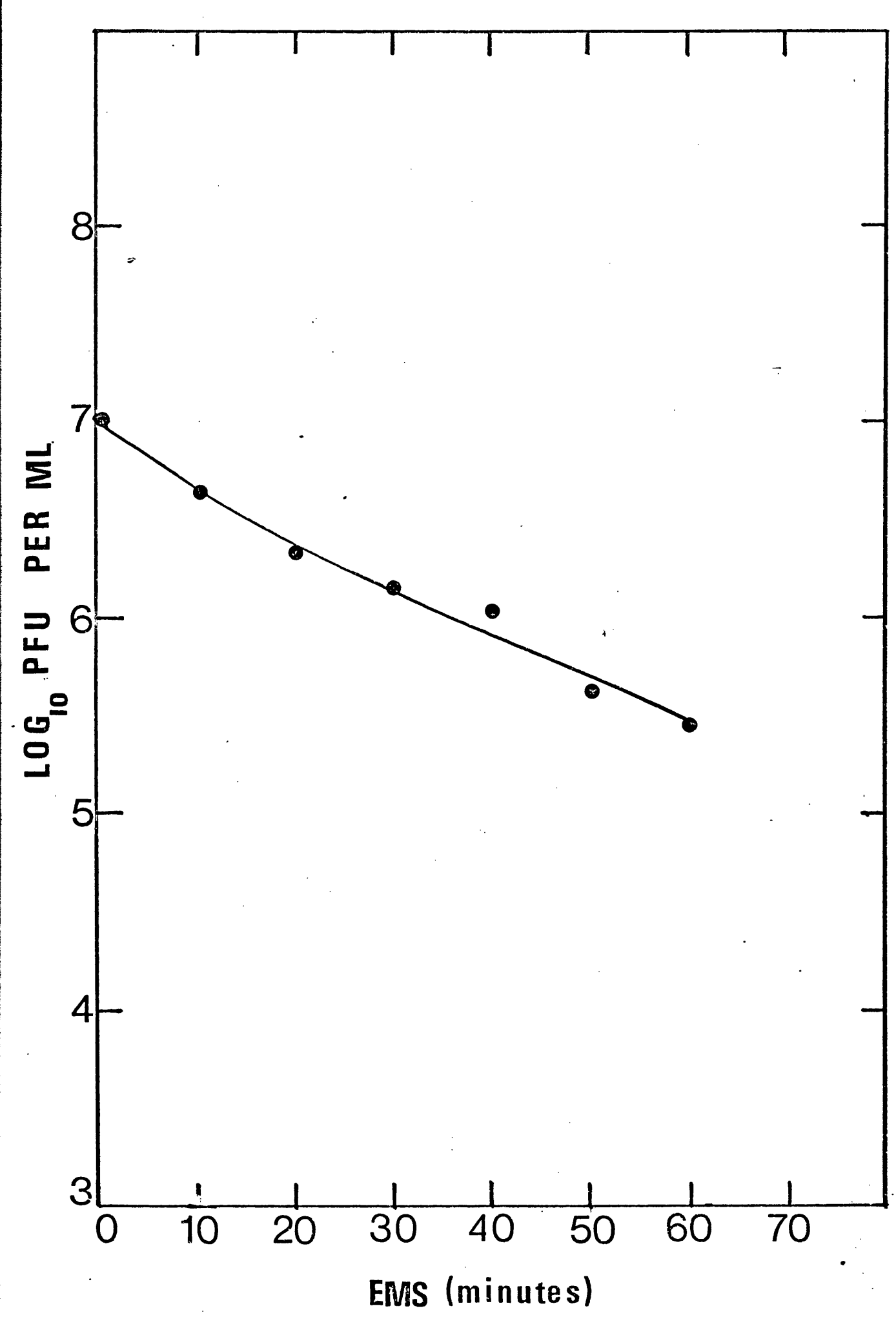

7
6
5
4
3
2
LOG10 PFU PER ML
0
5
10
15
NTG μg/ml

Figure 12 : The effect of EMS treatment on RS virus (RSN-2 strain) infectivity

Temperature : 31°C

RS virus treated with EMS.

EMS (1 IN 100 DILUTION)


8
7
6
5
4
3
LOG10 PFU PER ML
0
10
20
30
40
50
60
70
EMS (minutes)

3.5.2 Isolation of temperature-sensitive (ts) mutants of the RSN-2 strain of RS virus

Mutants were isolated by two procedures. The first was a conventional procedure (method I) of random isolation of clones followed by screening at two temperatures. This procedure had certain disadvantages which necessitated the development of a second improved procedure (method 2).

Method-I

Various dilutions of mutagenised virus were plated on BS-C-1 cells and incubated at 31°C for 6 to 7 days. Then clones were picked from plates containing a few well separated plaques. The clones were picked using Pasteur pipettes which were inserted into the agar overlay above the plaques, removing a plug of agar and infected cells. Then the plug of agar and infected cells was placed into medium (2 ml) in a vial (12 mm). The vials were frozen and thawed before the contents were titrated at 31°C (permissive temperature) and 39°C (non-permissive temperature).

This method was not suitable for the isolation of RS virus ts mutants because of the poor recovery of infectivity per clone (20-200 pfu/clone). About a thousand clones were screened using this method, however no ts mutants were isolated. To reduce the possibility of picking aggregated virus particles clones which had been screened were recloned. However, no ts mutants were isolated from this recloned virus (231 clones). Since freeze-thawing was an important cause of poor recovery of infectivity method 2 was devised to eliminate the necessity of freeze-thawing.

TABLE 15

Ts mutants of RSN-2 strain of RS virus

Mutagen	No. of clones isolated	No. of viable clones	*No. of clones for calculation of frequency	No. of ts mutants	Frequency of ts mutants	χ^2
Experiment 1 5-Fluorouracil (µg/ml)						
10	223	185	118	0	⩽ 0.8	ND
25	237	199	84	1	1.2	1.5
50	227	215	116	1	0.9	0.9
100	264	228	171	0	⩽ 0.6	ND
200	263	240	240	1	0.4	0.1
Experiment 2 5-Flurouracil (µg/ml)						***
50	240	168	115	3	2.6	7.8
100	172	113	100	0	⩽ 1.0	ND
Mutagenised clones 5-Flurouracil (50 µg/ml) Experiment 3						
Clone 11	101	45	45	0	⩽ 2.2	ND
Clone 12	144	128	124	0	⩽ 0.8	ND
Clone 14	87	57	57	0	⩽ 1.7	ND
Experiment 4						
Clone 10	396	305	258	1	0.4	0.1
Clone 15	392	304	299	6	2.0	** 5.3
Clone 38	316	232	138	2	1.5	2.7

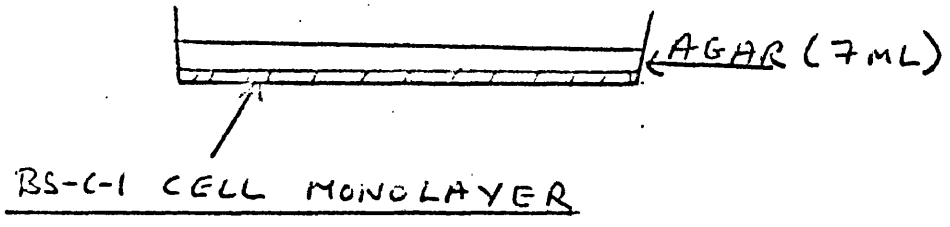
TABLE 15 continued

Mutagen	No. of clones isolated	No. of viable clones	*No. of clones for calculation of frequency	No. of ts mutants	Frequency of ts mutants	χ^2
EMS						
Experiment 1						
10 min	88	88	59	1	1.7	2.5
15 min	148	130	92	0	≤1.1	ND
20 min	79	64	14	0	≤6.7	ND
Experiment 2						
15 min	88	82	82	0	≤1.2	ND
NTG (µg/ml)						
0.1	151	148	99	0	≤1.0	ND
0.25	112	108	101	0	≤1.0	ND
0.50	123	122	122	0	≤0.8	ND
2.5	426	365	328	2	0.6	0.6
10	96	72	68	0	≤1.5	ND
Selected						
50 µg/ml						
5-azacytidine						
39°C						**
10 µg/ml 5-FU	651	564	453	9	2.0	5.5
50 µg/ml 5-FU	348	332	248	5	2.0	** 5.2
50 µg/ml						
5-azacytidine						
at 37°C						
Clone 7	391	313	259	2	0.8	1.0

A plaque of virus was picked using a Pasteur pipette from a plate containing a few well separated RS virus plaques.

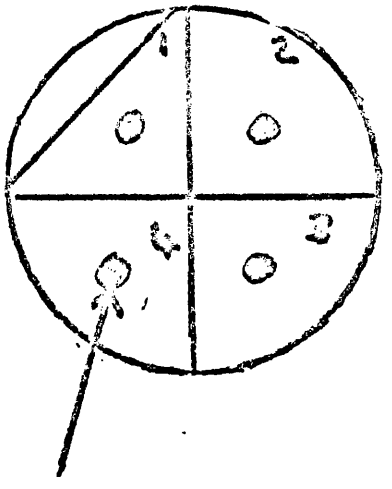
The Pasteur pipette c
was used to stab thro
agar (2 ml) covering
BSC-1 cell monolayer.
divided into 4 segment
the bottom of the plat
The same area in a d
also stabbed and scra
pipette.

3.



31°C

4.



The virus was allowed to adsorb for 1 hour at 31°C and then the plates were overlaid with agar (5 ml).

One of the plates was incubated at 31°C and the other plate was incubated at 39°C.

After 5 days incubatio
plates were fixed and
ments with and withou
noted. After 6-7 days
plates at 31°C were s
picked from plaques i
at 39°C had no viral

The clone was transfe
containing a BS-C-1 ce
grown at 31°C. Then
was titrated at 31°C a

before the plates were overlaid with more agar (5 ml). One of duplicate plates was incubated at 39°C for 5 days and the other for 7 days. The 39°C plates were fixed, stained and scored, t were picked from the sectors at 31°C whose duplicate sectors at showed no plaque formation. These clones were transferred to of BS-C-1 cells in bottles (1 oz) and the virus was allowed to before maintenance medium (5 ml) was added.

The infected cells were incubated at 31°C until CPE w 100%, then they were harvested by freeze-thawing or scraping. (infected cells + medium) was titrated at 31°C and 39°C to dete whether the virus was temperature sensitive.

This method represents a considerable improvement on The recovery of virus from clones was much better and ts mutant isolated. However, method-2 still had some disadvantages, abou (23%) of the clones picked had no detectable virus and most of (96%) which appeared to be ts in the first screening on growth further screening proved to be wild type rather than ts.

A total of 35 ts mutants of RS virus were isolated fr treated with different mutagens and one ts mutant, ts 24, was i from non-mutagenised virus (Table 15). Fifteen ts mutants (ts

TABLE 15 continued

Mutagen	No. of clones isolated	No. of viable clones	*No. of clones for calculation of frequency	No. of ts mutants	Frequency of ts mutants	χ^2
5-azacytidine (50 µg/ml) Clone 4	396	121	85	1	1.2	1.4
No mutagen	700	482	400	1	0.3	/
Total	6,859	5,313		36		

* The no. of viable clones is adjusted to take account of the number of clones lost due to contamination during screening.

**Significant value for χ^2 at 5% level (1 degree of freedom).

***Significant value for χ^2 at 1% level (1 degree of freedom).

ND - not done.

ts 15 - 17, ts 27 - 34, ts 36) were isolated from virus grown in various concentrations of 5-FU (25, 50, 200 μg/ml). The overall frequency of isolation of ts mutants was low (1.3%). However, the frequency of isolation of ts mutants from virus grown in the presence of 50 μg/ml of 5-FU in Experiment 1 and 4 (clone 15) was higher. There was no correlation between concentration of 5-FU and the frequency of isolation of ts mutants.

Two ts mutants (ts 19, ts 20) were isolated from virus grown in the presence of NTG (2.5 μg/ml). The frequency of isolation of ts mutants from virus grown in the presence of NTG was no higher than that for virus grown in the presence of 5-FU. There was also no correlation between concentration of mutagen and the frequency of isolation of ts mutants.

A ts mutant (ts 18) was isolated from virus treated with EMS for 10 minutes. There was no correlation between time of treatment and the frequency of isolation of ts mutants.

The frequency of isolation of the spontaneous ts mutant was low (0.25%). However there was no significant difference between this frequency and the frequency of isolation of ts mutants from most of the mutagen treatments as can be seen from the χ^2 values (Table 15). The values were calculated by comparing the number of clones screened to isolate ts mutants in a particular treatment with the number of plaques screened to isolate the single spontaneous ts mutant.

Since most of the frequencies of isolation of ts mutants from

different treatments are not significant and there is no correlation between frequency of isolation and concentration or time of mutagen treatment, it is possible that most of the ts mutants isolated are spontaneous rather than mutagen induced.

3.5.3 Selection procedure for early ts mutants of RSN-2 strain of RS virus

An attempt was made to isolate early ts mutants and also to increase the frequency of isolation of ts mutants using the following selection procedure.

Virus previously treated with 5-FU (10 or 50 µg/ml) was inoculated onto BS-C-1 cell monolayers in bottles (1 oz) and allowed to adsorb for 1 hr at 31°C. Then the cell sheets were washed twice and a final volume of 5 ml of maintenance medium added. The infected cells were incubated at 39°C for 5 hours. Then the maintenance medium was replaced with medium containing 50 µg 5-Azacytidine (5-ACR)/ml and the bottles retained at 39°C for 18 hours.

5-ACR when incorporated into the virus genome inactivates it. It was assumed that early ts mutants would not replicate at 39°C and therefore 5-ACR would only be incorporated into the RNA of wild type virus and late function ts mutants with lethal effect. After 18 hours incubation of the bottles at 39°C the medium containing 5-ACR was removed and the monolayers were washed twice and covered with maintenance medium containing no 5-ACR. The bottles were transferred to 31°C and incubated until the CPE was nearly 100%. The samples (infected cells + medium) were harvested by scraping and titrated at 31°C. Since 5-ACR inactivated

the genome of wild type virus and late function ts mutants a greater percentage than usual of the virus grown at 31°C was expected to be ts in an early function. Clones were picked from this virus and screened using the sector-plating method (method-2).

Fourteen ts mutants (ts 4-14, ts 21-23) were obtained using this procedure. The frequency of isolation of the selected ts mutants was 2% which was higher than the overall frequency of isolation of unselected mutants.

A modification of this selection procedure was also used. A clone of wild type RS virus was grown at 37°C for 5 hours and then 5-ACR was added to the maintenance medium. The virus was grown in the presence of 5-ACR for 18 hours and then the medium containing 5-ACR was removed. Following this the cell sheet was washed twice with medium containing no 5-ACR and a final volume of 5 ml added. The infected cells were transferred to 31°C until the CPE was nearly 100% and then they were harvested by scraping. The virus sample (infected cells + medium) was titrated at 31°C and clones picked from this virus. It was hoped that using this method early function ts mutants with a lower restrictive temperature (37°C) would be isolated. Two ts mutants (ts 20, ts 26) were obtained using this procedure. However the growth of these mutants was not restricted at 37°C. The frequency of isolation of these ts mutants was lower than that for the other selected ts mutants.

3.6 Growth and efficiency of plating of RS virus ts mutants

The growth and plating efficiency of RS virus ts mutants at 31°C, 37°C and 39°C was examined (Table 16). Some of the ts mutants grow at 39°C but do not plate at that temperature while a few ts mutants revert on passage at the permissive temperature (31°C). Most of the ts mutants grow and plate at 37°C; however some ts mutants (ts 5, ts 15, ts 16, ts 18) although they grow at that temperature do not form plaques. A few ts mutants (ts 2, ts 6, ts 19) seem to be ts at 37°C as well as 39°C, since they do not grow or form plaques at both temperatures.

In the growth experiment at the three different temperatures (31°C, 37°C, 39°C) a few of the ts mutants had low yields. This was due to low moi and in some cases perhaps to individual mutants having longer growth cycles than wild type virus.

Work using RS virus has three main problems, the low titres of virus, thermolability and the sensitivity of RS virus to freeze-thawing. These problems are equally applicable to work with RS virus ts mutants. RS virus must be stored at -70°C or below since infectivity is rapidly lost at higher temperatures. At -20°C 65% of RS virus infectivity was lost in 10 days. Virus stock could not be freeze-thawed more than twice, since a high proportion of virus infectivity was lost at each freeze-thawing. Some of the ts mutants are more sensitive to freeze-thawing than wild type virus.

TABLE 16

ts mutants code	Grown at 31, 37, 39°C Assayed at 31°C Titre (pfu/ml)			Grown at 31°C Assayed at 31, 36, 39°C Titre (pfu/ml)		
	31	37	39	31	36	39
1	3.4×10^4	4.2×10^3	3	7.0×10^6	2.7×10^6	0
2	8.0×10^5	5.0×10^1	5.0×10^1	3.5×10^6	7.5×10^5	0
3	2.5×10^5	2.4×10^3	$^+0$	2.1×10^6	2.0×10^6	3.5×10^1
4	3.5×10^5	1.0×10^3	$^+0$	1.4×10^5	4.2×10^4	5.0
5	1.5×10^5	4.7×10^3	1.0×10^1	1.6×10^7	0	0
6	1.6×10^6	1.5×10^1	5.0×10^1	4.8×10^6	0	0
7	3.9×10^5	9.0×10^3	5.0×10^1	3.3×10^6	1.7×10^6	0
8	1.5×10^5	9.0×10^3	4.3×10^3	2.2×10^6	1.2×10^6	0
9	1.5×10^5	1.0×10^4	5.0×10^1	1.5×10^5	9.5×10^4	0
10	ND	ND	ND	5.4×10^5	5.5×10^5	0
11	5.5×10^4	2.0×10^3	3.0×10^3	4.0×10^5	1.1×10^5	0
12	1.7×10^4	1.9×10^3	0	3.0×10^5	5.0×10^4	0
13	8.0×10^5	1.3×10^4	1.3×10^3	3.5×10^5	ND	0
14	7.5×10^4	3.2×10^4	2.1×10^2	3.0×10^5	ND	0
15	7.0×10^5	2.5×10^3	0	1.0×10^8	$^+0$	0
16	1.5×10^6	5.0×10^4	0	8.5×10^6	0	0
17	3.8×10^5	8.0×10^3	5.0	7.5×10^6	4.0×10^1	5.0
18	2.2×10^6	1.8×10^4	0	5.9×10^7	0	0
19	1.7×10^4	1.0×10^1	0	7.0×10^7	1.5×10^2	5.0
20	ND	ND	ND	1.5×10^5	ND	0
21	2.2×10^3	8.5×10^2	1.0×10^1	1.0×10^6	9.0×10^5	0
22	2.0×10^3	1.0×10^1	4.0×10^1	3.0×10^6	2.0×10^6	0
23	1.5×10^5	2.1×10^5	7.5×10^1	4.0×10^8	4.0×10^7	0
24	1.7×10^3	9.0×10^2	1.0×10^1	4.0×10^5	ND	0
25	4.4×10^4	2.2×10^4	2.5×10^1	1.1×10^6	ND	0
26	5.5×10^4	1.1×10^5	0	1.1×10^6	ND	0
27	3.0×10^3	1.7×10^2	0	1.0×10^5	ND	0
28	3.5×10^4	1.5×10^3	0	4.4×10^6	ND	5.0
29	1.7×10^5	1.4×10^4	2.7×10^2	2.6×10^6	ND	5.0

TABLE 16 continued

ts mutants code	Grown at 31, 37, 39°C Assayed at 31°C Titre (pfu/ml)			Grown at 31°C Assayed at 31, 36, 39°C Titre (pfu/ml)		
	31	37	39	31	36	39
30	ND	ND	ND	3.1×10^4	ND	0
31	1.3×10^4	1.3×10^2	0	1.5×10^5	ND	1.0×10^1
32	1.0×10^5	2.5×10^5	1.0×10^3	1.1×10^6	ND	2.0×10^1
33	4.5×10^2	3.9×10^2	0	1.2×10^6	ND	2.0×10^1
34	ND	ND	ND	4×10^4	ND	0
35	5.0×10^5	4.3×10^4	5.0×10^3	1.5×10^6	ND	1.5×10^1
36	6.5×10^2	8.0×10^1	0	3.0×10^4	ND	0
wild type virus	3.7×10^3	4.0×10^3	1.2×10^3	2.4×10^5	2.3×10^5	2.2×10^5

+ indicates cell change but has no plaque formation

3.7 Genetic studies using RS virus ts mutants

3.7.1 Complementation experiments using ts mutants of the RSN-2 strain of RS virus

Ts mutants of the RSN-2 strain were examined using the yield complementation test in an attempt to identify mutants affecting different genome functions.

Unless otherwise stated BS-C-1 cells were used in the yield complementation experiments. The complementation indices for each experiment were calculated using the highest titre of each single infection in that experiment. Complementation indices above the value of 10, an arbitrary figure, were considered to indicate complementation. A difficulty in work with RS virus is the low titres of virus obtained. Therefore, in order to obtain reasonable multiplicities of infection small numbers of cells had to be used.

Linbro wells (6 mm) were used in complementation experiments because fewer cells were required (1×10^5 cells/well) compared with other methods and consequently high ratios of pfu to cells could be obtained. The recovery of virus from Linbro wells was good but there was marked variation in yield between duplicate crosses in the same and different blocks. Yield complementation experiments using Linbro wells gave indications of complementation (Table 17, 18). However, since variation occurred between duplicate mixed and single infections it was difficult to interpret these results. Blocks of Linbro wells were incubated at 39°C in a CO_2-gassed incubator. In an attempt to have more accurate temperature control complementation methods 1, 3 and 4 using an

TABLE 17

Complementation using BS-C-1 cell monolayers in Linbro wells. Ts mutant infected cells incubated at 39°C for 7 days

Ts mutants	1	2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	(1×10^{1})	0.1	<0.1	[>62]	[11]	0.8	0.4	0.4	0.8	1.3	<0.1	>0.7	0.2	<0.3	<0.3	<0.3	<0.3	<0.3	[15]
2		(9×10^{2})	0.2	0.3	0.6	0.7	0.7	0.9	0.5	0.5	0.1	0.3	<0.1	1.3	1.7	<0.1	<0.1	1.1	<0.1
4			(4.5×10^{1})	<0.1	[10]	0.4	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	1.4	0.1
5				(<5×10^{0})	<0.1	1	1.3	1.2	[15]	1.3	5	[>45]	0.9	> 2	[>14]	[>20]	<0.1	0.3	<0.2
6					(6×10^{1})	0.4	0.5	0.6	1	1.3	2.5	[>20]	1.7	[>17]	0.2	>9.7	8	[17]	6.5
7						(2.5×10^{3})	<0.1	0.4	0.4	0.6	0.6	0.8	0.2	<0.1	0.4	<0.1	<0.1	0.3	1
8							(1×10^{3})	0.3	2	0.4	<0.1	1.2	0.6	0.6	0.7	0.9	1	0.9	0.8
9								(6.8×10^{2})	3	1	0.8	0.9	<0.1	1.2	1.6	1.8	2	0.7	0.6
10									(1.7×10^{2})	1.1	3.7	8	0.7	5.2	[19.5]	9.9	4	6.5	8.8
11										(2×10^{3})	0.8	1.3	0.7	1.3	<0.1	1.3	0.5	0.5	0.5
12											(5×10^{2})	5	<0.1	1.5	5	<0.1	<0.1	<0.1	<0.1
13												(<5×10^{0})	0.8	.	.	> 4	>0.2	.	> 1
14													(1.3×10^{3})	<0.1	0.3	0.8	0.3	0.3	0.2
15														(<5×10^{0})	.	.	.	.	.
16															(<5×10^{0})	.	.	.	.
17																(<5×10^{0})	.	.	.
18																	(7.5×10^{1})	.	.
19																		(<5×10^{0})	.
20																			(<5×10^{0})

( ) single-infection titre (pfu/ml)

. Complementation index cannot be calculated

TABLE 18

Complementation using monolayers of BS-C-1 cells. Ts mutant infected cells incubated at 39°C for 4 days

Ts mutants	4	7	8	9	10	11	12	15	16	17	18	19	20	21	23
	(5×10^{1})	(<5×10^{0})	(5.9×10^{3})	(9.7×10^{3})	(1.1×10^{4})	4.3×10^{3})	(1.1×10^{3})	(<5×10^{0})	(<5×10^{0})	(2.5×10^{1})	(5×10^{1})	(9×10^{2})	(2.3×10^{2})	(5.9×10^{3})	(1.2×1
1 (5×10^{1})	0.3	>138	0.9	0.9	0.4	0.4	0.5	>4.5	<0.1	5	0.3	<0.1	0.4	1.8	2.3
2 (1.3×10^{4})	0.3	0.7	0.5	0.5	0.2	0.6	0.5	>0.6	>0.2	0.5	0.2	1.3	0.2	0.7	0.3
5 (2.5×10^{1})	0.7	>333	1.1	0.7	0.5	1.7	<0.1	<0.1	>2.5	10.6	<0.1	0.6	0.3	2.1	0.8

( ) Single infection titre (pfu/ml)

accurate 39°C waterbath were used (section 2.7.1).

Complementation using vials, 12 mm in diameter, (method 3) had greater temperature control than that using Linbro wells (method 2) but viral recovery was poor. Only one mixed infection (ts 5 and ts 9) had a reasonable complementation index (i.e. above 10) (Table 19a).

The complementation experiments using bottles (method 1) suffered from the disadvantage of low moi due to the high number of cells used (1×10^6 cells/bottle). Some mixed infections which had high complementation indices in an earlier experiment (Table 17) now had lower complementation indices e.g. ts 4 and ts 6, ts 6 and ts 17. Experiments using three different methods (plates, vials and Linbro wells) were simultaneously performed. The plate method (method 4) seemed to be the best as infected cells could be removed efficiently by scraping with a piece of silicone whereas it was more difficult to harvest cells from vials. Also variation did not occur between duplicate mixed and single infections using this method unlike that using Linbro wells.

All of the complementation experiments irrespective of method used, were hampered by the leak yield of some of the ts mutants. This leak varied between experiments. Whether a mixed infection appeared to have a high complementation index (CI), depended often on the degree of leak of the ts mutants involved. For example, the ~~cross~~ MIXED INFECTION between ts 1 and ts 7 (Table 18) had a high complementation index value because in this experiment ts 7 had a lower leak value than in the other experiment using Linbro wells (Table 17). In the complementation experiment using vials, the complementation index between ts 5 and ts 9 was above 10 since the leak of ts 9 was lower in this experiment (Table 19a) than in previous

TABLE 19a

Complementation using BS-C-1 cell monolayers in vials (12 mm in diameter). Ts mutant infected cells incubated in a waterbath at 39°C for 72 hours

Ts mutants	1	2	3	4	5	6	9	13	15	17	18	2
	(2.5×10^{1})	(1.7×10^{2})	(1.5×10^{0})	($<5\times10^{0}$)	($<5\times10^{0}$)	(1×10^{1})	(1×10^{1})	(8×10^{1})	($<5\times10^{0}$)	($<5\times10^{0}$)	($<5\times10^{0}$)	(<5
1		0.6	<0.1	<0.1	<0.1	0.2	3.4	4.6	< 0.1	<0.1	0.2	<0
2			<0.1	<0.1	<0.1	0.1	1.8	0.9	/	/	/	
3				<0.1	<0.1	/	/	/	/	/	/	
4					.	/	/	/	/	/	/	
5						0.1	23	1.5	/	/	/	

TABLE 19b

Complementation using BS-C-1 cell monolayers in bottles (1 oz). Ts mutant infected cells incubated at 39°C for 48 hours

Ts mutants	4	6	17	25
4	($<5\times10^{0}$)	2	>0.5	>0.5
6		(5×10^{0})	>0.5	>5
17			(5×10^{0})	.
25				($<5\times10^{0}$)

( ) Self-infection titre (pfu/ml)

. Complementation index cannot be calculated. / Mixed infections not done.

experiments using Linbro wells (Tables 17, 18). The mixed infection between ts 1 and ts 20 and ts 1 and ts 6 in the same experiment (Table 19a) had low complementation indices which were contrary to the results in previous experiments (Table 17, 18).

Higher titres of virus had to be used in complementation experiments using plates, in order to obtain higher multiplicities of infection without altering the number of cells. The titre of the ts mutants used was increased by concentrating large volumes of stock (100 ml) using the PEG precipitation method (section 2.4.2). Although the moi was increased in these experiments (Table 20) there was no increase in the value of the complementation indices. Therefore lack of complementation in BS-C-1 cells did not seem to be due to the moi of infection of the ts mutants being too low. It is possible that the ts mutants tested were affected in the same genome function, which would result in them being unable to complement and the positive value obtained in these experiments being spurious. Another possibility is that the cell system used did not allow the detection of complementation. Complementation might be masked by the high leak of some ts mutants in these cells.

TABLE 20

Complementation using BS-C-1 cell monolayers on Petri dishes (35 mm). Ts mutant infected cells incubated in a waterbath at 39°C for 72 hours

Ts mutants	1	2	3	5	15	16	17	18	19	23
1	(1.7×10^3)	/	/	1.5	/	/	/	/	0.3	/
2		(3×10^5)	0.5	1.1	0.6	/	/	/	/	/
3			(2.3×10^5)	<0.1	0.1	/	/	/	/	/
5				(1.1×10^3)	<0.1	/	0.8	<0.1	2	<0.1
15					(2.8×10^2)	0.8	0.3	1	0.5	<0.1
16						(2.8×10^2)	0.3	<0.1	3.1	<0.1
17							(4.1×10^3)	1.7	<0.1	<0.1
18								(1.3×10^4)	<0.1	<0.1
19									($<5\times10^0$)	<0.1
23										(7.5×10^4)

( ) Single infection titre (pfu/ml)

/ Mixed infection not done.

3.7.2 Complementation between ts mutants of the A_2 and the RSN-2 strains of RS virus

To test whether lack of complementation between ts mutants of of RSN-2 strain was due to the mutants or to the cell system used, mutants of A_2 strain which complement in HeLa cells (Wright et al., 1973) were compared with these ts mutants in BS-C-1 cells. Before being used in complementation experiments they were passaged twice in BS-C-1 cells and their titres increased by concentration using the PEG precipitation method.

In the complementation experiment in BS-C-1 cells (Table 21a) using the ts mutants of the A2 and the RSN-2 strains of RS virus no complementation indices above 10 were obtained. Complementation indices above 1 were obtained in three mixed infections, ts A7 and ts 23, ts A7 and ts 15 and ts 19 and ts 15. These ts mutants had the same moi, approximately 3 pfu/cell, which was higher than those of the other ts mutants used. No complementation occurred between ts A1, ts A2, ts A7. This could be due to the cell system used (BS-C-1 cells) or to the low moi of ts A2 (0.14 pfu/cell) and ts A1 (1.4 pfu/cell) compared with ts A7 (3.3 pfu/cell).

To test whether the lack of complementation between the ts mutants was related to the cell system used, complementation experiments using the same ts mutants (ts A1, ts A2, ts A7, ts 15, ts 19, ts 23) were performed in HEp-2 (Table 21b) and HeLa cells (Table 21c). The moi of ts mutants in these cells was lower with the exception of ts A2 than those used in BS-C-1 cell experiment (Table 21a). Only one mixed infection in

TABLE 21

Complementation using different cell monolayers in Petri dishes (35 mm), Ts mutant infected cells incubated in a waterbath at 39°C for 48 hours

Table 21a

BS-C-1 cells used in complementation experiment

moi	1.40	0.14	3.30	3.20	1.50	3.30
Ts mutant	A1	A2	A7	15	19	23
A1	(3.4×10^{3})	<0.1	0.9	< 0.1	< 0.1	< 0.2
A2		(6×10^{4})	0.4	< 0.1	< 0.1	< 0.1
A7			(1.5×10^{4})	2.2	0.3	4.5
15				(7.5×10^{1})	< 0.1	2.0
19					(< 5×10^{0})	0.5
23						(3.4×10^{3})

Table 21b

HEp-2 cells used in complementation

moi	0.20	0.49	3.10	0.12	0.13	0.17
Ts mutant	A1	A2	A7	15	19	23
A1	(2.1×10^{3})	2.0	9.6	0.3	0.7	< 0.1
A2		(4.3×10^{4})	0.7	3.0	0.5	0.3
A7			(9×10^{4})	1.2	0.2	< 0.1
15				(2.4×10^{2})	[130]	< 0.1
19					(2.2×10^{2})	< 0.1
23						(4.5×10^{5})

Table 21c

HeLa cells used in complementation experiment

moi	0.19	0.46	2.90	0.11	0.12	0.16
Ts mutant	A1	A2	A7	15	19	23
A1	(1.5×10^{1})	4.3	0.2	0.3	0.9	0.8
A2		(1.5×10^{1})	[27]	<0.1	0.5	6.3
A7			(3.9×10^{2})	0.7	0.4	2.7
15				(1.6×10^{2})	[21]	[43]
19					(6×10^{1})	[63]
23						(1.5×10^{2})

( ) single infection titre (pfu/ml). moi = multiplicity of infection

HEp-2 cells had a complementation index above 10 (i.e. ts 15 and ts 19). However, the mixed infection between ts A1 and ts A2 had a CI of 2 and between ts A1 and ts A7 had a CI of 9.6. The CI of the mixed infection between ts A2 and ts A7 was low (0.7).

Mixed infections of ts mutants at the same moi in HeLa and HEp-2 cells had higher complementation indices in HeLa cells. Complementation indices above 10 in HeLa cells could be detected for mixed infections between ts A2 and ts A7, ts 15 and ts 19, ts 15 and ts 23 and ts 19 and ts 23. The CI of the mixed infection between ts A1 and ts A2 was 4.3 and that between ts A1 and ts A7 was 0.2 which was very low.

Complementation should have occurred between ts A1, ts A2 and ts A7 in HeLa cells, the variation in complementation indices between these mixed infections is perhaps due to the low moi of ts A1 (0.19 pfu/cell) and ts A2 (0.46 pfu/cell) compared with that of ts A7 (2.9 pfu/cell). In earlier experiments performed in HeLa cells by other workers complementation occurred between these ts mutants (Wright et al., 1973) each ts mutant had a moi of 5. Multiplicities of 5 pfu/cell could not be obtained with the number of cells used and the stock available in this experiment.

The difference in complementation indices obtained using the different cell systems could be due to the higher leak of ts mutants on HEp-2 and BS-C-1 cells than on HeLa cells (~~Figure~~ TABLE 21c). However, this is not true for all combinations, the leak of ts 15 and ts 19 was no higher in BS-C-1 cells than in HEp-2 or HeLa cells.

Complementation was detected in HeLa and HEp-2 cells and not in BS-C-1 cells even although moi was higher in BS-C-1 cells. Therefore

there could be some type of interference or suppression of complementation in BS-C-1 cells. HeLa cells seem to be the best cell system for complementation experiments as the leak in these cells was generally lower than in BS-C-1 or HEp-2 cells (Figure 13). However, it will be necessary to confirm these findings by future work.

The yield from two complementation mixed infections, one with a CI above 10, was examined (Table 22). All the viable clones recovered from both yields of virus were ts as expected if the increased yield was due to complementation. There was no indication of reversion or recombination.

3.7.3 Test for recombination between ts mutants of RS virus

A recombination experiment was performed using BS-C-1 cell monolayers on plates (35 mm) and PEG concentrated virus as described in section 2.4.2. The percentage of ts^+ virus recovered from the crosses was less than 0.05% (Table 23). However the percentage of ts^+ virus from the cross ts 1 and ts 5 was about 5 times greater than the sum of the self-crosses, suggesting low frequency recombination. A similar phenomenon was observed by Wright and his colleagues (1973), which on analysis of the progeny proved to be due to complementing heterozygotes.

Figure 13 : Complementation and leak in relationship to host cells

x and y are individual ts mutants of RS virus.

ts mutants grown in HeLa cells.

ts mutants grown in HEp-2 cells.

ts mutants grown in BS-C-1 cells.

Infectivity is measured as $\log_{10}$/ml.

TEMPERATURE : 39°C

COMPLEMENTATION INEX (VERTICAL AXIS) PLOTTED AGAINST SUM OF LEAK YIELD (HORIZONTAL AXIS)

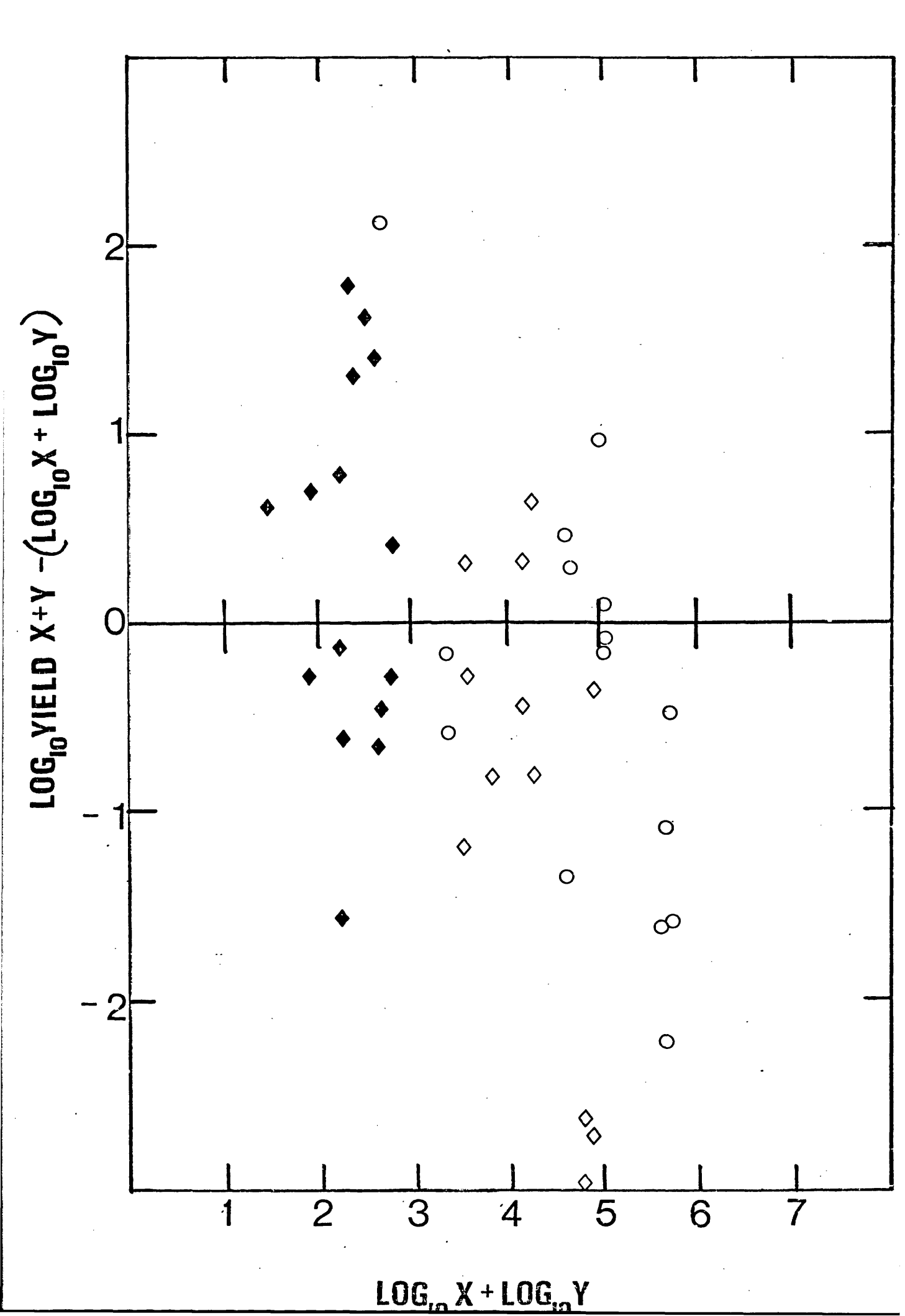

LOG10YIELD X+Y -(LOG10X + LOG10Y)
2
1
0
-1
-2
1
2
3
4
5
6
7
LOG10 X + LOG10Y

TABLE 22

Analysis of complementation yield

The complementation indices of the mixed infections examined are given in Table 19a

Mixed Infection	No. of clones isolated	No. of viable clones	No. of clones which grew at		% ts
			31°C	39°C	
ts 2 x ts 9	40	34	34	0	100
ts 5 x ts 9	40	27	27	0	100

Cross ts 2 x ts 9

Frequency of ts^+ clones $\leq$ 2.9%

Cross ts 5 x ts 9

Frequency of ts^+ clones $\leq$ 3.3%

TABLE 23

Recombination Experiment

CROSS of *ts* mutants	Titre (pfu/ml) 31°C	Titre (pfu/ml) 39°C	Percentage of ts^+ virus
1 x 0	5.9×10^5	5.0×10^1	0.008
5 x 0	3.3×10^6	2.5×10^0	< 0.001
19 x 0	6.5×10^5	1.5×10^1	0.002
1 x 5	6.1×10^6	2.7×10^3	0.044
1 x 19	2.7×10^6	2.8×10^1	< 0.001
5 x 19	2.2×10^6	0	0.001

3.8 Grouping of ts mutants of RSN-2 strain by immunofluorescence pattern

Since the ts mutants could not be grouped using genetic analysis other methods of classification had to be used. The ts mutants were grouped on the basis of their immunofluorescence pattern at 39°C (Table 24a, 24b). This work was a joint project with Dr. P. Shirodaria, Department of Microbiology and Immunobiology, The Queen's University of Belfast.

On the basis of number of fluorescing cells and amount of antigen present at 39°C as compared to 31°C, the ts mutants studied can be divided into 3 groups.

Group I

Mutants in this group show a marked reduction in the number of fluorescing cells at 39°C as compared to 31°C (Plates 18, 19). The amount of antigen in cells at 39°C is also reduced. Group I can be subdivided: I(a) Fluorescing cells which have no processes at 39°C; I(b) Fluorescing cells which have processes at 39°C.

Sixteen ts mutants are classified in Group I (Table 24a).

Group II

Mutants in this group have a similar pattern of fluorescence at 39°C to that at 31°C and resemble wild type virus (Plates 20-23). Inclusions and processes were present at both temperatures. The number of fluorescing cells at 39°C was only slightly reduced (Table 24a) and there was no reduction in the amount of antigen present. Fifteen ts mutants belong to this group.

Group III

Mutants in this group have a marked reduction in the amount of antigen present at 39°C but only a slight reduction in the number of fluorescing cells (Plates 24,25).No processes are detected at 39°C but inclusions are present. Three ts mutants belong to this group. (Table 24a)

Nuclear antigen can be detected in cells infected with many of the ts mutants. This antigen is only seen when bovine anti-bovine RS virus serum and not human RS virus convalescent serum is used to stain the infected cells. This is probably a consequence of the high titre of antibody in convalescent calf serum. Nuclear antigen had not previously been detected in RS virus infected cells. (Berthiaume et al., 1974; Kisch et al., 1962; Levine et al., 1971; Nagahama et al., 1970 and Shedden and Emery, 1965).

Table 24a

Ts mutants grouped according to immunofluorescence pattern at 39°C

Group I		Group II	Group III
a	b		
ts 4	ts 12	ts 1	ts 3
ts 5	ts 17	ts 2	ts 15
ts 16	ts 21	ts 6	ts 33
ts 18	ts 22	ts 7	
ts 19	ts 27	ts 8	
ts 25	ts 28	ts 9	
ts 31	ts 29	ts 10	
ts 35	ts 36	ts 11	
		ts 13	
		ts 14	
		ts 23	
		ts 24	
		ts 26	
		ts 30	
		ts 32	
Total No. of ts Mutants: 8	8	15	3

TABLE 24b

Quantitative immunofluorescence data supplied by Dr. P. Shirodaria, Department of Microbiology and Immunobiology, The Queen's University of Belfast.

GROUP	ts mutant	Number of fluorescing cells	
		31°C	39°C
Ia	ts 4	104 cells/43 fields	4 cells/coverslip
	ts 18	> 30 cells/field	67 cells/40 fields
	ts 19	380 cells/17 fields	8 cells/17 fields
	ts 35	40 cells/field	1 cell/17 fields
Ib	ts 36	163 cells/17 fields	3 cells/coverslip
II	ts 6	15 cells/65 fields	4 cells/29 fields
	ts 14	103 cells/8 fields	80 cells/8 fields
III	ts 3	> 30 cells/field	slight reduction

Plates 18 and 19 : BS-C-1 cells infected with a Group I ts mutant (ts 18)

The ts mutant infected cells were stained with bovine anti-bovine RS virus serum.

Plate 18 shows the Group I ts mutant infected BS-C-1 cells at 31°C.

Plate 19 shows the Group I ts mutant infected BS-C-1 cells at 39°C.

The magnification of these photographs was 480 X.

Plate 18

A large number of processes can be seen.

Plate 19

RS virus antigen can be seen in the nucleus. No viral processes were detected and there was a marked reduction in the number of fluorescing cells and amount of antigen detected.

PLATE 18

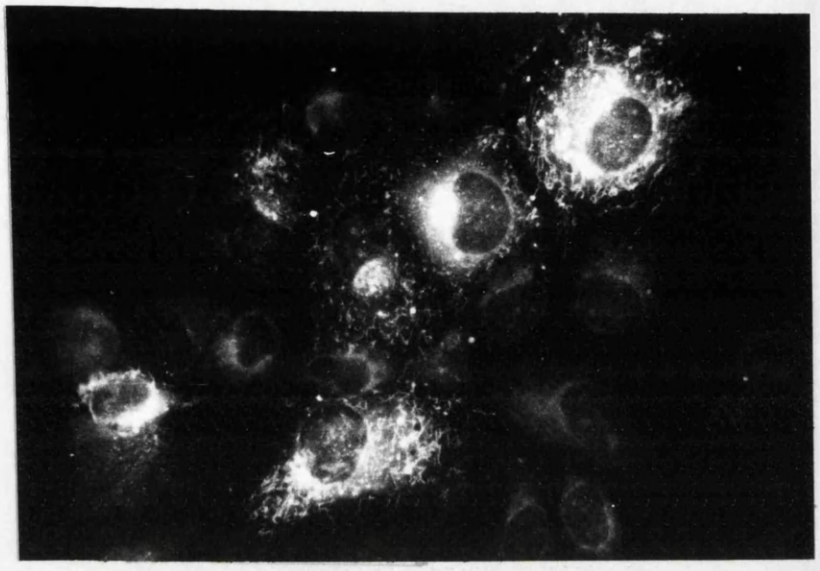
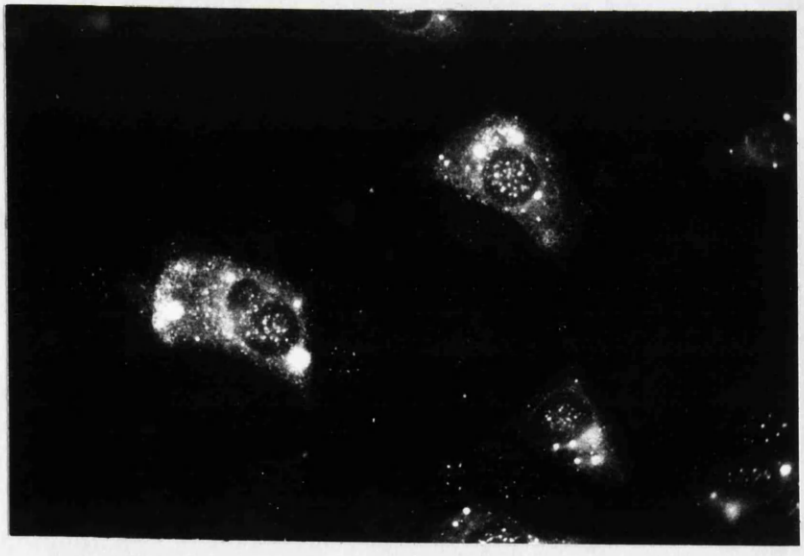


PLATE 19



Plates 20 and 21 : BS-C-1 cells infected with a Group II ts mutant (ts 8)

The ts mutant infected cells were stained with bovine anti-bovine RS virus serum.

Plate 20 shows the Group II ts mutant infected BS-C-1 cells at 31°C. A LARGE NUMBER OF PROCESSES WERE SEEN.

Plate 21 shows the Group II ts mutant infected BS-C-1 cells at 39°C. NO REDUCTION IN THE NUMBER OF PROCESSES SEEN AND AMOUNT OF ANTIGEN DETECTED.

The magnification of these photographs was 480 X.

PLATE 20

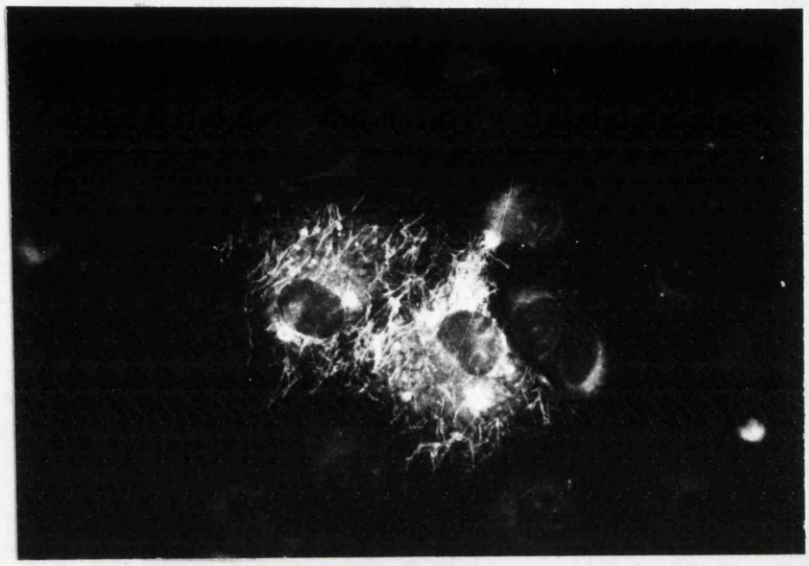
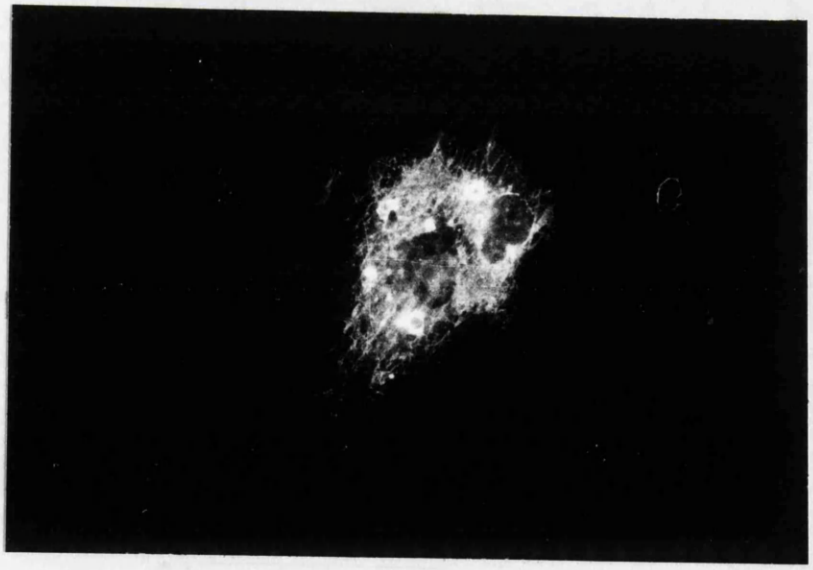


PLATE 21



Plates 22 and 23 : BS-C-1 cells infected with a Group II ts mutant (ts 14) and stained with human RS virus antiserum

The ts mutant infected cells were stained with convalescent sera from children with RS virus infection.

Plate 22 shows the Group II ts mutant infected BS-C-1 cells at 31°C.

Plate 23 shows the Group II ts mutant infected BS-C-1 cells at 39°C. NO REDUCTION IN THE NUMBER OF FLUORESCING CELLS AND AMOUNT OF ANTIGEN DETECTED.

The magnification of these photographs was 480 X.

PLATE 22

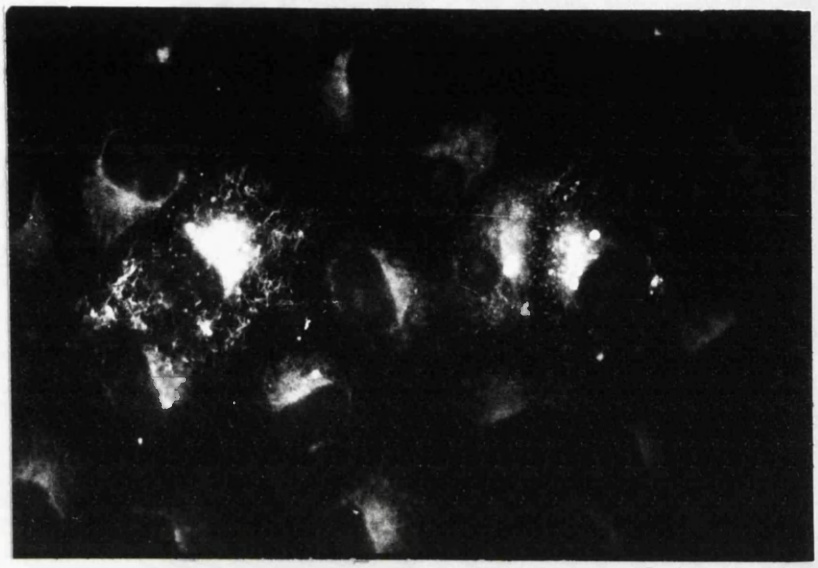
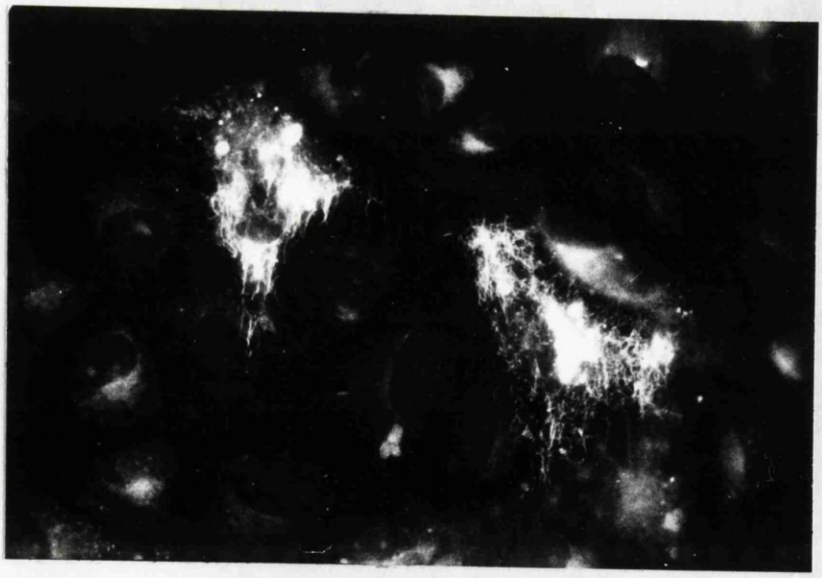


PLATE 23



Plates 24 and 25 : BS-C-1 cells infected with a Group III ts mutant (ts 3)

The ts mutant infected cells were stained with bovine anti-bovine RS virus serum.

Plate 24 shows the Group III ts mutant infected BS-C-1 cells at 31°C. This ts mutant produces a large number of viral processes at the permissive temperature.

Plate 25 shows the Group III ts mutant infected BS-C-1 cells at 39°C. ~~RS virus antigen can be seen in the nucleus.~~ NO PROCESSES WERE DETECTED.

The magnification of these photographs was 480 X.

PLATE 24

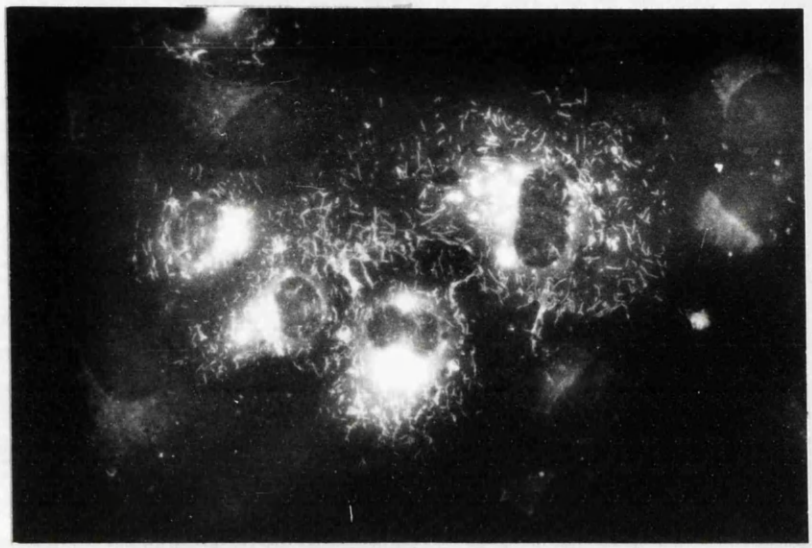


PLATE 25

3.9 Physiological properties of RS virus ts mutants

The physiological properties of RS virus ts mutants were studied at the biological rather than the biochemical level because of the difficulties involved in biochemical studies of wild type RS virus. For example the nucleic acid of RS virus has not yet been isolated and classification of mutants into RNA -ve and RNA +ve phenotypes would be premature.

3.9.1 Growth properties of ts mutants at 31°C and 39°C

The growth properties of wild type RS virus (Figure 14) and thirteen ts mutants of RS virus have been examined and found to fall into two main groups (Table 25).

Group A

This group includes those ts mutants which have no or very little ($< 2\%$) leak over 48 hrs at 39°C (Figure 15-21, 29a).

Group B

The ts mutants in this group have a high level of leak at 39°C ($> 10\%$) and their growth pattern at 39°C is similar to that at 31°C (Figure 22-25, 26a). The plating efficiency of these ts mutants at 39°C is low which suggests that they are defective in a cytolytic function.

The division of these thirteen ts mutants into groups based on growth pattern at 39°C corresponds very well with the immunofluorescence classification for these mutants. Group I and III ts mutants form Group A and Group II ts mutants form Group B. However, there is one exception ts 25 which although classified as a Group I ts mutant has the growth pattern at 39°C of a Group B ts mutant.

TABLE 25

Classification of *ts* mutants based on their growth at 39°C

Group A Poor or no growth at 39°C	Group B Growth at 39°C
ts 3	*ts* 1
ts 5	*ts* 2
ts 15	*ts* 9
ts 16	*ts* 23
ts 17	*ts* 25
ts 18	
ts 19	
ts 33	

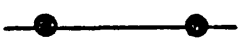
Different stocks of ts 25 were used in the immunofluorescence and growth cycle experiments. However, the difference in behaviour of ts 25 in these experiments was not due to reversion occurring during the growth cycle since this was less than 1%.

The effect of altering the moi of ts virus is shown in figures 23a, b and 24a, b. There is no difference in the yield of ts 23 in the two experiments although the moi is 2-fold higher in the first experiment (figure 24a). However, the yield increment of ts 2 is low (0.5 log units) in the experiment (Figure 23a) where the moi is high (4.8) and higher (1.5 log unit) when moi is low (0.48) (Figure 23b). Therefore, there appears to be multiplicity-associated interference of RS virus replication. This has also been noted by other workers (Beem and Treuhaft, 1972).

In some viruses (e.g. influenza, VSV) multiplicity associated interference is due to the replication of defective particles in multiply-infected cells.

Figure 14 : The growth of the RSN-2 strain of RS virus at 31°C and 39°C

Growth of RSN-2 strain at 31°C.



Growth of RSN-2 strain at 39°C.

MOI : 0·04 PFU/CELL

TIME : HOURS POST-ADSORPTION

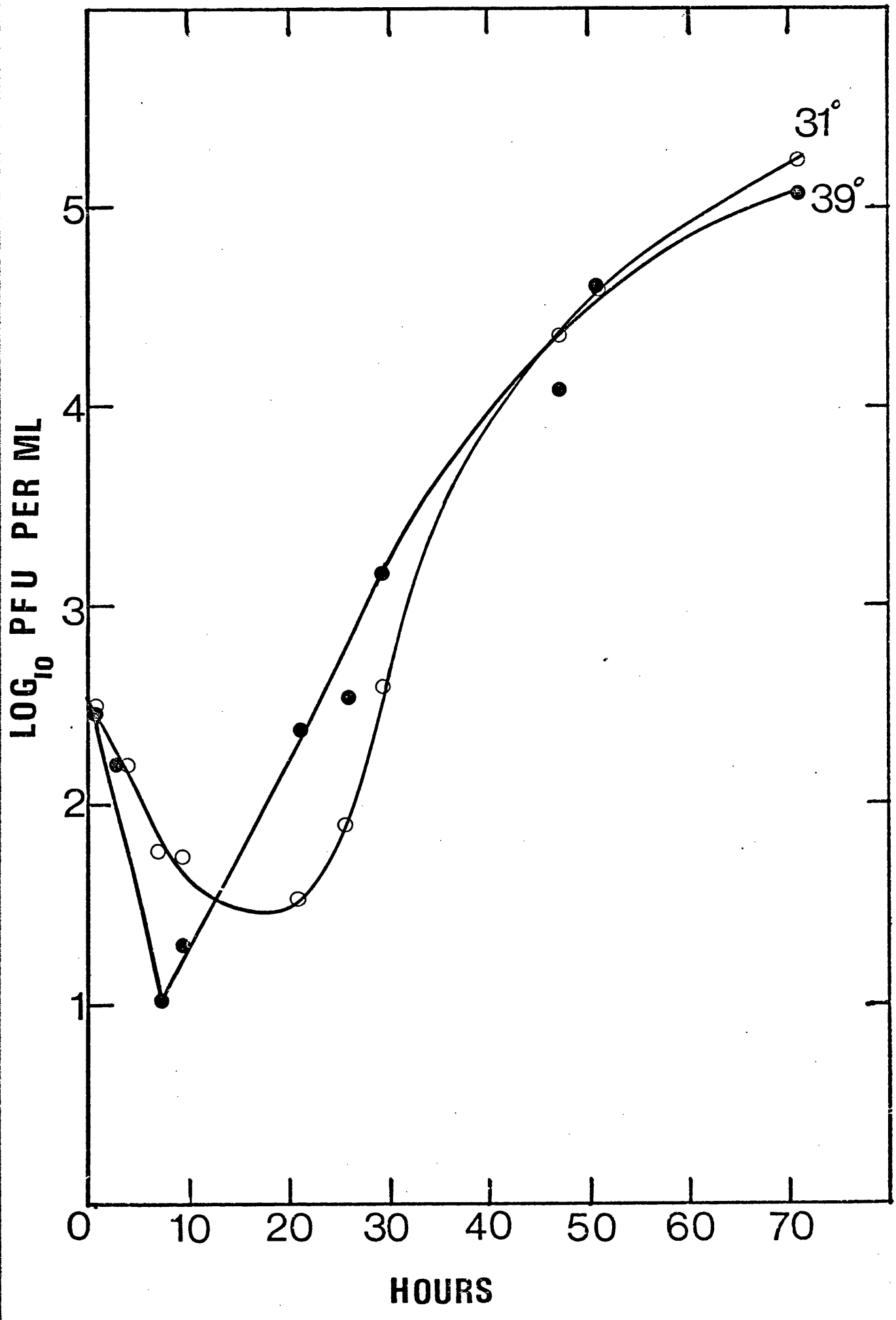

31°
39°
LOG10 PFU PER ML
HOURS
0
10
20
30
40
50
60
70
1
2
3
4
5

Figure 15 : The growth of ts 3 (Group III)

Growth of ts mutant at 31°C

Growth of ts mutant at 39°C.

MOI : 0.21 PFU/CELL

TIME : HOURS · POST-ADSORPTION

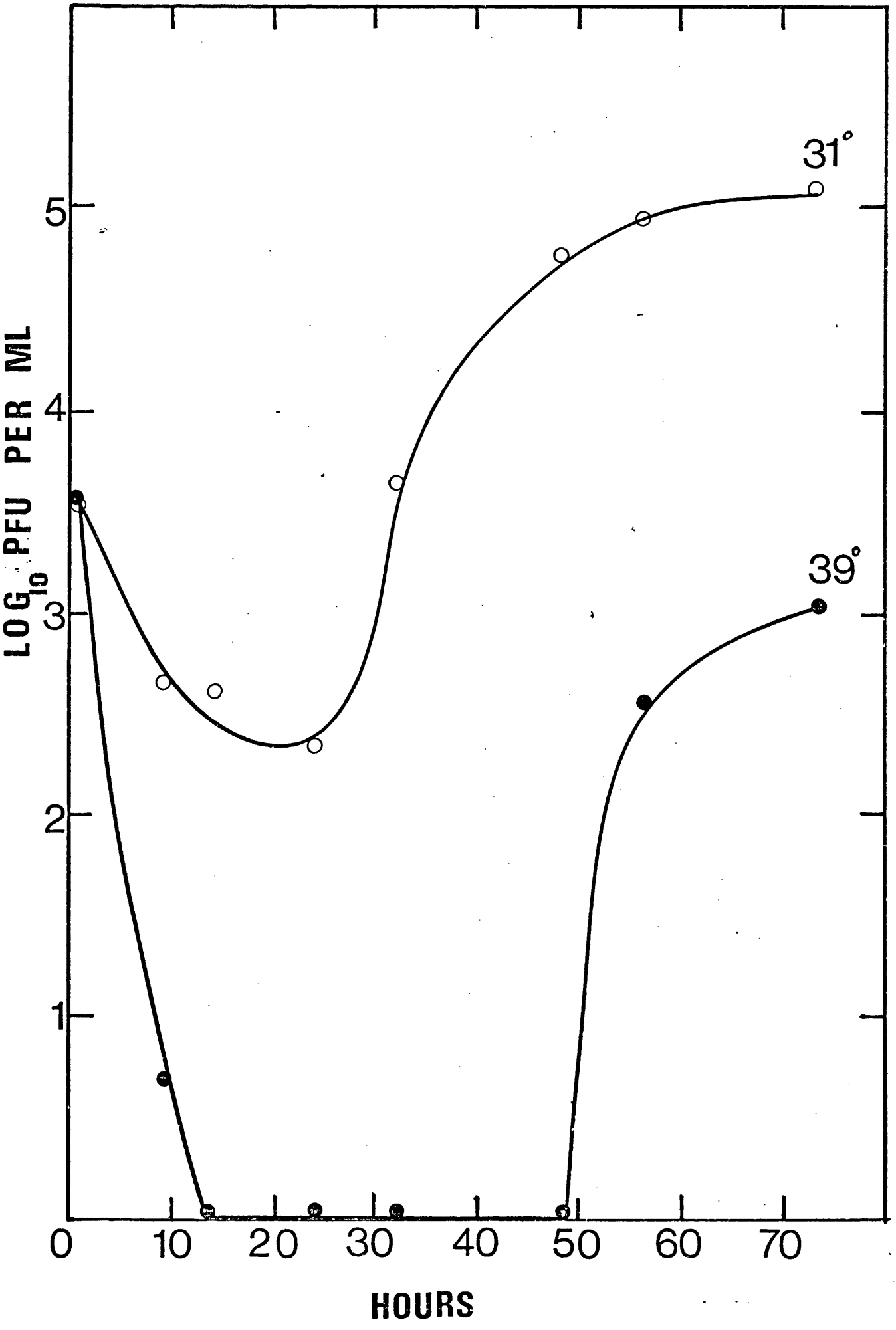
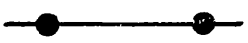

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 16 : The growth of ts 5 (Group I)

Growth of ts mutant at 31°C.



Growth of ts mutant at 39°C.

MOI : 0·04 PFU/CELL

HOURS : POST-ADSORPTION.

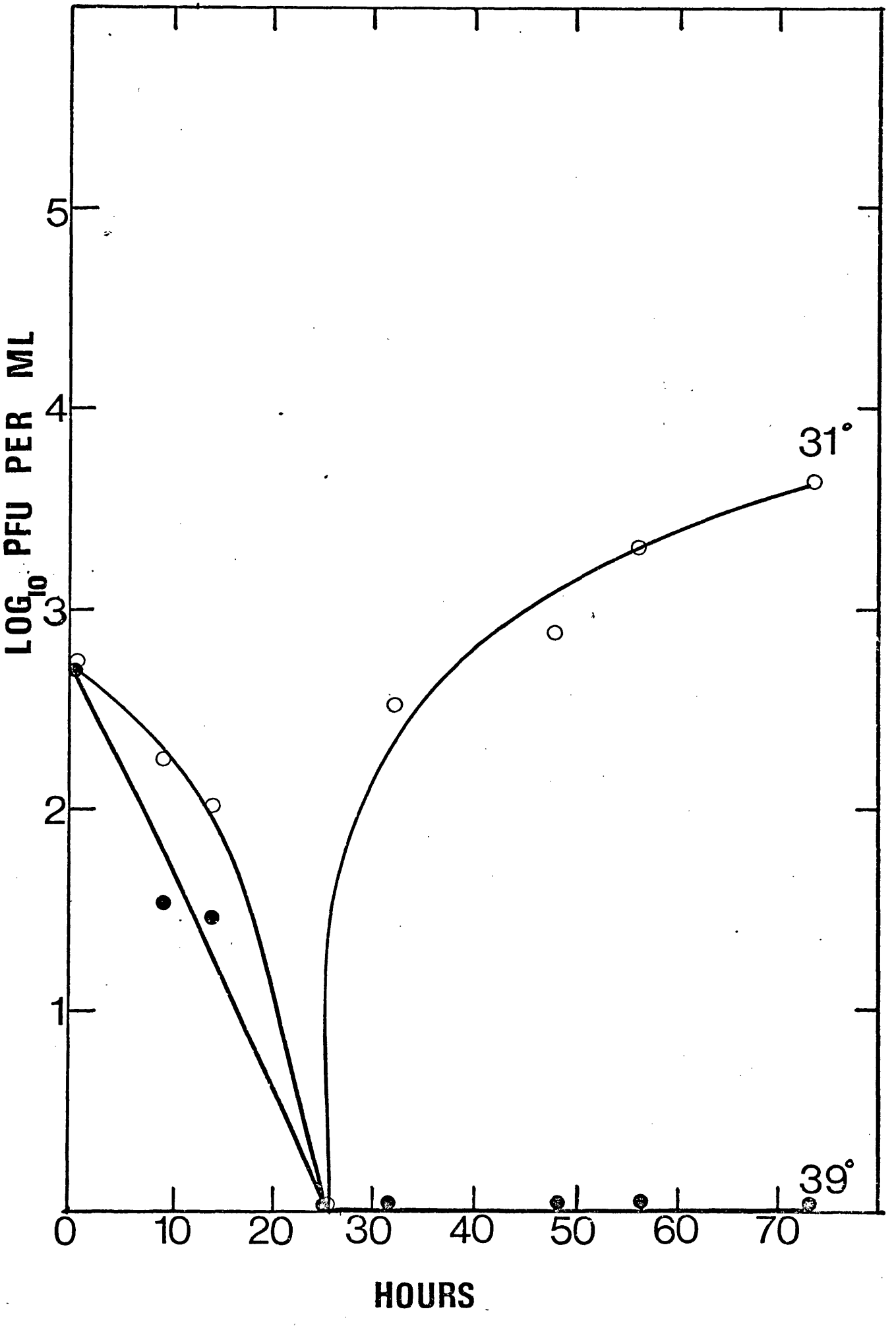
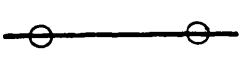

LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

Figure 17 : The growth of ts 15 (Group III)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

MOI : 0·15 PFU/CELL

TIME : HOURS POST-ADSORPTION

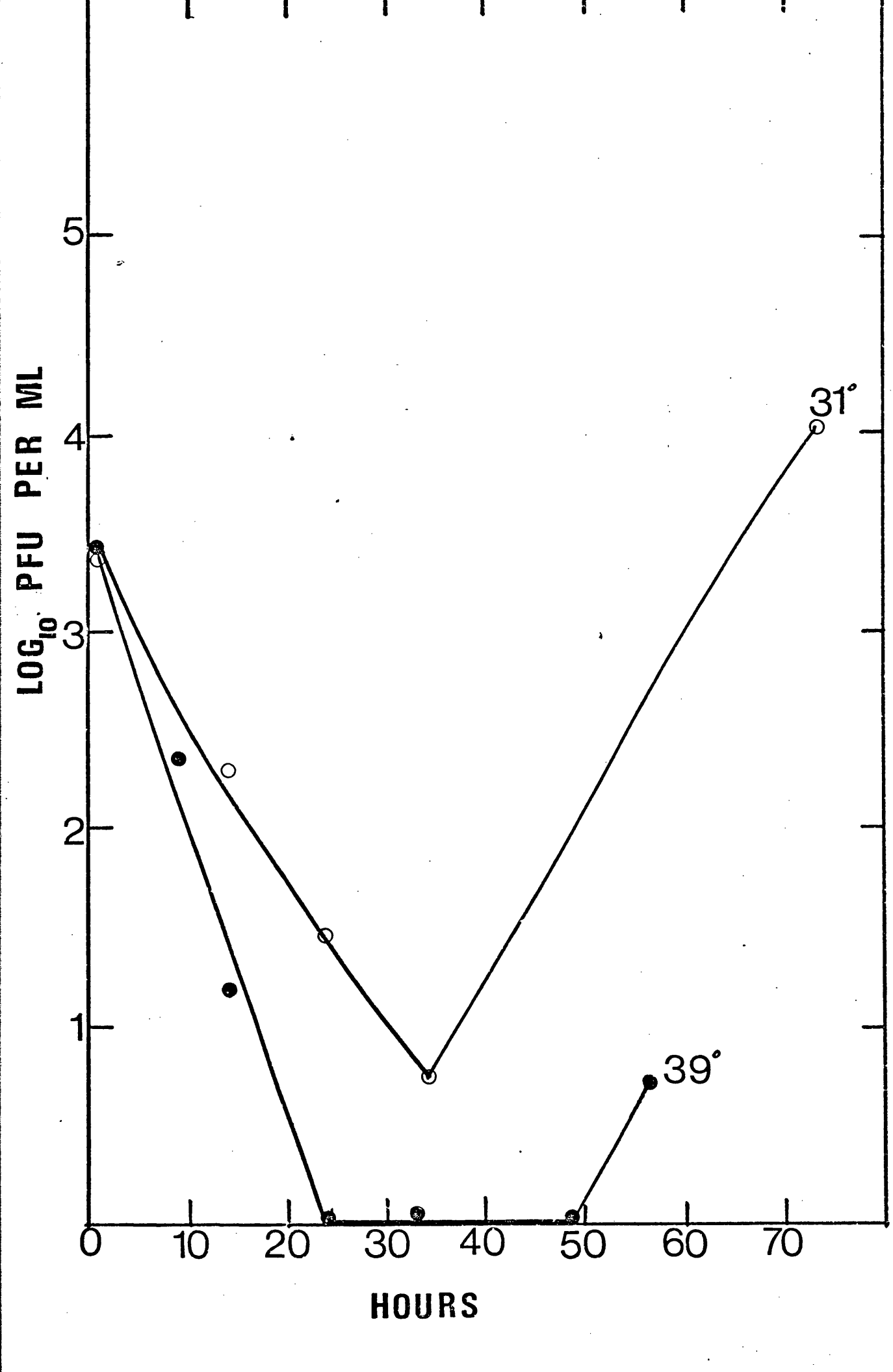
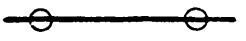
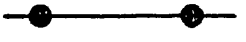

LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

Figure 18 : Growth of ts 16 (Group I)



Growth of ts mutant at 31°C.



Growth of ts mutant at 39°C.

MOI : 0.19 PFU/CELL
TIME : HOURS POST-ADSORPTION

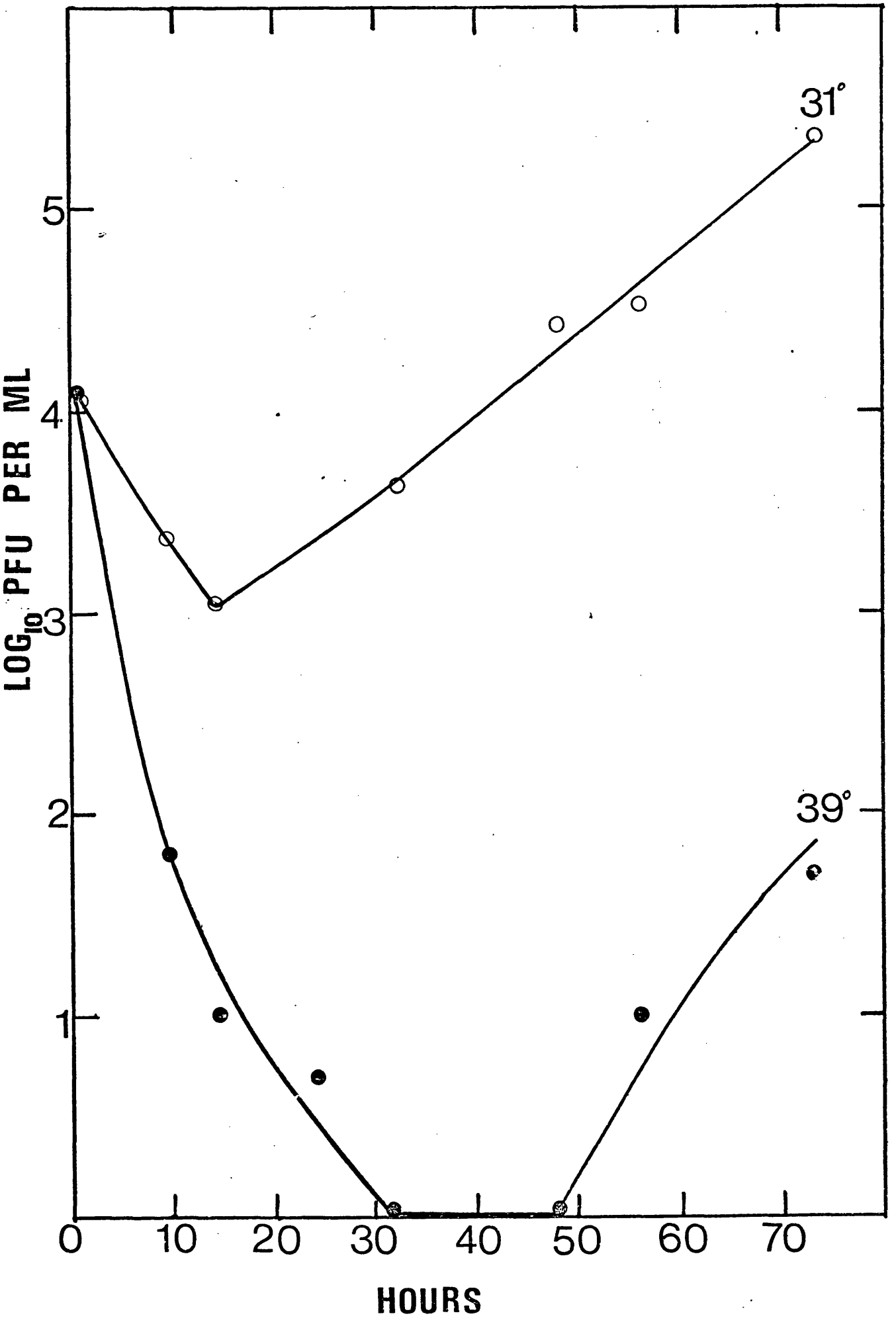
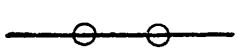
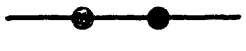

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 19 : The growth of ts 17 (Group I)



Growth of ts mutant at 31°C.



Growth of ts mutant at 39°C.

MOI : 0·27 PFU/CELL
TIME : HOURS POST-ADSORPTION.

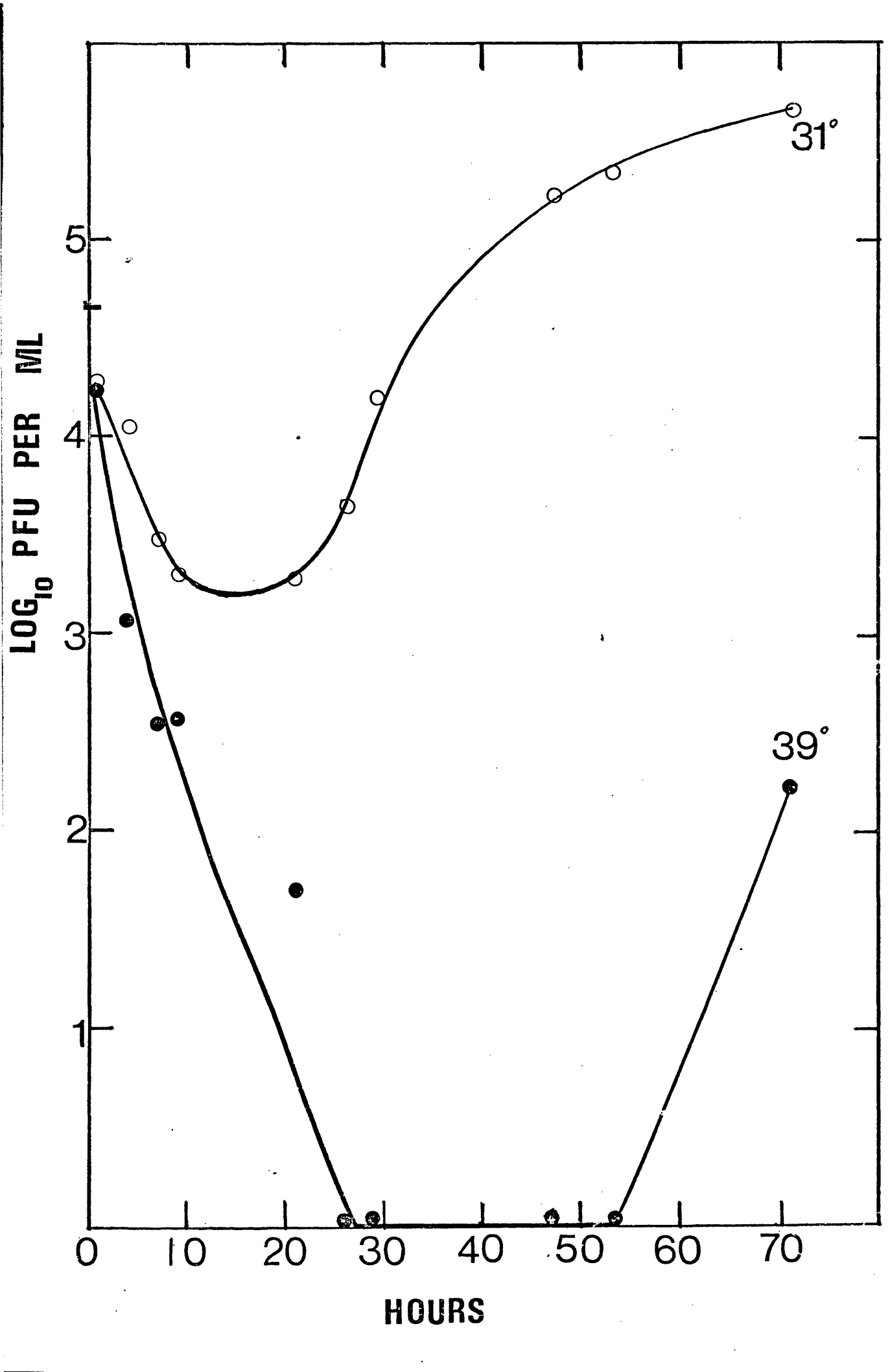
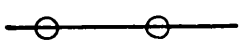

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 20 : The growth of ts 18 (Group I)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

MOI : 0.5 PFU/CELL

TIME : HRS. POST- ADSORPTION

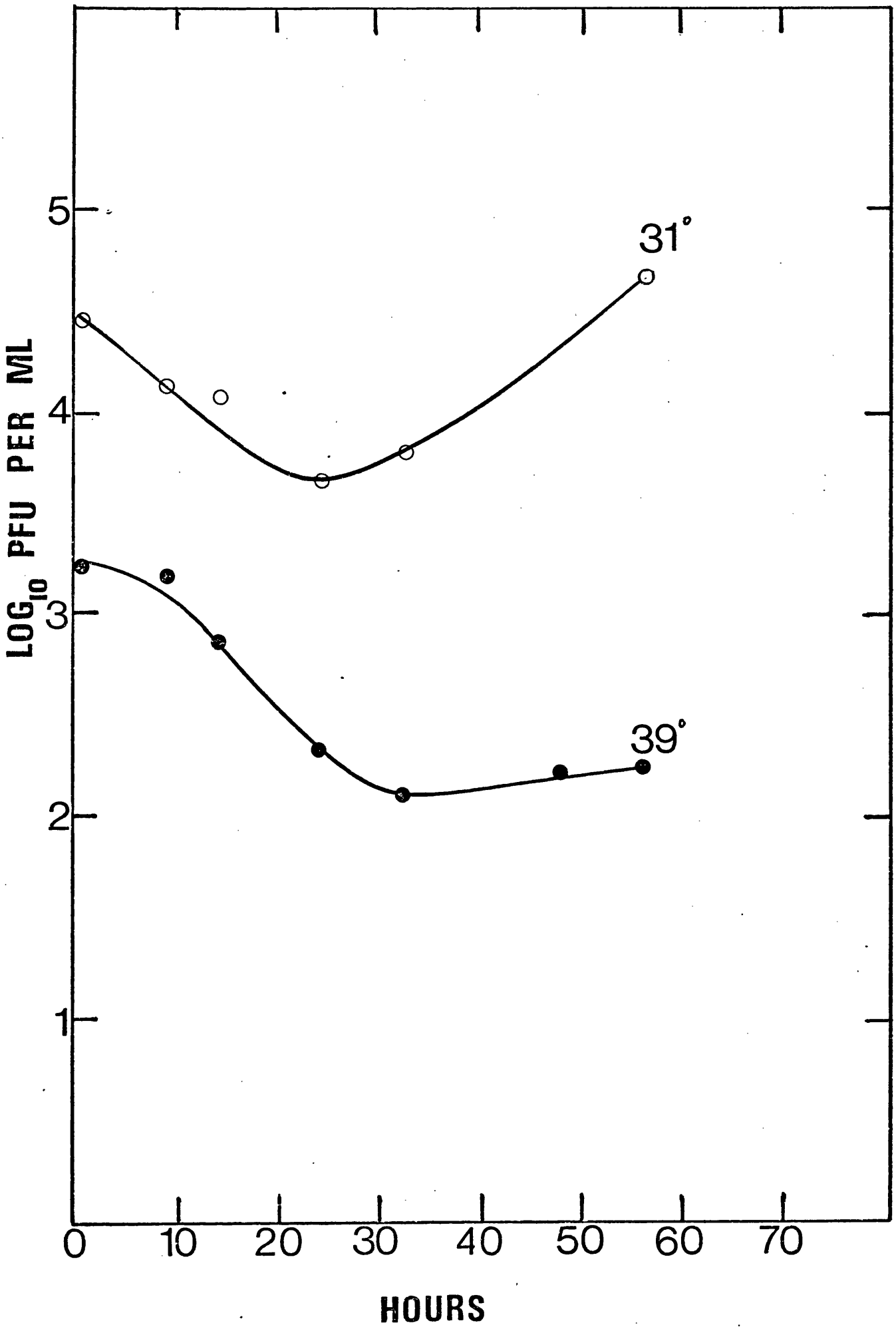
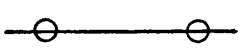

5
4
3
2
1
0
10
20
30
40
50
60
70
31°
39°
LOG10 PFU PER ML
HOURS

Figure 21 : Growth of ts 33 (Group III)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

MOI : 0.05 PFU/CELL
TIME : HOURS POST-ADSORPTION

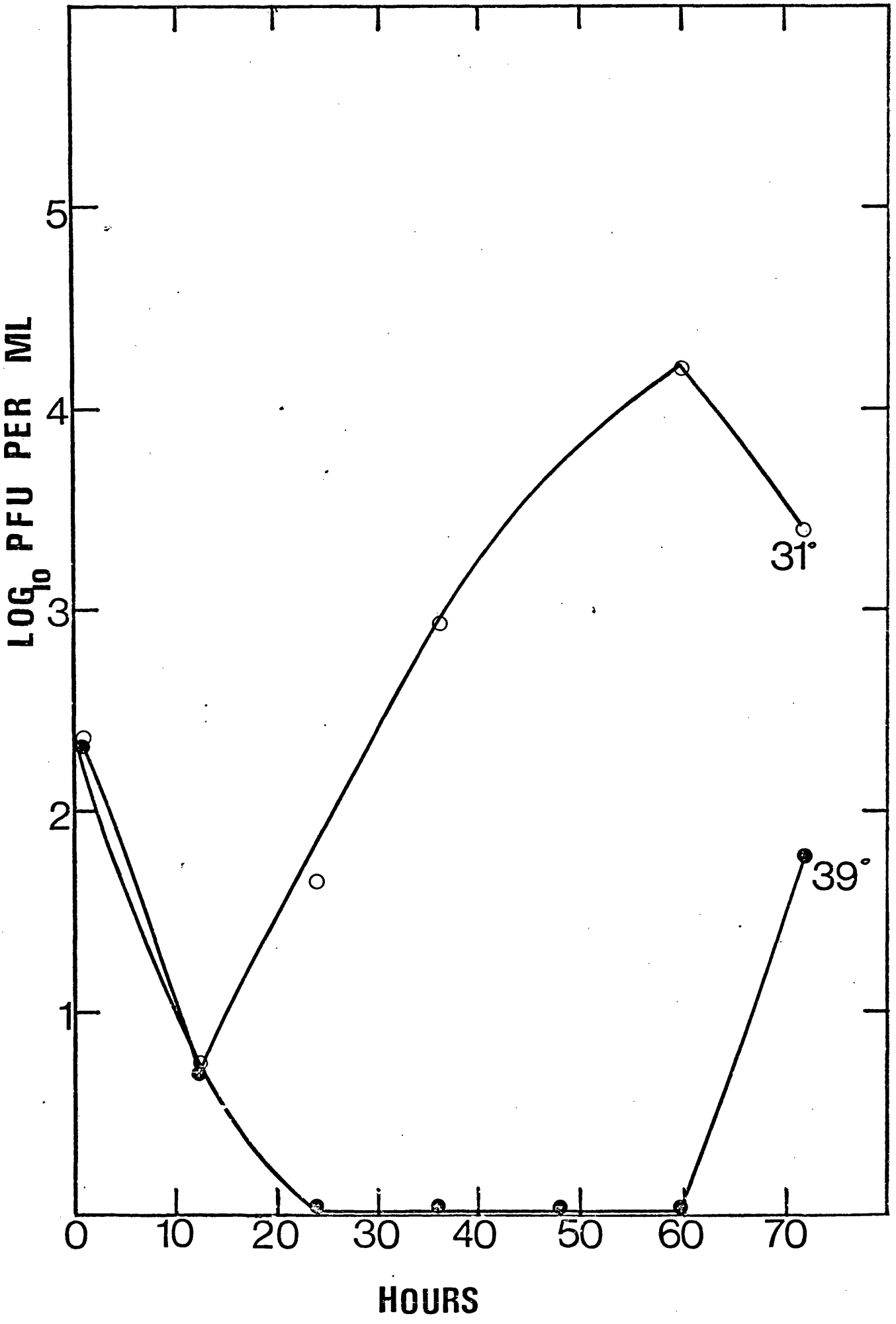
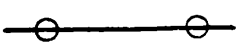

LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

Figure 22 : The growth of ts 1 (Group II)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

MOI : 0.12 PFU/CELL

TIME : HOURS POST-ADSORPTION.

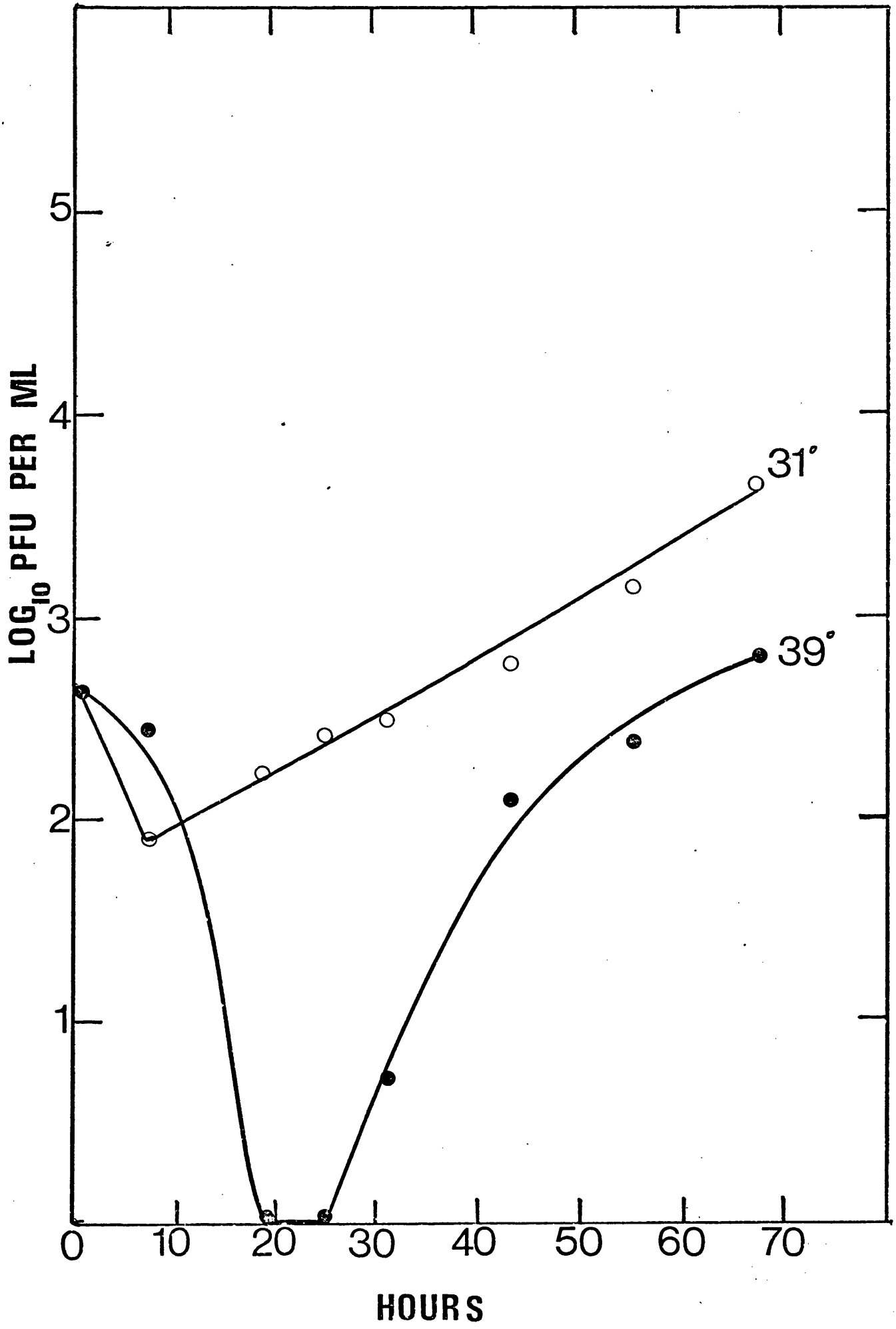
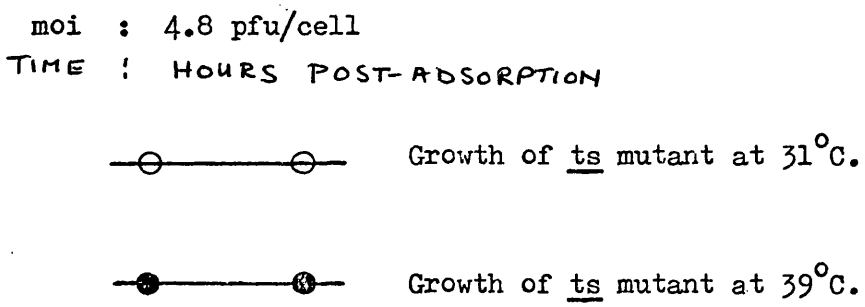

LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

Figure 23a : The growth of ts 2 (Group II)



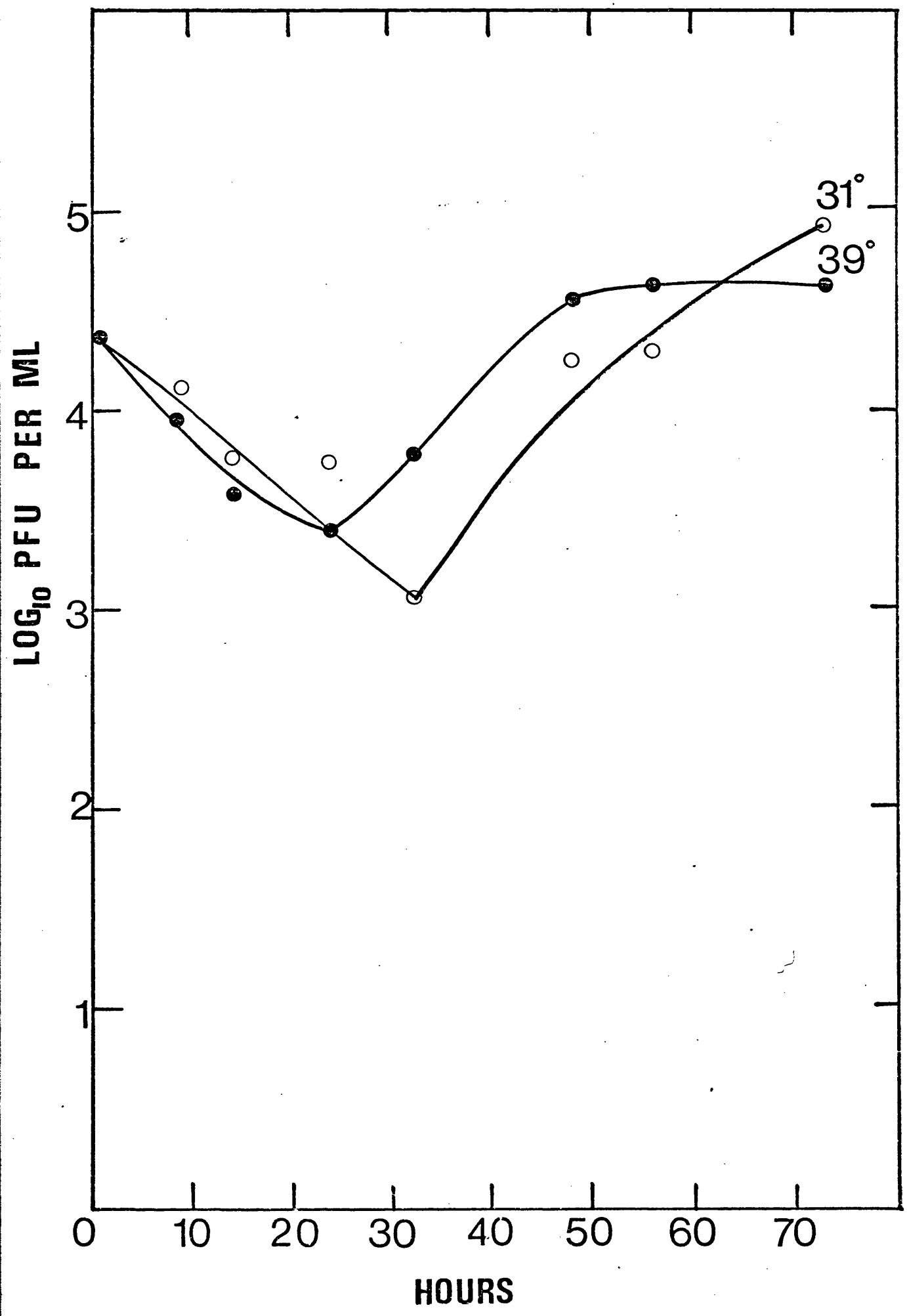
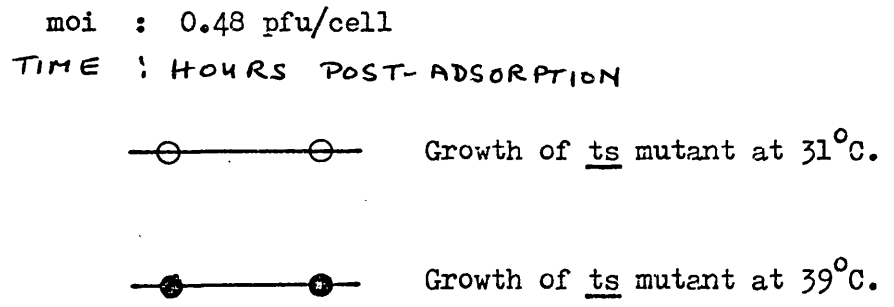

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 23b : The growth of ts 2 (Group II)



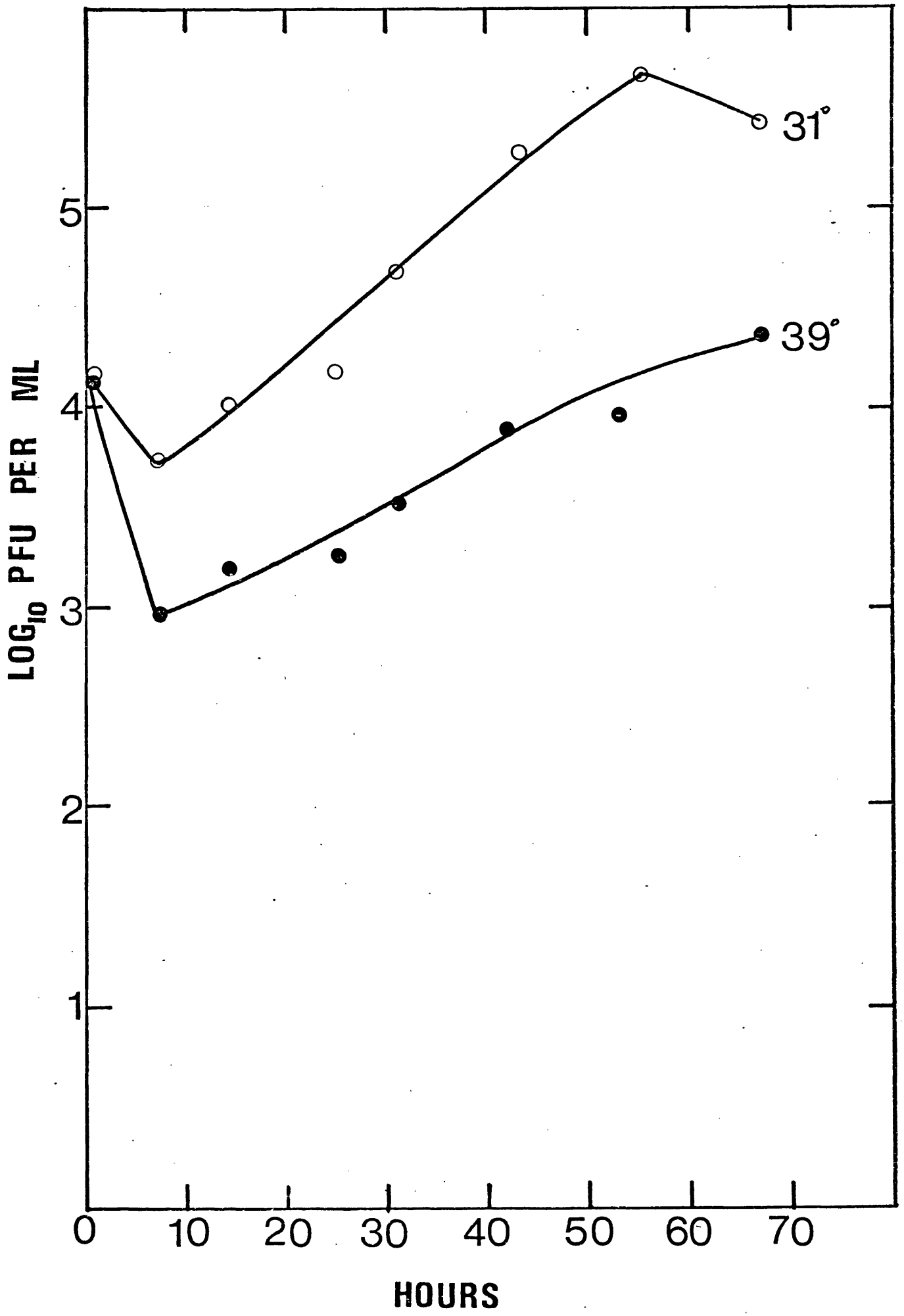
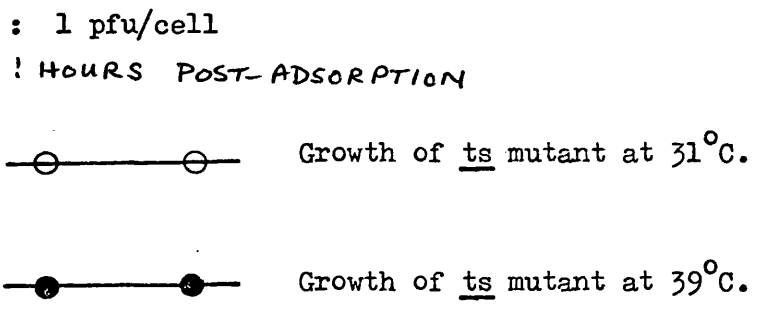

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 24a : The growth of ts 23 (Group II)



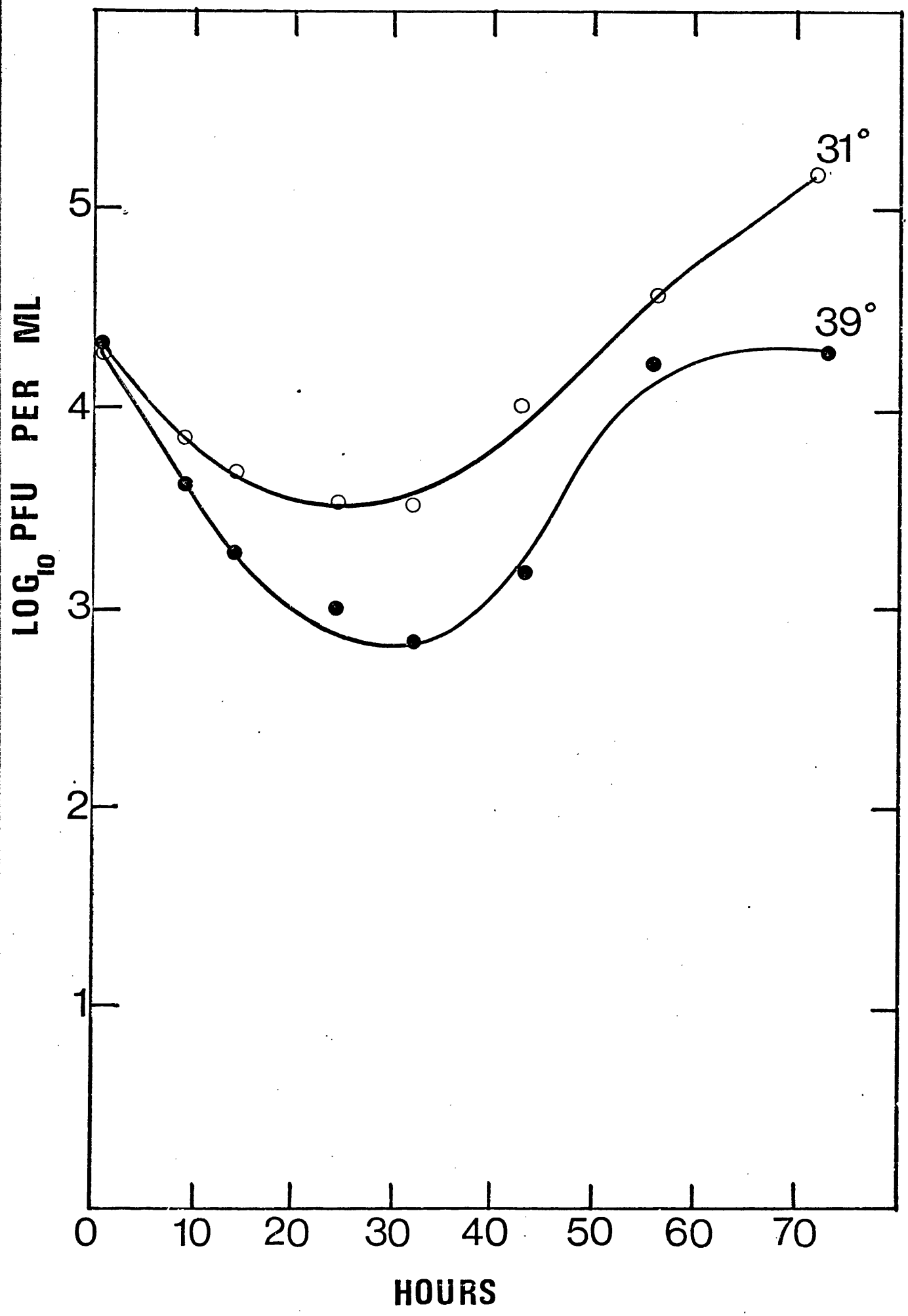
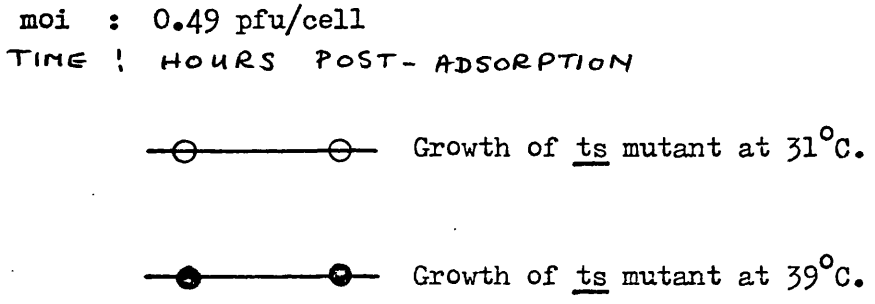

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 24b : The growth of ts 23 (Group II)



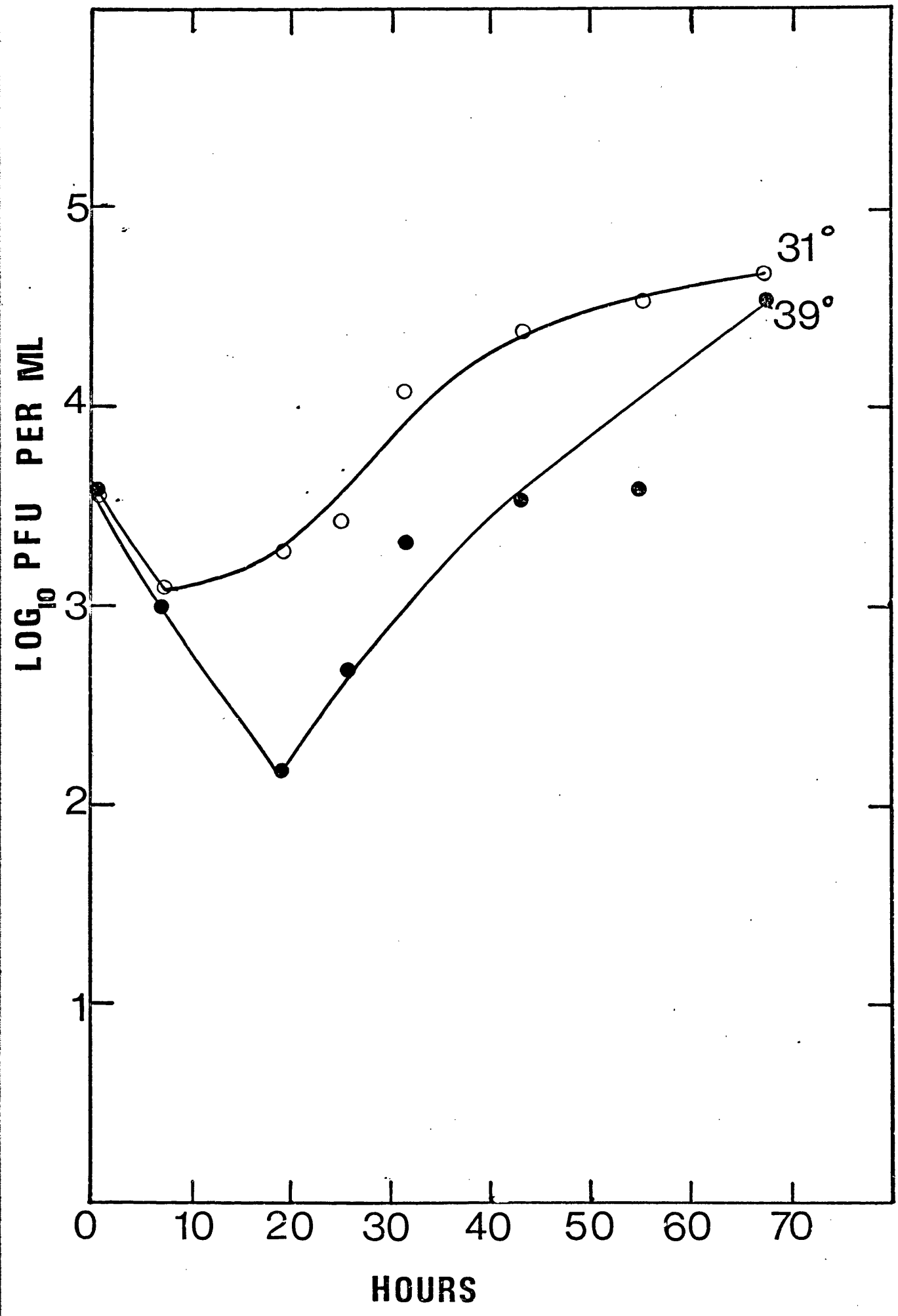
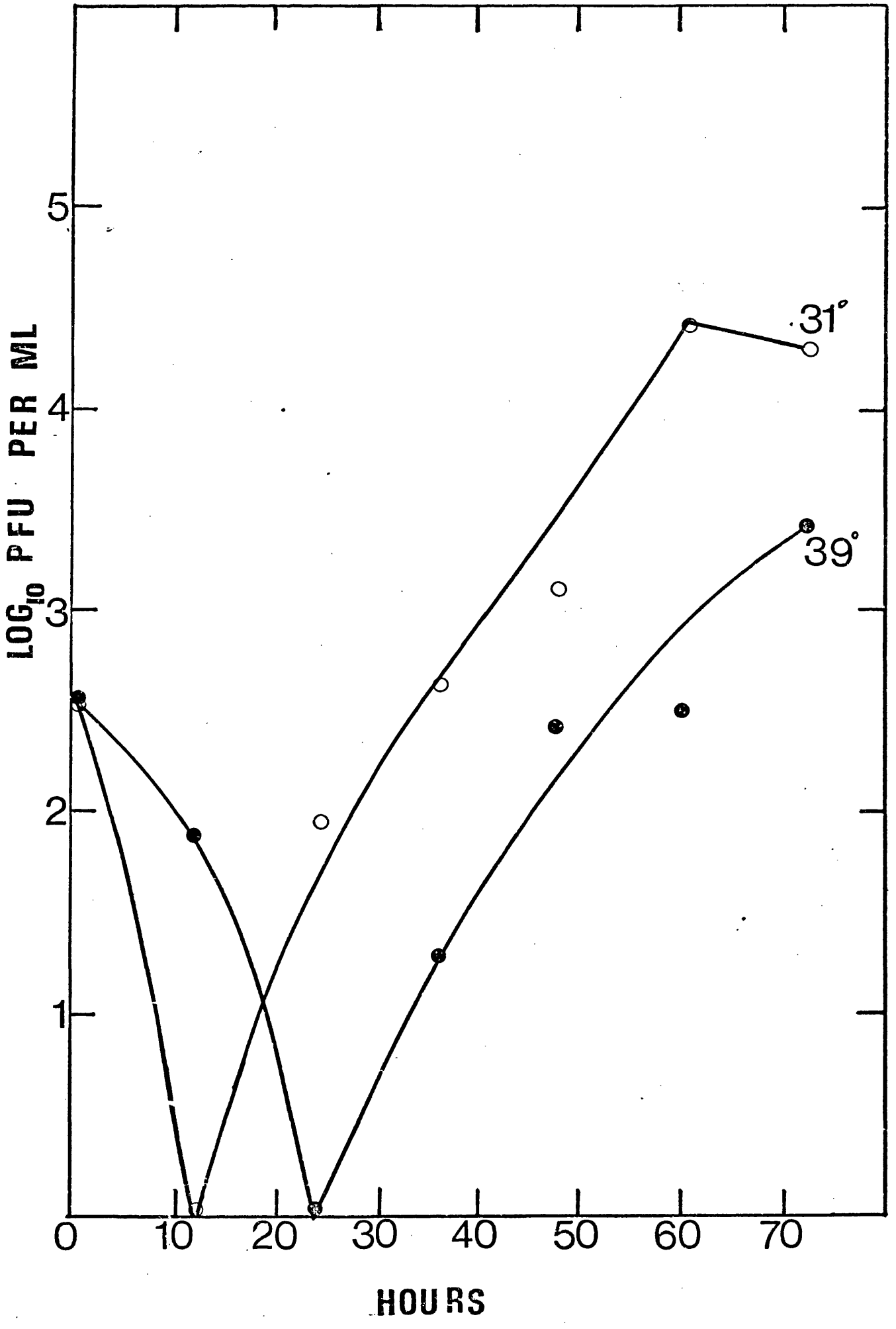

5
4
3
2
1
0
10
20
30
40
50
60
70
31°
39°
LOG10 PFU PER ML
HOURS

Figure 25 : The growth of ts 25 (Group II)

Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

MOI : 0·03 PFU/CELL
TIME : HOURS POST-ADSORPTION


LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

3.9.2 Temperature-shift experiments

Four ts mutants, three from Group A and one from Group B were studied by temperature-shift experiments (section 2.5.2, method 6) to determine whether their ts mutation affected a function which occurred early or late in the RS virus growth cycle.

1. Ts 9 (Group B)

Ts 9 is a very leaky ts mutant. For this reason these results are difficult to interpret. However, in the upshift (31°C to 39°C) experiment (Figure 26a) virus growth appears to be inhibited at all times during the growth cycle except at 60 hours. In the downshift experiment (39°C to 31°C) at 12, 24, 36, 48 and 60 hours there is immediate synthesis of ts 9 but a final reduction in yield at 36 and 48 hrs (Figure 26b).

Virus growth appears to be inhibited at 39°C but not at 31°C. This suggests that the mutated gene product in ts 9 infected cells is required throughout the growth cycle, and that its functional activity rather than its synthesis is temperature-sensitive.

2. Ts 15 (Group A)

In the upshift experiment (Figure 27a) the growth of ts 15 was inhibited when the virus was shifted-up at 12, 24 and 36 hours. However, no inhibition occurred at 48 and 60 hours. When ts 15 was shifted-down at 12 and 24 hours, virus growth was not inhibited whereas at 36 and 60 hours virus the yield was reduced (Figure 27b).

From these data it appears that ts 15 affects an early step in the RS virus growth cycle probably during the first 12 hours. The

affected gene product is required for at least 36 hours, as shift-up during that time results in inhibition of virus growth. Physiological factors may be responsible for the reduction in yield at 36 and 60 hours on shiftdown. It would appear that ts 15 kept for 36 hours or longer at 39°C is unable to recover when returned to the permissive temperature (31°C). This is probably partly due to the fact that ts 15 is very heat-labile (see Table 26).

3. Ts 16 (Group A)

When ts 16 was shifted-up to 39°C at 12 and 24 hours after infection, virus growth was inhibited (Figure 28a). However, when ts 16 was shifted-up after 24 hours there was no inhibition in virus growth. On downshift (Figure 28b) at 12 and 24 hours there was a slight reduction in virus yield which became marked at 36, 48 and 60 hours.

Ts 16 probably also affects a step in the RS virus growth cycle which occurs at or before 12 hours, and is required for at least 24 hours. The reduction in virus yield on downshift may indicate that ts 16 is unable to recover after incubation at 39°C. It is interesting to note that although on virus growth was not inhibited by upshift at 36 hours, there was a marked reduction in final yield of virus.

4. Ts 19 (Group A)

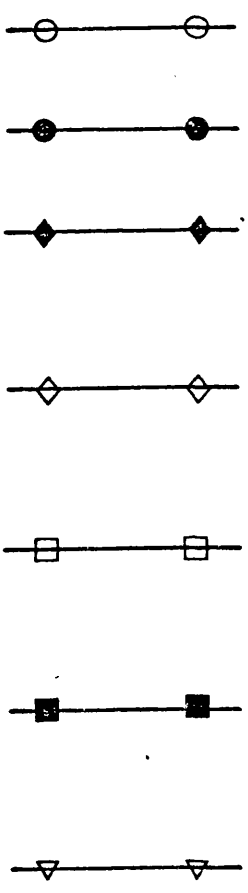
On upshift at 12 hour the yield of ts 19 was reduced and its growth delayed (Figure 29a). However, there was an enhancement of the yield on upshift at 24, 36, 48 and 60 hours. When ts 19 was shifted-down at 12, 24 or 36 hours there was no reduction in the final virus

yield (Figure 29b). A marked reduction in virus yield occurred when ts 19 was shifted-down at 48 and 60 hours.

Ts 19 probably affects a very early step in the RS virus growth cycle which occurs before 12 hours post-adsorption and is not required beyond 12 hours. The reduction in virus yield which occurs on shiftdown at 48 and 60 hours was probably due to inability of the virus to recover after exposure at 39°C, since ts 19 is very heat labile at 39°C (see Table 26).

The patterns exhibited by ts 15, ts 16 and ts 19 were similar. These Group A ts mutants appeared to affect early functions in the RS virus growth cycle, whereas ts 9, the group B ts mutant, appeared to affect a function required throughout the growth cycle. The ts 9 lesion may involve the functional activity rather than the synthesis of a gene product.

Figure 26a : Upshift growth curves of ts 9 (Group II)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 31°C, upshift at 12 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 24 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 36 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 48 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 60 hours p.a. to 39°C.

MOI : 0·04 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)

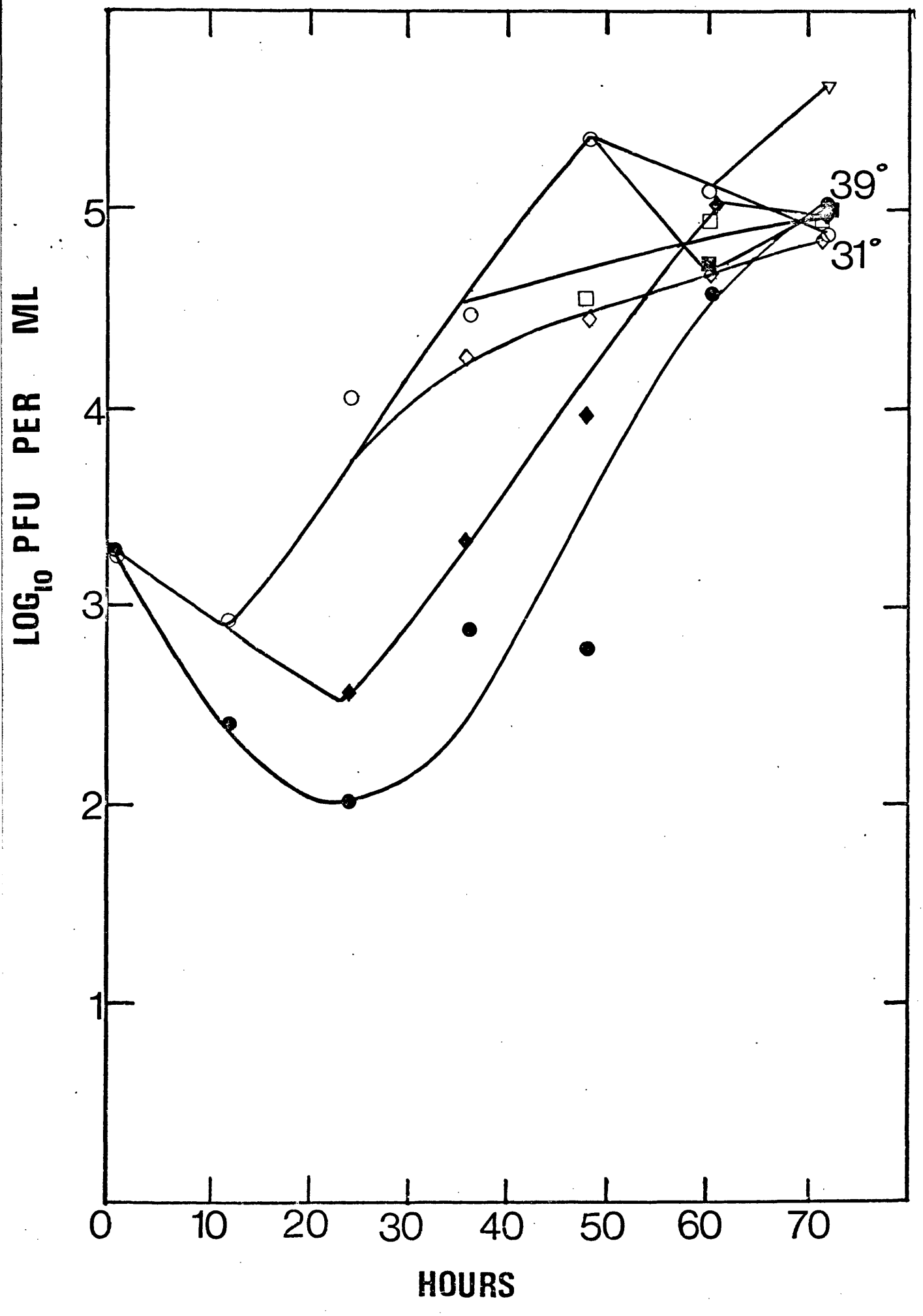
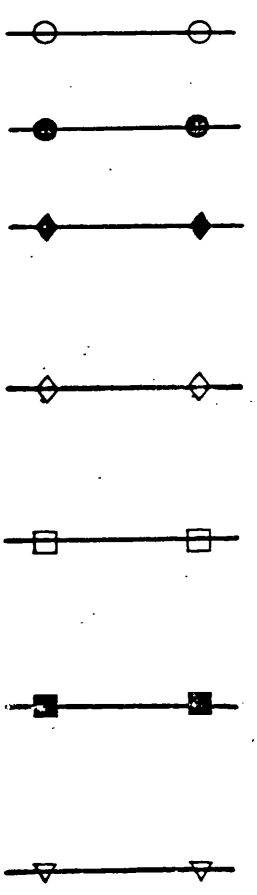

39°
31°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 26b : Downshift growth curves of ts 9 (Group II)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 39°C, downshift at 12 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 24 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 36 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 48 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 60 hours p.a. to 31°C.

MOI : 0·04 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)

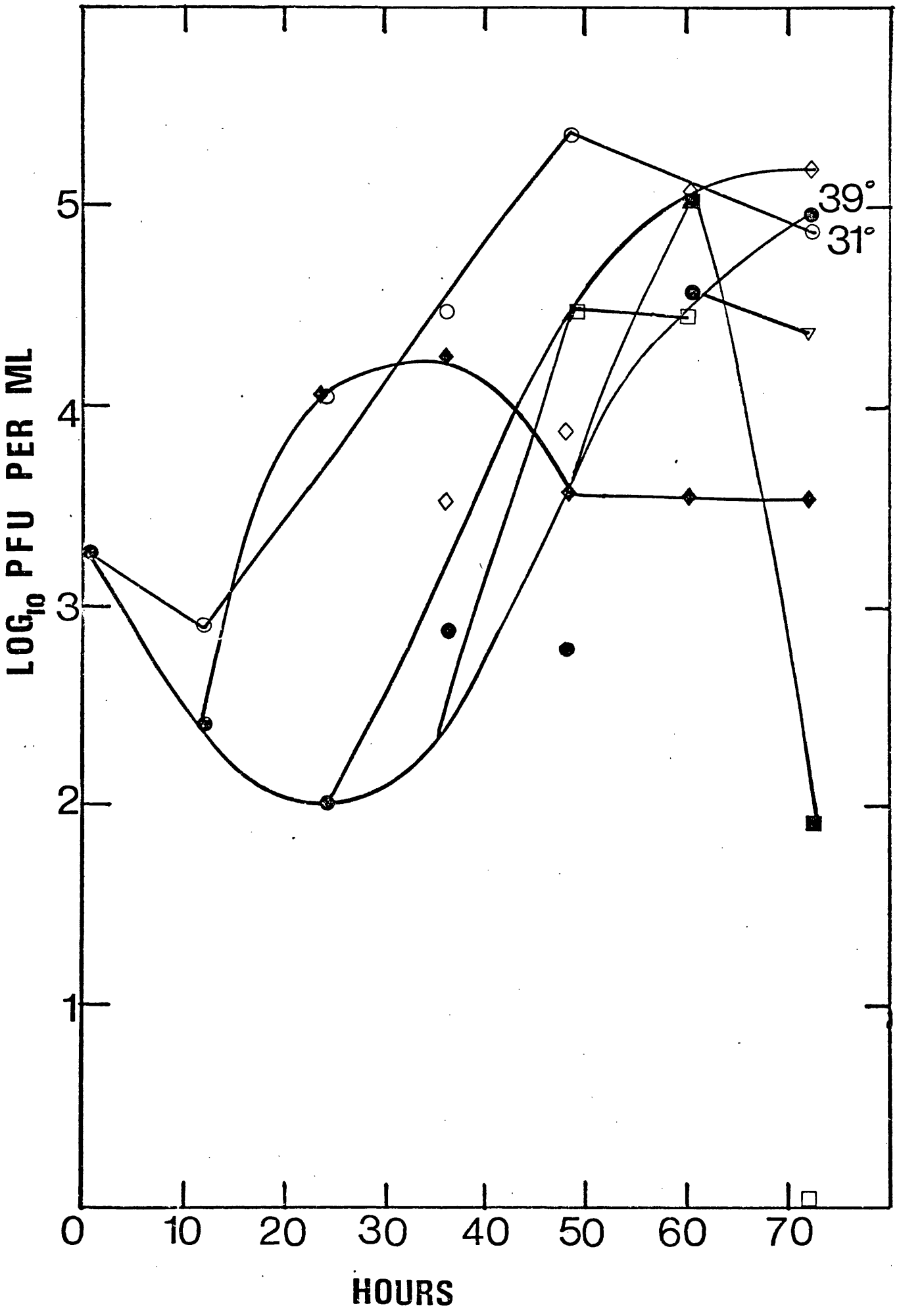
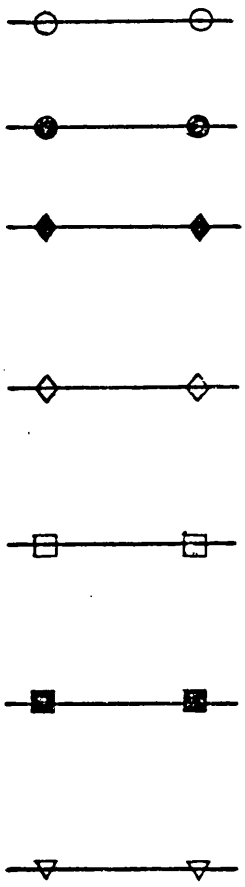

39°
31°
LOG10 PFU PER ML
1
2
3
4
5
0
10
20
30
40
50
60
70
HOURS

Figure 27a : Upshift growth curves of ts 15 (Group III)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 31°C, upshift at 12 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 24 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 36 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 48 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 60 hours p.a. to 39°C.

MOI : 0·32 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)

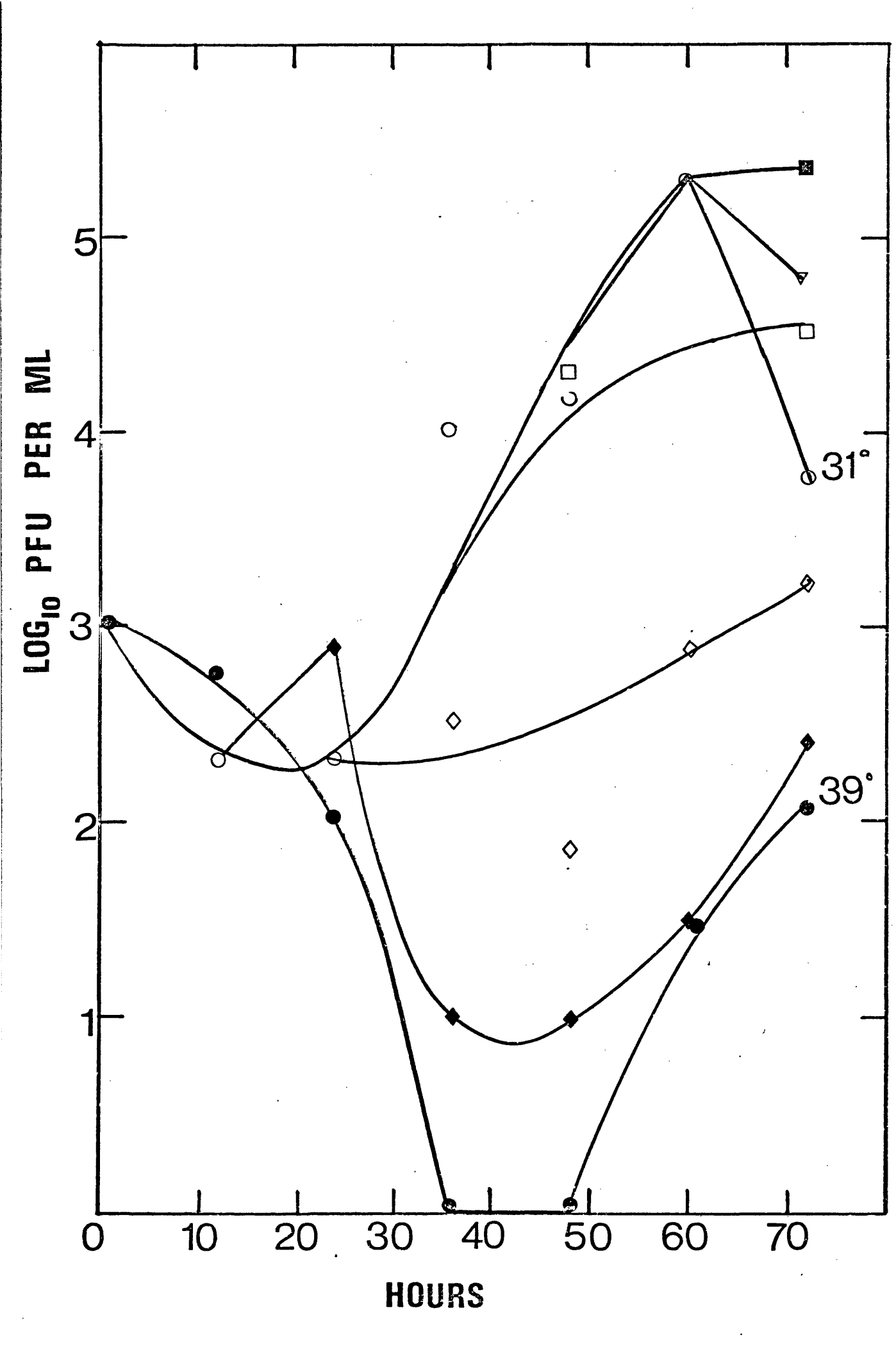
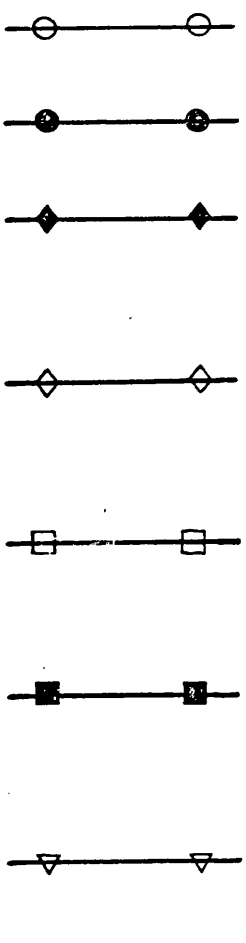

LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

Figure 27b : Downshift growth curves of ts 15 (Group III)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 39°C, downshift at 12 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 24 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 36 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 48 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 60 hours p.a. to 31°C.

MOI : 0·82 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)

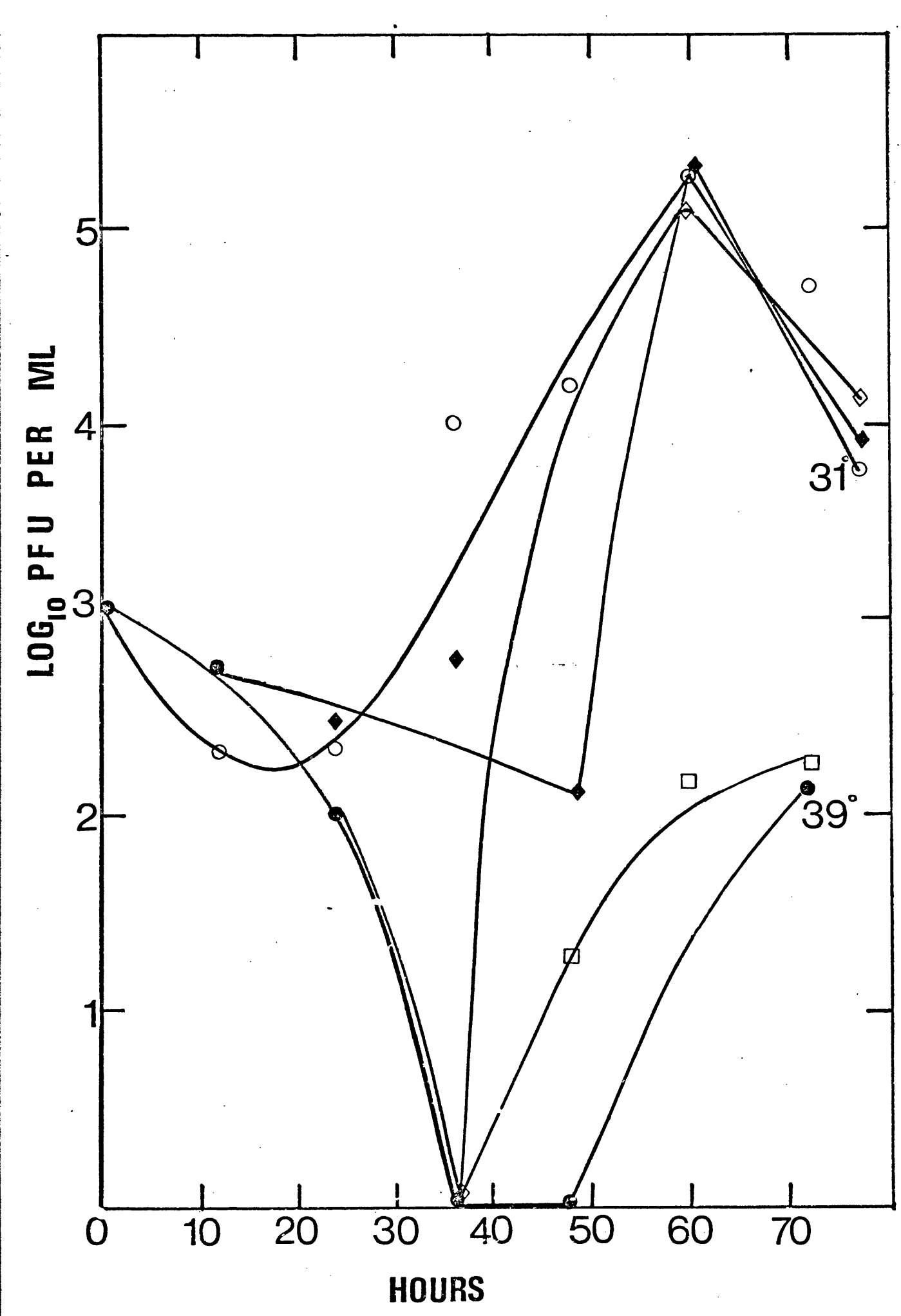
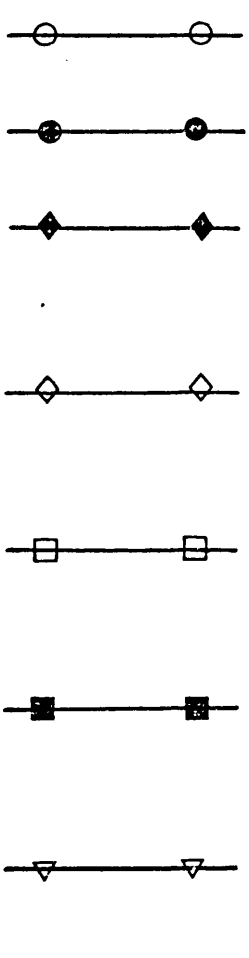

LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

Figure 28a : Upshift growth curves of ts 16 (Group I)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 31°C, upshift at 12 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 24 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 36 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 48 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 60 hours p.a. to 39°C.

MOI : 0·20 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)

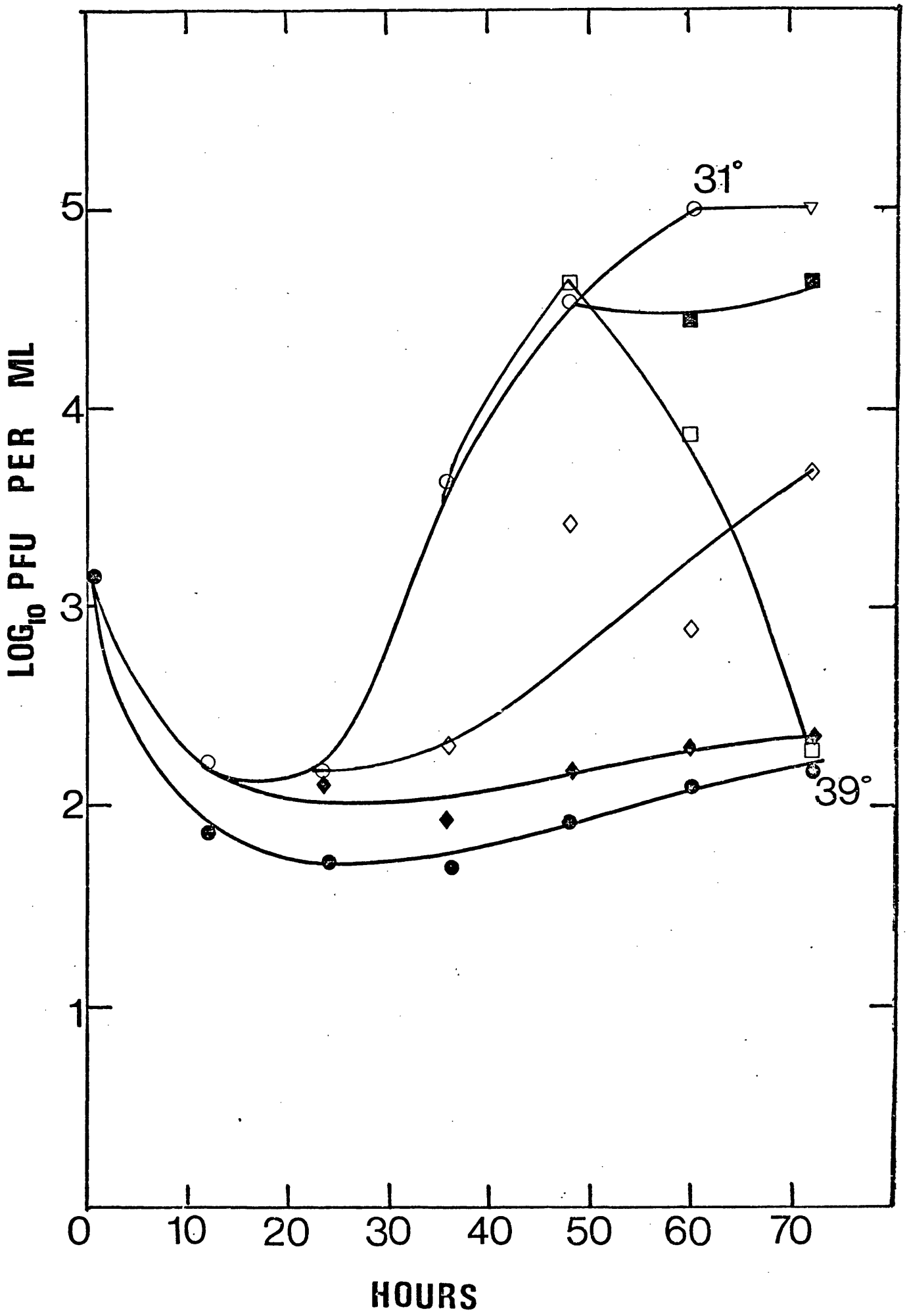
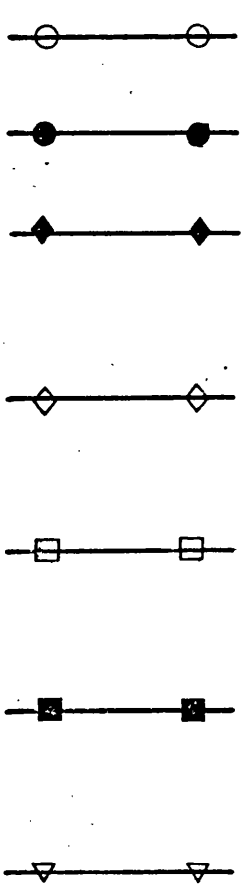

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 28b : Downshift growth curves of ts 16 (Group I)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 39°C, downshift at 12 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 24 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 36 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 48 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 60 hours p.a. to 31°C.

MOI : 0.20 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)

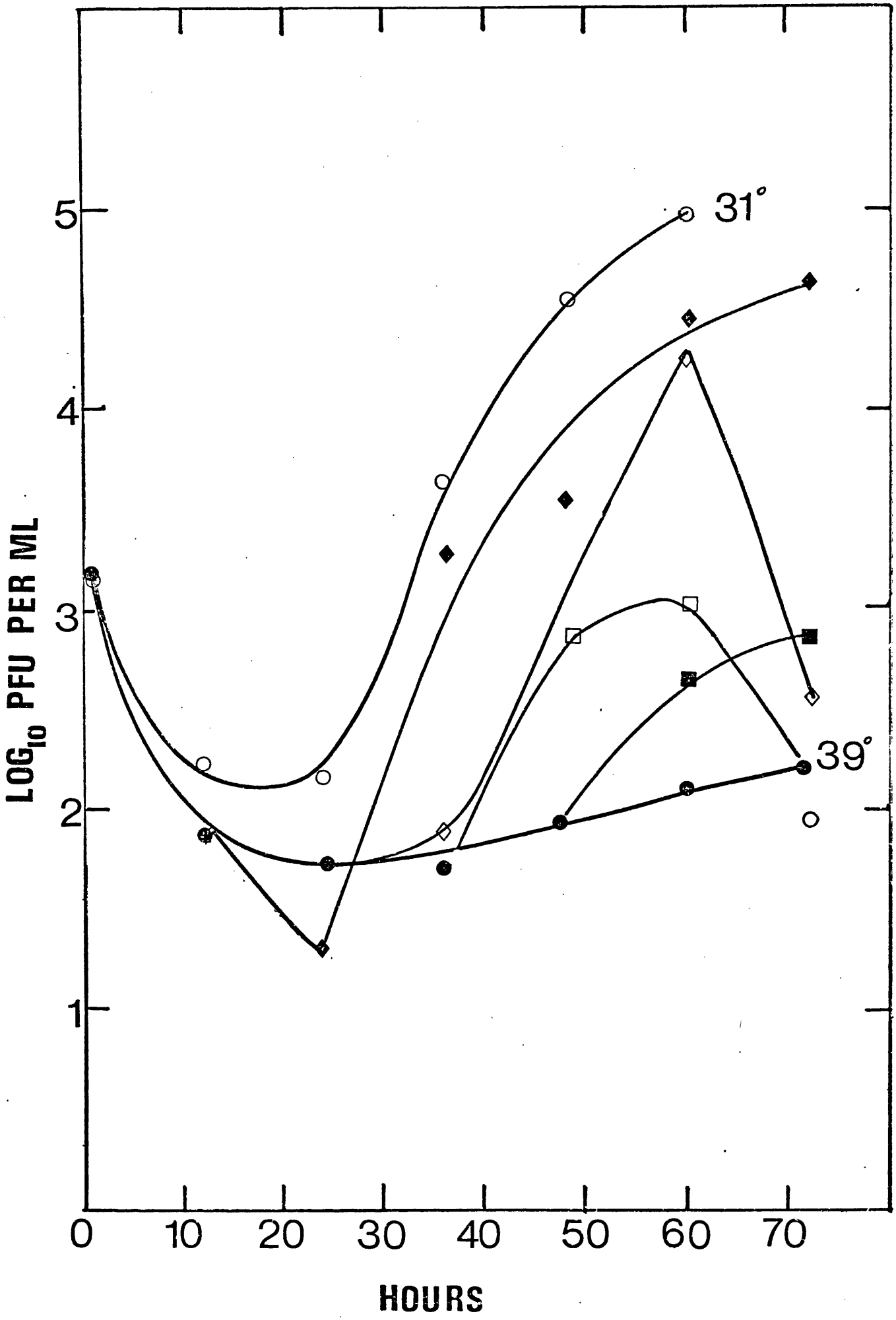
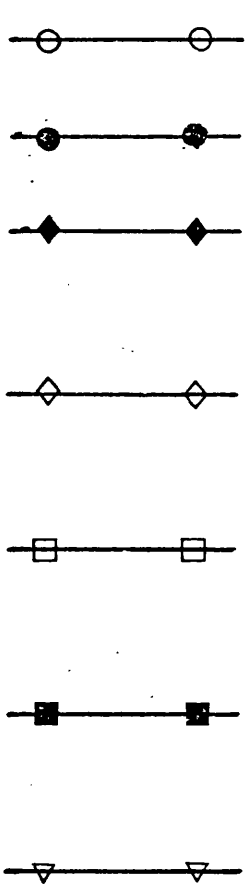

31°
39°
5
4
3
2
1
0
10
20
30
40
50
60
70
LOG10 PFU PER ML
HOURS

Figure 29a : Upshift growth curves of ts 19 (Group I)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 31°C, upshift at 12 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 24 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 36 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 48 hours p.a. to 39°C.

Growth of ts mutant at 31°C, upshift at 60 hours p.a. to 39°C.

MOI : 0.45 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)

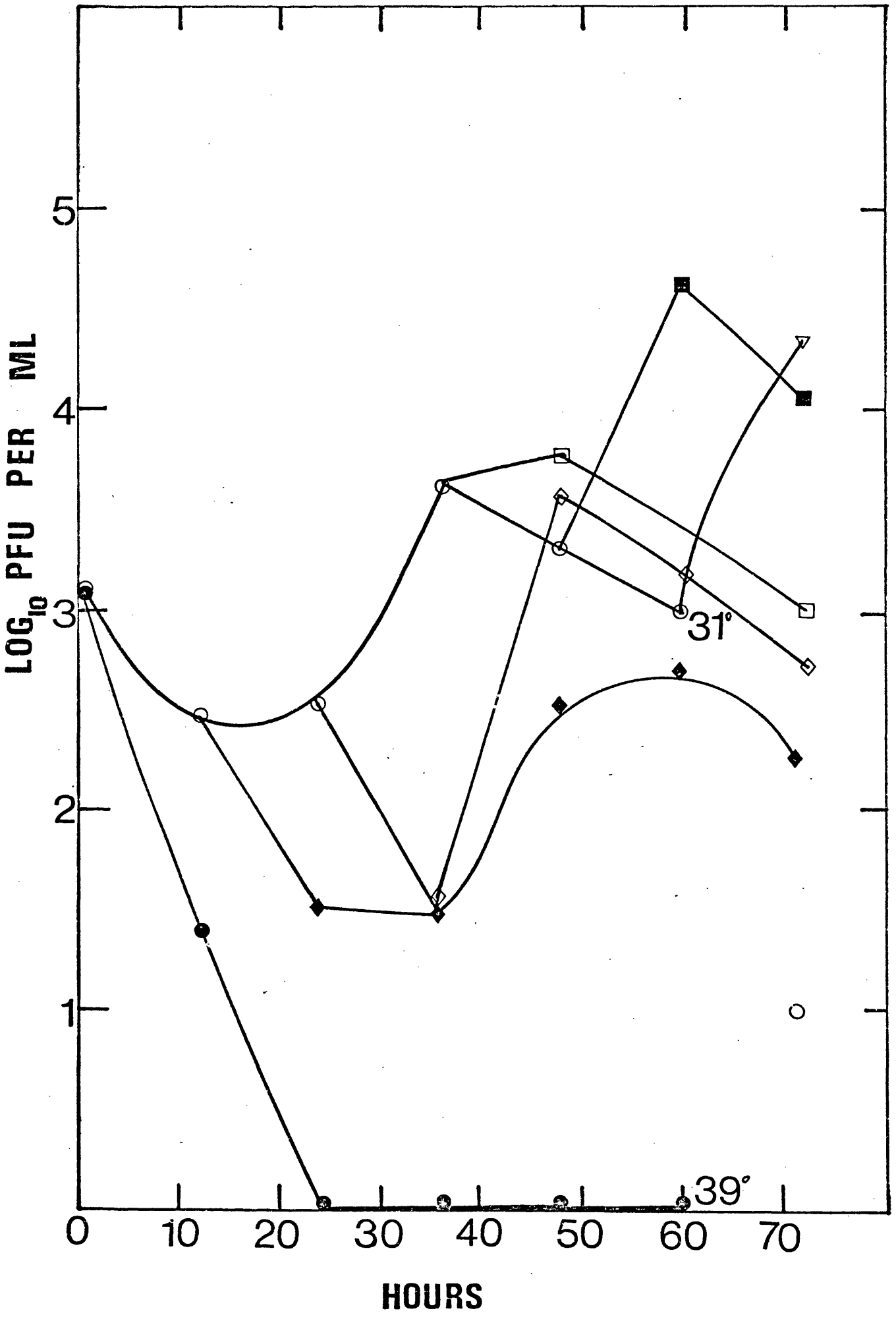
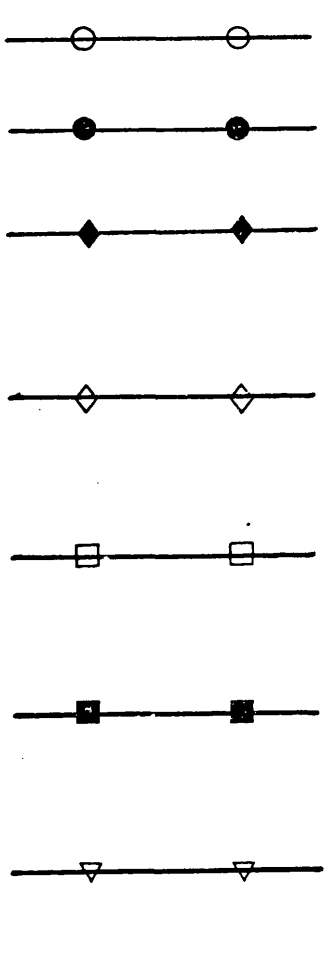

LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

Figure 29b : Downshift growth curves of ts 19 (Group I)



Growth of ts mutant at 31°C.

Growth of ts mutant at 39°C.

Growth of ts mutant at 39°C, downshift at 12 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 24 hours p.a. to 31°C.

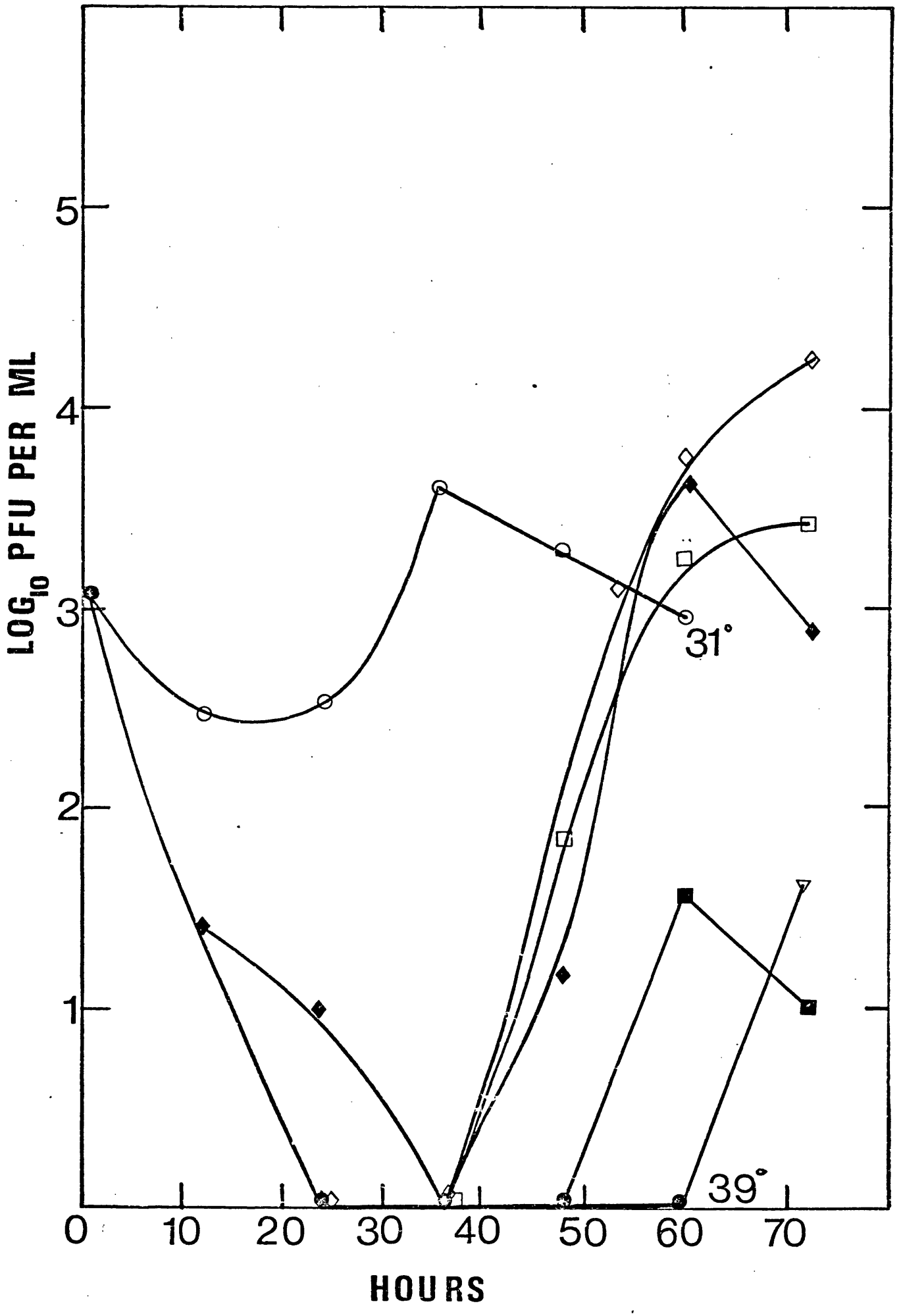
Growth of ts mutant at 39°C, downshift at 36 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 48 hours p.a. to 31°C.

Growth of ts mutant at 39°C, downshift at 60 hours p.a. to 31°C.

MOI : 0·45 PFU/CELL

TIME : HOURS POST-ADSORPTION (p.a.)


LOG10 PFU PER ML
5
4
3
2
1
0
10
20
30
40
50
60
70
HOURS
31°
39°

3.10 The heat stability of ts mutants of RS virus

The heat stability of the ts mutants was examined to determine whether the gene functions affected by the mutations were heat sensitive. If the ts lesion affects a structural protein the virion may be heat sensitive whereas if it involves a non-structural protein the virion is not expected to be heat sensitive.

The heat stability of some ts mutants (ts A2, ts A7, ts 9, ts 15, ts 16, ts 19, ts 23) was examined over a period of 2 hours at 39°C (Figure 30) and calculated as percentage of residual virus (Table 26). Ts A2 had a percentage of residual virus (58%) similar to that of wild type virus (52%) whereas ts A7 had a lower value (26%). These results are similar to those found by other workers (Kalica et al., 1973) for these ts mutants taking into account differences in experimental conditions.

The other ts mutants tested (ts 15, ts 19, ts 23) were very heat sensitive with the exception of ts 9 and ts 16. Ts 9 was a heat stable as wild type virus whereas ts 16 was more heat sensitive than wild type virus, although not as heat sensitive as the other ts mutants.

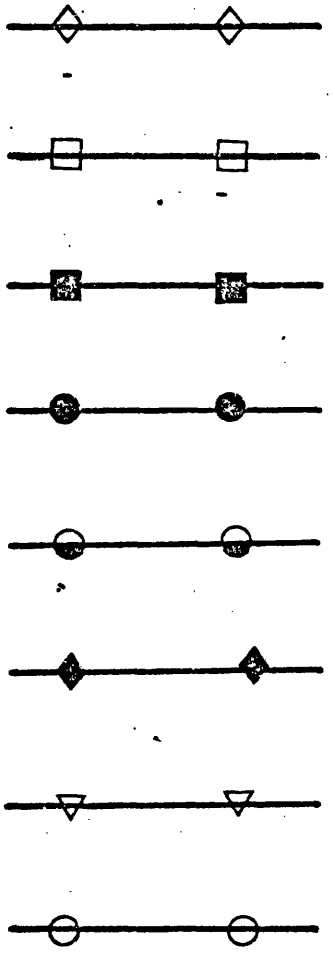
The ts mutants studied fall into three groups. In the first group are the ts mutants (ts 15, ts 19, ts 23) which are very heat sensitive at 39°C. Therefore the ts phenotype of these mutants could be due to their thermolability at 39°C. In the second group are those ts mutants (ts 16, ts A7) whose heat sensitivity at 39°C is intermediate between the level of heat sensitivity of wild type virus and the very sensitive group of ts mutants. The third group of ts mutants (ts A2,

TABLE 26

Heat stability of *ts* mutants of RS virus

ts mutant	Percentage residual virus after 2 hr at 39°C
ts A2	58
ts A7	26
ts 9	50
ts 15	0.6
ts 16	18
ts 19	1.5
ts 23	3.6
RSN-2 wild type virus	52

III) and Group B (i.e. Group II) ts mutants are heat sensitive.



ts 15

ts 19

ts 23

ts 16

ts A7

ts 9

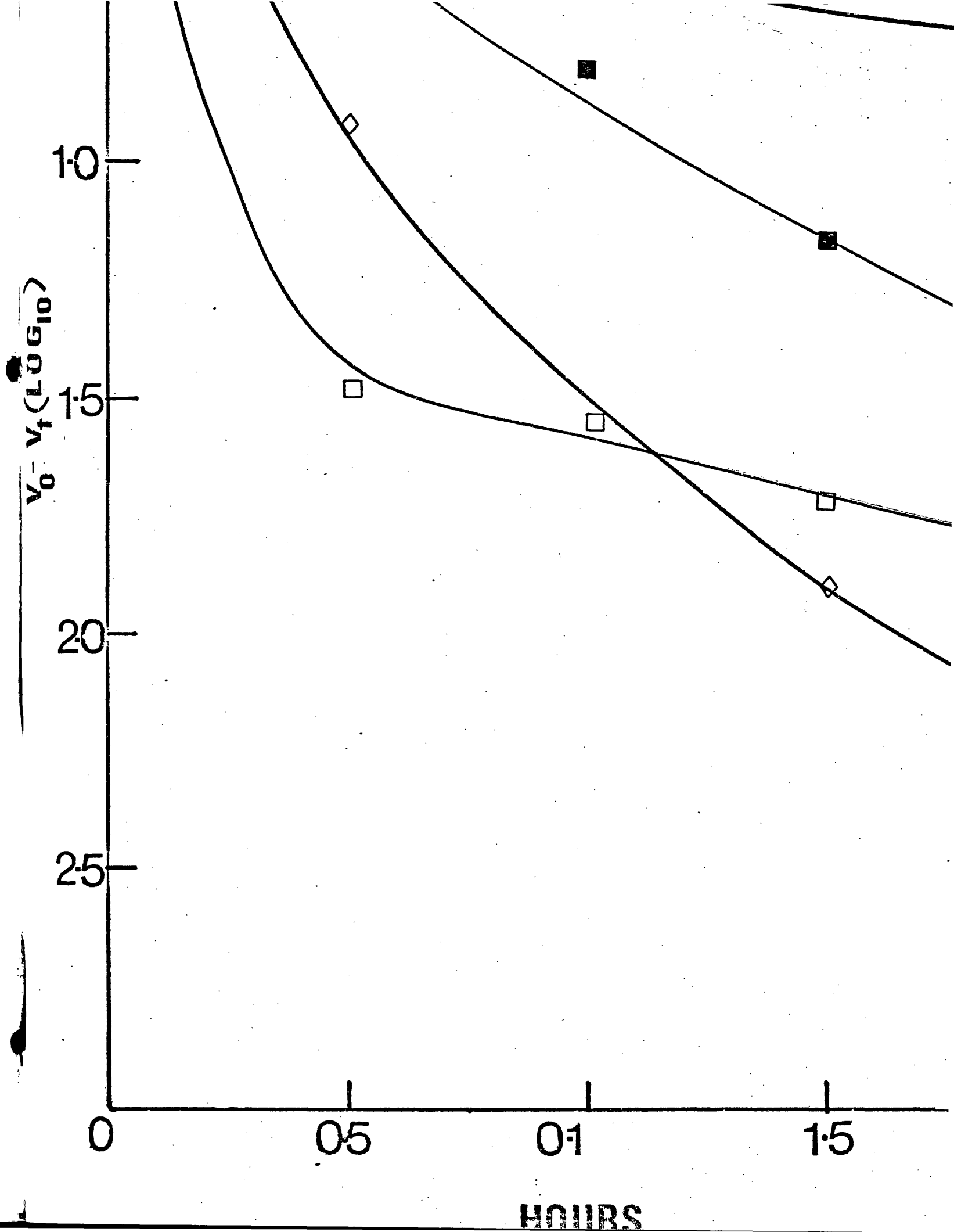
ts A2

wild type RS virus

(RSN-2 strai

V_0 - infectivity at 0 hours (log_{10}/ml).

V_T - infectivitv at > 0 hours (log_{10}/ml).


1·0
1·5
2·0
2·5
$V_0 - V_t (LOG_{10})$
0
0·5
0·1
1·5
HOURS

3.11 Polymerase activity associated with ts mutants of RS virus (RSN-2 strain)

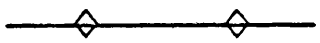
It has been suggested that RS virus may have viral associated RNA-polymerase activity (Szilagyi and Pringle, in preparation).

Experiments undertaken with wild type RS virus and ts mutants to detect polymerase activity were hampered by the high background activity found in uninfected BS-C-1 cells (Figure 31). Ts mutants ts 1, ts 2, ts 3, ts 5 and ts 19 were assayed for polymerase activity by Dr. J. Szilagyi, and ts 15, ts 16, ts 17, ts 19, ts 23 wild type virus and uninfected BS-C-1 cells by myself. The level of polymerase activity in the uninfected BS-C-1 cells studied was similar to that found associated with the wild type RS virus sample (Figure 31). However in some of the ts mutant samples the level of polymerase activity was 2-fold higher than that found associated with uninfected BS-C-1 cells. The polymerase activity was higher at 31°C than at 39°C in the ts 15 and ts 16 samples (Figure 31) and it declined more rapidly at 39°C than at 31°C. In the samples ts 2, ts 3, ts 5 and ts 17 (Figure 32) the polymerase activity was also 2-fold greater than in uninfected BS-C-1 cells but higher at 39°C than 31°C, declining more rapidly at 39°C. No polymerase activity above the background level was detected in the samples of ts 1, ts 18, ts 19 and ts 23 which were tested.

There was no correlation between enzyme activity and virus infectivity of the sample tested. The ts mutants with no polymerase activity above background level had the same (e.g. ts 1) and higher (e.g. ts 18, ts 19, ts 23) titres than those with greater polymerase activity. It is possible that cellular rather than viral polymerase

activity was measured since the virus preparations used were not pure but crude PEG precipitate suspensions.

Figure 31 : RNA-dependent RNA-polymerase assay



Polymerase activity at 31°C

(counts per minute x 10^3).

Polymerase activity at 39°C

(counts per minute x 10^3).

Infectivity (pfu) of polymerase sample (0.1 ml)

wild type virus : 8.0×10^5

ts 15 : 7.0×10^5

ts 16 : 8.5×10^5.

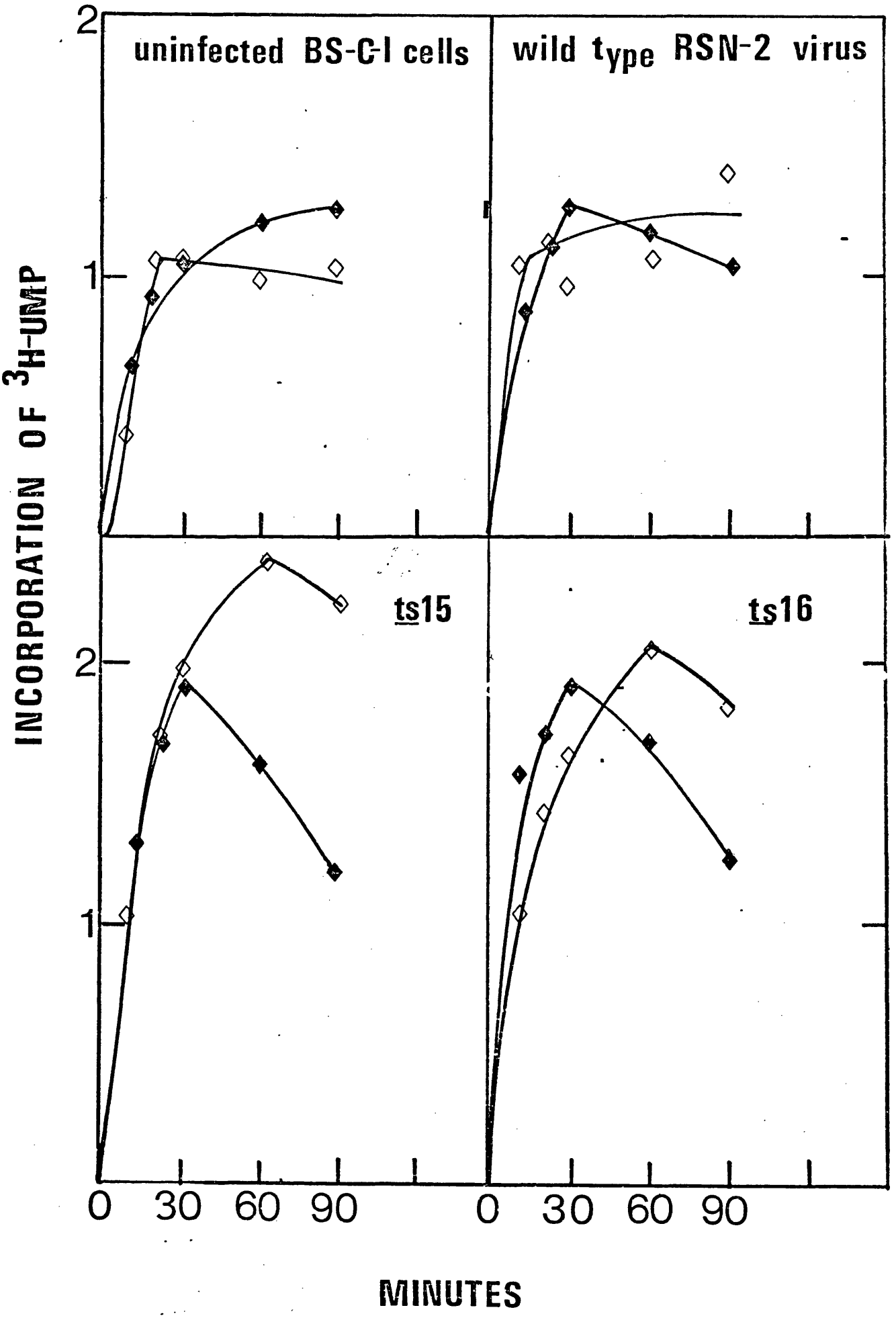
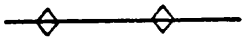

uninfected BS-C-1 cells
wild type RSN-2 virus
ts15
ts16
INCORPORATION OF ^{3}H-UMP
MINUTES
0
30
60
90
1
2

Figure 32 : RNA-dependent RNA-polymerase assay



Polymerase activity at 31°C

(counts per minute X 10^3).

Polymerase activity at 39°C

(counts per minute X 10^3).

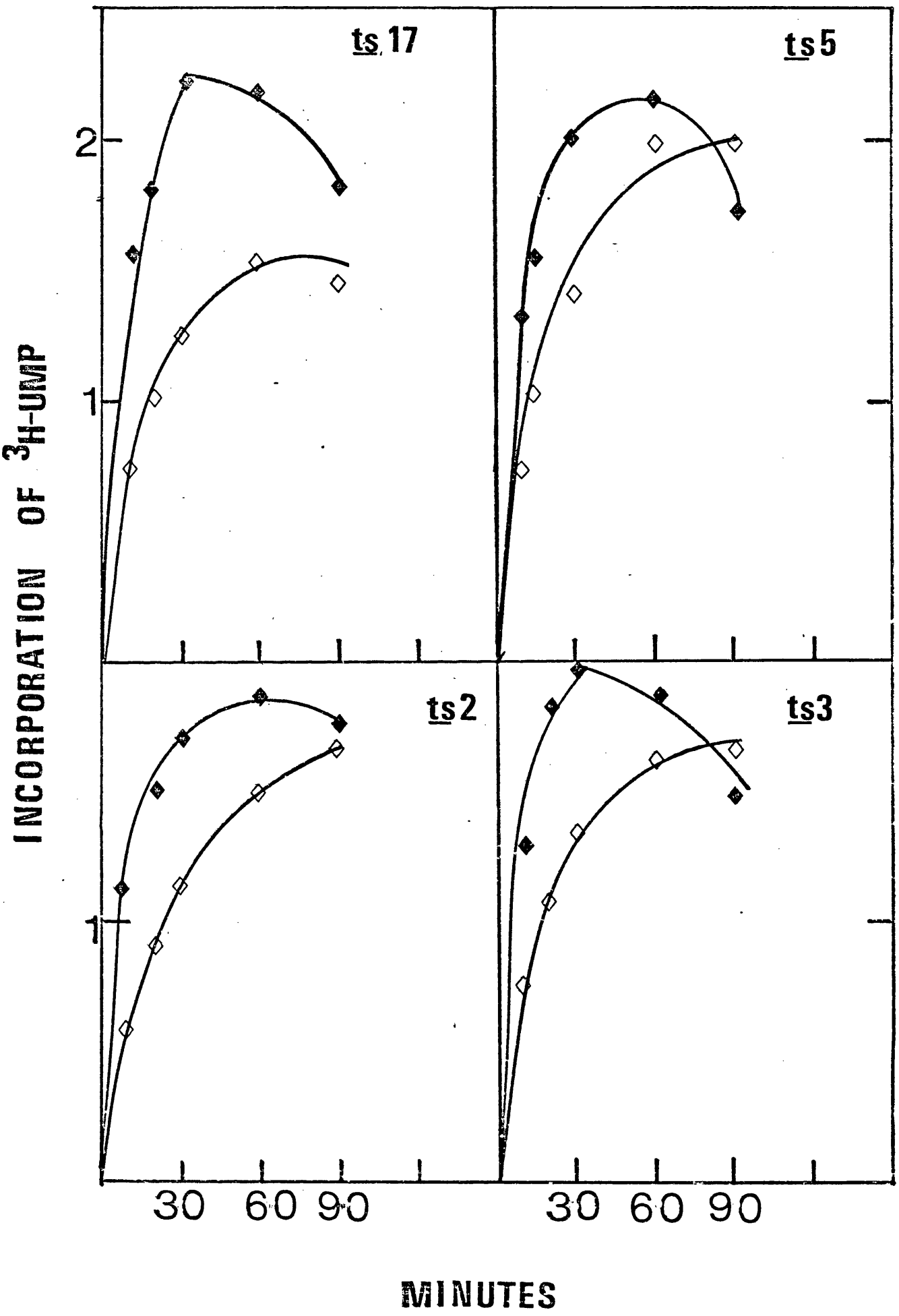
Infectivity (pfu) of polymerase sample (0.1 ml)

ts 2 : 3.5×10^6

ts 3 : 7.0×10^5

ts 5 : 1.3×10^6

ts 17 : 7.5×10^5.


ts 17
ts 5
ts 2
ts 3
2
1
1
30 60 90
30 60 90
INCORPORATION OF ^{3}H-UMP
MINUTES

Section 4

Discussion

Section 4 - Discussion

4.1 The biology of RS virus in BS-C-1 cells

In this discussion the biological properties of the RSN-2 strain of RS virus will be compared with those of other strains of RS virus in different cell lines.

The RS virus growth cycle is relatively long, 30 - 60 hours or longer depending on the host cell used. In BS-C-1 cells the latent period of the RSN-2 strain and the Long strain is similar (10 - 12 hours) and their growth cycles take 36 - 48 hours (see Figures 3 and 4). Levine and Hamilton (1967, 1969) found that the latent period of the Long strain in HeLa cells is similar to that in BS-C-1 cells but shorter than that in HEp-2 cells (12 - 16 hours).

In BS-C-1 cells the RS virus (RSN-2 and Long strain) growth cycle is longer than in HeLa cells (20 - 30 hours) but shorter than in HEp-2 cells (48 - 60 hours) (Bennet and Hamre, 1962; Richman and Tauraso, 1971; and Wilcyznski, 1971). Therefore the length of the RS virus growth cycle depends on the host cell used in the experiment.

The titre of RS virus is very poor irrespective of the cell line used, since it does not usually exceed 2×10^7 pfu/ml. This is probably due to the fact that the virus is very cell-associated. For example as much as 84% of the Long strain and 99% of the RSN-2 strain of the virus remain cell-associated in BS-C-1 cells. Levine and Hamilton (1967, 1969) also found the Long strain very cell-associated, 90% in HeLa cells and 96% in HEp-2 cells, although Jordan (1962) only found 50% of the Long strain cell-associated in HEp-2 cells. The degree

of cell-association of RS virus appears to depend on both the strain of virus and host cell used.

A wide variation in the rate of adsorption of RS virus to cells has been reported. This variation also appears to be related to the strain of virus and host cell used. Adsorption of the RSN-2 strain to BS-C-1 cell monolayers seems to be more efficient than that of the Long strain to HEp-2 or HeLa cell monolayers (see Table 27). However, these results are not directly comparable since the methods and RS virus strains used were different. It is known that variation in RS virus adsorption can occur between strains of RS virus. For example the Long and 18537 strains adsorbed more efficiently to HEp-2 cell monolayers (Coates et al., 1966) than the Randall strain (Bennet and Hamre, 1962). Adsorption to cells in suspension seems to be much faster than to cell monolayers, for example, the Long strain adsorbed faster to HeLa cells in suspension than to monolayers (see Table 27). However, the adsorption of the Burnett strain to HEp-2 cells in suspension (Richman and Tauraso, 1971) was slower than that of the Long strain to HEp-2 cell monolayers. This variation was probably due to the different virus strain used but may also be related to cell type as the Burnett strain adsorbed much faster to MA-160 cells (a continuous human heteroploid line derived from benign prostate) in suspension than to HEp-2 cells in suspension (Table 27).

The data presented for RS virus (RSN-2 strain) adsorption to BS-C-1 cells (1 hour at 25°C) was not adjusted for thermal inactivation as this was presumed to be negligible based on the results of earlier experiments (Hambling, 1964b; and Rechsteiner, 1969a). Thermal

inactivation of RS virus was taken into account in their data for the Burnett strain by Richman and Tauraso (1971). This data was adjusted for a 10-fold reduction in RS virus titre over 24 hours at 37°C based on earlier inactivation experiments by other workers (Beem et al., 1960; and Hambling, 1964b). However, the data for the adsorption of the Long strain to HeLa and HEp-2 cells at 37°C were not adjusted since between 91 and 113% of the original virus could be accounted for; thermal inactivation over 2 hours at 37°C was considered negligible (Levine and Hamilton, 1969).

Following adsorption (2 hours at 37°C) 45 minutes incubation was required for complete penetration of the Long strain inoculum into HeLa cells. Most of the cell-associated virus (90%) was neutralised by the addition of specific antisera therefore it was suggested that this virus was attached to the external surfaces of the cells (Levine and Hamilton, 1969).

The type of RS virus CPE depends on the host cell and medium used (Jordan, 1962). RS virus infected BS-C-1 cells under agar do not produce syncytia but dark-staining foci of heaped cells (plate 1). When examined using a scanning electron microscope uninfected BS-C-1 cells were seen to have some microvilli (plate 13). However, in RS virus infected cells the number and length of these processes increased markedly, as can be seen from the scanning electron microscope photographs, plates 14 - 17. These processes could correspond to the viral processes seen in thin sections of RS virus infected cells (plates 11, 12) and in RS virus infected cells (plate 22).

RS virus infection does not necessarily result in cell death since ts mutant infected cells can produce virus without visible CPE at the restrictive temperature (39°C). These ts mutants are probably defective in some cytolytic function since they grow but do not plaque at 39°C.

Multiplicity-associated interference was detected in experiments using ts mutants of RS virus (Figure 23a and b). A similar type of interference was noted in an earlier experiment by Beem and Treuhaft (1972) using HEp-2 cells infected with wild type virus at various multiplicities. The yield per cell of RS virus from these cells was low (0.2 pfu/cell) when a high moi (moi 7) was used but high (25 pfu/cell) when a lower moi (moi 0.02) was used. This interfering activity was not due to the action of interferon since it could be sedimented (30,000 rpm for 15 minutes) and was sensitive to acid and ether treatment. Therefore it is possible that defective RS virus particles are responsible for interference of RS virus replication.

Defective interfering particles usually contain normal viral structural protein but only part of the viral genome. Replication of these particles only occurs in the presence of normal virus. The interference of normal virus replication by defective interfering particles results in an inhibition or reduction in yield. If undiluted virus is passaged the proportion of defective particles in the progeny increases (Huang and Baltimore, 1970).

Host factors may be involved in RS virus growth since complementation between mixed infections of ts mutants (ts 15 and ts 23,

ts 19 and ts 23 and ts 15 and ts 23) could not be detected in BS-C-1 cells (Table 21a) but could be detected in HeLa cells (Table 21c). However, the requirement for these factors or factor may be variable since complementation may occur in BS-C-1 cells. The low frequency "recombination" detected between ts 1 and ts 5 (Table 23) is probably not true recombination but complementation. Aggregated virus particles or heterozygotes could complement at 39°C giving rise to virus with apparent ts^+ phenotype.

Nuclear antigen can be detected in ts RS virus infected cells at 39°C. The detection of RS virus specific material in the nucleus is interesting since it could possibly be related to the existence of a RS virus proviral DNA which has been recently reported (Simpson and Iinuma, 1975). This DNA was isolated from bovine embryonic kidney (BEK) cells which had been infected with a ts mutant of RS virus and passed at the non-permissive temperature (39°C) for more than 80 cell generations. No viral CPE was detected during the passage of these infected cells but infectious virus could be recovered from the cells by co-cultivation at the permissive temperature (31°C) or by treatment with halogenated pyrimidines. The DNA but not RNA extracted from the infected cells produced RS virus plaques when titrated on HEp-2 cells. When this extracted DNA was treated with DNase it lost its infectivity but not when it was treated with RNase. The DNA did not produce plaques in cells which had been pre-treated with actinomycin D or mitomycin C. RS virus proviral DNA was also recovered from HEp-2 cells persistently infected with RS wild type virus.

DNA intermediates have also been reported in cells persistently

infected with other RNA viruses (measles, tick borne encephalitis virus (TBE), Sindbis virus) (Zhadanov, 1975). More work needs to be done in this area in order to confirm these findings and to extend the search for proviral DNA to other RNA viruses. If RNA viruses have a DNA intermediate it cannot be essential for viral replication at least in the case of RS virus since it grows in enucleate cells. However the existence of proviral DNA in these RNA viruses would alter the present concept of replication and function of RNA viruses since only RNA tumour viruses were thought to have a DNA intermediate.

The inhibitory effect of actinomycin D on RS virus growth could be related to the existence of a proviral DNA. However, it could be due simply to the declining ability of BS-C-1 cells treated with actinomycin D to support RS virus growth. This is possible since the yield of VSV in the presence of actinomycin D was also reduced at 14 hours (Figure 7). Actinomycin D does not inhibit VSV virus replication directly (Hummeler, 1971). The reduction in yield is probably due to the effect of actinomycin D on the cells. Although RS virus growth is reduced in the presence of actinomycin D it is not affected to the same extent as PSRV (a DNA virus), which is completely inhibited. This was to be expected since RS virus does not require the presence of an intact nucleus for growth since it can grow in enucleate cells (Follet et al., 1975). However, the yield of RS virus from enucleate cells is only 40% of that from nucleate cells at 31°C since the enucleate cells deteriorate markedly before completion of the RS virus growth cycle.

Actinomycin D reduced the yield of RS virus when added immediately or a short time after adsorption (Table 13). This effect

RS virus yield is reduced in the presence of mitomycin C (10 μg/ml) to 59% whereas the yield of PSRV is less than 0.02% in the same concentration. Therefore RS virus does not require DNA replication. The reduction in RS virus yield could be due to a deterioration in the cell's ability to support virus growth due to the effect of mitomycin C.

RS virus protein synthesis can be inhibited by the removal of specific amino acids (i.e. arginine or phenylalanine) from the maintenance medium or by the addition of cycloheximide to the maintenance medium.

The growth of both the RSN-2 and the Long strains of RS virus is inhibited in arginine deficient medium. However, the addition of arginine reverses this effect (Figure 5). There is a maximum concentration of arginine (84 μg/ml) beyond which the addition of more arginine will not enhance virus growth (Figure 6). These results confirmed the findings of Levine and his colleagues (1971) who found that the growth of the Long strain of RS virus was inhibited in HeLa cells in phenylalanine (PA) or arginine deficient maintenance medium. In PA deficient medium (1 μg/ml PA) protein synthesis occurred at the normal rate for 6 hours and then the rate markedly decreased. This time was not long enough for the completion of the RS virus latent period therefore no RS virus was produced. However, RS virus production occurred within 2 hours of the addition of PA (5 μg/ml) to the maintenance medium of the infected cells which had been in deficient medium for 18 hours. This short latent period (2 hours) suggested that early events in the RS virus growth cycle had been completed during the time in PA deficient medium. PA was not a specific requirement since arginine depletion had a similar

effect on RS virus growth. It was suggested that at least one structural component was not made in RS virus infected cells in PA or arginine deficient medium. Also suggested was that the amino acid deficiency could block migration of RS virus antigen from the site of synthesis to the cell membrane (Levine et al., 1971). However, we found that the ratio of incorporation of ^{14}C-arginine to ^{3}H-leucine was high for all of the five RS virus polypeptides which were detected by PAGE. It was suggested that exogeneous arginine was a limiting factor because it was required for incorporation into RS virus polypeptides (Wunner et al., 1975).

Not all amino acids are essential for RS virus growth, however, since the virus grows well in methionine-free medium. However, 1% serum is required for good RS virus growth and at least 10% for optimal growth.

Cycloheximide (25 μg/ml) has been used by Levine and Hamilton (1969) to study RS virus protein synthesis. It inhibited RS virus production when added any time after infection. Therefore RS virus protein synthesis appears to be required throughout the growth cycle. It was suggested that continuous protein synthesis was required because there was no accumulation of RS virus structural proteins in the infected cells and that protein synthesis is essential for maturation of the RS virion, perhaps required in the budding process.

Evidence has been presented to suggest that RS virus is a negative strand virus (Wunner et al., 1975) based on the presence of a fast sedimenting RNA component (50S) and low molecular weight RNA (4S)

in the cytoplasm of infected cells. However, RS virus RNA has not yet been extracted and characterised. Therefore whether this RNA is complementary to m-RNA and non-infectious is not known. ^{3}H-thymidine is incorporated similarly into both RS virus-infected and uninfected cells indicating the virus did not contain DNA. Also incorporation of ^{3}H-uridine into virus specific RNA could be detected in the presence of actinomycin D (2.5 μg/ml) which was added late. The other feature of negative strand viruses is the possession of a virion associated RNA-dependent RNA-polymerase (transcriptase). RS virus may have a virion associated RNA-dependent RNA-polymerase (Szilagyi and Pringle, in preparation) but evidence for the existence of this polymerase in experiments detailed in this thesis is not conclusive. It is possible that the polymerase detected in these experiments (Figures 31, 32) was cellular rather than viral. RS virus is morphologically similar to the ortho- and para-myxoviruses, which are negative strand viruses. Its nucleocapsid is similar in structure and intermediate in width between that of the orthomyxoviruses and the paramyxoviruses. The evidence available at present favours the idea that RS virus is a negative-strand virus.

4.2 The temperature-sensitive mutants of RS virus

The ts mutants of the RSN-2 strain were classified and compared with the ts mutants of the A_2 strain of RS virus.

The RSN-2 ts mutants were isolated from virus treated with or grown in the presence of various mutagens. However, the overall frequency of isolation of these ts mutants was low and not significantly different from the frequency of isolation of the spontaneous ts mutant (Table 15). In two instances the frequency of isolation was significantly greater than the spontaneous frequency. In both cases the virus was grown in the presence of 50 µg/ml of 5-FU. The increased number of ts mutants may have been picked due to chance or due to effective mutagenisation increasing the proportion of ts mutants in the virus population. The ~~latter~~ former explanation is most probable as an increase in the proportion of ts mutants due to mutagenisation is unlikely since there is no relationship between concentration of mutagen and frequency of isolation of ts mutants. Therefore it must be presumed that all of the ts mutants isolated were spontaneous rather than induced.

Genetic analysis of the RSN-2 strain of ts mutants could not be undertaken in BS-C-1 cells since complementation between ts mutants was not reproducible. However, complementation could be detected in mixed infections of ts mutants in HeLa cells. BS-C-1 cells may be unsuitable for use in complementation analysis because they are large cells and RS virus particles may not complement unless in close proximity. From the data obtained from mixed infections in HeLa cells there appear to be at least three different complementation groups (Table 28) which correspond to Groups I, II and III of the immunofluorescence classification.

TABLE 28

Complementation between ts mutants representing Groups I, II and III

		BS-C-1			HeLa		
GROUP	ts mutant	ts 19	ts 23	ts 15	ts 19	ts 23	ts 15
I	ts 19		-	-		+	+
II	ts 23			-			+
III	ts 15						

\+ Complementation index above 10

\- Complementation index below 10.

Since genetic analysis using BS-C-1 cells was not feasible other methods of analysis were attempted. The results of the analyses are summarised in Table 29. The ts mutants can be classified into three groups by indirect immunofluorescence based on their growth pattern in BS-C-1 cells at 39°C (Table 24). The A_2 strain ts mutants can also be divided into three groups (Gharpure et al., 1969); however these groups do not correspond exactly to those of the RSN-2 strain. Ts A2 produced more antigen than wild type RS virus at 24 hours and about the same amount at 48 hours after infection at 39°C. This A_2 ts mutant shows the same pattern at 39°C as RSN-2 ts mutants belonging to Group II. Ts A4 produced no antigen in infected cells at 39°C. This ts mutant does not correspond to any RSN-2 strain ts mutant. The other ts mutants, ts A1 and ts A3 produced reduced amounts of viral antigen at 39°C. These mutants could correspond to those RSN-2 ts mutants belonging to Group III or I depending on whether the number of infected cells was also reduced as well as viral antigen. The pattern of complement-fixing (CF) antigen development at 39°C in individual ts mutants of A_2 strain was similar to that of their immunofluorescent antigen. For example, ts A2 produced as much CF antigen at 39°C as did wild type virus (Gharpure et al., 1969).

RS virus specific-staining was seen in the nucleus of some RSN-2 ts mutant infected cells at 39°C. Nuclear fluorescence had not previously been detected in any immunofluorescent experiments. No nuclear antigen was detected in cells infected with the ts mutants of the A_2 strain of RS virus. However, this is probably explained by the fact that these ts mutants were only stained with human anti-RS virus serum which does not have antibodies or have them in too low a

TABLE 29

Physiological classification of *ts* mutants

Method	GROUPS		
Fluorescence	I	III	II
Growth at 39°C	A	A	B
Temperature-shift experiment	"Early" function affected (*ts* 16, *ts* 19)	"Early" function affected (*ts* 15)	Function required throughout growth cycle. Functional activity rather than synthesis *ts* (*ts* 9)
Thermolability	labile (*ts* 16, *ts* 19)	labile (*ts* 15)	labile/stable (*ts* 23) (*ts* 9)

concentration to detect nuclear antigen. Nuclear antigen could only be detected when bovine anti-bovine RS virus serum was used to stain RSN-2 ts mutant infected cells, not when human anti-RS virus serum was used.

The high antibody titre of bovine convalescent serum is the result of a good immunological response in calves to parenteral inoculation. Human convalescent sera has a lower titre as natural infection does not evoke such a good response. Bovine RS virus and human RS virus are antigenically similar (Inaba et al., 1972 and Paccaud and Jacquier, 1970). The RS virus staining was specific since antiserum absorbed with RS virus infected cells when used did not stain the infected cells. There was one earlier report of RS virus having some kind of nuclear involvement. Armstrong and his colleagues (1962) detected nuclear inclusions in RS virus infected HeLa cells. However, this finding has never been confirmed.

From temperature-shift experiments the ts defects of the ts mutants ts 16, ts 19 and ts 15 classified in Groups I and III were found to occur in "early" functions. Therefore these ts mutants could be defective in viral nucleic acid synthesis. Since these ts mutants were also very thermolabile a particle-associated polymerase could be affected.

Since RS virus does not plaque and grow as well in primary cell ~~lines~~ as in continuous cell lines, a continuous cell line (BS-C-1 cells) had to be used initially to develop a good system for the isolation of ts mutants. A satisfactory system, i.e. the sector-plating

method, has been developed, therefore it will now be simpler to isolate RS virus ts mutants in a suitable cell line for vaccine work e.g. BEK cells. As the growth of RS virus ts mutants appears to be affected by the type of host cell used it would be valuable to evaluate possible ts vaccine mutants in human embryonic lung cells and in cultures of pieces of lung tissue.

Appendix 1

Media, Stains and Resin

1. Medium

Eagles MEM (Glasgow modification) was used (Busby et al., 1964). This medium contains twice the concentration of amino acids and vitamins present in Eagles MEM.

Growth medium

Eagles MEM (Glasgow modification) + 10% FCS.

Maintenance medium

Eagles MEM (Glasgow modification) + 5% FCS.

Arginine free medium

Eagles MEM (Glasgow modification) without arginine except that present in FCS. Normal Eagles MEM (Glasgow modification) contains 42 μg/ml of Arginine.

Methionine free medium

Eagles MEM (Glasgow modification) without methionine. Normal Eagles MEM (Glasgow modification) contains 15 μg/ml methionine.

2. Phosphate buffered saline (PBS)

The PBS used unless otherwise indicated was that described by Dulbecco and Vogt (1954).

Solution A	To make 12 litres
NaCl	120 g
KCl	3 g
Na_2HPO_4	17.25 g
KH_2PO_4	3 g

Solution B	To make 1.5 litres
$CaCl_2\ 2H_2O$	1.5 g

Solution C	To make 1.5 litres
$MgCl_2\ 6H_2O$	1.5 g

PBS is prepared when required by mixing 8 parts of solution A with 1 part of solution B and then by the addition of 1 part of solution C. The PBS is sterilised by autoclaving for 10 mins at 10 lb/sq. in.

PBS (0.01M) pH 7.2

This PBS was used in immunofluorescent experiments.

	To make 5 litres
NaCl	42.5 g
$Na_2H\ PO_4$	5.3 g
$NaH_2PO_4.2H_2O$	1.9 g

The chemicals were dissolved in distilled water (5,000 ml) and the pH was checked using a pH meter. Then the solution was dispensed in 500 ml amounts and this was sterilised at 10 lbs/sq. in for 10 minutes.

3. Buffered glycerol saline pH 8.3

Pure glycerine	80 ml
PBS (0.01M) pH 7.2	20 ml

The solutions were mixed thoroughly and the pH adjusted to 8.3. Then the solution was dispensed in 4 ml amounts and stored at $-4^{\circ}C$.

4. Trypsin

Trypsin was used at a concentration of 0.25% (w/v) in Tris saline for the removal of cells from glass.

5. Versene (Diaminoethanetetra-acetic acid disodium salt)

Versene (0.6 mM) was dissolved in PBS and 0.002% (v/v) phenol red added.

6. Formal saline

The formal saline used was a solution of 4% (v/v) formaldehyde which also contained 85 mM NaCl and 0.1M Na_2SO_4.

7. Giemsa

A suspension of 1.5% (v/v) of Giemsa in glycerol was heated at $56^{\circ}C$ for 90-120 minutes. Then the solution was diluted with an equal volume of methanol. (Dacie and Lewis, 1968).

8. Agar overlay

Difco-Bacto agar (3.6%) was melted and poured into warm 1.3 Eagles MEM (Glasgow modification) without phenol red. The final percentage of agar in the medium was 0.9% (w/v); the medium was supplemented with FCS (4%).

9. Epon

Solution A

6.2 ml Epikote resin

10 ml Dodecenyl succinic anhydride (DDSA)

Solution B

10 ml Epikote resin

8.9 ml Methyl nadic anhydride (MNA)

Solutions A and B were mixed in proportions 3:2 (i.e. 16.2 ml solution A and 10.8 ml of solution B). An accelerator BDMA was added to the mixture of solutions A and B. All of these products were obtained from Polaron Equipment Ltd., Watford, England.

Appendix 2

Preparation of samples for scanning electron microscope examination

The medium was poured off plates (50 mm) containing coverslips of infected and uninfected BS-C-1 cells.

Glutaraldehyde (2.5%) was added to the plates and left on the plates for 30 minutes to fix the cells. Then the glutaraldehyde was poured off and the plates washed with PBS (A, B, C). The PBS was removed and 1% osmic acid (1 ml) added and retained for 5 minutes before being poured off. PBS (A, B, C) was used to wash off the osmic acid. Then distilled water was added and the coverslips stored at $4^{\circ}C$ in distilled water until required.

The coverslips to be examined by the scanning electron microscope were transferred to a holder and placed in alcohol. These coverslips were transferred serially from low to high concentrations of alcohol (25%, 50%, 75%, 90%) and left 15 minutes in each concentration. The coverslips were left for 60 minutes in 100% alcohol. Alcohol treatment was used to dehydrate the cells. From 100% alcohol the coverslips were transferred to a 1:1 solution of 100% alcohol and iso-pentane and left for 15 minutes. Then the coverslips were dipped into warm isopentane for a few seconds before being attached to the microscope. Stubs were coated with carbon and gold. A JEOL, JSM-35 scanning electron microscope was used with permission of JEOL Ltd., London.

Dr. E. Follett took the scanning EM photographs and Mrs. P. McSevney prepared the coverslips using the above procedure.

References

Adams, J.M., Imagawa, D.T. and Zike, K. (1961a)
Epidemic bronchiolitis and pneumonitis related to respiratory syncytial virus. J. Am. med. Ass., 176, 1037-1039.

Adams, J.M., Imagawa, D.T. and Zike, K. (1961b)
Relationship of pneumonia in infants to respiratory syncytial virus. Journal-Lancet, Minneapolis, 81, 502-506.

Adams, M.O., Thompson, K.M. and Tobin, J.O'H. (1963)
Respiratory syncytial virus infection in northwest of England. Arch. ges. Virusforsch., 13, 268-271.

Aherne, W., Bird, T., Court, S.D.M., Gardner, P.S. and McQuillin, J. (1970) Pathological changes in virus infections of the lower respiratory tract in children.
J. clin. Path., 23, 7-18.

Anderson, J.M. and Beem, M.O. (1966)
Use of human diploid cell cultures for primary isolation of respiratory syncytial virus. Proc. Soc. exp. Biol. Med. 121, 205-209.

Andrew, J.D. and Gardner, P.S. (1963)
Occurrence of respiratory syncytial virus in acute respiratory diseases in infancy. Br. med. J., 2, 1447-1448.

Armstrong, J.A., Pereira, H.G., Valentine, R.C. (1962)
Morphology and development of respiratory syncytial virus in cell culture. Nature, Lond., 196, 1179-1181.

Augusten, K., Brunnemann, H., Gruhn, I. and Urbach, H. (1974)
The RS-infected cell in vitro. II Fluorescent photometrical study of RNA and DNA content. Exp. Pathol., 9, 113-117.

Bachi, T. and Howe, C. (1973)
Morphogenesis and ultrastructure of respiratory syncytial virus. J. Virol. 12, 1173-1180.

Bairan, Aurora, C., Cherry, J.D., Fagan, L.F. and Codd, J.E. (1974)
Complete heart block and respiratory syncytial virus.
Am. J. Dis. Child., 127, 264-265.

Barry, R.D., Ives, D.R. and Cruickshank, J.G. (1962)
Participation of deoxyribonucleic acid in the multiplication of influenza virus. Nature, 194, 1139-1140.

Beardmore, W.B. and Hebeka, E.K. (1967)
Evaluation of antibodies to respiratory syncytial virus in humans and experimental animals. Bacteriological Proceedings, 67, 151.

Beem, M. (1967)
Repeated infections with respiratory syncytial virus.
J. Immunol. 98, 1115-1122.

Beem, M., Egerer, R. and Anderson, J. (1964)
Respiratory syncytial virus neutralising antibodies in persons residing in Chicago, Illinois. Pediatrics, Springfield, 34, 761-770.

Beem, M.O. and Treuhaft, M.W. (1972)
Multiplicity associated interference observed in respiratory syncytial virus infections of HEp-2 cells. Pediat. Res. 6, 387.

Beem, M., Wright, F.H., Fasan, D.M., Egerer, R. and Oehme, M. (1962)
Observation on the etiology of acute bronchiolitis in infants.
J. Pediat., 61, 864-869.

Beem, M., Wright, F.H., Hamre, D., Egerer, R. and Oehme, M. (1960)
Association of chimpanzee coryza agent with acute respiratory disease in children. New Engl. J. Med., 263, 523-530.

Bennet, C.R. jr. and Hamre, D. (1962)
Growth and serological characteristics of respiratory syncytial virus.
J. infect. Dis., 110, 8-16.

Berglund, B. (1967)

Studies on respiratory syncytial virus infection.

Acta paediat. scand. (suppl.) 176, 1-40.

Berkovich, S. and Taranko, Y. (1964)

Acute respiratory illness in the premature nursery associated with respiratory syncytial virus infections.

Pediatrics, Springfield, 34, 753-760.

Berthiaume, L., Joncas, J., Boulay, G. and Pavilanis, V. (1973)

Serological evidence of respiratory syncytial virus infection in sheep.

Vet. Rec. 93, 337-338.

Berthiaume, L., Joncas, J. and Pavilanis, V. (1974)

Comparative structure, morphogenesis and biological characteristics of respiratory syncytial (RS) virus and pneumonia virus of mice (PVM).

Arch. ges. Virusforsch. 45, 39-51.

Bishop, D.H.L., Obijeski, J.F. and Simpson, R.W. (1971)

Transcription of the influenza ribonucleic acid genome by a virion polymerase. II Nature of in vitro polymerase product.

J. Virol. 8, 74-80.

Bloth, B.N.T. and Norrby, E.C.J. (1965)

Morphology of subunits of respiratory syncytial (RS) virus.

Arch. ges. Virusforsch., 15, 249-252.

Bloth, B.N.T. and Norrby, E.C.J. (1966)

Fractionation of respiratory syncytial (RS) virus components by centrifugation techniques.

Arch. ges. Virusforsch., 19, 385-392.

Bloth, B.N.T. and Norrby, E.C.J. (1967)

Electron microscopic analysis of the internal component of respiratory syncytial (RS) virus. Arch. ges. Virusforsch., 21, 71-77.

Bloth, B.N.T., Espmark, A., Norrby, E.C.J. and Gard, S. (1963)
The ultrastructure of respiratory syncytial (RS) virus.
Arch. ges. Virusforsch., 13, 582-586.

Bonissol, C. (1966)
Etude de cellules KB infectées par les Myxovirus parainfluenzae de type II et III et le virus respiratoire syncytial.
Annls. Inst. Pasteur, Paris, 111, 265-286.

Brandt, C.D., Kim, H.W., Arrobio, J.O., Jeffries, B.C., Wood, S.C., Chanock, R.M. and Parrott, R.H. (1973)
Epidemiology of respiratory syncytial virus infection in Washington D.C. III Composite analysis of 11 consecutive yearly epidemics.
Am. J. Epidemiol. 98, 355-364.

Brandt, C.D., Patrick, J.R., Chandra, R. and Parrott, R.H. (1970)
Infectious disease epidemiology. Infectious agents from cases of sudden infant death syndrome and from members of their community.
Clin. Proc. Child. Hosp. D.C. 26, 249-260.

Burnet, F.M. (1959)
Genetic interactions between animal viruses. In "The Viruses" volume 3, 275-306, ed. Burnet, F.M. and Stanley, W.M. Academic Press, New York and London.

Burton, J.F. (1964)
Respiratory syncytial virus and parainfluenza myxoviruses as causal agents of acute respiratory infections in Auckland children.
N.Z. med. J., 63, 372-374.

Busby, D.W.G., House, W. and Macdonald, J.R. (1964)
In "Virological Techniques". Churchill, London.

Caul, E.O., Jacobs, J.W. and Clarke, S.K.R. (1974)
Bovine testis cells for routine isolation of respiratory syncytial virus from infants. J. med. Microbiol., 7, 301-304.

Cavallaro, J.J. and Maassab, H.F. (1966)

Adaptation of respiratory syncytial (RS) virus to brain of suckling mice. Proc. Soc. exp. Biol Med. 121, 37-41.

Cavallaro, J.J. and Maassab, H.F. and Abrams, G.D. (1967)

An immunofluorescent and histopathological study of respiratory syncytial virus encephalitis in suckling mice.

Proc. Soc. exp. Biol Med., 124, 1059-1064.

Cavallaro, J.J. and Monto, A.S. (1972)

HL cells, a sensitive line for the isolation and propagation of respiratory syncytial virus.

Proc. Soc. exp. Biol Med., 140, 507-510.

Chanock, R.M. (1970)

Control of acute mycoplasmal and viral respiratory tract disease.

Science, 169, 248-256.

Chanock, R.M. and Finberg, L. (1957)

Recovery from infants with respiratory illness of virus related to chimpanzee coryza agent (CCA). II Epidemiologic aspects of infection in infants and young children. Am. J. Hyg., 66, 291-300.

Chanock, R.M., Kapikian, A.Z., Mills, J., Kim, H.W. and Parrott, R.H. (1970) Influence of immunological factors in respiratory syncytial virus disease. Arch. Environ. Health, 21, 347-355.

Chanock, R.M., Kim, H.W., Vargosko, A.J., Deleva, A., Johnson, K.M., Cumming, C. and Parrott, R.H. (1961)

Respiratory syncytial virus. J. Am. med. Ass., 176, 647-653.

Chanock, R.M. and Parrott, R.H. (1965)

Acute respiratory disease in infancy and childhood: Present understanding and prospects for prevention.

Pediatrics, Springfield, 36, 21-39.

Chanock, R.M., Parrott, R.H., Vargosko, A.J., Kapikian, A.Z., Knight, V. and Johnson, K.M. (1962)
Respiratory syncytial virus: Acute respiratory diseases of viral etiology, eighty-ninth meeting, Detroit, Michigan, Nov. 1961.
Am. J. publ. Hlth., 52, 918-925.

Chanock, R., Roizman, B. and Meyers, R. (1957)
Recovery from infants with respiratory illnesses of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterisation. Am. J. Hyg. 66, 281-290.

Chin, J., Magoffin, R.L., Shearer, L.A., Schieble, J.H. and Lennette, E.H. (1969) Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in the pediatric population.
Am. J. Epidemiol., 89, 449-463.

Coates, H.V., Alling, D.W. and Chanock, R.M. (1966a)
An antigenic analysis of respiratory syncytial virus isolates by a plaque reduction neutralisation test.
Am. J. Epidemiol. 83, 299-313.

Coates, H.V. and Chanock, R.M. (1962)
Experimental infection with respiratory syncytial virus in several species of animals. Am. J. Hyg. 76, 302-312.

Coates, H.V. and Chanock, R.M. (1964)
Clinical significance of respiratory syncytial virus.
Postgrad. Med. 35, 460-465.

Coates, H.V., Forsyth, B.R. and Chanock, R.M. (1966b)
Biophysical studies of respiratory syncytial virus. I. Density of respiratory syncytial virus and associated complement-fixing antigens in a cesium chloride density gradient.
J. Bacteriol., 91, 1263-1269.

Coates, H.V., Kendrick, L.K. and Chanock, R.M. (1963)
Antigenic difference between two strains of respiratory syncytial virus. Proc. Soc. exp. Biol Med., 112, 958-964.

Compans, R.W., Harter, D.H. and Choppin, P.W. (1967)
Studies on pneumonia virus of mice (PVM) in cell culture.
J. exp. Med. 126, 267-276.

Cooper, P.D. (1964)
The mutation of poliovirus by 5-Flurouracil. Virology, 22, 186-192.

Cooper, P.D. (1966)
The inhibition of poliovirus growth by actinomycin D and the prevention of the inhibition by pretreatment of the cells with serum or insulin. Virology, 28, 663-678.

Cooper, P.D. (1969)
The genetic analysis of poliovirus. In "The Biochemistry of Viruses", 177-218, ed. Levy, H.B. Macel Dekker, New York and London.

Coombs, R.R.A. and Gell, P.G.H. (1975)
Classification of allergic reactions responsible for clinical hypersensitivity and disease. In "Clinical aspects of immunobiology," 3rd edition, 761-781, ed. Gell, P.G.H., Coombs, R.R.A.and Lachman, P.J. Blackwell Scientific Publications, London.

Corbitt, G. (1971)
Interferon and respiratory syncytial virus. Lancet, 2, 492-493.

Corbitt, G. and Beswick, T.S.L. (1972)
The cytochemistry of inclusion bodies in cell cultures into which respiratory syncytial virus has been inoculated.
J. med. Microbiol. 5, 144-147.

Courtney, R.J., Steiner, S.M. and Benyesh-Melnick, M. (1973)
Effects of 2-deoxy-D-glucose on herpes simplex virus repliaction.
Virology, 52, 447-455.

Cradock-Watson, J.E., McQuillin, J. and Gardner, P.S. (1971)
Rapid diagnosis of respiratory syncytial virus infection in children by the immunofluorescent technique. J. clin. Path. 24, 308-312.

Crone, P.B., Heycock, J.B., Noble, T.C. and Patton, J.B. (1964)
Serologic evidence of infection by respiratory syncytial virus in outbreak of acute bronchitis. Br. med. J., 1, 1539-1540.

Dacie, J.V. and Lewis, S.M. (1968)
Basic haematological techniques. In "Practical Haematology", 4th edition, 55-77, Churchill, London.

Dahlberg, J.E. and Simon, E.H. (1969a)
Recombination in Newcastle disease virus (NDV): The problem of complementing heterozygotes. Virology, 38, 490-493.

Dahlberg, J.E. and Simon, E.H. (1969b)
Physical and genetic studies of Newcastle disease virus: Evidence for multiploid particles. Virology, 38, 666-678.

D'Alessio, D., Williams, S. and Dick, E.C. (1970)
Rapid detection and identification of respiratory viruses by direct immunofluorescence. Appl. Microbiol. 20, 233-239.

De Jong, J.C. and Harmsen, M. (1973)
Stimulated growth of respiratory syncytial virus in the presence of actinomycin D. Antonie van Leeuwenhock J. Microbiol. Serol. 39, 409-413.

De Jong, J.C. and Winkler, K.C. (1970)
The multiplication of measles virus in human amnion cells *in vivo*. J. gen. Virol., 7, 13-17.

Ditchburn, R.K., McQuillin, J., Gardner, P.S. and Court, S.D.M. (1971)
Respiratory syncytial virus in hospital cross-infection.
Br. med. J. 3, 671-673.

Doggett, Jennifer, E., Taylor-Robinson, D. (1965)
Serological studies with respiratory syncytial virus.
Arch. ges. Virusforsch., 15, 601-608.

Doggett, Jennifer, E., Taylor-Robinson, D. and Gallop, R.G.C. (1968)
A study of an inhibitor in bovine serum active against respiratory syncytial virus. Arch. ges. Virusforsch. 23, 126-137.

Downham, M.A.P.S., Gardner, P.S., McQuillin, J. and Ferris, J.A.J. (1975)
Role of respiratory viruses in childhood mortality.
Br. med. J. 1, 235-239.

Dreizin, R.S., Ponomareva, T.I., Rapoport, R.I. and Petrova, E.I. (1967)
Respiratory syncytial virus infection of diploid cell strains.
Acta Virol. (Praha), 11, 533-537.

Dulbecco, R. and Vogt, M. (1954)
Plaque formation and isolation of a pure line with poliomyelitis viruses. J. exp. Med., 99, 167-199.

Earle, W.R. (1943)
Production of malignancy in vitro. IV The mouse fibroblast cultures and changes seen in the living cells. J. Nat. Cancer Inst., 4, 165-212.

Edington, N. and Jacobs, J.W. (1970)
Respiratory syncytial virus in cattle. Vet. Rec., 87, 762.

Eckert, E.A., Maassab, H.F. and Cavallaro, J.J. (1965)
Inner component of respiratory syncytial virus.
J. Bacteriol. 90, 1784-1785.

Feldman, G.V. and Cradock-Watson, J.E. (1972)
Viruses and whooping cough. Lancet, 1, 379.

Fenner, F., McAuslan, B.R., Mims, C.A., Sambrook, J. and White, D.O. (1974) The multiplication of RNA viruses. In "Biology of animal viruses", 2nd edition, 221-273. Academic Press, New York and London.

Ferris, J.A.J., Aherne, W.A., Locke, W.S., McQuillin, J. and Gardner, P.S. (1973) Sudden and unexpected deaths in infants: histology and virology. Br. med. J., 2, 439-442.

Flamand, A. (1970)
Etude génétique du virus de la Stomatite Vésiculaire: Classement de mutants thermosensibles spontanés en groupes de complémentation. J. gen. Virol., 8, 187-195.

Follett, E.A.C., Pringle, C.R. and Pennington, T.H. (1975)
Virus development in enucleate cells: echovirus, poliovirus, pseudorabies virus, reovirus, respiratory syncytial virus and Semliki forest virus. J. gen. Virol., 26, 183-196.

Follet, E.A.C., Pringle, C.R., Wunner, W.H. and Skehel, J.J. (1974)
Virus replication in enucleate cells: Vesicular stomatis virus and influenza virus. J. Virol. 13, 394-399.

Forbes, J.A., Bennet,, N. McK. and Gray, N.J. (1961)
Epidemic bronchiolitis caused by respiratory syncytial virus: Clinical aspects. Med. J. Aust. 2, 933-935.

Formica, J.V., Gharpure, M. and Bellanti, J.A. (1969)
Biochemical characterisation of respiratory syncytial virus infected HeLa monolayer cultures. Federation Proceedings 28, 433.

Forsyth, B.R. (1970)
Identification of respiratory syncytial virus antigens by agar gel diffusion and immuno-electrophoresis.
Proc. Soc. exp. Biol. Med. 133, 568-572.

Forsyth, B.R., Coates, H.V. and Chanock, R.M. (1966)
Biophysical studies of respiratory syncytial virus. II Identification of two soluble complement-fixing antigens of respiratory syncytial virus. J. Bacteriol. 91, 1270-1276.

Foy, H.M. and Ray, C.G. (1973)

Epidemiology of sudden infant death syndrome and lower respiratory tract disease in young children: a comparison.

Am. J. Epidemiol. 98, 69-71.

Fransen, H., Sterner, G., Forsgren, M., Heigl, Z., Wolontis, S., Svedmyr, A. and Tunevall, G. (1967)

Acute lower respiratory illness in elderly patients with respiratory syncytial virus infection. Acta med. scand., 182, 323-330.

Friedewald, W.T., Forsyth, B.R., Smith, C.B., Gharpure, M.A. and Chanock, R.M. (1968)

Low-temperature-grown RS virus in adult volunteers.

J. Am. med. Ass. 204, 690-694.

Fulginiti, V.A. (1969)

Respiratory virus immunisation. I A field trial of two inactivated respiratory virus vaccines: an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine.

Am. J. Epidemiol., 89, 435-448.

Fulginiti, V.A., Eller, J.J., Downie, A.W. and Kempe, C.H. (1967)

Altered reactivity to measles virus.

J. Am. med. Ass., 202, 1075-1080.

Gardner, P.S., Elderkin, F.M. and Wall, A.H. (1964)

Serological study of respiratory syncytial virus infections in infancy and childhood. Br. med. J. 2, 1570-1573.

Gardner, P.S., McGuckin, R., Beale, A.J. and Fernandes, R. (1970c)

Interferon and respiratory syncytial virus. Lancet, 1, 574-575.

Gardner, P.S. and McQuillin, T. (1968a)

Application of immunofluorescent antibody technique in rapid diagnosis of respiratory syncytial virus infection. Br. med. J., 3, 340-343.

Gardner, P.S. and McQuillin, J. (1968b)

Viral diagnosis by immunofluorescence. Lancet, 1, 597-598.

Gardner, P.S., McQuillin, J. and Court, S.D.M. (1970a)

Speculation on pathogenesis in death from respiratory syncytial virus infection. Br. med. J., 1, 327-330.

Gardner, P.S., McQuillin, J. and McGuckin, R. (1970b)

The late detection of respiratory syncytial virus in cells of respiratory tract by immunofluorescence.

J. Hyg., Camb., 68, 575-580.

Gardner, P.S., Turk, D.C., Aherne, W.A., Bird, T., Holdaway, M.D. and Court, S.D.M. (1967) Deaths associated with respiratory tract infection in childhood. Br. med. J., 4, 316-320.

Gell, P.G.H. and Coombs, R.R.A. (1963)

The classification of allergic reactions underlying diseases. In "Clinical aspects of immunology", 1st edition, 317-337, ed. Coombs, R.R.A. and Gell, P.G.H. Blackwell Scientific Publications, Oxford.

Gernez-Rieux, C., Breton, A., Samaille, J., Goudier, B., Lefebore, G. and Lelong, M. (1963) Isolement du virus respiratoire syncytial (CCA de Morris) au course d'une épidémie de manifestations respiratoires bénignes chez des prématurés. Arch. ges. Virusforsch., 13, 265-267.

Gey, G.O., Coffman, W.D. and Kubicek, M.T. (1952)

Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res., 12, 264-265.

Gharpure, M.A., Wright, P.F. and Chanock, R.M. (1969)

Temperature-sensitive mutants of respiratory syncytial virus.

J. Virol., 3, 414-421.

Gottschalk, A. (1957) Neuraminidase: the specific enzyme of influenza virus and Vibrio cholerae. Biochim. Biophys. Acta 23, 645-646.

Grauballe, P.C., Johnsen, N.T. and Homsleth, A. (1974) Rapid diagnosis by immunofluorescence of viral infections associated with croup syndrome in children. Acta pathol. microbiol. scand. Sect. B Microbiol. Immunol., 82, 41-47.

Gray, K.G., MacFarlane, D.E. and Sommerville, R.G. (1968) Direct immunofluorescent identification of respiratory syncytial virus in throat swabs from children with respiratory illness. Lancet, 1, 446-448.

Greenham, L.W. and Poste, G. (1971) Role of lysosomes in virus-induced cell fusion. I. Cytochemical studies. Microbios, 3, 97-104.

Grist, N.R., Ross, C.A.C. and Stott, E.J. (1967) Influenza respiratory syncytial virus and pneumonia in Glasgow 1962-5. Br. med. J., 1, 456-457.

Hambling, M.H. (1964a) A survey of antibodies to respiratory syncytial virus in the population. Br. med. J., 1, 1223-1225.

Hambling, M.H. (1964b) Survival of the respiratory syncytial virus during storage under various conditions. Br. J. exp. Pathol., 45, 647-655.

Hambling, M.H. (1966) Antibody response in guinea pigs following intranasal inoculation of respiratory syncytial virus. J. Pathol. Bacteriol., 91, 625-629.

Hamparian, V.V., Ketler, A., Hilleman, M.R., Reilly, C.M., McCelland, L., Cornfeld, D. and Stokes, J. jnr. (1961) Studies of acute respiratory illnesses caused by respiratory syncytial virus. I Laboratory findings in 109 cases. Proc. Soc. exp. Biol Med., 106, 717-722.

Hamre, D., Lashof, J.C., Marshall, J.A., Cassidy, J.E., Smith, M. and Bennet, C.R. (1961) A study of viruses implicated in acute respiratory disease in Chicago from Oct. 1958 to June 1959. Am. Rev. resp. Dis. 83, 38-42.

Hamre, D. and Procknow, J.J. (1961) Viruses isolated from natural common colds in USA. Br. med. J., 2, 1382-1385.

Hayflick, L. (1965) The limited in vitro lifetime of human diploid cell strains. Exp. Cell. Res., 37, 614-636.

Hers, J.F.Ph., Kuip, L. v.d. and Masurel, N. (1971) Rapid diagnosis of respiratory virus infection in infected tissue cultures. Ann. N.Y. Acad. Sci., 177, 70-77.

Hill, D.A. and Baron, S. (1969) Effect of interferon inducer on human respiratory viruses. Bacteriological Proceedings, 69, 149.

Hill, D.A., Baron, S. and Chanock, R.M. (1969) Effect of an interferon inducer on influenza virus. Bull. Wld. Hlth. Org., 41, 689-693.

Hilleman, M.R., Hamparian, V.V., Ketler, A., Reilly, C.M., McCelland, L., Cornfeld, D. and Stokes, J. jnr. (1962) Acute respiratory illnesses among children and adults. J. Am. med. Ass., 180, 445-453.

Hirst, G.K. (1941) Agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. Science, 94, 22-23.

Hirst, G.K. (1962) Genetic recombination with Newcastle disease virus, poliovirus and influenza. Cold Spring Harbor Symp. Quant. Biol., 27, 303-309.

Hodes, D.S., Kim, H.W., Parrott, R.H., Camargo, E. and Chanock, R.M. (1974b) Genetic alteration in a temperature-sensitive mutant of respiratory syncytial virus after replication *in vivo*. Proc. Soc. exp. Biol Med., 145, 1158-1164.

Hodes, D.S. Schauf, Victoria and Chanock, R.M. (1974a) Isolation of RNA species from HeLa cells infected with respiratory syncytial virus. Proc. Soc. exp Biol Med., 146, 287-290.

Hodes, D.S., Schnitzer, T.J., Kalica, A.R., Camargo, E. and Chanock, R.M. (1975) Inhibition of respiratory syncytial, parainfluenza 3 and measles viruses by 2-deoxy-D-glucose. Virology, 63, 201-208.

Holzel, A., Parker, L., Patterson, W.H., White, L.L.R., Tompson, K.M. and Tobin, J.O'H. (1963) The isolation of respiratory syncytial virus from children with acute respiratory disease. Lancet, 1, 295-298.

Hopps, H.E., Bernheim, B.C., Nisalak, A., Tjio, J.H. and Smadel, J.E. (1963) Biologic characteristics of a continuous kidney cell line derived from the African Green Monkey. J. Immunol., 91, 416-424.

Horn, M.E.C., Brain, E., Gregg, I., Yealland, S.J. and Inglis, J.M. (1975) Respiratory viral infection in childhood. A survey in general practice. Roehampton 1967-1972. J. Hyg. Camb., 74, 157-168.

Hornsleth, A. (1966a) Acceleration of growth of respiratory syncytial virus by proflavine. J. Pathol. Bacteriol., 92, 210-217.

Hornsleth, A. (1966b) Effect of inhibitors and respiratory factors on the growth of respiratory syncytial (RS) virus in HEp-2 cell cultures. Acta pathol. microbiol. scand., 68, 293-304.

Hornsleth, A. (1967)

Respiratory virus disease in infancy and childhood in Copenhagen 1963-1965. An estimation of the etiology based on complement-fixation tests. Acta pathol. microbiol. scand., 69, 287-303.

Hornsleth, A. (1969)

Growth of respiratory syncytial (RS) virus in HEp-2 cell cultures. Difference in effect of proflavine and actinomycin D.

Acta pathol. microbiol. scand., 76, 637-645.

Hornsleth, A. and Volkert, M. (1964)

The incidence of complement-fixing antibodies to respiratory syncytial virus in sera from Danish population groups aged 0-19 years.

Acta pathol. microbiol. scand., 62, 421-431.

Huang, H.S. and Baltimore, D. (1970)

Defective viral particles and viral disease processes.

Nature, Lond., 226, 325-327.

Hummler, K. (1971)

Bullet-shaped viruses. In "Comparative Virology," 3rd edition, 361-386, ed. Maramorosch, K. and Kurstak, E. Academic Press, New York and London.

Hurwitz, J., Furth, J.J., Malamy, M. and Alexander, M. (1962)

The role of deoxyribonucleic acid in ribonucleic acid synthesis. III The inhibition of the enzymatic synthesis of ribonucleic acid by actinomycin D and proflavin.

Proc. Nat. Acad. Sci. USA, 48, 1222-1230.

Inaba, Y., Tanka, Y., Omori, T. and Matumoto, M. (1970b)

Isolation of bovine respiratory syncytial virus.

Jap. J. exp. Med., 40, 473-474.

Inaba, Y., Tanaka, Y., Sato, K., Omori,T. and Matumoto, M. (1972) Bovine respiratory syncytial virus. Studies on an outbreak in Japan 1968-1969. Jap. J. Microbiol., 16, 373-383.

Inaba, Y., Tanaka, Y., Sato, K., Ito, H., Omori, T. and Matumoto, M. (1970a). Nomi virus, a virus isolated from an apparently new epizootic respiratory disease of cattle. Jap. J. Microbiol., 14, 246-248.

Ito, Y., Tanka, Y., Inaba, Y. and Omori, T. (1973) Structure of bovine respiratory syncytial virus. Arch. ges. Virusforsch., 40, 198-204.

Jacobs, J.W. and Edington, N. (1971) Isolation of respiratory syncytial virus from cattle in Britain. Vet. Rec., 88, 694.

Jacobs, J.W., Peacock, D.B., Corner, B.D., Caul E.O. and Clarke, S.K.R. (1971) Respiratory syncytial virus and other viruses associated with respiratory disease in infants. Lancet, 1, 871-876.

Johnson, K.M., Bloom, H.H., Mufson, M.A. and Chanock, R.M. (1962) Natural reinfection of adults by respiratory syncytial virus. Possible relation to mild upper respiratory tract disease. New Engl. J. Med., 267, 68-72.

Johnson, K.M., Chanock, R.M., Rifkind, D., Dravetz, H.M. and Knight, V. (1961) Respiratory syncytial virus infection in adult volunteers. II Correlation of illness and clinical observations. J. Am. med. Ass., 176, 663-677.

Johnstone, J.W. and Lawy, H.S. (1966) Role of infection in cot deaths. Br. med. J., 1, 706-709.

Joncas, J.H., Berthiaume, L. and Pavilanis, V. (1969a)

The structure of respiratory syncytial virus. Virology, 38, 493-496

Jordan, jr. W.S. (1962)

Growth characteristics of respiratory syncytial virus.

J. Immunol., 188, 581-585.

Kalica, A.R., Wright, P.F., Hetrick, F.M. and Chanock, R.M. (1973)

Electron microscopic studies of respiratory syncytial temperature-sensitive mutants. Arch. ges. Virusforsch., 41, 248-258.

Kaluza, G., Scholtissek, C. and Rott, R. (1972)

Inhibition of the multiplication of the enveloped RNA viruses by glucosamine and 2-deoxy-D-glucose. J. gen. Virol., 14, 251-259.

Kapikian, A.Z., Bell, J.A., Mastroda, F.M., Johnson, K.M., Huebner, R.J. and Chanock, R.M. (1961) An outbreak of febrile illness and pneumonia associated with respiratory syncytial virus infection. Am. J. Hyg., 74, 235-248.

Kapikian, A.Z., Mitchell, R.H., Chanock, R.M., Shvedoff, R.A. and Stewart, C.E. (1969) An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol., 89, 405-421.

Kilbourne, E.D. (1968)

Recombination of influenza A viruses of human and animal origin. Science, 160, 74-75.

Kilbourne, E.D., Lief, F.S., Schulman, J.L., Jahiel, R.I. and Laver, W.G. (1967) Antigenic hybrids of influenza viruses and their implications. Perspect. Virol., 5, 87.

Kim, H.W., Arrobio, J.O., Brandt, C.D., Jeffries, B.C., Pyles, G., Reid, J.L., Chanock, R.M. and Parrott, R.H. (1973a) Epidemiology of respiratory syncytial virus infection in Washington D.C. I Importance of virus in different respiratory tract disease syndromes and temporal distribution of infection. Am. J. Epidemiol., 98, 216-225.

Kim, H.W., Arrobio, J.O., Brandt, C.D., Wright, P.W., Hodes, D.S., Chanock, R.M. and Parrott, R.H. (1973b) Safety and antigenicity of temperature sensitive (ts) mutant respiratory syncytial virus (RSV) in infants and children. Pediatrics, Springfield, 52, 56-63.

Kim, H.W., Arrobio, J.O., Pyles, G., Brandt, C.D., Camargo, E., Chanock, R.M. and Parrott, R.H. (1971) Clinical and immunological response of infants and children to administration of low-temperature adapted respiratory syncytial virus. Pediatrics, Springfield, 48, 745-755.

Kim, H.W., Bellanti, J.A., Arrobio, J.O., Mills, J., Brandt, C.D., Chanock, R.M. and Parrott, R.H. (1969b) Respiratory syncytial virus neutralising activity in nasal secretions following natural infection. Proc. Soc. exp. Biol Med., 131, 658-661.

Kim, H.W., Canchola, J.G., Brandt, C.D., Pyles, G., Chanock, R.M., Jensen, K. and Parrott, R.H. (1969a) Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol., 89, 422-434.

Kingston, D. (1968) Towards the isolation of respiratory syncytial virus from the environment. J. appl. Bacteriol., 31, 498-510.

Kisch, A.L. and Johnson, K.M. (1963)

A plaque assay for respiratory syncytial virus.

Proc. Soc. exp. Biol Med., 112, 583-589.

Kisch, A.L., Johnson, K.M. and Chanock, R.M. (1962)

Immunofluorescence with respiratory virus. Virology, 16, 177-189.

Klenk, H.D., Scholtissek, C. and Rott, R. (1972)

Inhibition of glycoprotein biosynthesis of influenza virus by D-glucosamine and 2-deoxy-D-glucose. Virology, 49, 723-734.

Klone, W., Kulemany, H., Ward, E.N. and Salk, J.E. (1966)

Fusion of LLC-MK_2 tissue culture cells induced by measles virus.

Arch. ges. Virusforsch., 19, 91-101.

Kravetz, H.M., Knight, V., Chanock, R.M., Morris, J.A., Johnson, K.M., Rifkind, D. and Utz, J.P. (1961) Respiratory syncytial virus infection in adult volunteers. III Prediction of illnesses and clinical observations. J. Am. med. Ass., 176, 657-662.

Lang, W.R., Howden, C.W. and Costello, J.M. (1964)

Respiratory disease associated with parainfluenza and respiratory syncytial virus. N.Z. med. J., 63, 508-510.

Larionov, A.S. and Soloneva, A.I. (1968)

Comparative study of the cytopathic activity of respiratory syncytial virus in various tissue cultures. Vop. Virusol., 13, 164-167.

Law, T.J. and Hull, R.N. (1968)

The stabilising effect of sucrose upon respiratory syncytial virus.

Proc. Soc. exp. Biol Med., 128, 515-518.

Levine, S., Buthala, D.A. and Hamilton, R.D. (1971)

Late stage synchronisation of respiratory syncytial virus replication.

Virology, 45, 390-400.

Levine, S. and Hamilton, R.D. (1967)

Respiratory syncytial (RS) virus growth in HEp-2 cells.

Bacteriological Proceedings, p147.

Levine, S. and Hamilton, R.D. (1969)
Kinetics of respiratory syncytial virus growth cycle in HeLa cells. Arch. ges. Virusforsch., 28, 122-132.

Lewis, F.A., Rae, M.L., Lehmann, N.I. and Ferris, A.A. (1961)
A syncytial virus associated with epidemic disease of the lower respiratory tract in infants and young children. Med. J. Aust., 2, 932-933.

Loda, F.A., Glezen, W.P., Collier, A.M. and Denny, F.W. (1972)
Recurrent respiratory syncytial virus infections. Clin. Res., 20, 103.

Macpherson, I.A. and Stoker, M.G.P. (1962)
Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. Virology, 16, 147-151.

MacWilliam and Herd, S. (1970)
Diagnosis of respiratory infections by an immunofluorescent technique. Lancet, 2, 102-103.

Mahy, B.W.J. and Barry, R.D. (1975)
In "Negative Strand Viruses," volume I. Academic Press, New York and London.

Manyjarvi, R., Berglund, B. and Salmi, A. (1967)
Precipitating antibody response of guinea pigs to respiratory syncytial virus. Acta Virol., 11, 467-469.

Marquez, A. and Hsuing, G.D. (1967)
Influence of glutamine on multiplication and cytopathic effect of respiratory syncytial virus. Proc. Soc. exp. Biol Med., 124, 95-99.

Matumoto, M., Inaba, Y., Kurogi, H., Sato, K., Omori, T., Goto, Y. and Hirose, O. (1974) Bovine respiratory syncytial virus: Host range in laboratory animals and cell cultures. Arch. ges. Virusforschung., 44, 280-290.

McCelland, L., Hilleman, M.R., Hamparian, V.V., Ketler, A., Reilly, C.M., Cornfeld, D. and Stokes, J. jnr. (1961) Studies of acute respiratory illnesses caused by respiratory syncytial virus. 2 Epidemiology and assessment of importance. New Engl. J. Med., 264, 1169-1175.

McIntosh, K., Arbeter, A.M., Stahl, M., Orr, I.A., Hodes, D.S. and Ellis, E.F. (1974) Attenuated respiratory syncytial virus vaccines in asthmatic children. Pediat. Res., 8, 689-696.

McIntosh, K., Ellis, E.F., Hoffman, L.S., Lybass, T.G., Eller, J.J. and Fulginiti, V.A. (1973) The association of viral and bacterial respiratory infections with exacerbations of wheezing in young asthmatic children. J. Pediat., 82, 578-590.

McQuillin, J. and Gardner, P.S. (1968) Rapid diagnosis of respiratory syncytial virus infection by immuno-fluorescent antibody technique. Br. med. J., 1, 602-605.

McQuillin, J., Gardner, M.S. and Sturdy, P.M. (1970) The use of cough/nasal swabs in rapid diagnosis of respiratory syncytial virus infection by the fluorescent antibody technique. J. Hyg., Camb., 68, 283-292.

Melnick, J.L. (1971) Classification and nomenclature of animal viruses. Prog. med. Virol., 13, 461-484.

Mills, J., Van Kirk, J.E., Wright, P.E. and Chanock, R.M. (1971) Experimental respiratory syncytial virus infections of adults. Possible mechanisms of resistance to infection and illness. J. Immunol., 107, 123-130.

Moehring, Joan M. and Forsyth, B.R. (1971) Role of the interferon system in respiratory syncytial virus. Proc. Soc. exp. Biol Med., 138, 1009-1014.

Monto, A.S., Bryan, E.R. and Rhodes, L.M. (1974)
The Tecumseh study of respiratory illness: virus and Mycoplasma pneumonia infections. Am. J. Epidemiol., 100, 458-468.

Morris, J.A., Blount, R.E. jnr. and Savage, R.E. (1956)
Recovery of cytopathogenic agent from chimpanzees with coryza. Proc. Soc. exp. Biol Med., 92, 544-549.

Moss, P.D., Adams, M.O. and Tobin, J.O'H. (1963)
Serological studies with respiratory syncytial virus. Lancet, 1, 298-300.

Mufson, M.A., Levine, H.D., Wasil, R.E., Mocega-Gonzalez, H.E. and Krause, H.E. (1973)
Epidemiology of respiratory syncytial virus infection among infants and children in Chicago. Am. J. Epidemiol., 98, 88-95.

Nader, P.R., Horwitz, M.S. and Rousseau, J. (1968)
A typical exanthem following exposure to natural measles: Eleven cases in children previously inoculated with killed vaccine. J. Pediat., 72, 22-28.

Nagahama, H. (1970)
Direct immunofluorescent studies of infection with respiratory syncytial virus. J. infect. Dis., 122, 260-271.

Neligan, G.A., Steiner, H., Gardner, P.S. and McQuillin, J. (1970)
Respiratory syncytial virus infection of the newborn. Br. med. J., 3, 146-147.

Nisevich, L.L., Konstantinova, L.A. and Stakhanova, V.M. (1972)
The effect of DEAE-dextran and dimethyl sulphoxide on the results of titration of parainfluenza, respiratory syncytial virus and rhinoviruses in cell cultures. Vop. Virusol., 17, 344-347.

Normura, S. (1968)

Interaction of respiratory syncytial virus with polyions: Enhancement of infectivity with diethyl-aminoethyl dextran. Proc. Soc. exp. Biol Med., 128, 163-166.

Norrby, E., Marusyk, H. and Orvell, C. (1970a)

Morphogenesis of respiratory syncytial virus in a Green Monkey kidney cell line (Vero). J. Virol., 6, 237-242.

Norrby, E., Marusyk, H. and Orvell, C. (1970b)

Ultrastructure studies of the multiplication of respiratory syncytial virus. Acta pathol. microbiol. scand. Sect B. Microbiol. Immunol., 78, 268.

Paccaud, M.F. and Jacquier, C. (1970)

A respiratory syncytial virus of bovine origin. Arch. ges. Virusforsch., 30, 327-342.

Parrott, R.H., Kim, H.W., Arrobio, J.O., Hodes, D.S., Murphy, B., Brandt, C.D., Camargo, E. and Chanock, R.M. (1973)

Epidemiology of respiratory syncytial virus infection in Washington, D.C. II Infection and disease with respect to age, immunologic status, race and sex. Am. J. Epidemiol., 98, 289-300.

Parrott, R.H., Vargosko, A.J., Kim, H.W., Cumming, C., Turner, H.C., Turner, H.C., Huebner, R.J. and Chanock, R.M. (1961)

Respiratory syncytial virus. II Serologic studies over a 34 month period of children with bronchiolitis, pneumonia and minor respiratory diseases. J. Am. med. Ass., 176, 653-657.

Peacock, D.B. and Clarke, S.K.R. (1961)

Respiratory syncytial virus in Britain. Lancet, 2, 466.

Pinto, C.A., Hoff, R.F. and Stewart, R.G. (1969)

Pathogenesis of and recovery from respiratory syncytial virus and influenza infections in ferrets. Arch. ges. Virusforsch., 26, 225-237.

Poste, G. (1971a)

Cell surface changes in virus-induced cell fusion. 2.Changes in cell coat thickness. Microbios, 3, 55-61.

Poste, G. (1971b)

The role of lysosomes in virus-induced cell fusion. 2. Modification of cell surface. Microbios, 3, 105-112.

Potash, L. and Gilbertson, E.M. (1967)

Plaquing of viruses in the absence of a carbon dioxide atmosphere. Appl. Microbiol., 15, 1496.

Pringle, C.R., Howard, D.K. and Hay, J. (1973)

Temperature-sensitive mutants of pseudorabies virus with differential effects on viral and host DNA synthesis. Virology, 55, 495-505.

Ray, G.C., Gravelle, C.R. and Chin, T.D.Y. (1967)

Circulating interferon in infants and children with acute respiratory illness. J. Pediat. 71, 27-32.

Rechsteiner, J. (1969a)

Thermal inactivation of respiratory syncytial virus in water hypertonic solutions. J. gen. Virol., 5, 397-403.

Rechsteiner, J. (1969b)

Inactivation of respiratory syncytial virus in air. Antonie van Leeuwenhoek, 35, 238.

Rechsteiner, J. and Winkler, K.C. (1969)

Inactivation of respiratory syncytial virus in aerosol. J. gen. Virol., 5, 405-410.

Reich, E. (1966)

Binding to DNA and inhibition of DNA functions by actinomycins. Symp. Soc. Gen. Microbiol., 16, 266-280.

Reich, E. and Goldberg, I.H. (1964)
Actinomycin and nucleic acid function. Progress in nucleic acid research and molecular biology 3, 184-230.

Reilly, C.M., Stokes, J. jnr., McCelland, L., Cornfeld, D., Hamparian, V.V., Ketler, A. and Hilleman, M.R. (1961) Studies of acute respiratory illnesses caused by respiratory syncytial virus. 3. Clinical and laboratory findings. New Engl. J. Med., 264, 1176-1182.

Richman, A.V., Pedreira, F.A. and Tauraso, N.M. (1971)
Attempts to demonstrate hemagglutination and hemadsorption by respiratory syncytial virus. Appl. Microbiol., 21, 1099-1100.

Richman, A.V. and Tauraso, N.M. (1971)
Growth of respiratory syncytial virus in suspension culture. Appl. Microbiol., 22, 1123-1225.

Rosenquist, B.D. (1974)
Isolation of respiratory virus from calves with acute respiratory disease. J. infect. Dis., 130, 177-182.

Ross, C.A.C., Pinketon, I.W. and Assaad, F.A. (1971)
Pathogenesis of respiratory syncytial virus disease in infancy. Arch. Dis. Child., 46, 702-704.

Ross, C.A.C., Stott, E.J., McMichaels, S. and Crowther, I.A. (1964)
Problems of laboratory diagnosis of respiratory syncytial virus infection in childhood. Arch. ges. Virusforsch., 14, 553-562.

Rossi, C.R. and Kiesel, G.K. (1974)
Serological evidence for the association of bovine respiratory syncytial virus with respiratory tract disease in Alabama cattle. Infect. Immuno., 10, 293-298.

Rowe, D.S. and Michaels, R.H. (1960)
Isolation of respiratory syncytial virus from patients with pneumonia. Pediatrics, Springfield, 26, 623-629.

Sandiford, B.R. and Spencer, B. (1962)

Respiratory syncytial virus in epidemic bronchiolitis of infants.

Br. med. J., 2, 881-882.

Schieble, J.H., Kase, A. and Lennette, E.H. (1967)

Fluorescent cell counting as an assay method for respiratory syncytial virus. J. Virol., 1, 494-499.

Scholtissek, C. (1974)

Glycoprotein synthesis of enveloped viruses.

Advances in Biosciences, 11, 193-204.

Scholtissek, C., Drzeniek, R. and Rott, R. (1969)

Myxoviruses. In "The Biochemistry of viruses" 2nd edition 220-258 ed. Levy, H.B. Marcel Dekker, New York and London.

Scott, R. and Gardner, P.S. (1970)

Respiratory syncytial virus neutralising activity in nasopharyngeal secretions. J. Hyg., Camb., 68, 581-588.

Scott, R. and Gardner, P.S. (1974)

The local antibody response to RS virus infection in the respiratory tract. J. Hyg., Camb., 72, 111-120.

Senterfit, L.B. and Baldridge, F.B. (1974)

Separation and concentration of respiratory syncytial virus (RSV) antigens. J. Immunol. Methods, 4, 349-357.

Shatkin, A.J. (1971)

Viruses with segmented ribonucleic acid genomes: multiplication of influenza versus reovirus. Bacteriological Reviews, 35, 250-266.

Shedden, W.I.H. and Emery, J.L. (1965)

Immunofluorescent evidence of respiratory syncytial virus infection in cases of giant-cell bronchiolitis in children.

J. Pathol. Bacteriol., 89, 343-347.

Siegert, W., Bauer, G. and Hofschneider, P.H. (1973)
Direct evidence for messenger activity of influenza virion RNA. Proc. Nat. Acad. Sci., USA, 7D, 2960-2963.

Simpson, R.W. and Hirst, G.K. (1968)
Temperature-sensitive mutants of influenza A virus: isolation of mutants and preliminary observations on genetic recombination and complementation. Virology, 35, 41-49.

Simpson, R.W. and Iinuma, M. (1975)
Infectious proviral DNA of a human ribovirus in eukaryotic cells. Microbial Genetics Bulletin, 38, 4.

Simpson, H., Matthew, D.J., Habel, A.H. and George, E.L. (1974b)
Acute respiratory failure in bronchiolitis and pneumonia in infancy. Modes of presentation and treatment. Br. med. J., 2, 632-636.

Simpson, H., Matthew, D.J., Inglis, J.M. and George, E.L. (1974a)
Virological findings and blood gas tensions in acute lower respiratory tract infections in children. Br. med. J., 2, 629-632.

Smith, M.H., Frey, M.L. and Dierks, R.E. (1975)
Isolation, characterisation and pathogenicity studies of a bovine respiratory syncytial virus. Archives of Virology, 47, 237-247.

Smith, C.B., Purcell, R.H., Bellanti, J.A. and Chanock, R.M. (1966)
Protective effect of antibody to parainfluenza type 1 virus. New Engl. J. Med., 275, 1145-1151.

Sommerville, R.G. (1963)
Respiratory syncytial virus in acute exacerbations of chronic bronchitis. Lancet, 2, 1247-1248.

Staiger, H.R. (1964)
Plaque-type recombination in fowl plague virus. Virology 22, 419-422.

Sterner, G. (1972)

Respiratory syncytial virus in hospital cross-infection.

Br. med. J., 1, 51-52.

Sterner, G., Wolontis, S., Bloth, B. and De Hevesy, G. (1966)

Respiratory syncytial virus. An outbreak of acute respiratory illnesses in a home for infants. Acta paediat. scand., 55, 273-279.

Strieder, D.J. (1974)

Pediatric origins of chronic obstructive lung disease.

Bull. Physiol. Pathol. Respir., 10, 273-279.

Subak-Sharpe, J.H. and Pringle, C.R. (1975)

Viral information controlling DNA and RNA animal virus replication.

In "Control Processes in virus multiplication," 363-403,

ed. Burke, D.C. and Russel, W.C. Cambridge University Press, Cambridge.

Suto, T., Yano, N., Ikeda, M., Miyamoto, M., Takai, S., Shigeta, S., Hinuma, Y. and Ishida, N. (1965) Respiratory syncytial virus infection and its serological epidemiology.

Am. J. Epidem. 82, 211-224.

Taylor-Robinson, D. and Doggett, J.E. (1963)

An assay method for respiratory syncytial virus.

Br. J. exp. Pathol., 44, 473-480.

Toolan, H.W. (1954)

Transplantable human neoplasms maintained in cortisone-treated laboratory animals: HS 1; H.Ep 1, H.Ep 2, H.Ep 3 and H. Emb. Rh. 1. Cancer Res., 14, 660-666.

Treuhaft, M.W. and Beem, M.O. (1972)

Polypeptides of respiratory syncytial virus.

Pediat. Res., 6, 392.

Urquhart, G.E. and Gibson, A.A.M. (1970)
Respiratory syncytial virus infections and infant deaths.
Br. med. J., 3, 110.

Urquhart, G.E. and Martin, K.W. (1970)
Respiratory syncytial virus tissue culture immunofluorescence as a laboratory aid. Arch. ges. Virusforsch., 32, 365-372.

Urquhart, G.E. and Walker, G.H. (1972)
Immunofluorescence for routine diagnosis of respiratory syncytial virus infection. J. clin. Path. 25, 843-845.

Waldman, R.H., Kasel, J.A., Fulk, R.V., Togo, Y., Hornick, R.B., Heiner, G.G., Dawkins, A.T. and Mann, J.J. (1968)
Influenza antibody in human respiratory secretions following subcutaneous or respiratory immunisation with inactivated virus.
Nature, 218, 594-595.

Waterson, A.P. and Hobson, D. (1962)
Relationship between respiratory syncytial virus and Newcastle disease - parainfluenza group. Br. med. J., 2, 1166-1167.

Weightman, D., Downham, M.A.P.S., and Gardner, P.S. (1974)
Introduction of a cross-infection rate in children's wards and its application to respiratory virus infections. J. Hyg., Camb., 73, 53-59.

Wellemans, G. and Leunen, J. (1971)
L'importance du virus V220/69 (virus respiratoire syncytial bovin) dans les troubles respiratoires des veaux en Belgique.
Ann. Med. Vet., 115, 35-39.

Wellemans, G., Leunen, J. and Blondel, H. (1971)
The role of RSB/220 virus in respiratory disorder of cattle in Belgium. Vlaams Diergeneesk Tydschr, 40, 252-258.

Wellemens, G., Leunen, J. and Luchsinger, E. (1970)
Isolement d'un virus (220/69) serologiquement semblable au virus respiratoire syncytial (RS) humain. Ann. Med. Vet., 114, 89-93.

Wilszynski, J. (1971)
Intravital staining of HEp-2 cells in the course of RS virus infection. Exp. med. Microbiol., 23, 47-54.

Winter, S.T. (1972)
The male disadvantage in diseases acquired in childhood.
Dev. Med. Child. Neurol. 14, 517-519.

Woodhour, A.F., Sweet, B.H., Tytell, A.A., Potash, S.J. jnr., Weibel, R.E., Metzgar, D.P. and Hilleman, M.R. (1966) Respiratory syncytial virus vaccines (4) heptavalent respiratory syncytial - parainfluenza mycoplasma - influenza vaccine in institutionalised persons.
Am. Rev. resp. Dis., 94, 350-361.

Wright, F.H. (1965)
Respiratory syncytial virus infection.
J. Iowa med. Soc., 55, 301-309.

Wright, P.F., Gharpure, M.A., Hodes, D.S. and Chanock, R.M. (1973)
Genetic studies of respiratory syncytial virus temperature-sensitive mutants. Arch. ges. Virusforsch., 41, 238-247.

Wright, P.F., Mills, J. and Chanock, R.M. (1971)
Evaluation of a temperature-sensitive mutant of respiratory syncytial virus in adults. J. infect. Dis., 124, 505-511.

Wright, P.F., Woodend, W.G. and Chanock, R.M. (1970)
Temperature-sensitive mutants of respiratory syncytial virus. *In vivo* studies in hamsters. J. infect. Dis., 122, 501-512.

Wulff, H., Kidd, P. and Wenner, H.A. (1964b)
Respiratory syncytial virus: Properties of strains propagated in monkey kidney cell culture. Proc. Soc. exp. Biol Med., 115, 458-460.

Wunner, W.H., Faulkner, G.P. and Pringle, C.R. (1975)

Respiratory syncytial virus: some biological and biochemical properties. In "Negative Strand Viruses," volume I, 193-201, ed. Mahy, B.W.J. and Barry, R.D. Academic Press, New York and London.

Yamamoto, K. (1969)

Stabilising effect of inorganic salts on respiratory syncytial virus. Jap. J. med. Sci. Biol., 22, 337-339.

Young, B.J.J. and Matthews, R.S. (1969)

A comparison of HEp-2 cells and a continuous line of human amnion cells for the primary isolation of respiratory syncytial virus. J. med. Lab. Technol., 26, 93-98.

Zakstelyskaya, L.Y., Almeida, J.D. and Bradstreet, C.M.P. (1967)

The morphological characterisation of respiratory syncytial virus by a simple electron microscope technique. Acta virol. Prague, 11, 420-423.

ZHDANOV
~~Zhadanov~~, V.M. (1975)

Integration of viral genomes. Nature, 256, 471-473.