BIOCHEMICAL AND GENETIC STUDIES ON VESICULAR STOMATITIS VIRUS

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To my wife,
Pick

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ABSTRACT

Twenty-five temperature-sensitive mutants have been isolated from a heat-resistant wild type clone of the Indiana strain of vesicular stomatitis virus by treatment with ethylmethane sulfonate, nitrous acid, 5-fluorouracil or proflavine hydrochloride. Three complementation groups have been identified. However fourteen mutants remain unclassified. No recombination could be demonstrated between mutants.

The group I mutants were able to synthesize RNA at 38° but in an amount considerably less than that at 30°. Furthermore, the amount of RNA synthesized at 38° was not affected by the presence of puromycin. These observations together with the fact that mutants of group I complemented those of group III suggest the possibility that the group I mutants may be defective in a polymerase which is required for the replication of viral RNA.

The group II mutants were found to synthesize virus-specific RNA at 38° in an amount comparable to that at 30° although no virus multiplication was observed at 38°, indicating that the defect of the group II mutants occurs after RNA synthesis, perhaps in some function involved in virion assembly or maturation.

The group III mutants failed to synthesize RNA at 38°. No ^{14}C-uridine uptake at 38° was observed in infected cells in the presence of puromycin with a high input multiplicity of virus. Furthermore, effect of temperature-shift from 30° to 38° on ^{14}C-uridine uptake with a high input multiplicity of ts11 showed that in the absence of puromycin ^{14}C-uridine uptake was observed whereas in its presence there was no

uptake. These observations suggest that the group III mutants are defective in the structural polymerase which is required for the transcription of viral RNA. In a complementation between a group I and a group III mutant, progeny of both parents were obtained which suggests that the defect in the structural polymerase could be circumvented by a group I mutant.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

The biochemical events which occur during the infection of animal cells by RNA viruses have not been completely defined. Our knowledge regarding these events may be advanced by means of modern biochemical techniques. However, investigation in this field would be greatly facilitated if a range of mutants each with a single mutated gene were available. By comparing a given characteristic of the mutant (e.g. ability to synthesize RNA) with that of the unaltered parent (wild type) under similar conditions, it would be possible to obtain information relating to the function of the affected gene. For a long time it was thought that the essential steps in virus replication would escape genetic analysis because the mutants defective in such functions would be lethal. However, the discovery of conditional-lethal mutants completely changed this attitude, because in conditional-lethal mutants the conditions under which the mutation is expressed can be controlled by the experimenter.

Two types of conditional-lethal mutants are known: the temperature-sensitive (ts) mutants and the suppressor-sensitive mutants. Temperature-sensitive mutants are mutants with an altered gene product which is formed and functional at a lower (permissive) temperature but at a higher (non-permissive) temperature the product is made but cannot assume or maintain a functional configuration (Wittman and Wittman-Liebold, 1966). Suppressor-sensitive mutants are host dependent and have been recognized so far only in bacteriophages. Consequently all

studies with conditional-lethal mutants in animal viruses have been carried out with ts mutants.

There are several advantages in using ts mutants for studying the events that occur when viruses infect animal cells:

i) Ts mutants are easy to isolate (given the appropriate permissive and non-permissive conditions) because it is not necessary to know which function is altered; any essential gene function may be involved. A set of ts mutants may therefore be expected to include some with altered viral proteins, some with altered essential enzymes and some with changed regulatory functions. By finding out the stage at which the viral development of each mutant is arrested under non-permissive conditions, the sequence of events that occur during replication may be obtained.

ii) With ts mutants, it is possible to alternate between permissive and non-permissive conditions during a single growth cycle which makes ts mutants particularly valuable for the analysis of the duration and sequence of events occuring in viral replication.

iii) Ts mutants provide the best available method for exploring the entire genome of an animal virus although there may be cistrons in which ts mutations cannot be isolated. Two approaches are available for genetic analysis:

a) Mixed infection of a suitable host cell by two different mutants, each defective in a different gene, permits viral growth by complementation under non-permissive conditions. If complementation occurs, it can be used for defining the viral genome into functional

units. This is done by sorting mutants into complementation groups such that mutants within a complementation group do not complement one another but do complement all mutants in other complementation groups. Each complementation group then represents a defect in a particular cistron. If a large enough series of mutants is available, the total number of functional units of the viral genome may thus be established.

b) If recombinants arise during mixed infection of an animal cell with two different mutants, by breakage and reunion of the mutant genomes, a recombination frequency may be determined. The recombination frequencies of many pairs of mutants could be used for the construction of a genetic map.

The preparatory step of isolating temperature-sensitive mutants of vesicular stomatitis virus (VSV) and preliminary characterization of the mutants has been undertaken (Wong, 1969). The present study is an attempt to elucidate the biochemical events which occur during the infection of L cells by VSV of the Indiana serotype using temperature-sensitive mutants. VSV has been selected as a model for the study of the replication of the Rhabdoviruses, a large family of single-stranded RNA enveloped viruses, because it is convenient to work with (Wong, 1969) and it is relatively non-pathogenic to humans.

LITERATURE REVIEW

LITERATURE REVIEW

REPLICATION OF VESICULAR STOMATITIS VIRUS (VSV)

The replication of RNA viruses was for a long time an enigma because it was argued that if cellular RNA was synthesized from a DNA template it seemed inconceivable that viral RNA could be made in any other way. However, during the last few years three different viral genetic systems have been discovered among the single-stranded RNA viruses each unique in the characteristics of its replication (Baltimore, 1971). These three systems may be represented by poliovirus, RNA tumor viruses and vesicular stomatitis virus (VSV). There is also the possibility that the influenza viruses may represent a fourth viral genetic system because these viruses are sensitive to inhibition by actinomycin D during the first two hours immediately following infection and before RNA synthesis has begun (Blair and Duesberg, 1970).

The purpose of this review is to present recent advances in our understanding of the mode of replication of VSV since VSV has been selected as a model for the study of the replication of the Rhabdoviruses in this investigation.

The molecular weight of the single strand of RNA in VSV is estimated to be about 3 to 4 x 10^6 daltons (Nakai and Howatson, 1968; Huang and Wagner, 1966). The RNA strand is packed very tightly with a large amount of protein to form a helical nucleocapsid. This internal component of the virion is surrounded by a lipoprotein membrane with spike-like surface projections (Howatson and Whitmore,

1962; Nakai and Howatson, 1968; Cartwright et al., 1969). The virus population consists of infectious bullet-shaped B particles and shorter, non-infectious T particles (Hackett et al., 1967; Huang et al., 1966). Two forms of T particles have been described: T particles which are one-third the length of the B particles, containing an RNA genome of approximately one-third the weight of the B particle genome (Huang and Wagner, 1966; Nakai and Howatson, 1968); and another form of T particle, only produced by a heat-resistant (HR) strain of VSV of the Indiana serotype, is approximately one-half the length of the B particles and contains an RNA genome one-half the weight of the B particle genome. These two forms of T particles have been designated "short T" and "long T" particles by Petric and Prevec (1970). Both the "short T" and "long T" particles are similar to the B particle in general morphology, antigenicity and protein constituents (Huang et al., 1966; Wagner et al., 1969; Kang and Prevec, 1969; Petric and Prevec, 1970) and may interfere with the growth of the infectious B particles (Huang and Wagner, 1966a; Hackett et al., 1967; Prevec and Kang, 1970).

The study of the process of viral replication requires an understanding of the chemical nature of the viral components, the manner in which these components are synthesized and the principles governing their interaction in the process of assembly into mature virus particles.

In recent years some advances have been made in understanding the nature of VS viral components but much less is known about the mechanisms involved in the synthesis of these components and the

principles governing their interaction in the process of assembly into mature virions.

In order to understand the synthesis and assembly processes of VS viral components, a possible approach that can be adopted is the isolation and characterization of the various successive intermediate stages as the virus components are made and assembled into virus particles. However this method of study is not always feasible because cells contain virus particles at all stages of replication. Occasionally some of the intermediate stages are sufficiently stable to permit their isolation and detailed study, but this is usually not the case. There are two ways of overcoming this difficulty. First, the use of inhibitors capable of arresting replication at specific intermediate stages: however, suitable inhibitors for specific intermediate steps are not always available. A second approach makes use of suitable mutants. In animal viruses, temperature-sensitive (ts) conditional-lethal mutants are available for such studies. The use of ts mutants to investigate the events occurring during VSV replication in animal cells has already begun. Several sets of ts mutants of VSV have been isolated (Pringle, 1970; Flamand, 1969, 1970; Holloway et al., 1970). Among the ts mutants isolated by Pringle (1970) and Flamand (1969; 1970) a total of five complementation groups have been identified (Flamand and Pringle, 1971). Preliminary characterization of the genetic defects of some of these

ts mutants has been attempted (Lafay, 1969; Lafay and Berkaloff, 1969; Holloway et al., 1970; Cormack et al., 1971; Pringle and Duncun, 1971; Printz and Wagner, 1971). From these studies it appears that ts mutants may serve as powerful and incisive probes in elucidating the events that occur when VSV infect animal cells.

The following is a review of the current state of knowledge of the mode of replication of VSV:

I. VS VIRAL RNA SYNTHESIS

1. Virus-specific RNA found in infected cells

During replication of VSV in animal cells several species of viral-specific RNA have been detected in cell extracts by various groups of workers (Table I). Some differences in results are noted which perhaps reflect differences in cell lines, virus strains and experimental techniques used by the different groups of workers. Nevertheless, it may be concluded from the results shown in Table I that replication of VSV in animal cells involves a large number of distinct species of viral-specific RNA which may be classified into i) viral RNA, ii) messenger RNA, ii) double-stranded forms and iv) partially double-stranded forms.

i) Viral RNA 38S, 40S, 42S and 43S RNA species are most likely B type virion RNA because of the similarity of sedimentation coefficient to that of RNA extracted from B particles (Huang et al., 1970; Schincariol and Howatson, 1970). 19S and 26S RNA species are most likely

TABLE I

VIRUS-SPECIFIC RNA SPECIES FROM INFECTED CELLS

REFERENCES	SCHAFFER ET AL. (1968)	NEWMAN & BROWN (1969)	STAMPFER ET AL. (1969) HUANG ET AL. (1970)	MUDD & SUMMERS (1970)	SCHINCARIOL & HOWATSON (1970)
CELL LINE / RNA SPECIES	VERO	BHK21	CHO	HeLa	L-CELL
SINGLE-STRANDED RNA's					
Viral RNA:					
B	43S	38S	40S	42S	38S
T	23S[3]		19S		26S & 19S[3]
mRNA			13S* & 28S	14S*	13S & 15S
Unidentified	31S, 15S, 6S	28S, 20S, 12S	6S		31S, 21S
DOUBLE-STRANDED RNA's	some present	7S to 11S	13S[1]		13S to 15S (T)[1] 19S to 20S (B)[1]
PARTIALLY DOUBLE-STRANDED RNA's			23S to 35S[2] 15S to 20S[2]	30S	13S to 50S

* Acrylamide gel electrophoresis showed that the 13S RNA fraction (Huang et al., 1970) is composed of at least two RNA species and the 14S RNA fraction (Mudd & Summers, 1970) consists of several RNA species.

1 Referred to as replicative form by authors quoted.

2 Referred to as replicative intermediate by authors quoted.

3 Relation to T particle uncertain.

the T type virion RNA. The 19S RNA has been isolated from cells infected with a high input multiplicity of wild type VSV (Stampfer et al., 1969) and the 26S RNA isolated from a heat-resistant clone of VSV (Schincariol and Howatson, 1970). These 19S and 26S RNA species probably belong to the "short" and "long" T particles respectively.

ii) Messenger RNA (mRNA) 13S to 15S and 28S RNA species are believed to be mRNA because they have the following properties:

a) their association with polyribosomes (Huang et al., 1970; Schincariol and Howatson, 1970),

b) their removal from the polyribosomal region of sucrose gradients by EDTA treatment (Huang et al., 1970; Mudd and Summers, 1970),

c) their sensitivity to ribonuclease (Schincariol and Howatson, 1970; Mudd and Summers, 1970),

d) their predominance over other viral-specific RNA species during the infectious cycle at each of the time intervals examined (Stampfer et al., 1969; Huang et al., 1970; Schincariol and Howatson, 1970),

e) their much smaller size than the viral RNA and complementarity to viral RNA (Huang et al., 1970; Mudd and Summers, 1970; Schaffer et al., 1968) and

f) their heterogeneous sizes* which are appropriate to code

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* See footnote on p. 8 (Table I)

for the polypeptides found in infected cells (Mudd and Summers, 1970; Schincariol and Howatson, 1970; Huang et al., 1970).

iii) Double-stranded RNA forms These are the completely double-stranded, RNase-resistant forms of RNA whose sedimentation coefficients were found to be 7S to 11S (Newman and Brown, 1969), 13S (Stampfer et al., 1969) and 13S to 15S, 19S to 20S (Schincariol and Howatson, 1970). According to Schincariol and Howatson, the sedimentation coefficients of the 13S to 15S and 19S to 20S RNA are consistent with their being replicative forms of the T and B particles respectively. The fact that Stampfer et al. isolated 13S only when more T than B particles were being produced in cells superinfected with T particles is in agreement with the above conclusion. Since at least two different sizes of T particles have been reported (Petric and Prevec, 1970) it is conceivable that the 7S to 11S RNA reported by Newman and Brown are also replicative forms of T particles.

iv) Partially double-stranded forms These are the 13S to 50S RNA, heterogeneous, partially RNase-resistant RNA complexes consisting of the replicative form and nascent single strands (Schincariol and Howatson, 1970). According to Schincariol and Howatson, these polydisperse RNA

molecules probably contain replicative intermediates for both B and T particles because their evidence indicates that both 13S to 15S and 19S to 20S double-stranded RNase-resistant RNA's are present.

2. Transcription of messenger RNA (mRNA)

The non-infectious nature of VS viral RNA (Huang et al., 1966) and the lack of association of viral RNA with polyribosomes led to the discovery of a structural polymerase in the virion of VSV (Baltimore et al., 1970). It was shown from in vitro studies that this virion-bound RNA polymerase makes use of the viral RNA as template for the synthesis of RNA product which is smaller in size than, but complementary to, the viral RNA (Baltimore et al., 1970; Huang et al., 1971; Bishop and Roy, 1971). This virion-bound RNA polymerase activity has also been demonstrated in vivo (Marcus et al., 1971; Cormack et al., 1971). Marcus et al. further showed that at least 55% of the virus-specific RNA made in VSV infected chick embryo cells under inhibition of protein synthesis by cycloheximide is complementary to viral RNA.

In vitro experiments by Bishop and Roy (1971) suggested that the viral RNA remains undegraded during product RNA synthesis because of the linear rate of incorporation of ^{32}P into acid insoluble RNA and the conservation of ^{3}H label during RNA synthesis in a reaction mixture with ^{32}P-UTP and purified ^{3}H-uridine labelled virus particles for the enzyme preparation. Huang et al. (1971) arrived at the same conclusion that the template is not degraded during

transcription by sucrose gradient analysis of the RNA product of VSV polymerase reaction mixtures. In vitro annealing experiments with ^{3}H-labelled RNA template and ^{32}P-labelled RNA product, showed that at least 94% of the template polynucleotide is transcribed in the reaction (Bishop, 1971). This was assumed to indicate complete transcription since it was difficult to determine whether the residual 6% was transcribed but not detected or not transcribed at all.

Bishop and Roy (1971) showed that, in a reaction mixture containing ^{32}P-UTP and ^{3}H-uridine labelled VSV particles for the enzyme preparation, the initial product is synthesized in an association with the viral RNA in the high molecular weight region determined by electromobility on polyacrylamide gel. Subsequently the product RNA accumulates in the low molecular weight region of the polyacrylamide gel. A pulse-chase experiment showed that the RNA product synthesized in association with VSV RNA is subsequently displaced and appears in the low molecular weight region of the polyacrylamide gel as free RNA species of 2×10^5 to 1×10^6 daltons. The association of RNA product and viral RNA template was further confirmed by resolving the template and RNA product complexes into free template and free product (Bishop and Roy, 1971).

That these low molecular weight RNA species are mRNA's is suggested by the association with polyribosomes of several similar complementary low molecular weight RNA species in the cytoplasmic extracts of VSV infected cells (Mudd and Summers, 1970; Huang et al., 1970; Schincariol and Howatson, 1970; Schaffer et al., 1968). Huang

et al. (1971) also observed that in infected cells the initial VSV polymerase products are associated with the viral RNA. Such partially ribonuclease-resistant RNA complexes were called transcriptive intermediates because it was found by in vitro studies that the VSV structural polymerase does not replicate the viral genome and it should therefore be described as a transcriptase rather than a replicase (Baltimore et al., 1970; Aaslestad et al., 1971).

It has been postulated by Mudd and Summers (1970) that the mRNA species may be derived either by the transcription of only certain portions of the VSV genome or by transcription of the whole genome, and subsequent cleavage by specific endonuclease to smaller mRNA species. The presence of partially-RNase-resistant RNA from VSV polymerase reaction mixtures (Huang et al., 1971) and the disproportionate synthesis of viral structural proteins over non-structural proteins in infected cells (Wagner et al., 1970; Mudd and Summers, 1970) seems to indicate that the former process is the more likely to occur.

The above observations introduce a number of interesting aspects concerning the replication of VSV. Firstly, the finding that in VSV the viral RNA is not the mRNA, but instead several species of small molecular weight RNA transcribed from the viral RNA serve as mRNA implies that replication and transcription of the viral RNA are separate events for this virus, as opposed to the system found in poliovirus where the mRNA is a single species identical to the virion RNA (Summers and Levintow, 1965). Among other viruses with single-stranded RNA as genome, only the paramyxoviruses, Sendai (Blair and Robinson,

1968) and Newcastle disease virus (Bratt and Robinson, 1967; Huang et al., 1971) have been reported to produce several single-stranded complementary RNA species which appear to be attached to polyribosomes and serve as mRNA. The recent report of the presence of a RNA-dependent transcriptase in Newcastle disease virus analogous to the polymerase of VSV (Huang et al., 1971) indicate a very similar mode of replication in VSV and the paramyxoviruses.

Secondly, the fact that different sizes of mRNA's all smaller than the viral RNA are transcribed implies the existence of an enzyme that can recognize stop and start signals on an RNA template, just as can the DNA-dependent RNA polymerase of cells.

Thirdly, the fact that the viral RNA is not translatable necessitates that an early event to occur after penetration of the virus into a host cell is the transfer of information to a new nucleic acid rather than the direct translation of the viral RNA.

3. Replication of viral RNA

Little is know as yet concerning the replication of the viral RNA in VSV. Double-stranded 13S (Stampfer et al., 1969) and 13S to 15S, 19S to 20S (Schincariol and Howatson, 1970) forms of RNA have been isolated from VSV infected cells. Partially RNase-resistant 13S to 50S forms of RNA have also been implicated as replicative intermediates by their preferential labelling during a short pulse with ^{3}H-uridine (Schincariol and Howatson, 1970).

In summary, the synthesis of VSV RNA components has two parts.

The transcription system appears to be packaged into the virion and can synthesize mRNA directly from viral RNA. The replication system, on the other hand, would appear to require protein synthesis. Additional details concerning this system are not known although it is thought that the double-stranded RNA represents a replicative form. Only a low percentage of the total virus-specific RNA would appear to be synthesized by the replicative system (Stampfer et al., 1969).

II. VS VIRAL PROTEIN SYNTHESIS

1. Virion proteins and virus-specific proteins in infected cells

The structural proteins of VSV and VSV-specified proteins in infected cells have been studied by various groups of workers (Table II). There is general agreement as to the number of structural proteins in the VS virion but conclusions as to the location and function of some of these proteins are sometimes at variance. Disparity in molecular weights of the structural proteins identified may be attributed to the different molecular weight standards used for the estimations. Burge and Huang (1970) however claimed that their estimations using poliovirus proteins as molecular weight standards gave more accurate determinations.

B and T particles have been found to contain the same proteins in essentially the same proportions (Kang and Prevec, 1969; Wagner et al., 1969a). The viral proteins of the New Jersey serotype differ from that of the Indiana serotype only in one of the coat proteins (VP4) in electromobility (Wagner et al., 1969).

TABLE II

VIRION PROTEINS AND VIRUS-SPECIFIC PROTEINS OF VSV IN INFECTED CELLS
MOLECULAR WEIGHTS OF THE PROTEINS AND THEIR CORRESPONDING STRUCTURAL COMPONENTS
IN THE VIRION

VIRUS-SPECIFIC PROTEIN	PROTEIN FRACTIONS		MOLECULAR WEIGHTS				
			KANG & PREVEC (1969, 1970, 1971)	BURGE & HUANG (1970)	MUDD & SUMMERS (1970)	WAGNER ET AL. (1969, 1969a, 1970)	CARTWRIGHT ET AL. (1970)
STRUCTURAL PROTEINS							
Aggregate	I*	(VP1)**	230,000	300,000	175,000	275,000 186,000 140,000	not determined
Coat (glycoprotein)	II	(VP2)‡	84,000	73,000	67,000	81,500	73,000
Nucleocapsid protein	III	(VP3)	64,000	49,000	52,000	59,500	60,000
	IV				40,000		
Coat	V	(VP4)	47,000	25,000	25,000	34,500	32,000
NON-STRUCTURAL PROTEINS		NS1***				55,000	
		NS2				30,000	

* Nomenclature after Mudd and Summers (1970)
** Nomenclature after Kang and Prevec (1969)
*** Nomenclature after Wagner et al. (1970)

‡ VP2 isolated from purified virion is of a higher molecular weight than VP2 isolated from VSV infected cells and VP2a of the 6S antigen released into extracellular fluid of VSV infected cultures. VP2a also has a higher ratio of glucosamine to amino acid than that in virion VP2.

Apart from Mudd and Summers (1970) all other workers obtained four structural proteins in SDS-polyacrylamide gel electrophoresis with purified virions. It has been suggested (Printz and Wagner, 1971) that the additional component identified by Mudd and Summers might be the structural polymerase reported by Baltimore et al. (1970). In addition to the structural proteins, two non-structural proteins designated NS1 and NS2 have been identified in VSV infected cells (Wagner et al., 1970). These proteins are presumed to be of viral origin because similar ^{3}H-labelled protein peaks could not be found in labelled uninfected cells. NS1 was produced in relatively large amounts, whereas NS2 was present in small amounts and sometimes could not be detected. NS1 appears to remain free in the cytoplasm unassociated with cellular organelles.

There is agreement among the various groups of workers that VP3 (using the terminology of Kang and Prevec (1969)) is the nucleocapsid protein and VP2 and VP4 are viral coat (envelope) proteins, VP2 being a glycoprotein (Kang and Prevec, 1969, 1970; Wagner et al., 1970; Burge and Huang, 1970). This virion-bound VP2 is of a higher molecular weight than VP2 isolated from VSV infected cells and VP2a of the 6S antigen released into extracellular fluid of VSV infected cultures (Kang and Prevec, 1970). The origin of VP1 which was present in small quantities was not determined. It has been suggested that this may be an aggregate of some of the smaller proteins. However, in a recent report, Kang and Prevec (1971) indicated that they have evidence that VP1 may not be an aggregational artifact but that VP1 is also a distinct

virus-specified polypeptide.

There is also some disagreement as to which of the two coat proteins induces production of neutralizing antibody in animals. According to Wagner et al. (1969) the lower molecular weight protein (VP4) is the surface protein which is split off when VS virions are exposed to digitonin. It is the only protein that is different in antigenicity in the New Jersey and Indiana serotypes, and it is this protein that is responsible for blocking the virus neutralizing activity of the type-specific antiserum of each serotype. The glycoprotein (VP2), which is the higher molecular weight coat protein, forms the "shell" which surrounds the nucleoprotein core. Wagner et al. (1970) expressed uncertainty as to whether the outer or the inner layer of the envelope comprises the spikes.

However, Cartwright et al. (1970) concluded from the complete degradation of the glycoprotein after treatment with trypsin together with the loss of immunizing activity of trypsin-treated viruses (Cartwright et al., 1969) that the glycoprotein is the spike bearing surface layer while VP4, the lower molecular weight protein, forms the "skeleton" housing the nucleoprotein.

The findings of Kang and Prevec (1970) that the 6S antigen (VP2a), present in the lysates of VSV infected cells, is a glycoprotein immunologically similar to the higher molecular weight coat protein (VP2), and antigenically distinct in the New Jersey and Indiana serotypes seems to support Cartwright et al's conclusion. Apparently from their studies on temperature-sensitive mutants of VSV which can

synthesize RNA at the non-permissive temperature (RNA^+ ts mutants), Printz and Wagner (1971) also became convinced that the glycoprotein (VP2) is the major antigen of VSV.

2. Synthesis of virus-specific protein

The kinetics of viral protein synthesis in infected cells have been studied by Wagner et al. (1970), Mudd and Summers (1970) and Kang and Prevec (1971). Their findings are summarized in the following paragraphs of this section.

Wagner et al. using ^{3}H-leucine and ^{3}H-tyrosine as labels, studied the kinetics of virus protein synthesis of VSV by three different techniques: continuous labelling of the virus-specific proteins, pulse-labelling of the virus-specific proteins and pulse-chase of the virus-specific proteins.

Using the first technique to examine the incorporation of label into different virus-specific proteins extracted from infected cells, Wagner et al. observed that, by one hour after infection, the predominant protein was nucleocapsid protein (VP3), and that possibly glycoprotein (VP2) and one of the non-structural proteins (NS1) could be detected. By two hours after infection all structural proteins (except VP1) and the two non-structural proteins were present.

In the pulse-labelling experiments with one hour pulses at hourly intervals after virus adsorption, maximal synthesis of all viral proteins appeared to be between 3 to 4 hours after infection although the amounts of each protein synthesized was markedly different. Protein synthesis continued for at least 5 to 6 hours.

With the third technique, the infected cells were incubated in medium without leucine and tyrosine for 3.5 hours then pulsed for 30 minutes and the labels chased by adding unlabelled leucine and tyrosine for times varying from 0 to 3 hours. At the end of the chase the virions were extracted and their proteins analysed by acrylamide gel electrophoresis. It was found that the label was present in greatest amount in the glycoprotein (VP2) after 0 hour chase but it did not increase with prolonged chase. The label associated with the nucleocapsid protein (VP3) and the coat protein (VP4) were barely detectable at 0 hour chase but increased with increased duration of chase, the rate of increase of VP3 being greater than VP4.

From the above experiments, Wagner et al. concluded that, there appeared to be no regulation in the order in which VS viral proteins are synthesized since all viral proteins were detected by 2 hours after infection. Maximal synthesis of all viral proteins appeared to be between 3 to 4 hours after infection although the amount of each protein synthesized was different. They also concluded that the nucleocapsid protein (VP3) and the coat protein (VP4) accumulated within the cell prior to their incorporation into virions whereas the glycoprotein (VP2) was rapidly incorporated into virions immediately after synthesis.

Mudd and Summers (1970), by means of acrylamide gel analysis of polypeptides synthesized during successive one-hour pulses in VSV infected cells, also found that all viral-specific proteins were synthesized in the same relative proportions throughout the infectious cycle. The non-structural proteins were present in small amounts relative to the structural proteins.

Kang and Prevec (1971) performed similar experiments to those of Wagner et al. (1970) and came to the same conclusions except that they found that VP4 was incorporated in the virions soon after synthesis while the incorporation of VP2 was delayed. The reason for this discrepancy was not known. An additional discrepancy was that the maximal rate of synthesis of NS1 occurred during the first two hours after infection and decreased at later times. From this observation the authors concluded that NS1 may have an early intracellular function in replication. They further suggested that the reason why Wagner et al. failed to observe the above result might be because the synthesis of NS1 was obscured by the large amount of cellular protein synthesized during the first two hours after infection in cells not pretreated with actinomycin D.

Differences in the amount of VS viral protein synthesized raise the interesting question of the mechanism of control of protein synthesis. Wagner et al. (1970) were of the opinion that this was determined at the translational level. However, recent findings that messenger RNA consists of a number of species, each coding for one polypeptide, suggested that protein synthesis may be regulated at the transcriptional level (Mudd and Summers, 1970a; Huang et al., 1971; Bishop and Roy, 1971). In other words, the disproportionate synthesis of the various proteins throughout the growth cycle is a consequence of selective transcription of the various monocistronic messages or by alteration of certain messenger RNA's so that initiation of translation is prevented. That viral RNA synthesis is required for the synthesis of

viral proteins is demonstrated in the failure of ts5, a RNA^- ts mutant of VSV, to synthesize all viral proteins at the non-permissive temperature (Printz and Wagner, 1971).

Several laboratories (Burge and Huang, 1970; Kang and Prevec, 1970; Wagner et al., 1970) have demonstrated that one of the two coat proteins is a glycoprotein but whether the structure of the viral carbohydrate is specified by the viral genome or the cell is not known. A comparison of the envelope glycoproteins and glycopeptides of Sindbis virus and VSV grown in the same type of cell (Burge and Huang, 1970) showed that the glycopeptides of the two virions differ principally in the number of sialic acid residues per glycopeptide. On removal of the sialic acid, the glycopeptides of the two viral proteins were found to be indistinguishable by exclusion chromatography. Burge and Huang argue from this evidence that the protein moiety is specified by the virus which is subsequently modified by host enzymes (nucleotide sugar transferases) by covalently linking the carbohydrate moiety to the virus-specified protein substrate.

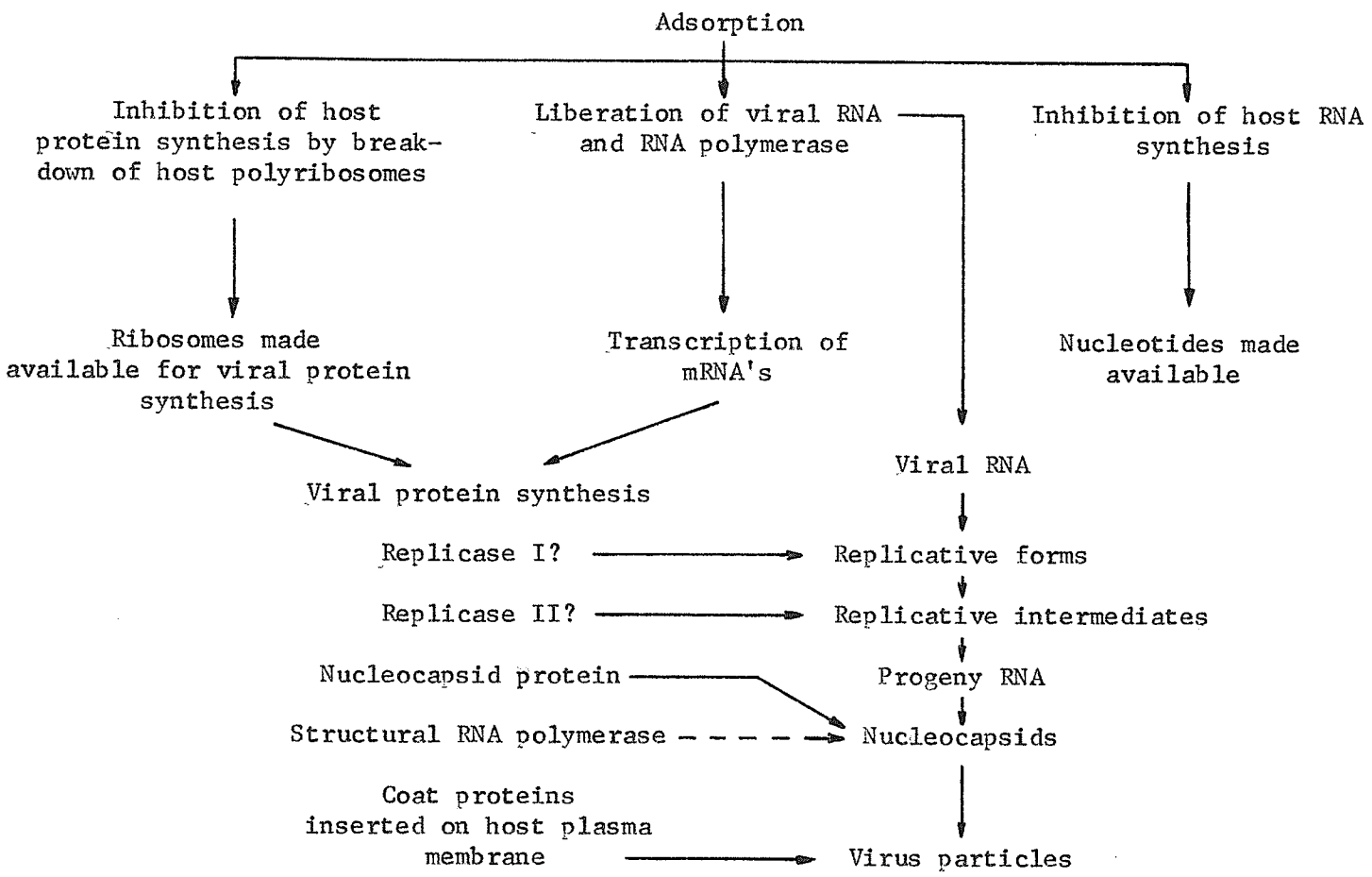
The above hypothesis appears to find support in the study of ts52, a RNA^+ ts mutant of VSV (Printz and Wagner, 1971). These authors found that ts52 synthesizes all the viral proteins. However, the glycoprotein is replaced by a lower molecular weight protein. They were able to show that ^{3}H-glucosamine was specifically incorporated into the viral glycoprotein at the permissive and non-permissive temperatures. This suggested to the authors that there is no inhibition of glycosylation at the cellular level, but that failure of ts52 to

glycosylate at the non-permissive temperature is due to a defect in the viral precursor glycoprotein which fails to serve as a sugar acceptor, presumably owing to conformational changes at the non-permissive temperature.

Wagner et al. (1970) suggested that both the coat proteins of VS virions are synthesized on, and inserted into, cellular membrane which envelopes a nucleocapsid core to form the virion. Their conclusion was based on experimental evidence that the two coat proteins were never found free in the cytoplasm and appear to be always associated with cell membranes, and the fact that exposure of infected cells to the membrane dissolving agent, digitonin, resulted in solubilization of most of the glycoprotein and all of the other coat protein but not the nucleoprotein. Recent investigations (McSharry and Wagner, 1971) which demonstrate the similar lipid composition of purified VSV to that of the plasma membrane of their host cell, and preferential adsorption of the coat proteins to plasma membrane isolated from uninfected HeLa cells, are consistent with the above hypothesis. How the coat proteins become inserted into the plasma membrane and how envelopment of the nucleocapsid core takes place have not yet been determined.

The possible events occurring during the replication of VSV are schematically summarized on the following page.

A SCHEMATIC SUMMARY OF POSSIBLE EVENTS OCCURING DURING VSV REPLICATION



MATERIALS AND METHODS

MATERIALS AND METHODS

Cells and Medium

Earl's mouse L cells (L60 line), obtained from Dr. G.F. Whitmore, Ontario Cancer Institute, were cultured in medium CMRL 1066 obtained from Schwarz BioResearch, Inc. The preparation of medium CMRL 1066 used throughout this study was as follows: Ten grams of the dried medium was suspended in 978 ml of triply distilled water. Twenty-two ml of a 10% sodium bicarbonate solution was added and the medium adjusted to pH 7.2 to 7.4. The medium was sterilized by passing it through a millipore filter with a porosity of 0.22μ. After sterilization the medium was supplemented with 5% foetal calf serum, Penicillin (100 I.U./ml) and Streptomycin (50μg/ml).

Cells were first subcultured in bottles and then transferred into spinner flasks which were kept in a 37^{o} water bath. The cells were kept in suspension by means of a magnetic stirrer and were maintained in exponential growth phase by diluting the cell suspension with fresh medium as required. The doubling time of the cells is about 18 hours. Clumping of cells was avoided by rapid stirring and limiting the concentration of cells to values below 4×10^{5} cells/ml.

Cell counts were carried out by adding 1 ml of cell suspension to 19 ml of phosphate-buffered saline (PBS) and cell number counted by means of a Coulter counter (Model A).

Possible contamination of cell cultures by aerobic PPLO (Pleuropneumonia-like organism) was tested several times during the course of this investigation and was found to be negative on every occasion.

Preparation of virus stocks

The Indiana serotype of vesicular stomatitis virus (VSV) was obtained from Dr. A.F. Howatson, Ontario Cancer Institute. A heat-resistant (HR) strain was selected by heat treatment of the wild type VS virion in medium at 43° for about 4 hours and regrowing the survivors to provide a stock for further heat-treatment at 43°. This heating cycle was repeated several times until the heat-resistance of the virus stock could not be further improved (Wong, 1969). All temperature-sensitive (ts) mutants described in this thesis were derived from this HR strain.

Stocks of virus were prepared by infecting monolayers of L cells in 4 oz Brockway bottles with an input multiplicity (i.m.) of about one plaque-forming unit (PFU) per cell in 0.5 ml of PBS. After allowing 30 minutes for adsorption at 38°, 10 ml of fresh medium was added and the cultures were incubated either for 18 hours at 38° or 24 hours at 30° if infected with HR or for 24 hours at 30° if infected with a ts mutant. After incubation, each infected culture was then centrifuged for 5 minutes at 500g and the supernatant assayed to determine the titre of the virus preparation. The virus preparation was then stored in sealed ampules of 1 ml each at -196° until required. Very little change in titre was detected upon prolonged storage at this temperature.

If a high concentration or a large pool of a virus stock was required, the above method was modified as follows: Cells were seeded in 16 oz screw-capped prescription bottles. Following formation of a

confluent monolayer, the medium was drained and 2 ml of virus stock added to give an input multiplicity of 1 PFU/cell. The virus suspension was allowed to adsorb for 30 minutes at 38°. After adsorption, 40 ml medium was added and the cultures incubated at 30° for 48 hours. At harvest, the contents of the prescription bottles were shaken and poured into 50-ml centrifuge tubes and centrifuged at 500g for 10 minutes. After removal of cells and debris the virus in the supernatant was pelleted by further centrifugation at 30,000g for 1 hour. The supernatant from each tube was discarded and the pellet resuspended in 1 ml of 0.1 M tris-HCl at pH 7.9. If the virus preparation was not required immediately it was assayed and stored as described above.

Assay of virus

The virus was assayed using the Dulbecco plaque method by adding 0.1 ml of a suitable VSV dilution to a monolayer of 2×10^6 L cells from which the medium has been removed. After allowing 30 minutes for virus adsorption at 38°, the cell monolayer was overlaid with 3 ml of medium containing 1.1% agar. It was then incubated in an atmosphere of 5% CO_2 and 95% air at 38° for 20 hours or at 30° for 44 hours. To make the plaques more readily visible, 1 ml of a 1:5,000 solution of neutral red was added at this time and the monolayers were incubated for a further 4 hours. The resulting plaques had a diameter of about 2 mm and were easily counted. It has been found that the plaque count within a range of 20 to 170 plaques per dish was directly proportional to concentration of virus preparation. Replicate plaque assays carried out on the same preparation indicated reproducibility with a standard deviation of ± 20%.

The virions of the parent strain (HR) and each of the mutants were negatively stained with 1% phosphotungstic acid at pH 7.2 and examined with an electron microscope. In the virus stocks used for the various tests most of the virions were normal, bullet-shaped B particles and 1 to 20% were the short, non-infectious T particles. No difference in the proportion of B and T particles were observed between the wild type (HR) and ts mutant stocks.

Preparation of infected cell suspension cultures

For the experiments reported in this thesis on viral growth, temperature-shift and uridine incorporation, viruses were grown in small suspension cultures as follows: 25 ml samples of L cells were withdrawn from a 1-litre suspension culture containing about 4×10^5 cells/ml. Each sample was centrifuged for 5 minutes at 500g and the supernatant discarded. The cells were resuspended in 1 ml of suitably diluted virus stock and incubated at 38° for 20 minutes. The cells were then washed with 40 ml of pre-warmed PBS to remove unadsorbed virus, resuspended in 25 ml of medium and incubated in a 100 ml bottle with magnetic stirring in a water bath either at $(30\pm0.2)^\circ$ or $(38\pm0.2)^\circ$. Inhibitors and radioactive labels were added to the cell suspension as specified in the detailed experimental procedure given in Results, IV.

Chemicals

Actinomycin D was supplied by the courtesy of Merck, Sharp and Dohme. Uridine-2-^{14}C with a specific activity of 53.3 mCi/mmole was obtained from Schwarz BioResearch, Inc. Puromycin dihydrochloride

was obtained from Nutritional Biochemicals Corporation. Cycloheximide (ACT-DIONE) was obtained from Calbiochem.

RESULTS

RESULTS

I. Selection of Temperature-sensitive (ts) Mutants

All mutants described in this thesis were derived from the heat-resistant (HR) strain of the Indiana serotype of VSV which grew to approximately the same titre at temperatures varying from 30° to 38°. In testing the temperature-sensitivity of possible mutants, 30° was chosen as the permissive temperature and 38° as the non-permissive temperature. The methods for mutagenization and for the isolation of temperature-sensitive mutant strains are similar to those used for other RNA animal viruses, such as poliovirus (Cooper, 1964), Sindbis virus (Burge and Pfefferkorn, 1966), influenza virus (Simpson and Hirst, 1968) and reovirus (Fields and Joklik, 1969; Ikegami and Gomatos, 1968).

Mutagenesis

Mutagenization by nitrous acid, ethylmethane sulfonate, proflavine and 5-fluorouracil was carried out separately, in various experiments as follows: For mutagenization with nitrous acid, approximately 5×10^8 virus particles, suspended in 5 ml of culture medium, were exposed to 2.0 M $NaNO_2$ in 0.25 M phosphate buffer (7 ml 0.25 M $NaHPO_4$ solution and 4 ml 0.25 M KH_2PO_4 solution) at pH 7 for 3 hours at 27°. This treatment reduced the titre of the virus by a factor of approximately 10^5 (Wong, 1969). For mutagenization with ethylmethane sulfonate (EMS), one volume of EMS was added to 150 volumes of PBS (pH 7) containing about 1×10^8 PFU of VSV per ml and incubated at 30°.

After treatment for an hour the titre of the virus population was reduced by a factor of 10^5 approximately (Wong, 1969). Mutagenization by proflavine or 5-fluorouracil was carried out by treating L cell monolayers with 100 µg/ml of proflavine or with 1 mg/ml 5-fluorouracil at the time of infection and incubating the infected cultures in the presence of the mutagen for 24 hours. At this time the virus yield was reduced by factors of 10^5 and 10^2 for treatment with proflavine and 5-fluorouracil respectively compared to infected control cultures incubated for the same period of time without the mutagen.

Isolation of ts mutants from mutagenized viral stocks

The mutagenized viral stock was diluted, plated on monolayers and incubated at 30^o for 24 hours. The dishes were then shifted to 38^o and incubated for a further 18 hours. Plaques showing little increase in size after temperature shift-up were considered possible ts mutants and were selected for further testing. Small, well-isolated plaques were picked using a Pasteur pipette and individually suspended in 3-ml volumes of PBS. These plaque suspensions were assayed at both the permissive (30^o) and the non-permissive (38^o) temperature. If the assay was approximately the same at each temperature the sample was discarded. If the 38^o assay was smaller by a factor of 10 or more, further plaques were picked from the 30^o assay plate and reassayed at both 30^o and 38^o. If the ratio of these plaque assays ($38^o/30^o$) was less than about 10^{-3}, the mutant strain was considered to be sufficiently temperature sensitive. A stock of the mutant virus was then grown

in a monolayer at 30° as described in Materials and Methods and a number was assigned to it. The stock was then sealed in ampules and stored at -196°.

In addition to the 8 ts mutants reported by Wong (1969), 17 ts mutants have since been isolated in our laboratory by treatment with EMS, nitrous acid, 5-fluorouracil and proflavine. With each of the mutagens used only about 1% of the plaques picked gave rise to acceptable mutant stocks.

Two properties of ts mutants have to be found satisfactory in order that the mutants may be considered useful for biochemical and genetic studies. These properties are reversion to wild type and "leakiness" of the mutant.

"Reversion" is the term used when the mutant genome undergoes a further mutation which results in the renewed ability of the virus to grow at the non-permissive temperature. Experimentally a measure of the number of revertants in a given stock may be determined by assaying that stock at the non-permissive temperature. Plaques similar to those produced by wild type virus will arise wherever a revertant virus infects a cell on a monolayer. The number of such plaques gives a measure of the number of revertants in the stock. The relative incidence of revertants may thus be obtained by assaying at both the permissive temperature (to give the total number of infectious virions, both revertant and ts) and at the non-permissive temperature (to give the number of revertants).

The ratio of the assay at 38° to that at 30° will be an

indication of the incidence of revertants in a given stock. The values of this are given in Table III for all of the mutants isolated in this laboratory.

In addition to the wild type looking plaques that may occur on assay plates at the non-permissive temperature, one sometimes sees very small plaques. While the former are clearly due to revertants, the latter plaques are due to the presence of some residual function in the mutant protein specified by the mutated gene so that some ts virus multiplication may take place even at the non-permissive temperature. This phenomenon is referred to as "leakiness" of the mutant.

To assess the degree of "leakiness" of a given mutant, one grows a stock of the mutant virus at permissive and non-permissive temperature. The growth of the mutant at the non-permissive temperature (corrected for the presence of revertants found in the progeny virus) is largely due to "leakiness" of the mutant. The degree of "leak" varies from mutant to mutant and from experiment to experiment. For a given mutant the variation in "leak" from experiment to experiment may be due to differences in input multiplicity, temperature control and condition of the cells. This variation in the degree of "leak" was particularly noted for those mutants which produced virus at the non-permissive temperature in number greater than 1% of that produced at the permissive temperature. Such mutants are ts9, ts12, ts17, ts18, ts22, ts23, ts25 and ts26.

TABLE III

PLAQUE RATIO OF MUTANTS

MUTANT	MUTAGEN	PLAQUE RATIO** $38^{o}/30^{o}$
2	EMS	1×10^{-4}
3	EMS	2×10^{-5}
4	NA	5×10^{-4}
5	EMS	5×10^{-4}
6	EMS	5×10^{-5}
7	NA	5×10^{-3}
8	EMS	1×10^{-5}
9	EMS	1×10^{-5}
10	EMS	3×10^{-4}
11	EMS	3×10^{-4}
12	NA	3×10^{-3}
14	EMS	1×10^{-4}
15	EMS	1×10^{-4}
16B+	EMS	3×10^{-4}
17	EMS	3×10^{-3}
18	EMS	2×10^{-3}
19	EMS	2×10^{-4}
20	EMS	2×10^{-4}
22	PRO	5×10^{-3}
23	5FU	3×10^{-3}
24	PRO	4×10^{-4}
25	PRO	2×10^{-4}
26	5FU	5×10^{-4}
28	5FU	3×10^{-4}
29	EMS	5×10^{-5}
HR		1×10^{-4}
		1

Mutagens: EMS = ethylmethane sulfonate
NA = nitrous acid
PRO = proflavine dihydrochloride
5FU = 5-fluorouracil

* Plaque ratio = Stocks assayed at 38^{o} and 30^{o}, and ratio of plaque numbers noted.

+ A non-leaky variant derived from ts16.

II. Grouping of Temperature-sensitive Mutants of VSV by Means of Complementation Tests

According to Edgar et al. (1964), the importance of the complementation test is in the definition of genes or cistrons* as functional units in the absence of any information about the chemical nature of the function. Complementation studies have been carried out on the 25 ts mutants isolated in this laboratory to provide a basis for characterization of the genetic defects of the mutants by sorting them into complementation groups with the view that each complementation group will correspond to a specific biochemical defect in the sequence of events which occur during viral replication.

When applied to ts mutants of viruses, complementation may be defined as the interaction in mixed infection at non-permissive temperature between two mutants, with different defects, which will give appreciable production of the mutant progeny. Complementation may be either inter- or intracistronic (Fincham, 1966; Drake, 1970). In intercistronic complementation, two mutants defective in different cistrons assist each other's multiplication by providing the gene product lacking in the other; while in intracistronic complementation, two mutants, mutated at different sites in the same cistron, help the replication of each other by forming active hybrid aggregates of the

---

*Although genes which are defined by means of cis-trans complementation tests (Lewis, 1951) were called cistrons by Benzer (1957), the word cistron has since come into common use as a substitute for gene. In this thesis, gene and cistron are used interchangeably. Furthermore, a cistron is assumed to correspond one-to-one with a specific polypeptide.

gene products of the mutated cistron. Wittman and Wittman-Liebold (1966) showed that in temperature-sensitive mutants of tobacco mosaic viruses, the defective protein is produced in normal amounts but cannot assume or maintain a functional configuration at the non-permissive temperature. Hybrid aggregates, however, may restore, at least partially, the activity of the protein.

Inter- and intracistronic complementation might be discriminated on a quantitative basis since intercistronic complementation is reported to give higher yields in mixed infections than intracistronic complementation (Fincham, 1966). However, Bernstein et al. (1965) showed that the production of mutant virus resulting from intercistronic complementation may not always be greater than that resulting from intracistronic complementation. Thus inter- and intracistronic complementation cannot be unambigously distinguished by complementation alone.

Complementation test

Complementation tests were performed on cell monolayers prepared by seeding each of a number of 60 x 15 mm petri dishes (Falcon Plastic) with 2.5×10^6 cells. The titre of each virus stock was obtained within a short time before the experiment. On a number of occasions the stock was reassayed at the time of infection and there was no significant difference in titre between the two assays. For mixed infections 2.5×10^7 PFU of each of the two mutants was added while for the single infections 5×10^7 PFU was added. For both mixed and single infections, therefore, the total input multiplicity was 20 PFU/cell. This input multiplicity was chosen because it was found

that with a total input multiplicity of either 10 or 20 PFU/cell, the complementation levels calculated as defined in the following paragraph were approximately the same (Table IV). The higher input multiplicity was chosen to make sure that mixed infection takes place in the cell. After inoculation the virus was allowed to adsorb for 25 minutes at 38° in a volume of 0.5 ml of PBS. Thirty-eight degrees was chosen for the temperature for adsorption irrespective of subsequent incubation temperature since no mutant has been found in this laboratory whose ts defect was in the adsorption process. The infected monolayers were then washed with 10 ml of PBS, 5 ml of medium was added, and the infected dishes were incubated at 38° for 6.5 hours. The medium was then removed from each dish and assayed on L cell monolayers at 30°.

The results of the complementation tests have been expressed in this thesis in terms of a complementation level (C.L.) which is defined as the ratio of the yield in the mixed infection to the greater of the two single infection yields using the same total input multiplicity. This definition differs somewhat from that adopted by other workers (Burge and Pfefferkorn, 1966; Cooper, 1966; Dahlberg, 1968; Eckhart, 1969; Flamand, 1969) who defined C.L. or complementation index as the ratio of the yield in the mixed infection to the sum of the yields of the single infections, where the input multiplicity of the single infections is half that of the total mixed infection.

We have chosen the above definition to avoid the possibility of false positive results because single infection yields at 38° (i.e. "leak") for several of our mutants was found to increase by as much as

TABLE IV

EFFECT OF INPUT MULTIPLICITY (I.M.) ON COMPLEMENTATION LEVEL (C.L.)*

MUTANT PAIRS	C.L. WITH I.M. OF 20 PFU/CELL	C.L. WITH I.M. OF 10 PFU/CELL
ts4 x ts15	0.4	0.6
ts4 x ts18	2.6	2.6
ts15 x ts17	2.4	1.5
ts17 x ts18	1.9	1.8
ts15 x ts18	1.3	1.9
ts16 x ts26	51.5	50.0

* C.L. see text on p. 37 for definition.

a factor of 5 when the input multiplicity was doubled (Table V). The relation of input multiplicity to production of virus of three mutants and HR after 6½ hours incubation at 38° is also shown in Fig. 1.

Values of complementation levels greater than 1 were taken as indication of complementation. Based on the above criterion, the mutants were tentatively classified into groups such that mutants within a group do not complement one another but do complement all mutants in other groups. Mutants which show irregular complementation behaviour were tentatively considered unclassified. However, it was necessary to ask whether the increased yield obtained in the mixed infections where C.L. is greater than 1 is significantly different from the yield of the single infection. To test the statistical significance of the positive complementation levels a representative from each group was chosen:ts16B (Group I), ts29 (Group II) and ts4 (Group III). Complementation tests were then repeated for all mutant pairs involving ts16B, ts29 or ts4 using three replicate monolayers for both single and mixed infections. The results were then analyzed by a Students' t test with a digital computer and the significance of the difference of the C.L. from 1 was determined. The results of one experiment are given in Table VI.

Results

The results of the complementation tests of the 25 ts mutants are shown in Table VII. Mutants ts10 and ts16B complement all other mutants tested but do not complement each other. These results lead to the unambiguous separation of these two mutants from the remainder to form a single complementation group, I. Mixed infections by other ts

TABLE V

EFFECT OF INPUT MULTIPLICITY (i.m.) ON THE LEAKINESS OF SOME TEMPERATURE-SENSITIVE MUTANTS OF VSV

MUTANT	VIRUS YIELD AFTER 6½ HOURS INCUBATION AT 38° (PFU/ML)		
	i.m. 10 PFU/CELL	i.m. 20 PFU/CELL	i.m. 20 PFU/CELL / i.m. 10 PFU/CELL
ts4	4.4×10^5	2.0×10^6	4.5
ts8	1.0×10^5	1.6×10^5	1.6
ts15	4.0×10^5	1.2×10^6	3
ts16B	2.5×10^5	7.5×10^5	3
ts17	9.0×10^5	2.4×10^6	2.7
ts18	3.4×10^5	1.6×10^6	4.7
ts20	2.5×10^5	1.2×10^6	5
ts29	1.2×10^5	1.7×10^5	1.4

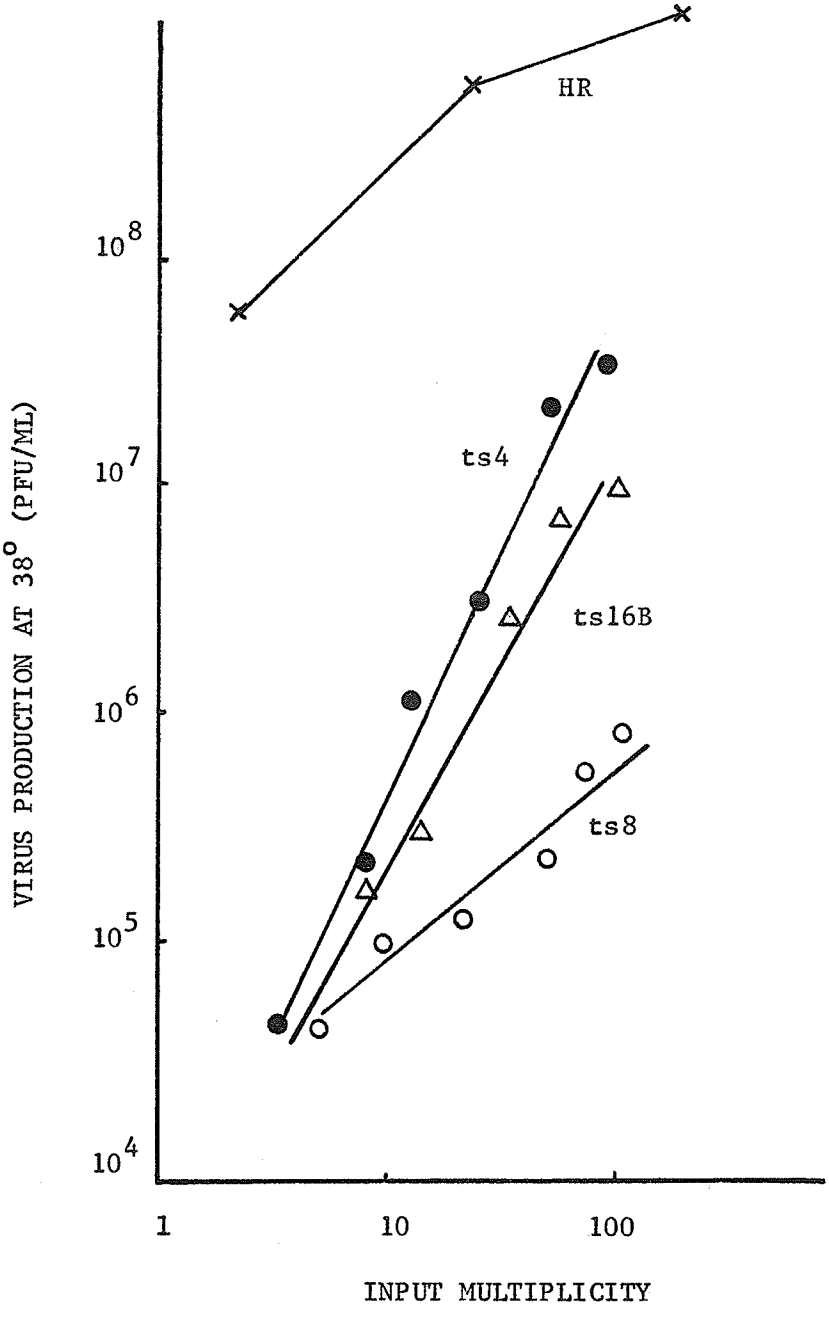


Fig. 1 Relation of virus production to different input multiplicities (i.m.) of a given stock of mutant or HR. Cells infected with different i.m. were incubated at 38° for 6½ hours. Virus production was assayed at 30°.

TABLE VI

DATA OF A COMPLEMENTATION TEST AND CALCULATION OF T VALUE

MUTANT	SAMPLE	ASSAY OF VIRUS PRODUCTION NUMBER OF PLAQUES COUNTED AT VARIOUS DILUTIONS 10^{-3}	10^{-4}	10^{-5}	INPUT DATA (VIRUS YIELD IN 10^6 UNITS OF PFU/ML)	CALCULATION OF C.L.*
ts29	1	~500	50, 55		5.2	
	2	~500	44, 54		4.9	
	3	~500	55, 53		5.4	
ts14	1	12, 18	1, 0			
	2	15, 16	2, 0			
	3	18, 10	1, 1			
ts29xts14	1		~500	40, 38	39	$\frac{39+46+69}{5.2+4.9+5.4}$
	2		~500	34, 58	46	
	3		~500	58, 80	69	= 10

COMPUTER PROGRAM:- (Computer Department for Health Sciences, Faculty of Medicine, University of Manitoba)

ST13 (TWO-SAMPLE T TEST WITH LOGARITHMIC TRANSFORMATION OF INPUT DATA: COMPLEMENTATION ts14 x ts29 VS ts29)

SAMPLE ONE
DATA....
39 46 69/

SAMPLE TWO
DATA....
5.2 4.9 5.4/

TRANSFORMATION 4 HAS BEEN MADE ON DATA (Transformation to $\log_e$)

		SAMPLE ONE	SAMPLE TWO
N (Number of samples)	=	3	3
MEAN	=	3.909	1.641
VAR (Variance)	=	0.086	0.002
SD (Standard deviation)	=	0.294	0.049
SDM (Standard deviation of mean)	=	0.170	0.028
DIFF (Hypothetical difference to be tested)	=	0.000	
DF (Degree of freedom)	=	4	
T (Calculated T value)	=	13.193	

*C.L. for definition see p. 37.

TABLE VII

COMPLEMENTATION LEVELS FOR PAIRS OF TEMPERATURE-SENSITIVE MUTANTS OF VSV

COMPLEMENTATION GROUP		I		II		III			UNCLASSIFIED MUTANTS																	
	MUTANT	ts10	ts16B	ts12	ts29	ts4	ts11	ts14	ts15	ts20	ts28	ts2	ts3	ts5	ts6	ts7	ts8	ts9	ts17	ts18	ts19	ts22	ts23	ts24	ts25	ts26
I	ts10																									
	ts16B	0.7±0.3																								
II	ts12	9	7*																							
	ts29	24*	10*	1.0±0.2																						
III	ts4	150*	50*	3	9.5*																					
	ts11	14*	60*	4*	10*	0.5±0.4																				
	ts14	8*	80*	0.8	15*	0.9±0.5	0.3																			
	ts15	9*	100	3	3*	0.4±0.2	1	0.9																		
	ts20	4*	30*	2.6*	2.2*	1	0.4	0.2	0.2																	
	ts28	30*	120	0.3	34*	0.4	1.0±0.7		0.5±0.1	0.4±0.3																
UNCLASSIFIED MUTANTS	ts2	8	55*	1.1	5*	14*	2.3			1.9	1.2															
	ts3	16*	30*	1	12*	12*	2.7	0.3	0.9	1.4	4															
	ts5	4.6*	10*	2	1.2±0.4	3	0.7	0.7	3	1.4	0.8		1													
	ts6	4*	13	1.5	2.5*	3	0.4	0.2	0.6	0.6	0.6		0.2	0.7												
	ts7	6*	22*	4	6*	4*	0.7	0.5	0.6	0.5	0.6		1.7	1	1.3											
	ts8	33	10*	1.4*	4*	2.1*	1.7	0.4		0.7	0.8		1		0.7	1.5										
	ts9	50	3.4*	1.6	4*	2.5*	1.2	0.7	1.3	7.4*	0.8		0.7				1.1									
	ts17	5*	8*	1.6	0.2±0.1	2.6	1.6	1	1.9	0.4	0.7				0.8	0.8	1.4									
	ts18	35*	12*	2	3*	4.3*	1.5	1	1.9	2.8*			0.2		2	0.5	1.8		1.4							
	ts19	4*	60	2	30*	3.4*	0.7		0.4	0.6				1	0.6	0.7	0.7		1.3	1.7						
	ts22	1.7*	23*	3	4*	1.6*	0.4		2.7	1	0.2	0.9	1.5	7	1.2	2*	1.4		1.5	1.4*	5					
	ts23	1.4	4.5*	1.3	0.3±0.2	2.4*	0.7	0.3	1.9	3.5	0.9		0.8	0.7	0.8	0.7	0.6		0.4	2	1.3	2				
	ts24	40*	6*	5	6*	1.0±0.3	4.8		1.1	0.8			1.9	0.7	0.3	0.7	0.7		1.2	0.8	0.6	2.4	0			
	ts25	4*	12*	2.4	1	4*	1.7		1.7	0.3	0.3	0.5	1	10	2	2	0.2		1	1.1	0.2	0.6	0	0		
	ts26		9*	4	3*	1.2±0.4	0.8		0.5	1			0.9		0.9		0.8		1.4	1	3	1.2	0	0	0	

* $p < 0.05$ level at which complementation (difference from 1) is statistically significant.

TABLE VII

COMPLEMENTATION LEVELS FOR PAIRS OF TEMPERATURE-SENSITIVE MUTANTS OF VSV

	II				III	UNCLASSIFIED MUTANTS																
ts16B	ts12	ts29	ts4	ts11	ts14	ts15	ts20	ts28	ts2	ts3	ts5	ts6	ts7	ts8	ts9	ts17	ts18	ts19	ts22	ts23	ts24	ts25	ts26
7*																							
10*	1.0±0.2																						
50*	3	9.5*																					
60*	4*	10*	0.5±0.4																				
80*	0.8	15*	0.9±0.5	0.3																			
00	3	3*	0.4±0.2	1	0.9																		
30*	2.6*	2.2*	1	0.4	0.2	0.2																	
20	0.3	34*	0.4	1.0±0.7		0.5±0.1	0.4±0.3																
55*	1.1	5*	14*	2.3			1.9	1.2															
30*	1	12*	12*	2.7	0.3	0.9	1.4	4															
10*	2	1.2±0.4	3	0.7	0.7	3	1.4	0.8		1													
13	1.5	2.5*	3	0.4	0.2	0.6	0.6	0.6		0.2	0.7												
22*	4	6*	4*	0.7	0.5	0.6	0.5	0.6		1.7	1	1.3											
10*	1.4*	4*	2.1*	1.7	0.4		0.7	0.8		1		0.7	1.5										
3.4*	1.6	4*	2.5*	1.2	0.7	1.3	7.4*	0.8		0.7				1.1									
8*	1.6	0.2±0.1	2.6	1.6	1	1.9	0.4	0.7				0.8	0.8	1.4									
12*	2	3*	4.3*	1.5	1	1.9	2.8*			0.2		2	0.5	1.8		1.4							
60	2	30*	3.4*	0.7		0.4	0.6				1	0.6	0.7	0.7		1.3	1.7						
23*	3	4*	1.6*	0.4		2.7	1	0.2	0.9	1.5	7	1.2	2*	1.4		1.5	1.4*	5					
4.5*	1.3	0.3±0.2	2.4*	0.7	0.3	1.9	3.5	0.9		0.8	0.7	0.8	0.7	0.6		0.4	2	1.3	2				
6*	5	6*	1.0±0.3	4.8		1.1	0.8			1.9	0.7	0.3	0.7	0.7		1.2	0.8	0.6	2.4	0.5			
12*	2.4	1	4*	1.7		1.7	0.3	0.3	0.5	1	10	2	2	0.2		1	1.1	0.2	0.6	0.7	5		
9*	4	3*	1.2±0.4	0.8		0.5	1			0.9		0.9		0.8		1.4	1	3	1.2	1	0.1	1	

plementation (difference from 1) is
ificant.

mutants with ts10 and ts16B at the non-permissive temperature not only gave high complementation levels but also total yields of 10 to 50% those of the wild type virus.

Mutant ts29 complemented all mutants tested except ts12, ts17, ts23 and ts25. Ts12 appeared to complement most of the other mutants which show complementation with ts29 except for a few pairwise crosses. Ts17, ts23 and ts25 not only failed to complement ts29 but also failed to complement most of the other mutants. Moreover, they seemed to complement ts12 and were therefore tentatively excluded from the same complementation group as ts12 and ts29, which have been assigned to group II.

Among the remaining 21 mutants, 6 mutants, ts4, ts11, ts14, ts15, ts20 and ts28 showed no complementation among themselves but complemented all members of groups I and II except for the pairwise crosses between ts12 and ts14 and ts12 and ts28. These mutants have been assigned to a third group, III.

Most of the crosses of the remaining 15 mutants failed to show complementation with one another but showed complementation with at least one member of each of the three complementation groups I, II and III. These mutants remain tentatively unclassified.

Interpretation of the complementation results will be given in the Discussion.

III. An attempt to Demonstrate Recombination between ts Mutants of VSV

Having assigned some of the ts mutants of VSV into complementation groups, an attempt was made to demonstrate recombination between mutants from each complementation group, hoping to determine from the recombination frequencies the size and arrangement of these genes in the VS viral genome.

In RNA animal viruses, genetic recombination between ts mutants has been demonstrated with influenza viruses (Simpson and Hirst, 1968), poliovirus (Cooper, 1968) and reoviruses (Fields and Joklik, 1969). Among these viruses recombination by breakage and reunion between two genomes has only been demonstrated with poliovirus (Cooper, 1968). Recombination in influenza viruses and reoviruses appears to take place by the reassortment of genetic fragments (Shatkin, 1971). Recombination has been looked for but not found in Sindbis virus, Semliki Forest virus and Newcastle disease virus (Fenner, 1970). Pringle (1970) reported recombination in mixed infections between ts mutants from different complementation groups of VSV, but the genotype of the recombinants was not tested.

In the search for recombinants carried out in this laboratory, it was found that no recombination could be demonstrated. There were, however, some plaques in the 38^{o} assay plates which at first sight looked as if they could have been produced by recombinants. But further analysis showed that these plaques contained progeny virus of both ts parental types used for the mixed infection instead of ts^{+}

recombinants. The plaques formed at 38^{o} might therefore be due to the complementation effect brought about by clumping of the virus particles or by the formation of "heteropolyploids". A detailed report of our recombination studies on ts mutants of VSV has been published (Wong et al., 1971). A reprint of this report can be found in the appendix.

IV. An Attempt to Characterize the Genetic Defects of the Complementation Groups

As an aid in identifying the defect represented by each complementation group, the following properties were tested:

1. Ability of the mutants to synthesize virus-specific RNA at the non-permissive temperature.
2. Time of expression of the temperature-sensitive defect.
3. Heat-lability of the virion.
4. Virus-specific RNA synthesis induced by the structural RNA polymerase (Baltimore et al., 1970) in vivo.

1. Virus-specific RNA synthesis

This experiment was performed in an attempt to find out whether the temperature-sensitive defect of the mutant occurs before, during or after virus-specific RNA synthesis by examining the ability of the ts mutant to incorporate ^{14}C-uridine at both the permissive and non-permissive temperature in infected cells treated with actinomycin D. Stampfer et al. (1969) and Schincariol and Howatson (1970) showed that cytoplasmic extracts prepared from uridine labelled uninfected cell cultures in the presence of actinomycin D had no radioactivity except for a negligible amount of slowly sedimenting 4S RNA which was presumably due to terminal labelling of transfer-RNA (Franklin, 1963). However, in cytoplasmic extracts prepared from VSV infected cells in the presence of actinomycin D, incorporation of radioactive label was observed. This radioactive incorporation was demonstrated to be due to

synthesis of virus-specific RNA.

Method

Cells were treated with actinomycin D (2 μg/ml) for 2 hours before infection, as well as during and after infection. Cells were then infected, washed and incubated as described in Materials and Methods. ^{14}C-uridine was added 0.5 to 2 hours after infection and samples were removed periodically for determination of the acid-insoluble activity as follows: Aliquots of 1 ml, each containing approximately 3×10^5 cells, were removed from the 100-ml bottles and diluted immediately with 2 volumes of PBS at 0^o containing 100 μg/ml of unlabelled uridine. The cells were then collected on a membrane filter (Millipore HAWP, pore size 0.45 μ), washed once with cold PBS and treated twice with 5 ml of trichloro-acetic acid at 0^o. The filter was washed with water and with ethyl alcohol and placed in a vial containing 10 ml toluene-methanol-POPOP counting fluid. Each vial was then placed in a liquid scintillation counter and counted until at least 2,000 counts had been accumulated. The counts were then corrected by subtracting the background count obtained with a vial containing no radioactivity. Replicate samples from the same experiment gave reproducibility with a standard deviation of ±10%, and the activity per cell was independent of the number of cells in the sample within the range used in these experiments. Samples were also taken from time to time for plaque assay and to determine the cell number by means of a Coulter counter.

Results

Uridine uptake data for the parent HR strain are given in

Fig. 2 (a). In this experiment, and in others with HR where uridine uptake was measured, the cumulative uridine incorporation at 30^{o} continued to rise later than 6 hours after infection, while at 38^{o} the cumulative uridine uptake reached a plateau at 4 to 6 hours after infection. A comparison of RNA synthesis of HR at 30^{o} and 38^{o} with two different input multiplicities (10 and 100 PFU/cell) on the basis of the rate of uridine incorporation during a 2 hour period beginning at half an hour after the addition of label, showed that the rate of RNA synthesis of HR at 38^{o} was approximately equal to that at 30^{o} (Fig. 2 (a)). In 10 separate experiments with HR the ratio of the rates of uridine uptake had a mean value of 0.98 with a standard deviation of 0.25.

The uridine uptake patterns for representative mutants of the three complementation groups, ts16B, ts29 and ts11 are shown in Fig. 2 (b-f). Growth curves obtained at 30^{o} and 38^{o} of these mutants may be represented by those shown in Fig. 5, p.63, except that with a high input multiplicity (>100 PFU/cell) the eclipse period for the 30^{o} cultures was shortened by ½ to 1 hour.

For ts16B (Group I) the uridine uptake was investigated under three different conditions. In Fig. 2 (b) and (c) uridine uptake was examined in cells infected with the same input multiplicity but radioactive label was added at different times after infection. In Fig. 2 (d) the radioactive label was added at the same time as in Fig. 2 (c) but the cells were infected with an input multiplicity 16 times higher than that in Fig. 2 (c).

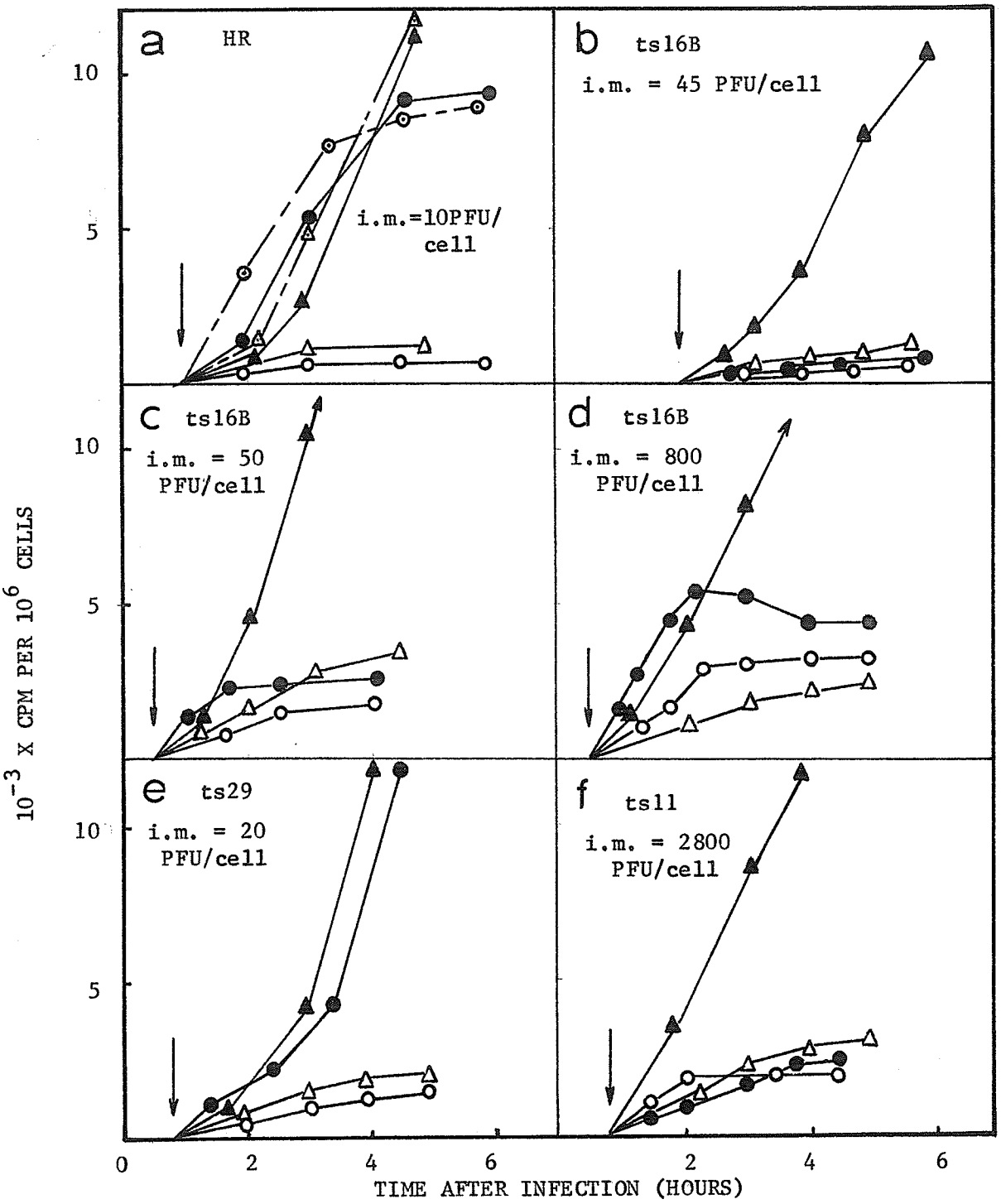


Fig. 2 Incorporation of ^{14}C-uridine by cells infected with HR (a), ts16B (b-d), ts29 (e) or ts11 (f) in the presence of actinomycin D (final concentration 2 µg/ml). Time of addition of ^{14}C-uridine (final concentration 0.03 µCi/ml) as indicated by arrows.

▲—▲, ●—● , Infected cells at 30° and 38° respectively;
△—△, ○—○ , Uninfected controls at 30° and 38° respectively;
△--△, ⊙--⊙ , HR infected cells with input multiplicity (i.m.) = 100 PFU/cell at 30° and 38° respectively.

In Fig. 2 (b) with an input multiplicity of 50 PFU/cell when label was added 2 hours after infection, the uridine uptake at 38° was not significantly higher than in the uninfected, actinomycin D treated control. However in Fig. 2 (c) when uridine was added half an hour after infection, there was some uridine uptake during the first hour following addition of label in the 38° cultures when compared to that of the uninfected control, but therewas no further increase of incorporation after this early phase. With an input multiplicity of 800 PFU/cell, Fig. 2 (d), uridine uptake continued for about 2 hours after addition of label. The amount of uridine incorporated was also greater than in Fig. 2 (c).

The uridine uptake pattern of ts29 (Group II) as shown in Fig. 2 (e) was very similar to that of HR. The amount of uridine uptake at 38° was comparable to that of the 30° culture. There is no evidence of apparent cessation of RNA synthesis after 4 hours incubation at 38° noted with HR.

In the case of ts11 (Group III), uridine uptake in the 38° culture was not significantly higher than in the uninfected control even though the label was added relatively early and a high input multiplicity was used. Uridine uptake at 30° was found to be comparable to that of HR.

The uridine uptake pattern was also examined for other members of the three complementation groups. Other members of groups II and III showed uridine uptake pattern similar to that of the representative mutant of each group. In the case of ts10, the other mutant of

group I, using high input multiplicity, uridine uptake continued for at least 4 hours at 38° instead of levelling off at 2 hours after the addition of label.

In order to provide an index for comparing the uridine incorporation of the ts mutants at the non-permissive temperature with one another the following measure, referred to as index of relative ^{14}C-uridine incorporation (%), was used. This index was defined as the ratio of the relative uridine uptake of the mutant at 38° compared to 30° divided by the relative uridine uptake for HR, the ratio being expressed as a percentage. The relative uridine uptake is defined for mutant (or for HR) as the net uridine incorporation during a 2 hour period beginning half an hour after addition of ^{14}C-uridine to mutant (or HR) infected cells at 38° divided by the incorporation at 30° during the same period.

The values of the index of relative ^{14}C-uridine incorporation for each mutant assigned to complementation groups I, II and III are listed in the right hand column of Table VIII. For each mutant the time of addition of label and the input multiplicity are also listed in this table.

The following conclusions may be drawn from the values of the index of relative ^{14}C-uridine incorporation for each mutant:

i) For group I mutants, when label was added at 2 hours after infection, no RNA synthesis at 38° was observed. However when label was added half an hour after infection a small amount of RNA synthesis was observed in the 38° culture when compared to that of the RNA

TABLE VIII

INDEX OF RELATIVE ^{14}C-URIDINE INCORPORATION OF MUTANTS OF COMPLEMENTATION GROUPS I, II AND III

COMPLEMENTATION GROUP	MUTANT	INPUT MULTIPLICITY	TIME AFTER INFECT. ^{14}C-URIDINE ADDED (HRS)	INDEX OF* RELATIVE ^{14}C-URIDINE UPTAKE (%)
I	ts10	100	1	10
		1000	1	20
	ts16B	20	2	0
		45	2	0
		75	2	0
		50	0.5	5
		800	0.75	12
II	ts12	20	1	40
		50	1	50
	ts29	20	1	50
		700	1	60
III	ts4	20	0.75	0
		50	1	0
	ts11	30	1	0
		250	1	0
		700	0.75	0
		2800	1	0
	ts14	25	1	0
		70	1	0
		300	0.5	0
	ts15	30	1	0
	ts20	30	1	0
		100	0.75	0
	ts28	3000	0.5	0

* See text on p. 52 for definition.

synthesized at 30°. The amount of RNA synthesized at 38° appeared to depend on but is not proportional to input multiplicity. The lack of linearity might be due to the possibility that the number of virus particles which penetrate into the cell during adsorption may not have been proportional to the input multiplicity of infection.

ii) The values of the index for group II mutants suggests that there is a partial defect in RNA synthesis at 38°. However it is noted that for both ts12 and ts29, RNA synthesis at 30° is excessively high. This high incorporation may be responsible for the low value of the index. The amount of RNA synthesis at 38° relative to HR appears to be normal.

iii) For ts11, ts14, ts20 and ts28 there is no evidence of RNA synthesis even though high input multiplicities were used and label was added early in the growth cycle. In the case of ts4 and ts15, only low input multiplicities were used since it was found that these mutants were "leaky" with high input multiplicities. Since infectious particles were produced under such conditions of high input multiplicities (>100 PFU/cell) at 38°, one could expect that some RNA would be synthesized. This was in fact found to be the case and was attributed to "leakiness" of the ts function, related to RNA synthesis, observed at 38°.

In summary, the results of the virus-specific RNA synthesis experiments suggest that the group I mutants are able to synthesize some RNA at 38° during the early phase of growth but the amount of RNA synthesized is considerably less than that at 30°. This observation

indicates that the mutants may be defective in some function involved in RNA synthesis. The group II mutants are able to synthesize RNA at 38° and in an amount comparable to that of HR at 38°, thus indicating that the defect of the group II mutants is not in RNA synthesis, but perhaps in some process involved in virion assembly or maturation. The group III mutants failed to synthesize RNA at the non-permissive temperature suggesting that they are defective in a function which occurs before or during RNA synthesis.

2. Time of expression of temperature-sensitive function

Virus production following temperature shift from 30° to 38° at various times was investigated for each mutant of the three complementation groups in order to find out during which part of the replicative cycle the temperature-sensitive step of the mutant is expressed.

Method

A temperature-shift experiment was carried out as follows: A number of cell suspension cultures were infected with virus at an input multiplicity of 20 PFU/cell as described in Materials and Methods. These cultures were initially incubated at 30°. At various time intervals, for example, 0 hour (that is, at the beginning of the experiment), 2 hours, 4 hours and so on, one culture was transferred from the 30° to the 38° bath. At each time of shift a sample was taken from the culture for plaque assay of the growth curve which is indicated by closed circles in Fig. 3. At 8 hours after infection all the cultures were assayed. The open circles and the dotted lines in Fig. 3 indicate

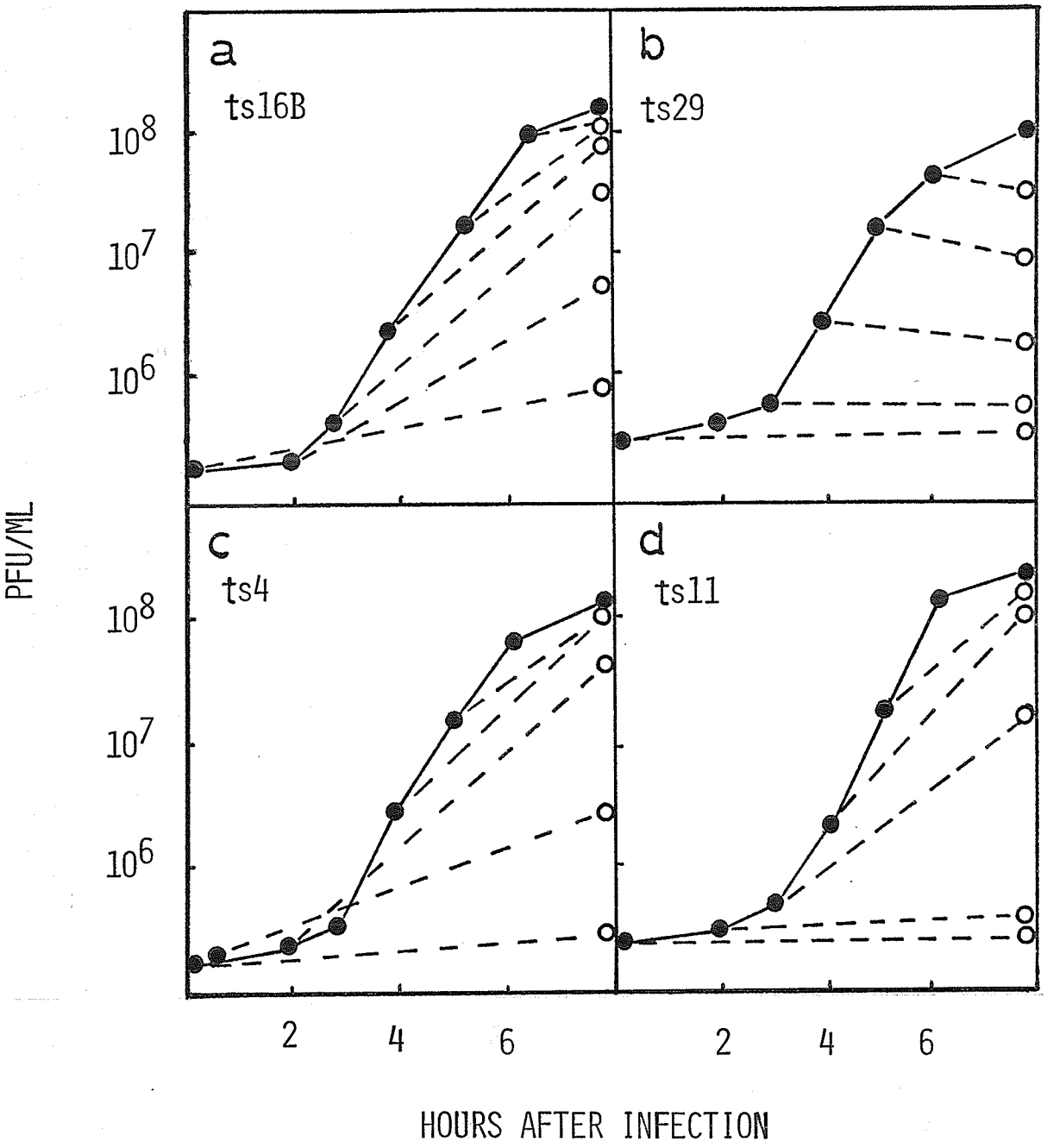


Fig. 3 Virus production of ts mutants in L cells after transfer from 30° to 38° at different times after infection.
●——● , Growth curves at 30°;
●- - -○ , Virus production at 38° following "shift-up" at the times indicated by the solid circles.

8-hour virus titre after different initial incubation times at 30°, and the time of incubation of the cultures at 38° respectively. In these temperature-shift experiments one would expect no more than a very low yield of virus (1% of 30° 8-hour titre) if the culture was kept at 38° for the total 8 hours. On the other hand, if the culture was left for the total time of 8 hours at 30° with no time at 38°, one would expect a normal production of virus. The virus production obtained from the remaining cultures would depend on the time at which the defect was expressed by each of the mutants tested. If the defective function of a mutant was expressed early in the growth cycle, for example, before 2 hours, then a shift from the permissive (30°) to the non-permissive temperature (38°) after 2 hours would no longer inhibit growth at 38° because the defect had already been overcome by putting the culture at 30° for 2 hours. Therefore one would expect a near normal yield for the 8-hour assay after an initial 2 hours at 30°. If the defective function of the mutant was expressed later than 4 hours in the growth cycle, a shift to 38° at 4 hours would inhibit the growth of the mutant and one would expect a low titre after a further 4 hours incubation at 38°.

Results

Fig. 3 shows the results of the temperature shift-up experiments performed on representative mutants of the three complementation groups: ts16B (group I), ts29 (group II) and ts4 and ts11 (group III). The temperature shift-up for each mutant was repeated at least once and these results confirmed.

With ts16B, a group I mutant (Fig. 3 (a)), the 8-hour assays

showed that some virus multiplication had occurred during incubation at the non-permissive temperature but that the amount of multiplication was dependent on the length of the initial incubation period at the permissive temperature (30^{o}). With a 2 hour initial incubation at 30^{o} and subsequent incubation at 38^{o} for 6 hours about 5% of the virus production of the 30^{o} 8-hour culture was obtained. Nevertheless it required about 4 hours initial incubation at 30^{o} for the virus yield of the 8-hour assay to reach 50% that of the 8-hour culture incubated throughout at 30^{o}. Similar results were obtained for ts10 the other mutant of group I. These observations suggest that the temperature-sensitive defect is expressed for at least 4 hours from the beginning of the growth cycle. It appears that the products made during the initial permissive period were used later for the production of some virus particles under non-permissive conditions.

With ts29, a mutant of group II (Fig. 3 (b)), the production of progeny virus ceased whenever the infected cells were transferred to 38^{o} even after 6 hours initial incubation at 30^{o}. This suggests that the temperature-sensitive defect occurs late in the growth cycle.

Among the group III mutants, ts4 infected cultures maintained at 30^{o} for half an hour then transferred to 38^{o} for the remainder of the 8-hour incubation period showed significant increase in production of virus over the culture incubated at 38^{o} throughout (Fig. 3 (c)). With 2 hours initial incubation at 30^{o} and subsequent incubation at 38^{o} for 6 hours virus production was about 50% that of the culture kept at 30^{o} throughout the 8-hour incubation period. In the case of ts11

(Fig. 3 (d)) there was no increase in viral yield in the 8-hour assay when the cultures were shifted to 38° after a period of up to 2 hours at 30°. But viral production as measured by the 8-hour assay increased considerably when the shift to 38° occurred after an initial 3 hour incubation at 30°, and with an initial 4 hour incubation at 30° the 8-hour assay showed a virus yield nearly 50% that of the culture kept at 30° for the entire 8-hour incubation period. For the rest of the mutants of group III the period of incubation at 30° required to enable virus yield to reach 50% that of the culture kept at 30° throughout the 8-hour incubation period varied from 2 to 4 hours. It appears from these observations that with the group III mutants, although the temperature-sensitive defect seems to be overcome at different time periods varying from half to four hours after infection, once the defect begins to be overcome an additional period of about 1.5 hours at the permissive temperature is sufficient to bring the virus yield to 50% that of the 8-hour 30° culture.

The fact that the temperature-sensitive defect of the group III mutants appears to be overcome at various times after infection suggeststhat they may not share the same defect. However, shift-up experiments only determine the time when the defect of a given mutant is overcome but not the time when the defect begins to be expressed during the growth cycle of the mutant. In order to test the time of onset of the temperature-sensitive defect(s) of the group III mutants, temperature "shift-down" experiments were performed for mutants ts4, ts11 amd ts14. It was argued that if a mutant has a temperature-sensitive defect in a

function required shortly after infection, a period of incubation at 38^{o} longer than the length of the time required for the expression of the defect, would delay subsequent growth at 30^{o} for a period of time corresponding to the difference between the length of time of incubation at the non-permissive temperature and the length of the growth cycle up to the time of expression of the defect.

Temperature "shift-down" experiments were carried out by initially incubating the cultures at 38^{o} for 2 hours, then transferring them to 30^{o} for the remaining 8-hour incubation period.

Growth curves of ts4 and ts11 following temperature "shift-down" to 30^{o} after 2 hours at 38^{o}, shown in Fig. 4, (result of ts14 is not shown because it is essentially similar to that of ts4 and ts11) indicate that for the group III mutants tested, viral growth was delayed about 2 hours thus indicating that the defect begins to be expressed very shortly after infection.

From the results of the temperature-shift experiments it appears that the defect(s) of the group III mutants is expressed shortly after infection but the defect(s) is overcome at various times during the growth cycle. There are two possible explanations for the above observations. Firstly, these mutants share the same defect. However the degree of seriousness of the defect is different among them so that it is overcome at different times during the growth cycle. Alternatively, some of these mutants e.g. ts11 and ts14 besides sharing the same defect as ts4 may also be defective in some other function which is overcome later (about 4 hours after infection) in the growth cycle.

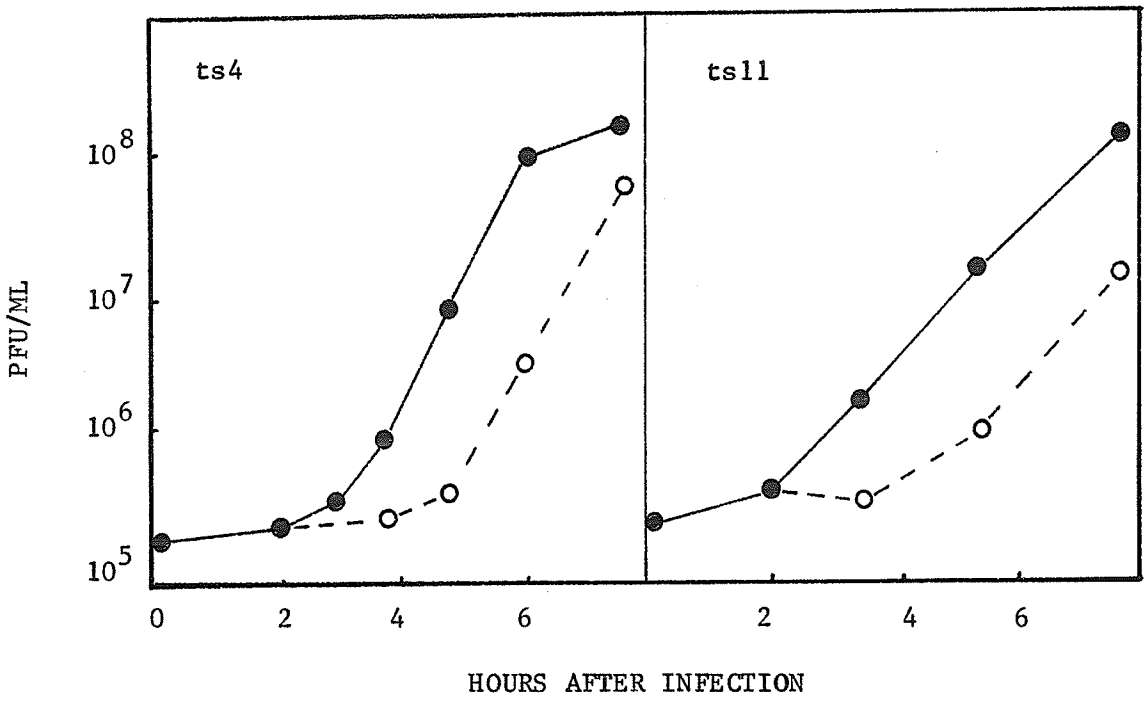


Fig. 4 Growth curves of ts mutants in L cells transferred from 38° to 30°. The infected cultures were transferred to 30°:-
●——● , immediately after infection;
O- - -O , 2 hours after infection.

Another approach in finding out the nature of the defects of the mutants was by investigating the effect of temperature-shift on virus-specific RNA synthesis.

In this experiment, host cell RNA synthesis was suppressed by adding actinomycin D as described for the experiments on virus-specific RNA synthesis. For each mutant tested, 3 out of 6 cell suspension cultures were infected with the same input multiplicity of virus as described in Materials and Methods while the other 3 cell suspension cultures were uninfected controls. One infected and one control culture were placed in the 38^{o} bath while the rest were kept at 30^{o}. ^{14}C-uridine was added to every culture 1 to 2 hours after infection. An infected and a control culture were transferred from the 30^{o} bath to the 38^{o} bath at 2 hours after infection. At various time intervals, samples were removed from each culture for assay of virus yield and rate of ^{14}C-uridine incorporation. The latter was measured as described for experiments on virus-specific RNA synthesis.

The results of this experiment are shown in Fig. 5 (a-d) and demonstrate the effect of temperature-shift on virus-specific RNA synthesis for mutants ts16B (Fig. 5 (a)), ts29 (Fig. 5 (b)), ts4 (Fig. 5 (c)) and ts11 (Fig. 5 (d)). The left hand panel (Fig. 5_1) shows the ^{14}C-uridine incorporation at various times by the infected and the uninfected control cultures at 30^{o} and 38^{o} as well as the growth curves for the mutants at these two temperatures. The right hand panel (Fig. 5_2) shows the ^{14}C-uridine incorporation by the infected and uninfected control cultures incubated initially for 2 hours at 30^{o} then at 38^{o} as

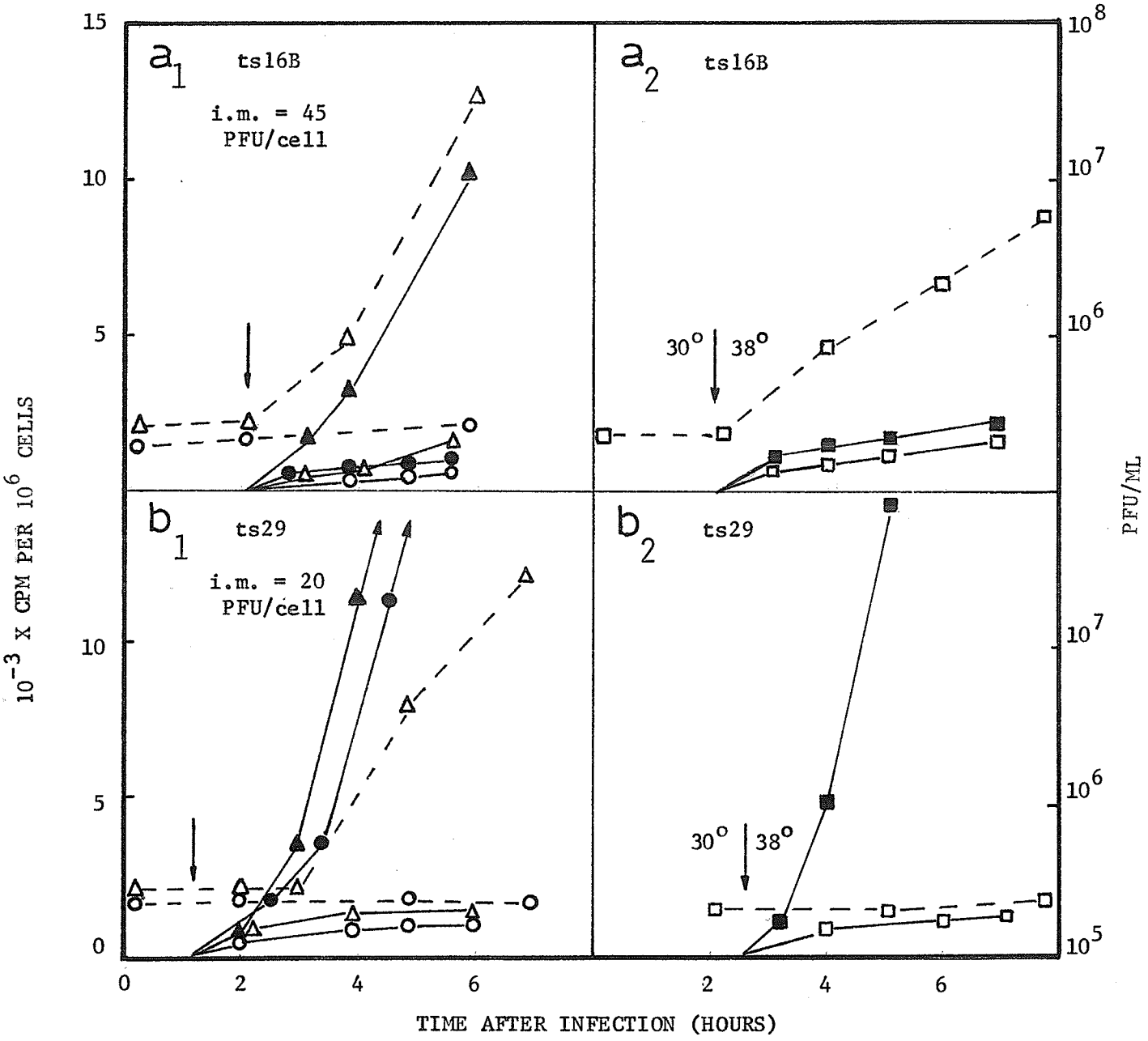
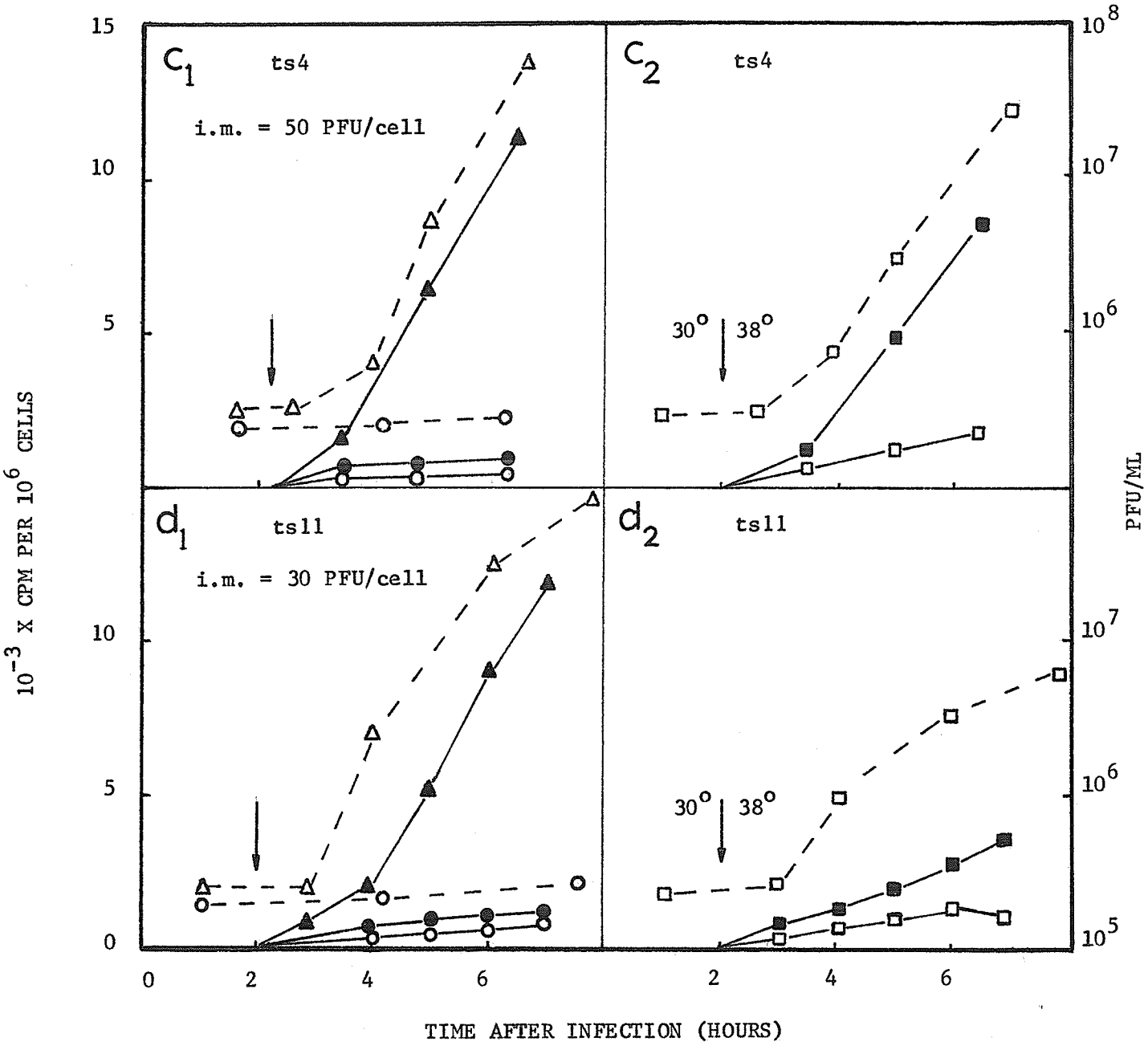


Fig. 5 ^{14}C-uridine incorporation and virus replication of selected ts mutants at 30°, 38° and after shift from 30° to 38°.
Uridine incorporation in infected cells:
▲——▲ at 30°, ●——● at 38°, ■——■ after shift from 30° to 38°;
Uridine incorporation in uninfected controls:
△——△ at 30°, ○——○ at 38°, □——□ after shift from 30° to 38°;
Growth curves of ts mutants:
△- -△ at 30°, ○- -○ at 38°, □- -□ after shift from 30° to 38°.
Arrows indicate time of addition of label and, in the right hand panels, time of temperature-shift as well.
Actinomycin D concentration, 2 µg/ml; ^{14}C-uridine, 0.03 µCi/ml.

Fig. 5 continued



well as the growth curves of the mutants under the same conditions.

In Fig. 5 (a), the results indicate that with ts16B, a group I mutant, ^{14}C-uridine incorporation at 38°, with label added 2 hours after infection, was unaffected by the temperature prevailing during the previous 2 hours. There is, however, a continuation of production of virus as seen in Fig. 5 (a_2), suggesting that the RNA made prior to temperature shift was used in the production of virus that occurred after shift. The time of shift for group I mutants was chosen at 2 hours after infection in order to avoid measuring RNA synthesized at 38° during the early phase of the growth cycle as shown in Fig. 2 (c), p. 50.

With the group II mutants, represented by ts29 (Fig. 5 (b)), a large amount of RNA is synthesized after shift from 30° to 38° but there is no virus multiplication. These findings are consistent with the late expression of the temperature-sensitive defect and the ability of the group II mutants to synthesize RNA at the non-permissive temperature. It seems therefore that the group II mutants may be defective in a process not involving viral RNA synthesis, but in some process in virion assembly or maturation.

Among the group III mutants, variations in uridine uptake patterns were observed. These variations, as shown by ts4 and ts11, are consistent with the results obtained from the temperature shift-up experiments memtioned above. In ts4 (Fig. 5 (c)) the rate of uridine uptake increases with time following a shift to 38° after a 2 hour incubation at 30° and is nearly as great as that of the corresponding

culture kept at 30°. As for ts11 (Fig. 5 (d)), although uridine uptake continues for several hours after shift to 38° following a 2 hour initial incubation at 30°, the rate of uridine uptake after shift was considerably below the uptake in the corresponding culture kept at 30°.

Although there are variations in the pattern of uridine uptake after shift among the group III mutants, some RNA appears to be synthesized at the non-permissive temperature following a period under permissive conditions. These observations also indicate that with a 2 hour period under permissive conditions, the temperature-sensitive defect is almost completely overcome in the case of ts4 and is at least partially overcome in the case of ts11. These observations are consistent with the results of the virus-specific RNA synthesis experiment and the temperature-shift experiments. Taken together the results of these two types of experiments indicate that the defect of the group III mutants is in a function which occurs before or during virus-specific RNA synthesis and that this defect is expressed for sometime after infection.

3. Heat-lability of the virions

In an attempt to distinguish between defects in viral components and those defects in the viral non-structural proteins induced in the infected cell, a study was made of the rate of heat inactivation of the various virus stocks. A stock of the virus to be tested was suspended in 5 ml of culture medium at a concentration of about 1×10^8 PFU/ml and was placed in a 43° bath and periodically assayed at 30°

for survivors. The inactivation kinetics of HR and of all the mutants were found to be approximately exponential for at least 3 hours and were fitted with an expression of the form $S=\exp(-Kt)$ where S is the surviving fraction after a time t and K is an inactivation rate constant.

The heat-lability test was repeated at least once for most mutants and the results were similar. The inactivation rate constant obtained for each mutant is shown in Table IX and the survival curves, after heating at 43^{o}, are shown in Fig. 6 for HR and representative mutants.

The results show that the group I mutants are not different in heat-lability from HR while mutants of group III appear to be significantly more heat-labile than HR. Since the group II mutants, ts12 and ts29, could synthesize RNA at the non-permissive temperature and are defective in a late function they might have been expected to be defective in a structural protein. However, there is no evidence of heat-lability significantly greater than HR in these two mutants.

The heat-lability of ts^{+} revertants obtained from ts4 and ts11, which had regained their ability to replicate at 38^{o}, was also investigated to find out whether the increased thermolability of the mutants was due to the same defect as that responsible for the ts behaviour of the mutant. To show this, it is necessary to demonstrate that the mutation responsible for the increase in heat-lability is in the same gene as that responsible for the ts defect. This can be shown with reasonable certainty if ts^{+} revertants have also reverted to the

TABLE IX

INACTIVATION CONSTANTS (K) OF MUTANTS OF COMPLEMENTATION GROUPS I, II & III

COMPLEMENTATION GROUP	MUTANT	NUMBER OF EXPERIMENTS	K ($HOUR^{-1}$) *
	HR	18	0.72 ± 0.02
I	ts10	1	0.8
	ts16B	6	0.75 ± 0.02
II	ts12	2	1.0
	ts29	1	1.0
III	ts4	10	2.1 ± 0.07
	ts11	4	1.6 ± 0.08
	ts14	3	1.5 ± 0.4
	ts15	2	1.2 ± 0.1
	ts20	4	2.0 ± 0.2
	ts28	1	2.2
	ts4 revertant 1	2	0.8 ± 0.15
	revertant 2	2	0.65 ± 0.05
	ts11 revertant 1	1	0.7
	revertant 2	1	0.7

* Data fitted by $S = \exp(-Kt)$ where S is the surviving fraction after a time t and K is an inactivation rate constant.
Confidence limits are standard errors of the mean.

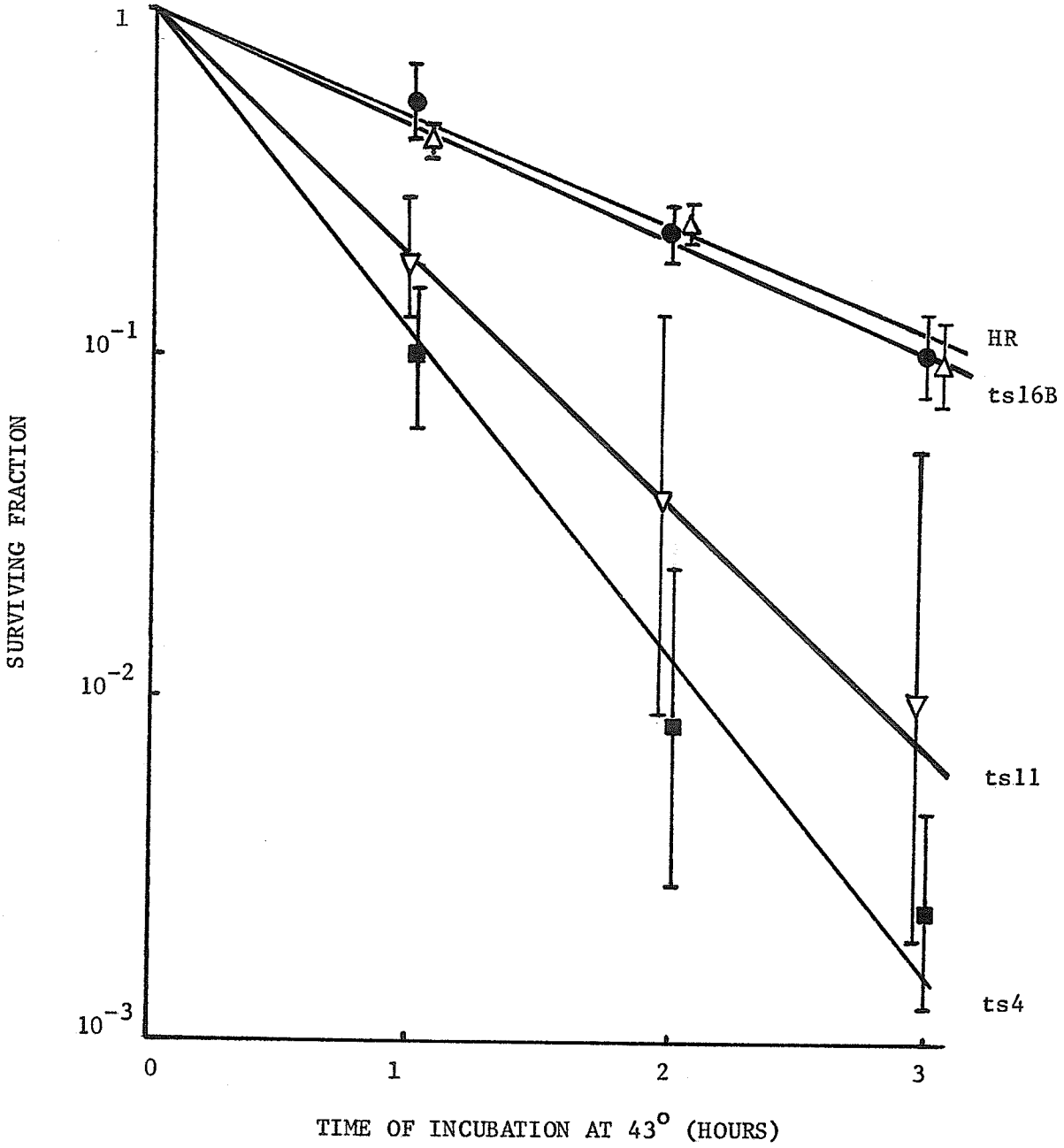


Fig. 6 Surviving fraction of HR strain and ts mutants of VSV at 43°. Virus stock was suspended in medium and incubated at 43°. At times indicated, 0.1 ml of the virus suspension was diluted with cold PBS and assayed at 30°.
●——● HR, △——△ ts16B,
■——■ ts4, ▽——▽ ts11.
The bars indicate 95% confidence limits.

same degree of heat-lability as the wild type parent (HR), as it is unlikely that two such independent changes could occur together by chance. It should be stressed, however, that an absence of such co-reversion need not indicate that the original defects are in different genes.

Results of the investigation on the heat-lability of two ts^+ revertants of each of the mutants ts4 and ts11 indicate that the heat-lability of these revertants is similar to that of HR (Table IX). It thus appears that the defect in ts4 and ts11 which prevents its replication at 38^o is due to an altered structural protein.

4. Virus-specific RNA synthesis induced by the structural RNA polymerase of the virion

Another approach for investigating the temperature-sensitive defect of the mutants was suggested by the heat-lability of the group III mutants, together with the fact that they appear to have a temperature-sensitive defect which is expressed early in the growth cycle and is involved with RNA synthesis. It seems possible that these mutants may be defective in the structural RNA polymerase reported by Baltimore et al. (1970).

In order to test for structural polymerase activity, selected mutants of groups I, II and III as well as HR were tested as to whether these mutants could synthesize RNA in cells in which puromycin had been added to inhibit the formation of new polymerase.

Investigation of uridine incorporation in cells infected with

different input multiplicities of HR (Fig. 7) (details of method to be described on page 73) shows that there is considerable puromycin-resistant incorporation (that is, incorporation when puromycin has been present throughout infection) in infected cells at both 30^{o} and 38^{o} which suggests that RNA is being synthesized in the absence of protein synthesis. Furthermore, at both temperatures, in the presence of puromycin, there is increased rate of uridine incorporation with increase in input multiplicity of virus. Since it has been demonstrated by in vivo experiments that the structural polymerase acts on the viral RNA template to synthesize complementary RNA (Marcus et al., 1971), these results suggest that the RNA synthesized in the presence of puromycin is due to the activity of the structural polymerase of the infecting virus particles. However, the rate of uridine incorporation in the absence of puromycin is much greater than in its presence which further suggests that a portion of the RNA synthesized during a growth cycle requires prior protein synthesis.

The question arises whether the uridine incorporation observed in experiments using very high input multiplicities represents abnormal RNA synthesis associated with the production of T particles (Stampfer et al., 1969). It has recently been shown however (Stampfer et al., 1971), that a high multiplicity passage from a clonally purified VSV stock does not give rise to a large amount of T particles nor to an altered spectrum of RNA species. The stocks of HR used in the experiments reported here were derived from isolated plaques and contained only a small percentage of T particles (<5%). Even at input multiplicities of 3,000

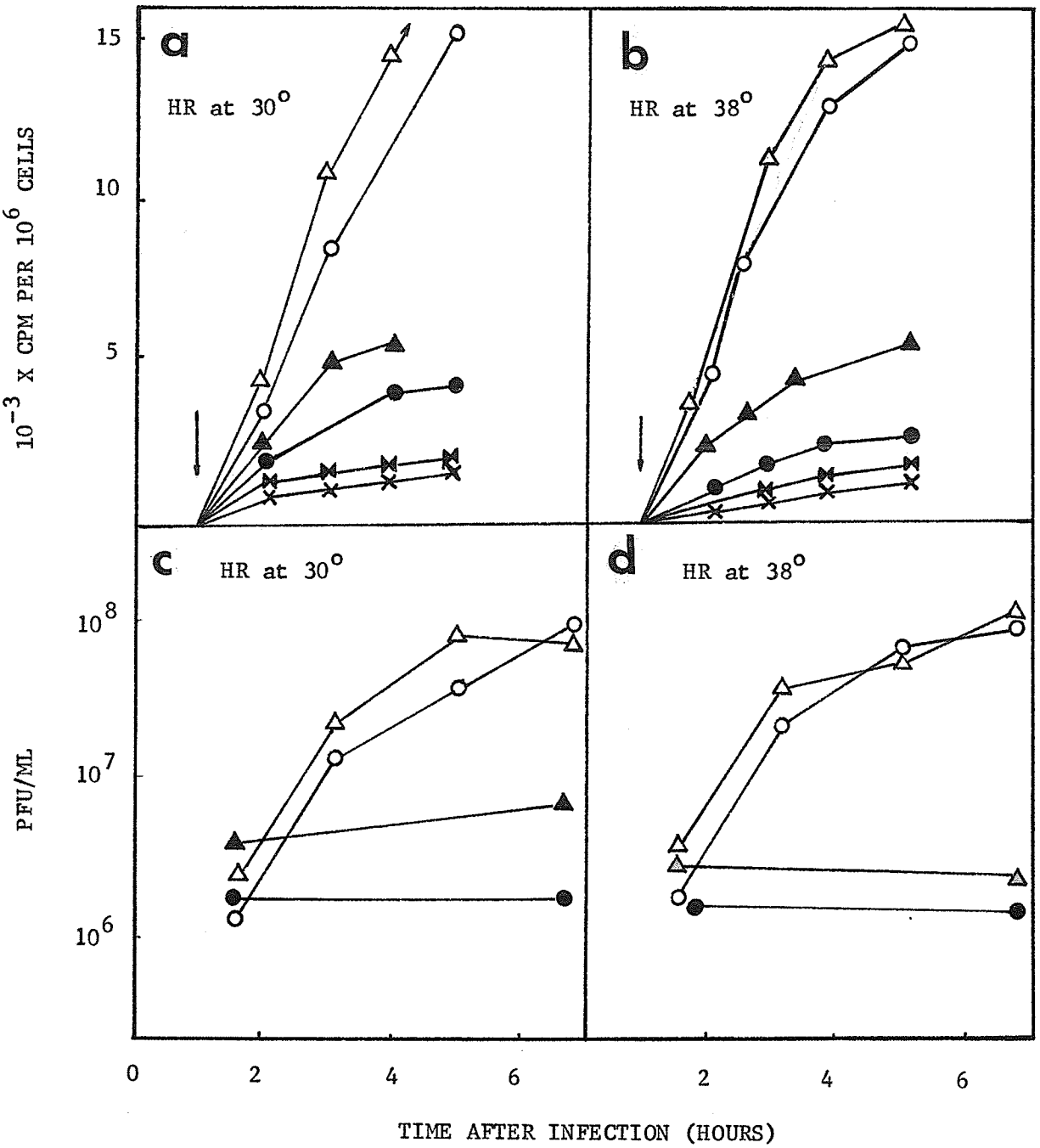


Fig. 7 Incorporation of ^{14}C-uridine (a,b) and virus growth curves (c,d) in L cells infected with HR with two different input multiplicities.
Medium containing 2 µg/ml actinomycin D:
O——O 500 PFU/cell, △——△ 1500 PFU/cell, ×——× uninfected control.
Medium containing 2 µg/ml actinomycin D and 60 µg/ml puromycin:
●——● 500 PFU/cell, ▲——▲ 1500 PFU/cell, ⋈——⋈ uninfected control.
Arrows indicate time of addition of label.

PFU/cell an 8-hour incubation gave virus production of 100-1,000PFU/cell with no increase in the percentage of T particles. Thus it appears that the patterns of uridine incorporation observed at high input multiplicities probably reflect RNA synthetic processes involved in viral replication.

Method

The RNA polymerase activity of HR and ts mutants were tested as follows: Cells were pretreated for 2 hours before infection with 2 μg/ml of actinomycin D. Infection of the cells with ts mutants was usually at high input multiplicity, the value of which is noted in Fig. 8. Approximately the same input multiplicity of HR as that of the mutant tested was used in each experiment. Where cells were infected in the presence of puromycin, puromycin was added at a concentration of 60 μg/ml* at the beginning of infection while ^{14}C-uridine was added to a final specific activity of 0.02-0.05 μCi/ml at the times indicated in Fig. 8. Experiments were carried out in suspension cultures using the methods previously described in Materials and Methods, and the methods used in determining the amount of ^{14}C-uridine incorporated into the acid-insoluble fraction are as described for experiments on virus-specific RNA synthesis.

*It was found that in infected cells with low concentrations of puromycin the rate of uridine uptake decreased with increased concentration of puromycin, but no further decrease in the rate of uridine uptake was observed by increasing the puromycin concentration from 50 to 100 μg/ml. With these puromycin concentrations no virus production was observed in the 8-hour assays. Although cycloheximide is frequently used for the suppression of protein synthesis, it was found that in our experiments with VSV, puromycin at concentrations of 60 μg/ml gave the same reduction of uridine uptake as concentrations of 300 μg/ml of cycloheximide.

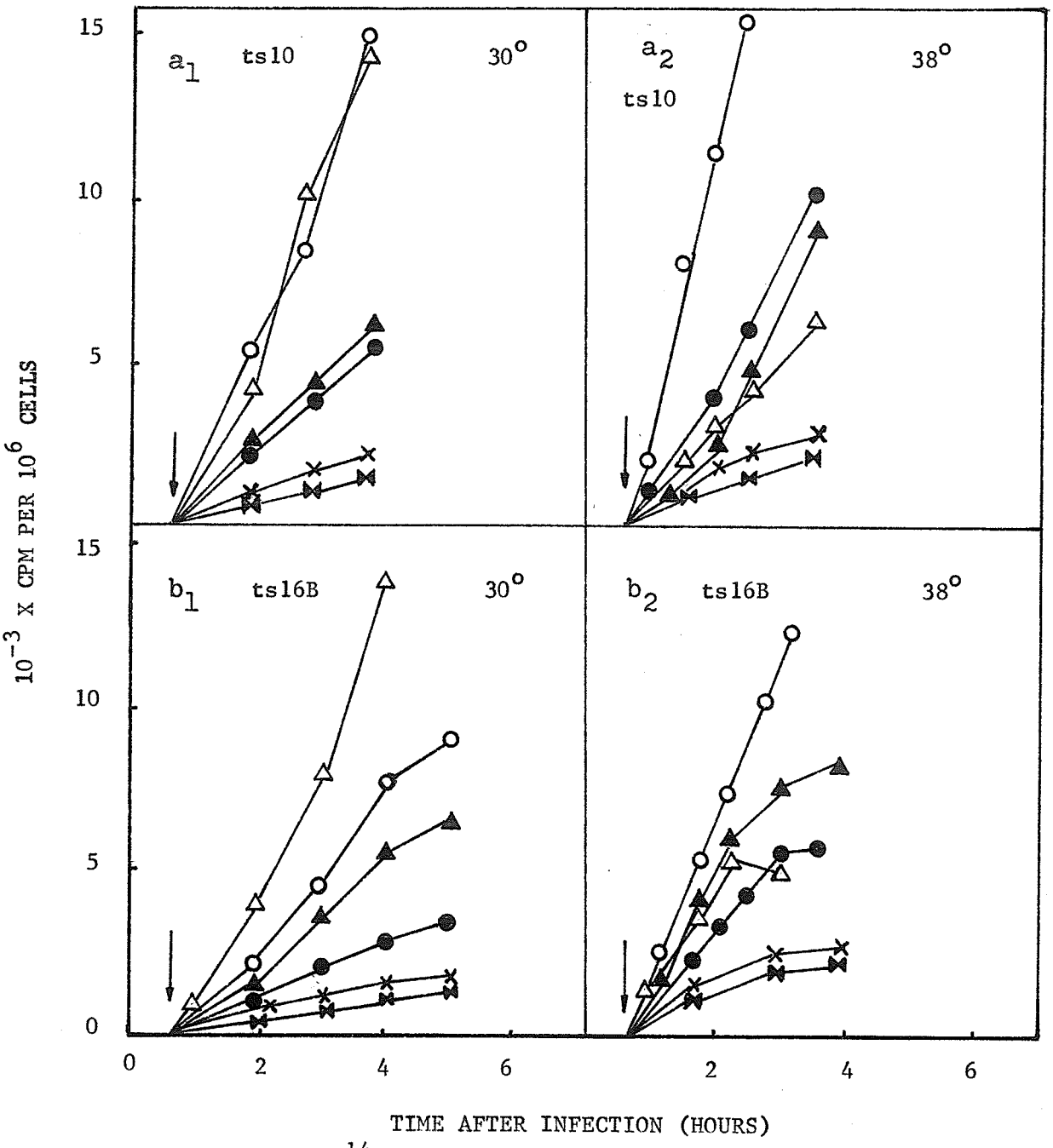
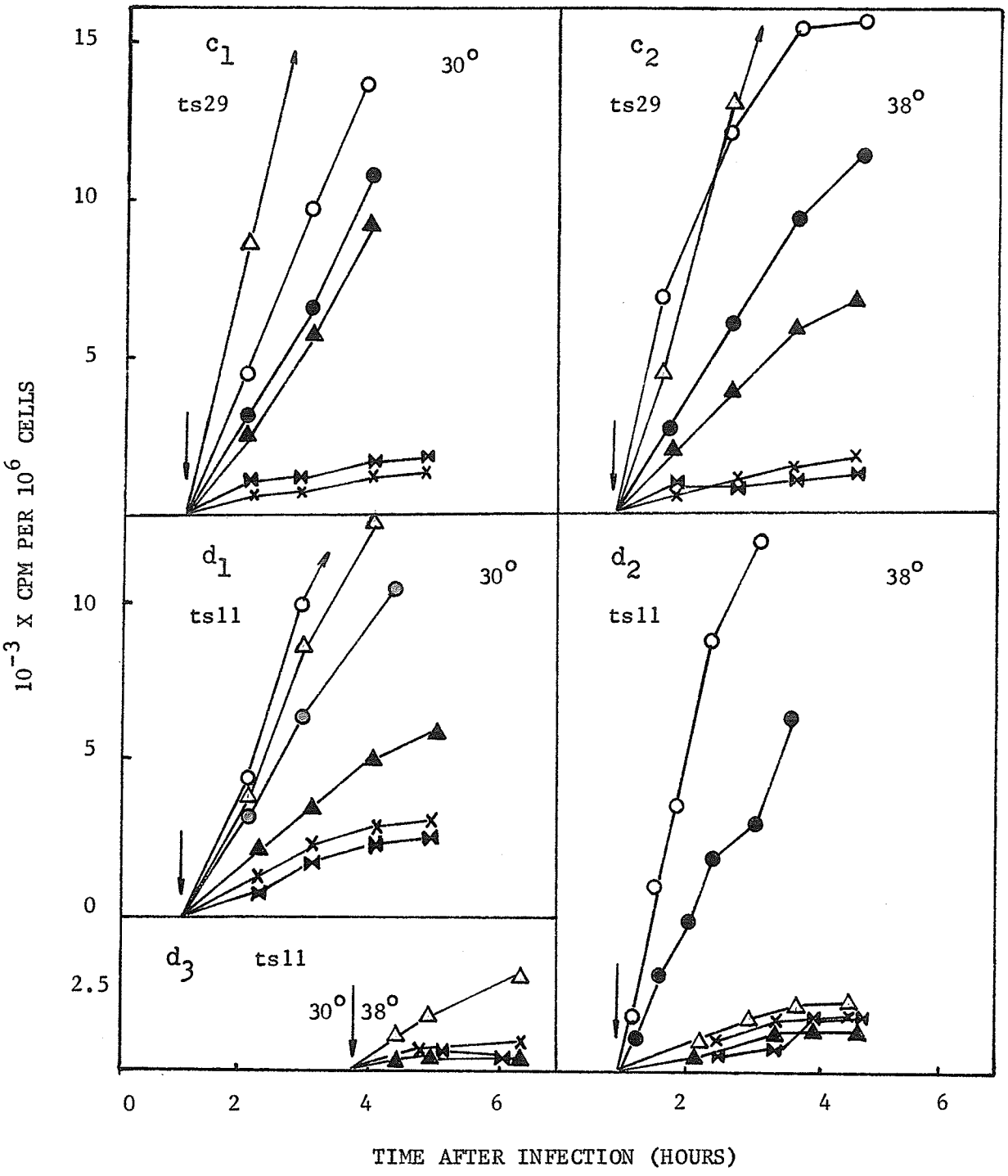


Fig. 8 Incorporation of ^{14}C-uridine in infected L cells at 30° and 38° for selected mutants. The incorporation of uridine for HR infected cells was also determined under the same conditions used for each mutant.
Medium containing 2 µg/ml actinomycin D:
O——O HR, Δ——Δ ts mutant, ✕——✕ uninfected control.
Medium containing 2 µg/ml actinomycin D and 60 µg/ml puromycin:
●——● HR, ▲——▲ ts mutant, ⋈——⋈ uninfected control.
^{14}C-uridine added at times indicated by arrows (final concentration, 0.03 µCi/ml).
Input multiplicities used were:
ts10: 2000 (HR:2500) PFU/cell, ts16B: 800 (HR:400) PFU/cell,
ts29: 1000 (HR:700) PFU/cell, ts11: 2000 (HR:2800) PFU/cell.

Fig. 8 Continued



Legend on Fig. 8, p. 74 applies. For the experiment indicated on d_3, the culture was incubated at 30° for 3.8 hours and then transferred to 38°.

Results

For the group I mutants (ts10 and ts16B) at 30°, uridine uptake in the presence of puromycin is considerably less than in its absence (Fig. 8 (a-b)). At 38° (Fig. 8 (a_2-b_2)), the amount of uridine uptake in ts10 or ts16B infected cells is very similar in the presence or absence of puromycin during the first 2 hours after infection, and in amounts comparable with that incorporated by HR in the presence of puromycin with about the same input multiplicity of virus. These results indicate that the group I mutants are not defective in the structural polymerase but are defective in RNA synthesis which requires protein synthesis.

For ts29, a group II mutant, results similar to those observed for HR were obtained (Fig. 8 (c)). At both 30° and 38°, there is considerably greater uridine uptake in cells infected with ts29 in the absence of puromycin than in cells where puromycin has been added. These observations are consistent with the previous suggestion that the group II mutants are not defective in RNA synthesis but probably in some function following RNA synthesis.

In the case of group III mutants, represented by ts11 as shown in Fig. 8 (d), no uridine uptake was observed at 38° in the presence or absence of puromycin. Similar results were obtained for the other group III mutants tested, ts14, ts20 and ts28. However, when ts4 was tested with an input multiplicity of 500 PFU/cell at 38°, some uridine uptake which is significantly lower than that obtained in the 30° culture was observed. The 8-hour viral production at 38° was 14%

that of a similarly infected spinner culture maintained at 30^{o} which suggests that the low uridine incorporation at 38^{o} in the presence of puromycin was due to "leakiness" of the mutant. Thus all group III mutants so far tested show a defect in structural polymerase activity at 38^{o}.

Uridine incorporation in cells infected with ts11 in the presence of puromycin was also measured following a temperature shift from 30^{o} to 38^{o}. No detectable puromycin-resistant incorporation after shift-up was observed (Fig. 8 (d_3)) supporting the belief that the temperature-sensitive defect is in the polymerase activity per se rather than in some preceding function (e.g. "uncoating"). In the absence of puromycin however, there is some incorporation by ts11 after shift to 38^{o} in contrast to its behaviour when the cultures are held at 38^{o} throughout the infection (Fig. 8 (d_2)). Thus a period under permissive conditions (30^{o}, in the absence of puromycin) allows the development of a polymerase activity which can continue to function at 38^{o}, unlike the activity of the structural polymerase. An interpretation of this behaviour is that the period of incubation at 30^{o} allows ts11 to transcribe some viral message which is then translated to form polymerase molecules differing from those of the structural polymerase in their ability to function at 38^{o}.

Having established that the group III mutants are defective in their structural polymerase one would like to find out whether their defect could be circumvented by a mutant which is not defective in the structural polymerase. In order to answer the above question

a complementation experiment was carried out between ts11, a mutant of group III, and ts16B, a mutant of group I, which is not defective in the structural polymerase. If the ts11 genotype were found in the progeny of such a mixed infection, then the defect in the structural polymerase must have been circumvented.

Such an experiment was carried out as follows: Ten well isolated plaques were picked at random from the 30° assay of the virus production from a mixed infection with ts11 and ts16B at 38°. Stocks grown from all these plaques showed no growth at 38° but growth at 30°. Each stock was then mixedly infected with either parent at 38° to find out which parental genotype it possessed. Five of these stocks complemented with ts11 but did not complement ts16B, while the other 5 failed to complement with ts11 but complemented with ts16B, thus indicating that such a mixed infection gave rise to progeny of both parents. These results suggest that whatever is defective in the ts11 structural polymerase, it is circumvented by a diffusible material from ts16B. The defect of ts16B is similarly circumvented by ts11.

DISCUSSION

DISCUSSION

One of the principal objectives of the present investigation of ts mutants of VSV is to sort these mutants into complementation groups with the hope that each complementation group will correspond to a specific biochemical defect in the sequence of events which occur during the infection of L cells by VSV. The subsequent identification of the function that is defective in each complementation group by biochemical studies may lead to a more precise understanding of discrete events that occur in the replication of VSV.

In the absence of any information about the biochemical nature of the defective function of each complementation group, it is essential to examine the evidence arising from the results of the complementation experiments to ensure that each complementation group corresponds to a specific viral cistron. If every mutant tested can be unambiguously assigned to one and only one complementation group, the complementation is said to be non-overlapping. That is, mutants belonging to a complementation group will complement every mutant outside the group but not those inside it. A non-overlapping pattern observed with a sufficiently large number of mutants provides evidence that each complementation group corresponds to one cistron.

The results of complementation tests for the 25 mutants isolated in this laboratory indicate that mutants ts10 and ts16B complement all other mutants but do not complement each other (Table VII, p. 43). This leads to the unambiguous separation of ts10 and ts16B from the remainder to form a single complementation group, I.

Mutant ts29 complements all mutants tested except ts12, ts17, ts23 and ts25, which indicates the possibility that these mutants may belong to the same complementation group and thus have defects in the same function. Ts12 appears to complement most of the other mutants which show complementation with ts29 except for a few pairwise crosses. Ts17, ts23 and ts25 not only fail to complement with ts29 but also fail to complement most of the other mutants. However, they do seem to complement ts12 and are therefore tentatively excluded from the same complementation group as ts12 and ts29, which have been assigned to group II.

Among the remaining mutants ts4 complements all mutants apart from ts11, ts14, ts15, ts20, ts24, ts28 and possibly ts26. Mixed infections with ts4 and ts26 give a C.L. of 1.2, a value whose uncertainty makes one hesitate to exclude ts26 from the ts4 group unless this exclusion is shown to be warranted by additional evidence. These mutants ts4, ts11, ts14, ts15, ts20, ts24, ts26 and ts28 do not show complementation among themselves except for the pairwise crosses between ts11 and ts24, which give rise to a C.L. of 4.8 and ts15 and ts24 to a C.L. of 1.1. Based on the fact that ts24 complements ts11 and possibly ts15, ts24 has been excluded from this group. Therefore, it appears that ts4, ts11, ts14, ts15, ts20, ts28 and possibly ts26 are defective in the same function and may be tentatively assigned to another complementation group, III.

The group III mutants complement all the members of group I and group II except for the pairwise crosses of ts14 and ts12 and,

ts28 and ts12. A possible explanation to account for this discrepancy could be that mutants ts12 and ts29 (group II) and mutants ts14 and ts28 (group III) are defective in different polypeptides which in their wild type state of conformation, would form a subunit of a protein complex required during the maturation of the virion. Ts12 and ts29 are mutated differently in the same cistron so that the defective polypeptide of ts29 cannot combine with the defective polypeptide of ts14 or ts28 to form a subunit thus leaving the wild type polypeptide of ts29 and ts14 or ts28 to form the normal subunits which assemble to form the functional protein complex. On the other hand, the mutated gene product of ts12 could combine with the mutated gene product of ts14 or ts28, but the subunits so formed, when assembled with the wild type subunits, give rise to a non-functional protein complex.

Another explanation could be that ts12, ts14 and ts28 are double mutants and share a common defect in addition to the defect of ts29 in the case of ts12, and in addition to the defect of the rest of the group III mutants in the case of ts14 and ts28. However the possibility that these are double mutants appears unattractive because reversion to wild type has been demonstrated for ts14, and ts12 shows complementation with most of the mutants tested.

It therefore appears that we have identified three complementation groups, each group defective in a different cistron.

When one considers the remaining 14 ts mutants, one finds it difficult to sort them into any of the three complementation groups without ambiguity. However there are two possibilities for classify-

ing these 14 mutants:

i) Some of these mutants may belong to other as yet unrecognized complementation group(s). For example, ts22 complements group I, group II and two mutants, ts4 and ts15, of group III. The failure of ts22 to complement other members of group III may be attributed to the same type of phenomenon as proposed for failure to demonstrate observable complementation between ts12 and ts14 and, ts12 and ts28.

ii) On the other hand, the complementation observed between the unclassified mutants and some of the members of group II or group III may be intracistronic rather than intercistronic. For example, ts17, ts23 and ts25 may belong to group II and the remaining mutants may belong to group III. The fact that all the unclassified mutants except ts24 complement with ts4 is not inconsistent with the possibility that intracistronic complementation occurs between these mutants and ts4 since this high frequency of intracistronic complementation has been shown to occur in gene 37 of phage T4 (Bernstein et al., 1965). However at present by complementation tests alone we have no means of distinguishing inter- and intracistronic complementation. It seems therefore best to treat the mutants with occasional and random complementation as an unclassified group until additional evidence becomes available.

Biochemical tests on the mutants assigned to the three complementation groups appear to support the classification based on complementation tests. It appears, therefore, that although complementation tests alone cannot unambiguously separate all the ts mutants of VSV isolated in this laboratory into complementation groups, each group

defective in a single cistron, it has served as a useful tool in the preliminary sorting out of some of these mutants into groups which has provided a basis for characterization of the defect represented by each group.

Since we began our studies on complmentation between ts mutants of VSV, similar investigations have been reported (Flamand, 1969; 1970; Pringle, 1970; Flamand and Pringle, 1971).

Flamand (1969, 1970) in her studies on 71 spontaneous ts mutants of VSV, isolated from the wild type Indiana strain, identified 5 complementation groups. The mutants of the "ts5" and "ts100" groups failed to make RNA at the non-permissive temperature (RNA^-), while the other three groups represented by ts23, ts45 and ts52 can synthesize RNA at the non-permissive temperature (RNA^+).

Pringle (1970), using the 175 ts mutants of VSV obtained by using mutagens, found four complementation groups. Reciprocal complementation studies with Flamand revealed four complementation groups in common among the mutants isolated from the two laboratories (Flamand and Pringle, 1971). Complementation groups I to IV of Pringle's classification correspond to Flamand's groups which are represented by ts4(=ts5 group), ts52, ts23 and ts100. The group represented by ts45 (Flamand's) belongs to a fifth group not represented among Pringle's mutants.

We have identified three complementation groups. The determination of correspondence between the three complementation groups identified in this laboratory with those of Pringle and Flamand will

either have to await reciprocal complementation tests with Pringle's or Flamand's mutants or identification of the genetic defects of the complementation groups themselves. Even if all our three groups correspond to three groups identified by Flamand and Pringle, then according to their complementation studies, the VSV genome consists of at least five cistrons.

Four structural proteins (II, III, IV and V) from purified virions (Mudd and Summers, 1970) and two non-structural proteins (NS1 and NS2) from VSV infected cells (Wagner et al., 1970) have been identified with polyacrylamide gel electrophoresis (See Literature Review, Table II, p. 16). According to Printz and Wagner (1971) these six proteins account for only two-thirds of the potential genetic information of VSV even if one takes into account the 10 to 20% discrepancies in molecular weights of the structural proteins reported by the different laboratories (See Literature Review). Recently a protein kinase has also been found in the virion of VSV (Strand and August, 1971). Presumably there are still undiscovered VS viral functions.

However, Kang and Prevec (1971) indicated that they have unpublished evidence that VP1 (= I of Mudd and Summer's virion protein classification) is not an aggregational artifact made up of a number of smaller virus-specific proteins but that VP1, VP2, VP3, VP4 and NS1 constitute the full complement of virus-specific proteins of VSV. According to these authors, the molecular mass of 3.6×10^6 daltons of a single RNA molecule from B particles would just be sufficient to code for these five proteins using the molecular weights of these proteins

as determined by Mudd and Summers (1970).

An obvious approach to investigating the number of cistrons in the VSV genome is the isolation of more ts mutants if necessary and correlation of the function of each complementation group with a viral specific polypeptide identified by acrylamide gel electrophoresis. This assumes, of course, that there is no cistron of VSV coding for RNA which is not subsequently translated into polypeptide.

There is now considerable evidence (See Literature Review) that the viral RNA of VSV acts as a template for the transcription of messenger RNA, by the structural polymerase. Recent work by Marcus et al. (1971) showed that RNA polymerase activity of VSV can be demonstrated in vivo. They showed that a linear rate of synthesis of viral RNA persisted for 5 to 6 hours at 34^{o} in infected monolayers of chick embryo cells treated with cycloheximide and actinomycin D and were able to show with annealing experiments that at least 55% of the RNA made is complementary to the virion RNA. We have used a similar technique to study the behavior of the VSV structural polymerase in infected L cells and obtained evidence that our group III mutants are defective in this polymerase (See section IV-4). This finding has been confirmed in in vitro experiments in our laboratory (Cormack et al., 1971; Cairns, J. personal communication). In addition, from temperature-shift experiments we have found that the structural polymerase is needed early in the growth cycle and possibly functions continuously for at least the first four hours of the growth cycle. We have also found from the complementation-rescue experiments

that whatever is defective in the structural polymerase of group III mutants, the defect can be circumvented by a diffusible substance from a group I mutant.

Little is known about the replication of the viral RNA apart from the demonstration that double-stranded RNA appropriate in size for the replicative forms of the B and T particles has been isolated from VSV infected cells (Schincariol and Howatson, 1970). Since the viral RNA remains undegraded after transcription of the messenger RNA (Bishop and Roy, 1971; Huang et al., 1971), presumably it is again used for the synthesis of a complementary (minus) strand which is then used for the synthesis of new plus strands (viral RNA). There is as yet no experimental evidence to confirm the above hypothesis. Besides, nothing is known as to whether a different polymerase other than the structural polymerase is required for the synthesis of the replicative intermediate and whether the same structural polymerase, a modified form of it or a second virus-induced replicase is needed for the synthesis of new plus strands.

Our present studies seem to indicate that second polymerase is required for VS viral RNA replication. The ability of the group I mutants to synthesize some RNA at 38° during the early phase of growth together with the fact that this RNA synthesized is not reduced in amount in the presence of puromycin at 38° indicate that the structural polymerase is functional. Nevertheless, the amount of virus-specific RNA synthesized by the group I mutants at 38° is considerably less than that at 30° which suggests that the mutants are defective in some

function involved with RNA synthesis. It seems possible that the group I mutants are defective in a polymerase (not the structural polymerase) which replicates viral RNA using the undegraded viral RNA template after messenger RNA synthesis. That the RNA made at 38^{o} by the group I mutants is messenger RNA could be tested by extracting the ^{14}C-labelled product and annealing it to unlabelled virion RNA. Furthermore, one could test the hypothesis that the group I mutants may be defective in a polymerase required for viral RNA replication by examining the spectrum of virus-specific RNA species after infecting cells with a high input multiplicity of a group I mutant. According to our speculation, if group I mutants are not defective in the structural polymerase, messenger RNA synthesis would not be hindered at 38^{o}, then 13S to 15S RNA species complementary to the viral RNA should be observed in an amount comparable to that of HR. On the other hand, if group I mutants are defective in the replicase, viral RNA synthesis will be blocked. As a result, no 40S viral RNA species would be obtained in the 38^{o} culture. Studies along this line will be carried out in this laboratory.

Flamand (1970) found three RNA^{+} complementation groups. The genetic defects of these three groups were recently investigated by Printz and Wagner (1971) using one representative from each group, namely, ts52, ts23 and ts45. Printz and Wagner demonstrated using the protein profile of ts52 that the peak of radioactivity representing the glycoprotein migrated more rapidly on gels then ^{3}H-glycoprotein extracted from cells infected at the permissive temperature. Further investigation showed that the primary defect in ts52 at the non-permissive

temperature is failure of the glycoprotein precursor to glycosylate which led to failure of insertion of the non-glycosylated protein into cell membrane.

Ts23 was found to produce a small amount of the S protein (one of the two coat proteins) at 38^{o} than at 30^{o}. Printz and Wagner further observed that all the newly synthesized structural proteins were largely found in a sedimentable form and viral antigen was readily detected on the surface of plasma membrane. From these observations they concluded that the deficiency in the S protein probably resulted in maturation arrest at a stage in virion assembly beyond insertion of the glycoprotein into cell membrane. We question the validity of the finding that ts23 actually produces a smaller amount of S protein at 38^{o} than at 30^{o} because it has been demonstrated by Wittman and Wittman-Liebold (1966) that ts mutants of tobacco mosaic virus are "mis-sense" mutants in which the defective protein is produced in normal amounts but cannot assume or maintain a functional configuration at the non-permissive temperature.

The genetic defect of ts45 was not apparent from their investigation.

The investigations of Printz and Wagner (1971) is a first attempt at characterizing the defects of RNA^{+} ts mutants of VSV. It appears that considerable difficulty is involved since only the defect of ts52 has been partially identified. Yet such studies are necessary in order to unravel some of the complex processes of virion assembly and maturation of VSV. A host of problems relating to virion assembly

and maturation is already apparent. For example, although there is evidence that the viral RNA associates with the nucleocapsid protein to form the nucleocapsid core (Wagner et al., 1970; Huang et al., 1970) nothing is yet known as to the mechanism involved in its formation. From electron microscopical studies, Nakai and Howatson (1968) postulated that the nucleoprotein consists of discrete subunits whose continuity is maintained by the viral RNA. Whether this is in fact the case has never been confirmed, and if it were so, is the assembly of the subunits spontaneous or is it effected by host or virus-induced enzymes? Furthermore, although it has been suggested that the two coat proteins are inserted on host plasma membrane, how this is brought about and what triggers the virus to bud from the host membrane remain open questions.

Our group II mutants can synthesize RNA at the non-permissive temperature, but these mutants are not heat-labile and their defect is expressed very late in the growth cycle. These characteristics suggest that these mutants may be defective in some function involved in maturation. It should be a useful tool for probing into the processes involved in VSV maturation. The initial step we could take to further investigate the defect of our group II mutants is to confirm whether these mutants have an altered structural protein although they are no more heat-labile than HR. This can be done by comparing the protein profile of our mutants at 30^{o} to that at 38^{o} or to that of HR using ^{3}H-labelled amino acid.

From the above discussion it seems that considerable complexity

appears to be involved in the replication of VSV. Nevertheless, using ts mutants we have helped resolve some of these problems especially in the area of virus-specific RNA synthesis. We have for the first time isolated and characterized a group of ts mutants (group III) defective in the RNA structural polymerase. That these mutants are defective in the structural polymerase has been confirmed by in vitro tests. We have also shown that whatever is defective in the structural polymerase, it could be circumvented by a diffusible material from a mutant not defective in the structural polymerase. Furthermore, we have also isolated another group of ts mutants (group I) defective in RNA synthesis which requires prior protein synthesis. From this finding we suggest the possibility that VSV requires an additional polymerase, other than the structural polymerase, for replicating its viral RNA. Our mutants which are able to synthesize RNA at the non-permissive temperature (group II) will be useful for elucidating events involved in virion assembly or maturation.

SUMMARY

Twenty-five temperature-sensitive (ts) mutants have been isolated from a heat-resistant (HR) parental wild type clone of the Indiana strain of vesicular stomatitis virus (VSV) by treatment with ethylmethane sulfonate, nitrous acid, 5-fluorouracil or proflavine hydrochloride.

From the complementation studies carried out on these 25 ts mutants, three complementation groups have been identified. Fourteen mutants remain unclassified.

No recombination could be demonstrated between mutants from each complementation group. Instead, the studies showed a complementation effect brought about by clumping or the formation of "heteropolyploids".

The group I mutants were able to synthesize some RNA at 38° but the amount of RNA synthesized was considerably less than that at 30°. Results of temperature-shift experiments showed that the defect occurs during the first four hours after infection and no RNA synthesis was observed after shift from 30° to 38° after 2 hours incubation at 30°. The mutants were not found to be significantly more heat-labile than HR. Furthermore, RNA synthesized at 38° in the presence of puromycin was not reduced in amount when compared with that synthesized in the absence of puromycin. These observations together with the fact that the group I mutants complemented group III mutants which are thought to be defective in the structural polymerase suggest the possibility that the group I mutants may be defective in a polymerase

which is different from that of the group III mutants and is required for the replication of viral RNA.

The group II mutants were found to have the following properties: i) they synthesize virus-specific RNA at the non-permissive temperature in an amount comparable to that of the 30° culture although no virus multiplication was observed at 38°, ii) they cease to produce virus whenever the infected cells were transferred to 38° up to 6 hours after infection, iii) they synthesize RNA after shift from 30° to 38° although no virus is produced and iv) in the presence of puromycin their rate of RNA synthesis is comparable to that of HR. These observations suggest that the defect of the group II mutants occurs after RNA synthesis, perhaps in some function involved in virion assembly or maturation.

The group III mutants fail to synthesize RNA at the non-permissive temperature. Experiments on the effect of temperature-shift on virus production and on RNA synthesis show that the defect begins to be expressed shortly after infection and the duration of expression lasts for at least four hours after infection. Group III mutants were also found to be more heat-labile than HR. No ^{14}C-uridine uptake at 38° was observed in infected L cells in the presence or absence of puromycin with a high input multiplicity, even up to 3,000 PFU/cell, of virus. Furthermore, effect of temperature-shift from 30° to 38° on ^{14}C-uridine uptake with a high input multiplicity of ts11 shows that in the absence of puromycin ^{14}C-uridine uptake was observed whereas in its presence there was no uptake. These observations suggest that the

group III mutants may be defective in the structural polymerase reported by Baltimore et al. (1970).

In a complementation between a group I mutant, ts16B and a group III mutant, ts11, progeny of both parents were obtained which suggests that whatever is defective in the ts11 structural polymerase, it is circumvented by a diffusible material from ts16B. The defect of ts16B is similarly circumvented by ts11.

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APPENDIX

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A Search for Recombination between Temperature-Sensitive Mutants of Vesicular Stomatitis Virus

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Studies of genetic recombination have been of considerable value in elucidating the structure and function of bacterial viruses and have also been of value for certain animal viruses (e.g. see Fenner, 1970). Pringle (1970) has reported recombination between *ts* mutants of vesicular stomatitis virus (VSV) when mutants from different complementation groups were used in mixed infections. We have looked for recombination in our collection of VSV mutants (Holloway, Wong & Cormack, 1970), also, and have obtained the results presented in this paper.

L-cell and virus stocks were maintained and assayed using the methods previously described (Holloway *et al.* 1970). For the present experiments, cell monolayers were prepared by seeding each of a number of 2 oz. Brockway bottles with $2{\cdot}5 \times 10^6$ cells. For mixed infection $2{\cdot}5 \times 10^7$ p.f.u. of each mutant were added in a total volume of 0·5 ml, giving a total m.o.i. of 20. For the single infections m.o.i. of 20 was used. The virus was allowed to adsorb for 25 min. at 38°, the monolayer was then washed, 5 ml of medium was added and the infected bottles were incubated for 8·5 hr at 30°, the permissive temperature for virus replication. The medium was then centrifuged at 500*g* for 5 min. to remove floating cells which might be mixedly infected and able to form a plaque at 38° by complementation.

For convenience in the following discussion the plaques observed in the assay plate incubated at 30° will be referred to as 30° plaques and those observed at 38° as 38° plaques. Fig. 1 shows results obtained for single and mixed infections using mutants *ts* 14 and *ts* 16B and are expressed as the number of 38° plaques observed per dish plotted against the number of 30° plaques per dish. Mutants *ts* 14 and *ts* 16B have been shown to complement in mixed infections at 38° (P. K. Y. Wong, A. F. Holloway & D. V. Cormack, unpublished results). The points in Fig. 1 (A) are for the medium harvested from cells mixedly infected with *ts* 14 and *ts* 16B and indicate that 0·8 % of the progeny are able to form plaques at 38°. Results (B) and (C) were obtained from single infections with *ts* 14 and *ts* 16B respectively. Re-plating of suspensions of these 38° plaques from the single infections showed that the viruses they contained were ts^+, i.e. able to form plaques equally well at 38° and 30° and are presumably revertants of the *ts* parent. The number of 38° plaques is directly proportional to the number of 30° plaques and accounts for 0·02 % and 0·001 % of the total progeny for *ts* 14 and *ts* 16B respectively. In addition to those due to revertants, 38° plaques might be expected in the mixed infection yield by random co-infection of some cells on the assay plate by two complementing *ts* mutants. To investigate this effect with mutants *ts* 14 and *ts* 16B, stocks with equal titres were mixed and various dilutions were plated at 30° and 38°. The results are shown in Fig. 1 D. Whereas the data in A, B and C indicate a direct proportionality between 38° plaques and 30° plaques the data in D are closely fitted by a 'two-hit' curve, i.e. one in which the number of 38° plaques is proportional to the square of the number of 30° plaques. Such a relation is expected since at least two virus particles are required per cell in order to have complementation. The straight line in D was calculated assuming that there are 3×10^6 cells per assay plate and that every mixedly infected cell gives rise to a plaque. The actual number of cells per dish is about 2×10^6 cells and the dif-

ference between this and the value of 3×10^6 used in fitting the data in D probably reflects the fact that not all mixedly infected cells give rise to observable plaques. A comparison of curves D and A indicate that only a small percentage of the 38° plaques observed in the products of mixed infection are due to complementation in cells on the assay plates.

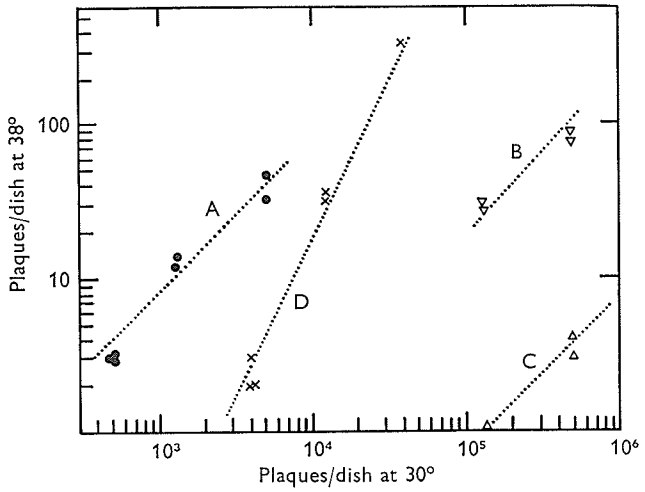

Fig. 1. Number of plaques formed in monolayers incubated at 38° as a function of the number of plaques observed or expected if the monolayer were incubated at 30°. ●——●, Mixed infection *ts* 14 × *ts* 16B; ▽——▽, infection with *ts* 14; △——△, infection; with *ts* 16B; × — ×, *in vitro* mixture of *ts* 14 and *ts* 16B made before plating.

Table 1. *Complementation levels and* 38°/30° *plaque ratios in mixed infections*

Group	*ts* mutants	*ts* 10	*ts* 16B	*ts* 12	*ts* 4	*ts* 8	*ts* 11	*ts* 14	*ts* 18
I	*ts* 10	0·001	**1·0**	**40**	**1000**	**26**	**20**	**30**	**9**
	ts 16B	0·001	0·001	**11**	**100**	**300**	**400**	**100**	**20**
II	*ts* 12	0·9	0·9	0·001	**5**	**1·2**	**3**	**0·8**	**3**
III	*ts* 4	0·7	1·0	0·002	0·001	**1·3**	**0·6**	**0·5**	**5**
	ts 8	0·6	1·2	0·01	0·002	0·004	**0·8**	**0·6**	**1·7**
	ts 11	0·9	1·3	0·001	0·002	0·02	0·01	**0·3**	**1·2**
	ts 14	0·5	1·0	0·001	0·001	0·001	0·01	0·006	**1·0**
	ts 18	0·7	0·8	0·01	0·001	0·02	0·001	0·01	0·001

The numbers in bold type are ratios of yields at 38° in mixed infection and single infection. The other numbers give the ratios of number of 38° plaques to 30° plaques in the yield of a mixed infection at 30°.

The temperature-sensitivity of the viruses in the 38° plaques from the mixed infection was then examined. Plaques arising from recombination would be expected to consist primarily of ts^+ particles. To our surprise, however, more than 90 % of the viruses in the plaques tested (9/9) were unable to produce plaques when re-plated and incubated at 38°. Further analysis showed that most of these plaques contained virus of both mutant types, *ts* 14 and *ts* 16B. These results show that with these mutants few, if any, of the 38° plaques are the result of genetic recombination during the mixed infection. Recombination at a frequency of 0·1 % or less might, however, have been undetected. The linear relation between the 38° and 30° assays indicate, however, that they can be produced by single infectious

units. These units may be mixed aggregates of virions of the two parental types or 'heteropolyploids', i.e. particles in which genomes of both parents are enclosed within one viral envelope (Fenner, 1970). These aggregates or 'heteropolyploids' may be related to the elongated particles and chains of particles observed during budding by Galasso (1967) and by Howatson (1970). In an attempt to dissociate possible aggregates the medium from the mixed infection was subjected to sonication or incubation at 37° for 1 hr. These treatments failed, however, to produce appreciable changes in either the slope or position of curve A. It has therefore not been possible to distinguish between aggregates and 'heteropolyploids'.

Similar experiments were performed with various other pairs of *ts* mutants and the results are shown in Table 1. The values below the diagonal give the ratio of 38° plaques to 30° plaques after correcting for revertants. Complementation data (P. K. Y. Wong, A. F. Holloway & D. V. Cormack, unpublished results) for these same pairs of mutants are given above the diagonal, expressed as complementation levels (ratio of the yield at 38° in mixed infection to that in single infection). Mutant *ts* 10 and *ts* 16B form a distinct complementation group, since they complement all other mutants and do not complement each other. The remaining mutants show some complementation among themselves but generally at a much lower level. The table shows a very similar pattern in the ratios of 38° and 30° plaques in the yields of mixed infection at 30°. Here, also, *ts* 10 or *ts* 16B, when combined with any mutant outside their group give yields with net plaque ratios of about 1 % whereas all other pairs give plaque ratios of 0·02 % or less. As in the *ts* 14 and *ts* 16B mixed infection, the 38° plaques from mixed infections with either *ts* 10 or *ts* 16B were found on re-plating to be made up largely of temperature-sensitive virus. There appears, therefore, to be no evidence of genetic recombination within this group of eight mutants representing the three complementation groups to which we have tentatively assigned our 21 *ts* mutants of VSV. The high proportion of 38° plaques for mixed infections involving either *ts* 10 or *ts* 16B is probably another manifestation of the complementation behaviour of these mutants brought about by aggregation of virus particles or the presence of heteropolyploids.

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