CHARACTERIZATION OF ts MUTANTS OF VESICULAR STOMATITIS VIRUS WITH RESPECT TO THEIR *IN VITRO* VIRION-ASSOCIATED POLYMERASE ACTIVITY

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To Lorraine,

Mom, and Dad

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SUMMARY

The temperature dependence of the virion-associated polymerase activity of seven temperature sensitive (ts) mutants of vesicular stomatitis virus (VSV)(ts10,4,11,14,16B, 28 and 29) has been examined *in vitro* and compared to the heat-resistant parent (HR). The polymerase of six of the mutants (ts10,4,11,14,16B and 28) appears to be significantly more temperature-sensitive than that of HR. Because certain pairs of these mutants can complement each other's *in vitro* polymerase activity, it appears that *in vitro* some components involved in the polymerase of one virion can be utilized by another virion. Examination of 19 revertants of ts11 and ts16B which had regained their ability to replicate at 38°C showed that their *in vitro* polymerase activity had also become less temperature sensitive. Furthermore, it was found that the pairs of mutants which showed *in vitro* complementation of polymerase activity were those which had shown complementation in yielding infectious progeny in mixedly-infected cells. These two observations suggest that the temperature sensitive behavior of the *in vitro* polymerase activity of the five mutants is related to their inability to replicate at the non-permissive temperature.

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Introduction

As a model system for studying the interaction of an enveloped single-stranded RNA virus with a mammalian cell, our laboratory has chosen vesicular stomatitis virus (VSV) and Earle's L cells. This system has several advantages. For example, L cells are a serial cell line which can be grown either in suspension or in monolayers. In addition, VSV has a relatively short growth cycle, can be easily assayed by its ability to form plaques on cell monolayers, and can grow to high titres over a wide temperature range (Holloway et al, 1970). Furthermore, the virus has a high degree of plating efficiency (Galasso, 1968; Katz, personal communication), and it is easy to obtain reproducible one-step growth curves (Wong, 1969). Moreover, VSV'S relatively small genome whose molecular weight has been estimated to be 3 to 4×10^6 daltons (Huang & Wagner, 1966; Brown et al, 1967; Nakai & Howatson, 1968; Mudd & Summers D.F., 1972) codes for relatively few proteins. For example, Kang & Prevec (1971) suggest that only five species constitute the total number of different VSV-specific proteins.

Since 1967, a number of virion-associated activities have been reported for animal viruses, including VSV. One of these activities which is present in VSV is an RNA-dependent RNA-polymerase (Baltimore et al, 1970). This enzyme appears to catalyze the synthesis of messenger RNA rather than virion RNA (Baltimore et al, 1970; Huang et al, 1970), that is, it appears to be a transcriptase rather than a replicase.

Our laboratory has isolated temperature-sensitive (ts) mutants of VSV and is presently attempting to characterize these mutants with respect to their virion-associated transcriptase activity. If a mutant has a temperature-sensitive defect in some activity, then one can switch this activity on or off merely by changing the temperature of the mutant's environment. Hence, such a mutant provides a valuable tool for studying the nature of the transcriptase activity and its importance in the process of infection. For example, by using ts mutants, one would expect to demonstrate whether or not the virion-associated polymerase must be functional in order for the virus to replicate. One might then determine, by means of temperature shift experiments, the period in the growth cycle in which this activity must operate. Furthermore, one might be able to discover relationships of the transcriptase activity to other activities. For example, if one causes transcriptase activity to stop, perhaps another activity will cease concurrently. Mutants with a temperature-sensitive transcriptase might also be used to elucidate the mechanism of an anti-viral agent since the effect of the agent on viral activities could be studied in the presence or absence of transcriptase activity. Furthermore, by the use of complementation studies, one might be able to show whether the transcriptase molecules can be exchanged among virions.

Studies of the virion-associated transcriptase of some of our ts mutants in cells infected in the presence of actinomycin

D and the protein inhibitor, puromycin, have been described by Wong (1972), while this thesis concerns the characterization of our ts mutants by the *in vitro* polymerase assay described by Baltimore *et al* (1972). Wong (1972), on the basis of the ability of pairs of mutants to complement each other in a mixed infection of L cells at the nonpermissive temperature to produce infectious progeny, also classified most of the mutants into three complementation groups. It was found that the members of one of the complementation groups appeared to have a temperature-sensitive virion-associated polymerase activity. Furthermore, the reciprocal nature of the complementation suggested that in mixedly infected cells, an exchange of components concerned with the transcriptase activity could occur. The *in vitro* studies described here support these conclusions and also suggest that the virions of another complementation group have a temperature sensitive defect in another component of the transcriptase.

LITERATURE REVIEW

I Some Properties of Vesicular Stomatitis Virus

Because of its characteristic shape and structure, VSV has been designated the prototype of the Rhabdovirus family (Melnick & McCombs, 1968). A comparison of the three serotypes of VSV (Indiana, New Jersey, and Cocal) indicates that these three serotypes are morphologically similar (Braddish and Kirkham, 1966; Bergold and Munz, 1967; Thormar, 1967).

VSV is a large single stranded RNA-containing virus (Prevec & Whitmore, 1963) and stocks usually contain two kinds of particles, designated B (bullet-shaped) and T (transmissible) particles (Cooper & Bellet, 1959). Infectivity appears to be associated with the B particles which are approximately 180 mμ long and 65 mμ wide and contain an internal striated component surrounded by an envelope with projecting spikes (Howatson & Whitmore, 1962, Reczko, 1962). This striated component is a helically wound nucleoprotein (Klimenko et al, 1966; Simpson and Hauser, 1966; Bergold & Munz, 1967; Nakai & Howatson, 1968).

The T particle appears to be a truncated from of the B particle and has a similar width, ultrastructure, buoyant density, antigenicity, ability to incorporate ^{3}H-uridine during replication in the presence of B particles, capacity to inhibit cellular RNA and interferon synthesis (Huang et al, 1966), and protein composition (Kang & Prevec, 1969; Wagner et al

1969a, 1969b; Mudd & Summers, 1970, 1970b). Although the T particles described by Huang et al (1969) appear to be approximately one-third of the length of B particles, another type of T particle whose size was intermediate between the other two types of particles was found by Petric & Prevec (1970) who named the particles long and short T particles. Two sizes of T particles have also been observed by Bishop & Roy (1971a). Both these laboratories found that the molecular weight of the RNA of the long T particle was approximately 1/2 that of the B particle while the molecular weight of the RNA of the short T particle was approximately 1/4 that of the B particle. Furthermore, hybridization studies performed by Bishop and Roy (1971b) indicate that the RNA species extracted from the long T, short T, and B particles are not complementary to each other.

T particles are not infectious and are produced preferentially in cells infected at high multiplicity with undiluted passage (Cooper & Bellet, 1959; Huang & Wagner, 1966a; Huang et al, 1966). They also interfere with the growth of infectious B particles (Hacket, 1964; Huang & Wagner, 1966a; Huang et al, 1966; Crick et al, 1966; Hacket et al, 1967; Brown et al, 1967).

If cells are infected with a preparation of B particles free of T particles, examination of the infected cell extracts reveals that only three species of single-stranded RNA are produced: 40S, 28S, and 13S(Stampfer et al, 1969). This is

observed even if input multiplicities as high as 100 PFU per cell are used (Stampfer et al, 1971). If T particles are also present in the infection, there are at least two additional species of single-stranded RNA detected by sucrose gradient analysis, and this suggests that the mechanism of interference of B particles of VSV by T particles is at the level of RNA synthesis (Stampfer et al, 1969).

The 40S RNA species which appear in cells infected by B particles has the same sedimentation constant as RNA extracted from B particles (Huang & Wagner, 1966b) and is indistinguishable from virion RNA by base ratio analysis (Newman & Brown, 1969). On the other hand, 28S and 13S RNA are associated with polysomes and are complementary to the virion (Huang et al, 1970). Hence, 28S and 13S RNA are probably messenger RNA species. Due to the lack of resolution on sucrose gradients, it is not known if more than one species of messenger RNA was in each of these fractions. However, preliminary gel electrophoresis studies indicate that there are at least two RNA species in the 13S fraction (Huang et al, 1971).

Pulse labelling experiments indicate that, in VSV replication, there are no precursor polypeptides which are cleaved to form several individual polypeptides (Wagner et al, 1970, Mudd & Summers, 1970b). Since there appear to be at least five species of proteins found in VSV virions, as indicated in Table I, one would expect at least five different species of

TABLE I

PROTEINS OF VSV:-NOMENCLATURE AGREED UPON AT RHABDOVIRUS SYMPOSIUM, ROSCOFF, FRANCE IN JUNE, 1972(j)

NAME ADOPTED	FORMER NAMES				M.W.(daltons)
	(a)	(b)	(c)	(d)	
L (large protein)	VP1	I	-	-	190,000
G (glycoprotein)(e)	VP2	II	G	P2	69,000(i)
N (nucleocapsid protein)(f)	VP3	III	N	P3	50,000
NS ("non-structural" protein)(g)	NS1	MS	NS1	-	45,000
M (matrix protein)(h)	VP4	V	S	P4	29,000

(a) Kang & Prevec (1969, 1970, 1971)

(b) Mudd & Summers (1970b)

(c) Wagner et al (1970)

(d) Cartwright et al (1970)

(e) forms the spikes of VSV

(f) This protein is found associated with the nucleoprotein cores produced by treating virions with sodium deoxycholate.

(g) is "non-structural" in that only 5%of the NS protein synthesized in infected cells is incorporated into the virions. This protein also appears to be located in the cores.

(h) is associated with the viral coat.

(i) the M.W. does not include the sugar residue.

(j) A.F. Holloway, personal communication.

messenger RNA. There may be more than five different VSV-specific proteins, but if more exist, they are probably small polypeptides because a comparison of the sum of the molecular weights of the five proteins with the molecular weight estimated for the viral genome suggests that practically all of the genome is used in coding for these five proteins (Kang & Prevec, 1971). As the next section of the literature review describes, five enzyme activities associated with VSV virions have been detected. It has not been proven whether any or all of the five proteins found in VSV virions are responsible for these activities. However, experiments reported at the Roscoff Symposium (A. F. Holloway, personal communication) suggest that the proteins NS and N are required for <u>in vitro</u> transcriptase activity.

II Virion-Associated Enzyme Activities in VSV

(1) RNA-dependent RNA Polymerase

This activity was first detected in VSV by Baltimore et al (1970). Two observations led the Baltimore laboratory to predict the presence of this enzyme in VSV virions. First, RNA of VSV virions (Huang & Wagner, 1966b), unlike the RNA from arboviruses (Wecker & Schafer, 1957) and from picornaviruses (Colter et al, 1957; Koch et al, 1958) is non-infectious. Second, in VSV-infected cells, the polysomal RNA involved in the synthesis of VSV-specific protein is complementary to virion RNA. This suggested that the virion RNA served as a template for messenger RNA synthesis using either a polymerase found in the virions or the host cell.

Baltimore observed the virion-associated polymerase activity by measuring the ability of a suspension of VSV virions to convert ^{3}H-GTP into an acid-insoluble product. In order to detect the activity, it was necessary to remove the virion envelope and expose the nucleocapsid by treating the virion with a nonionic detergent such as Triton N-101. The nonionic detergent could not be replaced by sodium deoxycholate. The requirement for the nonionic detergent, Triton N-101, was confirmed by Bishop and Roy (1971a) who also reported that this detergent could be substituted by Nonidet P-40.

Baltimore also showed that the reaction was dependent

upon Mg^{++}, and on the presence of all four nucleoside triphosphates. Furthermore, Mn^{++} could not replace Mg^{++}, and the optimum Mg^{++} concentration was found to be 4-6mM. The reaction was stimulated in the presence of NaCL (or KCL) and also by β - mercaptoethanol. In a subsequent publication (Huang et al, 1971), Baltimore's laboratory found that the pH optimum of the reaction was 7.3 and the optimum temperature was 32°C. Bishop's laboratory confirmed the requirements for the polymerase (Bishop & Roy, 1971a) but found different optimum conditions (Aaslestad et al, 1971). For example, they found an optimum Mg^{++} concentration of 8 mM, a pH optimum of 8.2, and an optimum temperature of 28°C. In both laboratories, however, it was found that the polymerase ceased to function after an incubation of approximately one hour at 37°C, while linear incorporation at the optimum temperature occured for several hours.

Baltimore et al (1970) found, as expected for the properties of an RNA-dependent RNA polymerase, that the reaction was insensitive to DNase, actinomycin D, or rifampicin, but was sensitive to RNase. Morever, the enzyme was shown to be associated with the virions by centrifuging a purified preparation of VSV through a sucrose gradient and demonstrating that the $O.D_{240}$ (mainly light scattering) peak of the virions coincided with the peak of polymerase activity. The template for the activity was assumed to be that of the virion since no template RNA was added to the reaction mixtures. In fact, Bishop and Roy (1971a) have demonstrated that the addition

of 28S Hela RNA, polio RNA, Q RNA, or RNA from B⁻ or T⁻ particles, poly rI, poly rG, poly rC, poly rA, or poly U either depresses or has no effect on this *in vitro* polymerase activity.

Bishop (1971) has also examined the storage properties of this activity. He reports that 10% of the enzyme activity is lost per week of storage at -20°C in 30% glycerol. He was unable to conserve activity whether he used isotonic, hypertonic, or hypotonic salt conditions; 10% dimethylsulfoxide; 30% glycerol; or whether he stored the preparations at -70°C, -20°C, or 4°C.

On examining the product, Baltimore *et al* (1970) found that although it was digested by RNase, it was 94% RNase resistant after it had been annealed in the presence of excess virion RNA. Since both the product of the virion-associated polymerase and the RNA from polysomes of VSV-infected cells is complementary to the RNA of the virion (Huang *et al*, 1970), it appears that this polymerase is a transcriptase rather than a replicase, that is, it catalyzes the synthesis of messenger RNA rather than virion RNA.

If transcription is allowed to proceed for 3 hours at 28°C, the resulting product, when annealed to the endogenous template, renders the template 94% RNase resistant (Bishop, 1971). Bishop interprets this observation as complete transcription of the virion RNA. At 37°C, only 1/3 of the virion RNA appears to be transcribed (Bishop & Roy, 1971b). Hence,

complete transcription is not a prerequisite to the release of product species. Furthermore, Bishop and Roy also demonstrated that after a polymerase reaction has proceeded for 60 min at 37°C, not all of the product anneals to endogenous template. However, if additional virion RNA is added before annealing, all of the "excess" product becomes RNA'se resistant. This indicates that transcription is repetitious and that the product species do not replicate autonomously.

By performing a gel electrophoresis study on ^{32}P-labelled products of a polymerase reaction in which the RNA of the virus had been ^{3}H-labelled, Bishop and Roy (1971a) also demonstrated that the product was initially associated with the template. Huang et al (1971) have examined the products of the polymerase reaction by analysis on sucrose gradients. These studies also indicate an association of the polymerase product with the template in the form of an RNA'se resistant complex. Moreover, a study by Bishop and Roy (1971a) of the incorporation of a pulse of ^{32}P-UTP, which was subsequently chased by unlabelled UTP, demonstrated that after being associated with the template, the product was released as a free species. Freed products were detected after 7 min incubation at 28°C or after 5 min incubation at 37°C (Bishop, 1970). Although all of the 5 min product is associated with the template, limited RNA'se digestion solubilizes approximately 50% of the product label (Bishop & Roy, 1971b). This suggests that the complex is multistranded containing single-stranded

tails. Moreover, Bishop and Roy (1971b) have demonstrated that product-template complexes migrate at a different rate during gel electrophoresis than do free template or free product molecules. Furthermore, gel electrophoresis experiments demonstrated that the complex can be melted by heat treatment into undegraded free template and product molecules. This indicates that the product is not covalently linked with the template.

By using the criterion that only those virions which can form product-template complexes have polymerase activity, Bishop (1970) reports that only 30% of the particles in the VSV preparation which he was using had polymerase activity. These preparations consisted of virions whose template was ^{3}H-labelled. Since the product was ^{32}P-labelled, and since the product-template complexes can be separated by gel electrophoresis from the freed product and template species, the percentage of templates involved in forming complexes can be estimated.

Furthermore, by using studies of this kind, Bishop calculates that at 28°C, it takes approximately one hour to completely transcribe the template. Bishop also reports that the preparation of purified VSV used in this study had a particle to PFU ratio of 500:1. Since approximately 30% of the particles had transcriptase activity, it appears that not all particles having transcriptase activity are infectious. Transcriptase activity also appeared to be associated with

the non-infectious long T particles (Bishop & Roy, 1971b). Moreover, all of the product formed at 37°C could anneal to the RNA extracted from these particles while this product did not appear complementary to the short T particles. In a recent publication, Roy and Bishop (1972) demonstrate that there is no transcriptase activity associated with the short T particle. Furthermore, under the conditions which permit the complete transcription of the viral genome, they report that the early product of the transcriptase cannot anneal with the RNA extracted from the short T particles, while RNA synthesized after 120-180 min at 28°C can be hybridized with the short T particle RNA. This suggests that *in vitro* transcription is sequential in that not all regions of the RNA of the B particle are transcribed at the same time.

The virion-associated polymerase has also been studied *in vivo* by infecting cells in the presence of a protein inhibitor. This kind of study was first performed by our laboratory (Cormack *et al*, 1971). Later, Marcus *et al* (1971) using a similar method, reported a study on the effects of interferon on the virion-associated transcriptase. Their studies indicated that in cells infected with VSV in the presence of cycloheximide, 55% of the RNA synthesized could be annealed to virion RNA. This was probably a conservative estimate of the amount of RNA that was complementary to the virion RNA because the amounts of unlabelled cellular RNA present may have interfered with the annealing of the viral

RNA molecules. Nevertheless, it was found that the virion-associated polymerase was inhibited not only in cells pre-treated with interferon derived from an homologous cell system but also in cells treated with polyriboinosinate • polyribocytidylate.

Recently, Huang and Manders (1972) have studied this method of detecting the virion-associated polymerase and used it to help elucidate the mechanism by which T particles interfere with the replication of B particles. In their studies, they found that in the presence of a protein inhibitor, such as cycloheximide, RNA synthesis depends on the input multiplicity (i.m.) of the virus, while in the absence of the protein inhibitor, RNA synthesis reaches a maximum at an i.m. of 40 PFU/ cell. Comparable observations have been made in our laboratory (D.V. Cormack, personal communication). Moreover, Huang and Manders (1972) also demonstrated that only 28 S and 13-15S RNA species are synthesized in the presence of cycloheximide. These species are single-stranded as indicated by their sensitivity to RNA'se and are completely complementary to the 40S found in the virion. (Ninety-seven percent of the product was RNA'se resistant when annealed in the presence of virion RNA.).

Since the 40S RNA of the virion is not synthesized in the presence of cycloheximide, these conditions provided an opportunity,for studying the effect of the presence of T particles on the virion transcriptase activity of B particles.

When Huang and Manders compared the virion-associated transcriptase activity in cells infected in the presence of cycloheximide by a mixture of B and T particles (in a 1:1 proportion) with the virion-associated transcriptase activity of the same number of B particles in the absence of T particles, no difference was detected in the amount of RNA synthesized or in the types of RNA species formed. Simdlar results were observed if the cells were pre-infected with the T particles one hour before the addition of B particles. However, in an experiment performed under the same conditions, but in the absence of cycloheximide, the T particles inhibited the growth of the B particles by 90% and caused a 90% inhibition of RNA synthesis. Hence, it appears that the interference of B particles is probably associated with replicase activity rather than transcriptase activity.

It is interesting to compare the results of Huang and Manders with some of the results of experiments studying the in vitro behaviour of the virion-associated transcriptase. For example, it appears that the products of this enzyme synthesized in vivo differ in size with those produced in vitro. The molecular weights of the RNA products observed in the gel electrophoresis studies of Bishop and Roy (1971a) indicate that only 13-15S species are produced in vitro. Furthermore, the absence of the 28S species in the in vitro polymerase assay is probably not due to nucleases in the preparation since tests for the presence of such enzymes were negative.

Bishop and Roy's studies also indicated that during the *in vitro* reaction, the template was undegraded. Another difference between the *in vitro* and the *in vivo* systems is the optimum temperature. The optimum for *in vitro* transcription was found to be 28-32°C (Huang *et al*, 1971, Aalestad *et al*, 1971), while the optimum temperature *in vivo* appears to be 34-37°C (Huang & Manders, 1972). The reason for these differences between the *in vivo* and the *in vitro* reaction has not been established.

(2) Protein Kinase

This activity was found in VSV virions by Strand and August (1971). It was detected by incubating virions in the presence of γ-^{32}P ATP and measuring the incorporation of ^{32}P into an acid-insoluble product. The reaction was stimulated by the addition of detergent and is dependent on the presence of Mg++. Adding cyclic AMP to the reaction mixture did not stimulate activity. Although Strand and August indicated that all five of the VSV proteins which they detected were phosphorylated, reports at the Roscoff symposium indicate that only the NS protein is phosphorylated (A.F. Holloway, personal communication). Although protein kinase appears to be involved in the regulation of metabolic processes in cells, its role in VSV infections has not yet been determined.

(3) Nucleotide Triphosphate Phosphohydrolase (NTP'ase)

NTP'ase activity associated with VSV virions has been found by Roy and Bishop (1971). This activity was measured by incubating virions with a γ-^{32}P NTP and determining the amount of free phosphate released by means of paper electrophoresis. Additional experiments using the ^{32}P-labelled substrate indicated that the NTP is degraded to a nucleotide diphosphate (NDP) but not to a nucleotide monophosphate (NMP). Adding unlabelled NTP's which were different than the labelled one, inhibited the reaction. This indicates that all the NTP's are hydrolysed at a common site. NTP'ase activity appears to be supressed by the presence of a nonionic detergent. A role of this enzyme in VSV infections has not been determined.

(4) Nucleoside Triphosphate Phosphotransferase

This enzyme activity in VSV virions was also detected by Roy and Bishop (1971). The activity involves the exchange of the γ-phosphate between NTP's. The reaction was detected by incubating one γ-^{32}P labelled triphosphate (ATP, for example) with detergent-treated VSV virions in the presence of different unlabelled NTP'S, (GTP, UTP, and CTP, for example). The exchange was measured by using the techniques of paper chromatography and Dowex-1 (Cl^-) column chromatography.

Phosphotransferase activity involving CTP was not detected

while UTP was involved to a lesser extent than ATP and GTP. The transfer of the γ-^{32}P phosphate does not appear to be associated with the breakdown of the donor triphosphate. Rather, the exchange appears to occur directly between triphosphates since labelled free phosphates and pyrophosphates were not incorporated into unlabelled NTP. Experiments using (β, γ-^{32}P) ATP as a substrate indicated that only the γ-^{32}P was transferred. The reaction was stimulated by Triton N-101. No role was suggested for this enzyme in VSV infections.

Roy and Bishop (1971) state that they will demonstrate, in a future communication, that both the phosphotransferase and NTP'ase can be separated from the nucleoprotein cores and the polymerase of VSV.

(5) Proteinase

Holland et al (1972) have reported the presence of a proteinase activity in several highly purified preparations of animal viruses, including VSV. This activity was detected in VSV by incubating detergent-treated virions in tris buffer containing dithiothreitol for 20 hours at 37°C. The activity was measured by examining the proteins after incubation by gel electrophoresis. Heat shocking the virion or treating it with a nonionic detergent is required to demonstrate this activity. Pre-treating the virions with irreversible inhibitors of proteinases does not inhibit the VSV-associated proteinase

activity. It was also shown that the nucleoprotein was the most stable of the five proteins of VSV detected in the gel electrophoresis examination. Whether or not this activity has some biological role in VSV infections, such as in the mechanism of fusion and penetration, has not been determined.

III Virion-Associated Enzymes in Other Viruses

(A) Kern Canyon Virus (KCV), another Rhabdovirus

Aaslestad et al (1971) found an RNA-dependent RNA polymerase activity in KCV and compared this activity with the VSV transcriptase. As in VSV, the RNA synthesized by the virion-associated polymerase is complementary to the virion RNA. Furthermore, the optimum conditions of these two viruses are similar with respect to pH, temperature, and Mg^{++}, Na^{+}, and detergent concentration. Mn^{++} could not substitute for Mg^{++} for either virus. On the other hand, the polymerase of KCV was stimulated to a greater extent than that of VSV by sulfhydryl agents. In KCV, dithiothreitol was found to cause a greater stimulation than mercaptoethanol and dithioerythritol. Under optimum conditions, the specific activity of the enzyme of KCV was only 4% of that of VSV.

(B) Paramyxoviruses

A virion-associated RNA-dependent RNA polymerase activity has been described for Sendai virus (Stone et al, 1971, Robinson 1971, 1972; Hutchinson et al, 1972) and for Newcastle disease virus (NDV) (Huang et al, 1971). Robinson's studies on Sendai virions are especially interesting since he has tested the virion-associated polymerase both in vivo

(in the presence of cycloheximide) and in vitro. As in the studies with the VSV virion-associated transcriptase (Huang & Manders, 1972), THE RNA species produced in vivo in the presence of cycloheximide were complementary to virion RNA and were similar in size to the RNA species complementary to virion RNA produced in the absence of cycloheximide (Robinson, 1971). On the other hand, the RNA species produced by the enzyme in vitro (Robinson, 1972), although complementary to the virion RNA, were smaller than those species produced in vivo. However, some nuclease activity was detected in the Sendai virus preparations.

In the studies of NDV polymerase by Huang et al (1971), a comparison was made with the virion-associated polymerase of VSV. The properties of the NDV polymerase were similar to that of VSV except that the specific activity of the NDV polymerase was only 1-3% of the specific activity of the VSV polymerase. Both viruses had the same optimum pH, temperature, and Mg^{++} concentration. Neither virus had polymerase activity if Mn^{++} was substituted for Mg^{++} in the reaction mixture. Furthermore, in the initial period of the reaction, the RNA product was found in a complex with the virion RNA template.

Other virion-associated enzyme activities have been detected in paramyxoviruses. For example, NDV and Sendai virus appear to have a nuclease which can degrade poly A and poly I but not poly C nor poly U, (Rosenbergová and

Pristašorá, 1972).

In addition, protein kinase activity has been detected in virions of parainfluenza virus (Hatanaka et al, 1971).

(C) Myxoviruses

RNA-dependent RNA polymerase has been detected in influenza virus (Chow & Simpson, 1971; Penhoet et al, 1971; Skehel, 1971) It is interesting to note that like VSV, which also has a virion-associated polymerase, the RNA of influenza virus is non-infectious but ribonucleoprotein of influenza virus (which probably contains polymerase) is infectious (Hirst & Pons, 1972).

The study of Chow and Simpson (1971) is also interesting since the properties of the polymerase of influenza virus were compared to the polymerase of VSV. They found that, unlike VSV, Mn^{++} rather than Mg^{++} was required for polymerase activity. The polymerases of both viruses, however, required the presence of a nonionic detergent for activity.

Influenza virus is also associated with the following enzyme activities: NTP'ase and nucleotide phosphotransferase (Roy & Bishop, 1971), protein kinase (Hatanaka et al, 1971), proteinase (Holland), and a nuclease (Rosenbergova & Pristašorá, 1972) which can degrade poly C, poly A, poly U, poly I,and denatured and native DNA. Rosenbergova and Pristašorá (1972) also detected this nuclease activity in fowl

plague virus.

(D) Double-stranded RNA viruses

An RNA-dependent RNA polymerase activity has been found in the virions of REO virus (Borsa & Graham, 1968; Shaktin & Sipe, 1968) and in wound tumor virus (Black & Knight, 1970). In addition to an RNA polymerase, an NTP'ase activity was found associated with purified REO virions (Borsa et al, 1970).

(E) RNA tumor virus

The largest variety of virion-associated enzymes are found in RNA tumor viruses. Implications with respect to the biological roles have been made for only five of the virion-associated enzymes that have been detected in tumor viruses, namely, RNA-dependent DNA polymerase (Baltimore, 1970; Temin & Mizutani, 1970); DNA-dependent DNA polymerase (Spiegelman et al, 1970; Mizutani et al, 1970); DNA ligase and exonuclease (Mizutani et al, 1971) and endonuclease (Mizutani et al, 1970). Some of the other enzyme activities which have been detected are as follows: protein kinase (Hatanaka et al, 1971; Strand & August, 1971), nucleotide kinase, phosphatase, hexokinase, and lactic dehydrogenase (Mizutani & Temin, 1972); NTP'ase and nucleotide triphosphate phosphotransferase (Roy & Bishop, 1971); transfer ribonucleic acid synthetase (Erikson & Erikson, 1971); and ribonucleic acid methylase (Grantt et al, 1972).

(F) DNA Tumor Viruses

Endonuclease activities have been reported to be associated with the virions of adenovirus (Burlingham et al, 1971; Burlingham and Doefler, 1972), of SV40 (Kaplan et al, 1972), and of polyoma (Cuzin et al, 1972).

(G) Pox Viruses

The following five enzyme activities have been reported in Vaccinia virions: DNA-dependent RNA polymerase (Kates et al, 1967; Munyon et al, 1967), nucleotide phosphorylase (Gold & Dales, 1968), endonuclease, and two exonucleases (acid and neutral DNA'ase) (Aubertin & McAuslan, 1971). Except for endonuclease activity, all of the above activities have also been reported in Yaba virions (Schwartz & Dales, 1971). Moreover, Aubertin & McAuslan (1971) reported that the three nuclease activities were also in rabbitpox. In addition, DNA-dependent RNA polymerase and the exonuclease activities also have been demonstrated in insect Poxviruses (Pogo et al, 1971; Lewandowski et al, 1969).

(H) Bacteriophages

Most of the enzymes associated with bacteriophages appear to be concerned with attachment and penetration. For example, a lysozyme-like enzyme has been reported to be present in

T- even phages (Barrington & Kozloff, 1954; Koch & Weidel & Primosigh, 1958), and phage λ (Fisher, 1959), and in phage G (Murphy, 1960). Furthermore, an endo-glycosidase activity, also probably associated with attachment, has also been found to be associated with a bacteriophage (Strim et al, 1971). However, Datta & Franklin (1971) have detected a DNA-dependent RNA polymerase associated with the bacteriophage PM2.

IV Ts Mutants of VSV

In order to increase the understanding of the biochemical and macromolecular events which occur during the infection of a mammalian cell by VSV, our laboratory has isolated temperature-sensitive (ts) mutants of the virus (Holloway et al, 1970). At least two other laboratories (Flamand (1969); Pringle (1970)) are also studying ts mutants of VSV. By studying the ability of the mutants to complement each other to produce infectious progeny in a mixed infection at the non-permissive temperature, Flamand's laboratory has classified their mutants into five complementation groups (Flamand, 1969, 1970). Two of these groups could not synthesize RNA at the non-permissive temperature (RNA^-) while the other three groups were able to synthesize RNA at the non-permissive temperature (RNA+).

Pringle (1970) found four complementation groups, two of which were consistently RNA^- (Pringle & Duncan, 1971).

Our laboratory has found 3 complementation groups (Wong, 1972, see Table Ia), two of which are RNA^- when i.m.'s of 10-40 PFU/ cell are used.

Five virion-associated enzyme activities have been found in VSV virions (see section II of Literature Review). It has not been established whether or not it is the virus or the host cell which supplies the genetic information for the synthesis of these five enzymes. However, our laboratory has found that one of our ts mutants, ts11 has a defective

TABLE Ia

COMPLEMENTATION LEVELS[a] FOR PAIRS OF TEMPERATURE SENSITIVE MUTANTS OF VSV

COMPLEMENTATION GROUP		IV		III	I			
	MUTANT	ts10	ts16B	ts29	ts4	ts11	ts14	ts28
IV	ts10							
	ts16B	0.7±0.3						
III	ts29	24*	10*					
I	ts4	150*	50*	9.5*	0.5±0.4			
	ts11	14*	60*	10.0*	0.9±0.5			
	ts14	8*	80*	15.0*	0.4±0.2	0.3		
	ts28	30*	120	34.0*	0.4±4	1.0±0.7	0.8	

(a) Complementation level 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. (Wong, 1972)

* Values marked with an asterisk have been shown to be significantly greater than 1($p < 0.05$).

virion-associated polymerase activity (Cormack et al, 1971). This finding indicates that the VSV genome codes for at least part of this enzyme activity.

Although no other laboratory has published any studies on the characterization of this enzyme or any other virion-associated enzymes in ts mutants of VSV, J. Szylagyi of Pringle's laboratory has reported at the Roscoff conference that the virion-associated polymerase of two of their mutants appear to be temperature-sensitive (Szylagyi & Holloway, personal communication). These two mutants belong to complementation group I which corresponds to the complementation group to which our tsll belongs (Pringle, Cormack, & Holloway, personal communication).

Additional studies regarding the behaviour of the virion-associated polymerase of our ts mutants in L cells have been described by Wong (1972), while this thesis reports the in vitro behaviour of this enzyme in some of our ts mutants.

MATERIALS AND METHODS

Cells and Medium

Earle's L cells obtained from Dr. G. F. Whitmore of the Ontario Cancer Institute were used in growing all virus preparations. The cells, grown in continuous suspension cultures at 37°C, were maintained between $5x10^4$ and $4x10^5$ cells/ml in medium CMRL 1066 obtained from Schwartz Bioresearch. The medium was prepared as follows: Twenty-two ml of a 10% sodium bicarbonate solution and 10 gm of dried medium were mixed in sufficient triply distilled water to bring the final volume of the solution to one litre. After the pH of the medium had been adjusted to 7.2-7.4, the medium was sterilized by millipore filtration through a pore size of 0.22 μ. Before use, sterilized medium was supplemented with 5% foetal calf serum, Penicillin (100 I.U./ml) and Streptomycin (50 gm/ml).

In the preparation of L cell monolayers, the culture was first diluted by approximately 25% by the addition of fresh medium. Sixty mm Falcon petri dishes, which were used for plaque assays, were seeded with 5 ml (approximately $2x10^6$ cells) of the diluted culture, while 16 oz. Brockway bottles, which were used in growing virus preparations were seeded with 25 ml (approximately $1x10^7$ cells). The cells were incubated twenty-four hours prior to use in a humidified 37°C incubator with an atmosphere of 5% CO_2 and 95% air.

Preparation of virus stocks

The Indiana serotype of VSV was obtained from Dr. A. F. Howatson, of the Ontario Cancer Institute. An HR strain of this VSV stock and ts mutants derived from this stock were obtained from Dr. P. K. Y. Wong (Wong, 1969). The permissive temperature used for the mutants is 30°C, while the non-permissive temperature is 38°C. In order to distinguish ts mutants isolated in Winnipeg from those isolated elsewhere, a "W" may be inserted after the ts. If no symbol appears after the ts, it may be assumed that the mutant referred to was a Winnipeg isolate.

Virus preparations were grown and harvested as follows: Monolayers of L cells in 16 oz. Brockway bottles were infected with 1 ml of the appropriate dilution of a virus stock in phosphate buffered saline (PBS), pH 7, to achieve an input multiplicity (i. m.) of 10 plaque-forming units (PFU) per cell. After a 30 min absorption period, 25 ml of fresh medium were added per bottle, and the bottles were incubated at 30°C in a 5% CO_2 incubator for 24 hours. The resulting lysate was clarified by centrifugation for 10 min at 10,000 rpm in the #870 head of an International centrifuge. The virus in the clarified lysate was pelleted by centrifugation in the International centrifuge for 60 min at 16,000 rpm, resuspended in 0.5 ml of 0.1 M tris buffer, pH 7.9 (approximately an 80 fold concentration), and dispersed by ultrasonication.

Virus preparations of the same type were pooled and assayed for PFU and in vitro polymerase activity. A suspension of virus prepared in this way will be referred to as a standard virus preparation. Standard virus preparations were used in all experiments except where noted in the text. If the virus preparation was not used immediately, it was stored at 4°C. In most virus preparations, except those of complementation group I, neither polymerase activity nor the amount of infectivity decreased significantly after two weeks of storage at 4°C. However, both infectivity and polymerase activity in the preparations of the group I mutants began to decrease after 1 week of storage. In general, the experiments described in this thesis were performed before any signigicant decrease had occurred in the infectivity and polymerase activity of the preparations. Some preparations were further purified by rate zonal centrifugation followed by isopycnic centrifugation. The details of these procedures are described in the appendix.

The virus stocks were derived from isolated plaques and were examined, after negative staining, by electron microscopy to ensure that the content of T particles was less than 5%.

Plaque assay

The virus preparations were assayed using a technique similar to that of Dulbecco and Vogt (1954). A suitable

dilution of the virus was made in PBS, pH 7, and 0.1 ml of the dilution was added to a monolayer of $2x10^6$ L cells from which the medium had been removed. After the virus was allowed to absorb for 30 min at 38°C, an overlay of 3 ml of medium containing Penicillin, Streptomycin, serum, and 1.1% agar were added to the monolayer. It was then placed in a CO_2 incubator for 20 hours at 38°C or for 44 hours at 30°C. To enhance the visibility of the plaques which had formed, 1 ml of a 1:5,000 solution of neutral red in PBS buffer, pH 5, was added and the monolayer was incubated for an additional 3 hours. The plaques whose size were now approximately 2mm were then counted.

In vitro polymerase assay

The virion-associated RNA polymerase activity was assayed essentially according to the method of Baltimore et al (1970). To a series of test tubes, 50 µl of a standard virus preparation were added and mixed with 50 µl of tris buffer (unless otherwise stated). After the tubes had been placed in an ice bath, 200 µl of a stock reaction mixture were added so that the final reaction mixture in each tube consisted of, in addition to virus, the following: 25 µmoles tris-HCl, pH 7.9; 1.6 µmoles of $MgCl_2$; 1 µ mole β- mercaptoethanol; 30 µ moles NaCl; 0.2 µ moles ATP, CTP, UTP; 0.02 µ moles of ^{3}H-GTP (containing 1 µ Ci); and 0.25 mg Triton N-101. All reaction mixtures tested for polymerase activity had a final volume of 300 µl. Tubes were then removed from the ice bath, and

placed in either a 30°C or a 38°C water bath. At various times, tubes were removed from the water baths, and the reaction was terminated by rechilling the tubes in the ice bath, adding 2 drops of yeast RNA (4 mg/ml) and 2.5 ml of cold 5% trichloroacetic acid (TCA) in 0.02 M sodium pyrophosphate, and mixing. After 10 min at 4°C, the samples were filtered through Whatman glass filters and washed in 30 ml of cold 5% TCA. The filters were placed in glass scintillation vials, dried, and 0.5 ml of a 1:3 mixture of NCS tissue solubilizer and toluene were added to each vial. After 30 min at room temperature, 7 ml of Liquiflour-toluene mixture containing 1% glacial acetic acid were added. The vials were then counted in a liquid scintillation counter for 10 min or 2000 counts (2 standard deviations).

The radioactivity due to non-specific background was determined by measuring a sample which had not been incubated. The average value of this background calculated from 96 determinations done over 34 experiments was approximately 50 cpm (standard deviation = 25 cpm) and this value was subtracted from the experimental results to give a measurement in net cpm.

Chemicals

^{3}H-GTP was obtained from Schwartz Bioresearch Inc., Orangeburg, N. Y.; ATP, GTP, UTP, CTP from P. L. Biochemicals, Milwaukee, Wisconsin; NCS tissue solubilizer, and L amino

acid-^{14}C mixture from Amersham / Searle, Arlington Heights, Illinois; Liquiflour from New England Nuclear, Pilot Chemicals Division, Boston, Massachusetts; Triton N-101 from Sigma Chemical Co., St. Louis, Missouri.

RESULTS

I Ability to Detect RNA-dependent RNA Polymerase Activity by the Method of Baltimore *et al* (1970)

The experimental data shown in Table II demonstrate that RNA-dependent RNA polymerase activity can be detected in our VSV preparations by means of the incorporation of ^{3}H-GTP into an acid-insoluble product. The table shows that this acid-insoluble product is RNA since it appears to be sensitive to RNase. The product does not appear to be produced by an enzyme like polynucleotide phosphorylase because incorporation cannot be demonstrated if UTP is omitted from the reaction mixture. Because incorporation occurs in the presence of Actinomycin D, the product is not due to the presence of DNA-dependent RNA polymerase. Furthermore, Table II shows that this RNA-dependent RNA polymerase activity occurs only when a preparation of VSV virions is added to the reaction mixture. The three types of experiments in Table II have been repeated once.

In experiment I of Table II it is seen that there was slighty less incorporation when Actinomycin D was in the reaction mixture. A similar small degree of inhibition in the presence of Actinomycin D was observed in the same experiment when the polymerase activity of the virions of ts 16B were tested. It could be interpreted that the small decrease in

TABLE II

RNA-DEPENDENT RNA POLYMERASE ACTIVITY

IN PREPARATIONS OF HR VIRIONS

	NET CPM AFTER 20 MIN AT 30°
EXP. I :	
complete reaction mix[(a)]	3330
complete plus 5 μg of actinomycin D	2770
EXP.11 :	
complete reaction mix[(a)]	4145
complete plus 25 μg RNase	25
complete minus virions	25
EXP. 111 :	
complete reaction mix[(a)]	4830
complete minus UTP	35

(a) A complete reaction mix is essentially that described by Baltimore et al (1970) and has been described in materials and methods. The titres of the three HR preparations used in these experiments are all 2×10^{11} PFU/ml.

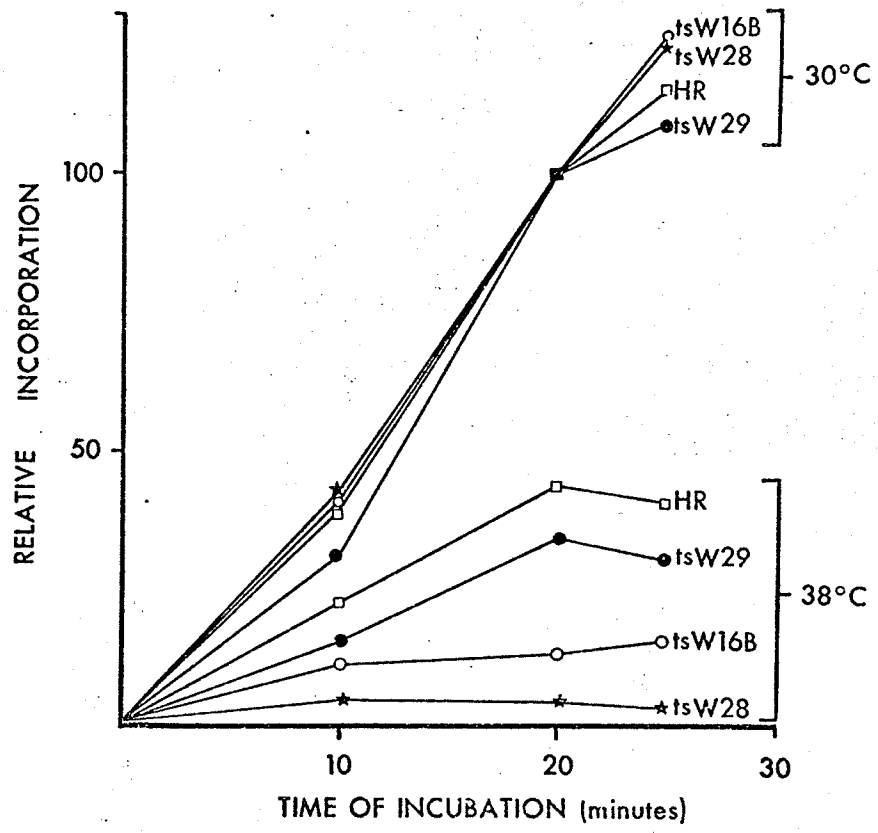
incorporation in the presence of Actinomycin D is due to the inhibition of contaminating cellular DNA-dependent RNA polymerase. However, when the experiment was repeated with a preparation of HR virions, there was essentially no difference in the amount of incorporation in the presence and in the absence of Actinomycin D. Furthermore, since all virion preparations compared in an experiment are usually grown in cells from the same spinner culture, and are harvested under the same conditions, it is likely that all the preparations would contain the same amount, if any, of cellular DNA-dependent RNA polymerase. Therefore, in comparisons of the polymerase activities of mutant virions and their parent, HR, any small contribution to the total RNA polymerase activity due to cellular polymerase would be the same in both types of virus preparations.

II Temperature Dependence of the Virion-associated RNA Polymerase Activities of HR and ts Mutants

The temperature dependence of the virion-associated RNA polymerase activity of HR and of several of our ts mutants was examined by comparing their ability to incorporate ^{3}H-GTP into an acid-insoluble product at 38°C and 30°C. Fig. 1-3 are three examples of experiments which show the kinetics of incorporation at these two temperatures for HR and some ts mutants. In Fig. 1 and Fig. 2, in order to facilitate the comparison of the ability of the virus preparations to synthesize RNA at 38°C relative to 30°C, the curves were normalized at the twenty min point at 30°C. In Fig. 3, in order to demonstrate, in addition, the low activity of the polymerase of ts11 compared to that of HR, this normalization was not done. Instead, the amount of incorporation of ts11 and of HR were plotted on different scales.

In Fig. 1, it can be seen that, relative to the incorporation at 30°C, the incorporation by the polymerase of ts16B and of ts28 at 38°C is considerably less than HR, while the temperature dependence of the polymerase activity of ts29 appears similar to HR. In Fig. 2, the virion-associated polymerase of ts16B (a different preparation than the one used in Fig. 1) again appears to be more temperature-sensitive than HR, while the temperature dependence of the

Fig. 1 Kinetics of incorporation by the virion-associated polymerases of HR, ts29, ts16B, and ts28. The net cpm of the four preparations have been normalized to the 20 min point at 30°C. The PFU in each reaction mixture were as follows: HR ($1x10^9$ PFU); ts29 ($3x10^9$ PFU); ts16B ($3x10^9$ PFU); ts28 ($7x10^9$ PFU). The cpm of each preparation after 20 min of incubation before normalization were as follows: HR (770 cpm); ts29 (900 cpm); ts16B (1660 cpm); ts28 (780 cpm).


tsW16B
tsW28
HR
tsW29
30°C
100
50
RELATIVE INCORPORATION
HR
tsW29
38°C
tsW16B
tsW28
10
20
30
TIME OF INCUBATION (minutes)

(W = Winnipeg)

Fig. 2 Kinetics of incorporation by virion-associated RNA polymerases of HR, ts16B, and ts10 at 30° and 38°C. The experimental data were treated as described in Fig. 1. The PFU in each reaction mixture were as follows: HR ($1x10^{10}$ PFU); ts16B ($1x10^{9}$PFU); ts10 ($2x10^{9}$ PFU). The net cpm after 20 min incubation at 30°C before normalization were as follows: HR (6,240 cpm); ts16B (1,870 cpm); ts10 (1,930 cpm).

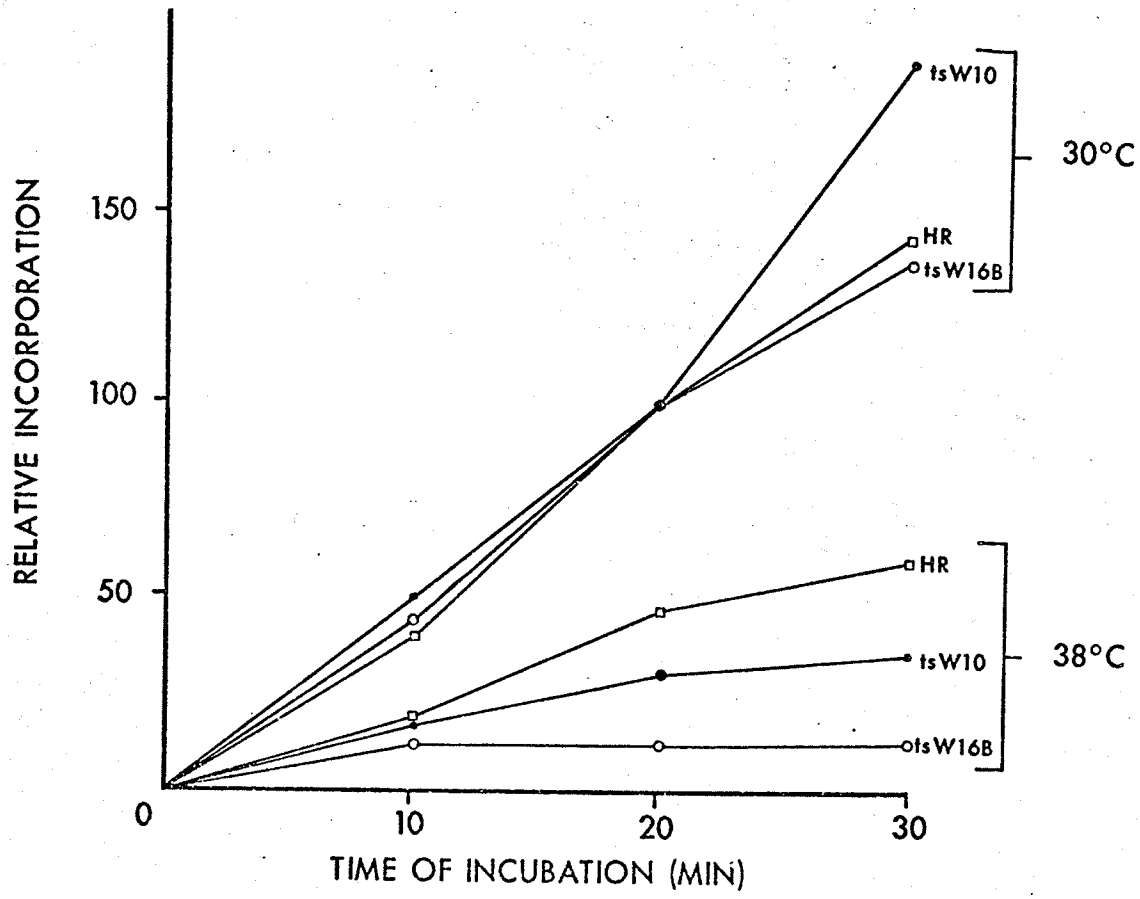
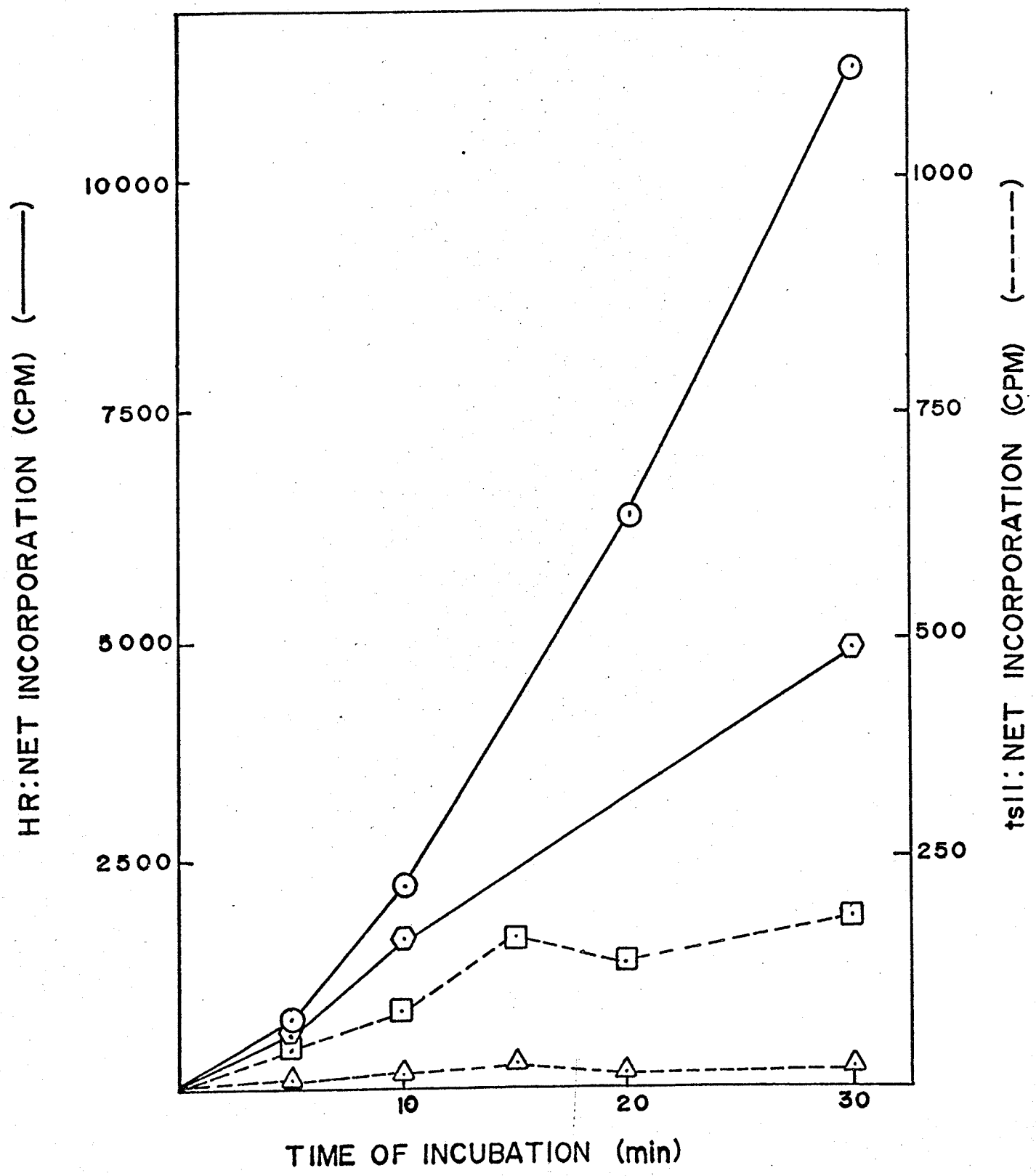

RELATIVE INCORPORATION
150
100
50
0
10
20
30
TIME OF INCUBATION (MIN)
tsW10
HR
tsW16B
30°C
HR
tsW10
tsW16B
38°C

Fig. 3 Kinetics of incorporation of virus-associated RNA polymerase of HR and of ts11 at 30°C and 38°C.

⊙——⊙, ⊙——⊙ HR at 30°C and 38°C, respectively;

⊡---⊡, △---△ ts11 at 30°C, and 38°C, respectively.

The PFU in each reaction mixture were as follows: HR (4×10^9 PFU); ts11 (9×10^{10} PFU).


10000
7500
5000
2500
HR: NET INCORPORATION (CPM) (——)
1000
750
500
250
ts11: NET INCORPORATION (CPM) (- - - -)
10
20
30
TIME OF INCUBATION (min)

polymerase of ts10 is intermediate between HR and ts16B. Fig. 3 demonstrates that the polymerase to ts11 appears more temperature-sensitive than HR.

In order to calculate an average value for the temperature dependence of the polymerase activity of a mutant tested in several different experiments, a temperature dependence index (TDI) was calculated as follows:

$$TDI = \frac{\text{net incorporation (cpm) in 20 min at } 38^{\circ}C}{\text{net incorporation (cpm) in 20 min at } 30^{\circ}C} \times 100\%$$

The index is based on a 20 min incubation since this is the longest time at 38°C during which the polymerase activity of HR always remained linear. Table III shows an example of experimental results for 30°C and 38°C incubations, and the TDI's which were calculated from these results. The data suggest that the virion-associated polymerase activities of all the mutants in the table except ts29 are more temperature-sensitive than HR. The observation that even HR shows a TDI of less than 100% is consistent with the other observations of temperature dependence of the virion-associated polymerase activity (Aaslestad et al, 1971; Huang et al, 1971). Most of these mutants have been tested sufficiently often for meaningful statistical analyses, and the average TDI of these viruses is shown in Table IV. This table demonstrates that the temperature-sensitivity of the polymerase activities of ts11, ts28, ts10, and ts16B, but not ts29 is significantly greater than HR.

TABLE III

EXAMPLES OF EXPERIMENTAL RESULTS FOR POLYMERASE ACTIVITIES OF HR AND ts MUTANTS AFTER A 20 MINUTE INCUBATION

VIRUS	PFU/ REACTION MIX	PLAQUE RATIO[a]	NET[b] CPM 30°C	NET[b] CPM 38°C	TDI[c]
HR	$2x10^9$	1	2910	1790	61%
ts29	$3x10^9$	$3x10^{-4}$	2200	1260	57%
ts10	$2x10^{10}$	$1x10^{-2}$	2130	655	31%
ts16B	$3x10^9$	$1x10^{-3}$	620	70	11%
ts4	$5x10^9$	$1x10^{-4}$	230	13	6%
ts11	$1x10^{10}$	$1x10^{-3}$	100	10	10%
ts14	$4x10^9$	$1x10^{-3}$	360	40	11%
ts28	$3x10^9$	$3x10^{-4}$	600	40	7%

(a) The ratio of the number of plaques formed in 24 hours at 38°C to that formed in 48 hours at 30°C.

(b) Experimental results have been corrected for background activity as described in materials and methods.

(c) $$TDI = \frac{\text{incorporation after 20' at 38°C}}{\text{incorporation after 20' at 30°C}} \times 100\%$$

TABLE IV

AVERAGE TDI[a] VALUES

MUTANT	AVERAGE TDI	NO. OF EXPERIMENTS
HR	60± 6%	40
ts29	52± 21%	6
ts10	34± 3%	8
ts16B	13± 2%	36
ts11	8± 11%	16
ts28	5± 2%	8

(a) $$TDI = \frac{\text{Incorporation in 20 min at } 38^{\circ}C}{\text{Incorporation in 20 min at } 30^{\circ}C} \times 100\%$$

The relative ability of the mutants to synthesize RNA at 30°C was also studied. This was measured by using the following ratio:

$$A = \frac{\text{incorporation (cpm) in 20 min at } 30^{\circ}C}{\text{PFU/ml at } 30^{\circ}C} \times 10^{10}$$

This ratio was calculated for the mutants, and then in order to compare them with the preparations of HR grown in cells from the same spinner and tested in the same experiment, the ratio; $A_{mutant}:A_{HR}$ was calculated. Data was used from experiments which had been performed within a week after the virus was harvested. If the ability (measured in cpm/PFU) of a mutant to synthesize RNA at 30°C was similar to the ability of HR, then the ratio would approximately equal unity, while the value would be less than unity for those mutants which made relatively little RNA at 30°C compared to HR.

Table V shows the average ratio of the mutants shown in Table III and, wherever possible, 95% confidence limits were assigned. The data indicates that the polymerase activities of ts4, ts11, ts14, and ts28 have changed from HR since these mutants synthesize relatively little RNA per PFU even at 30°C. This change appears to be especially severe in ts4, ts11, and ts14 since incorporation smaller than 150 cpm over background is typical for these mutants even in preparations containing as many as 1×10^{11} PFU/ml. When the activity at 30°C is so low, small variations in the

TABLE V

ABILITY OF MUTANTS TO SYNTHESIZE RNA AT 30°C

	$A^{(a)}_{MUTANT}/A_{HR}$	
MUTANT	GEOMETRIC MEAN	95% CONFIDENCE LIMITS
ts29	0.86	0.31-2.8
ts10	1.50	0.99-2.2
ts16B	1.30	0.72-2.50
ts11	0.02	0.01-0.04
ts28	0.22	0.12-0.43
ts4	0.19	b
ts14	0.06	b

(a) $A = \frac{\text{INCORPORATION IN 20 MIN AT } 30^{\circ}}{\text{PFU/ml AT } 30^{\circ}} \times 10^{10}$

(b) INSUFFICIENT DATA TO DETERMINE

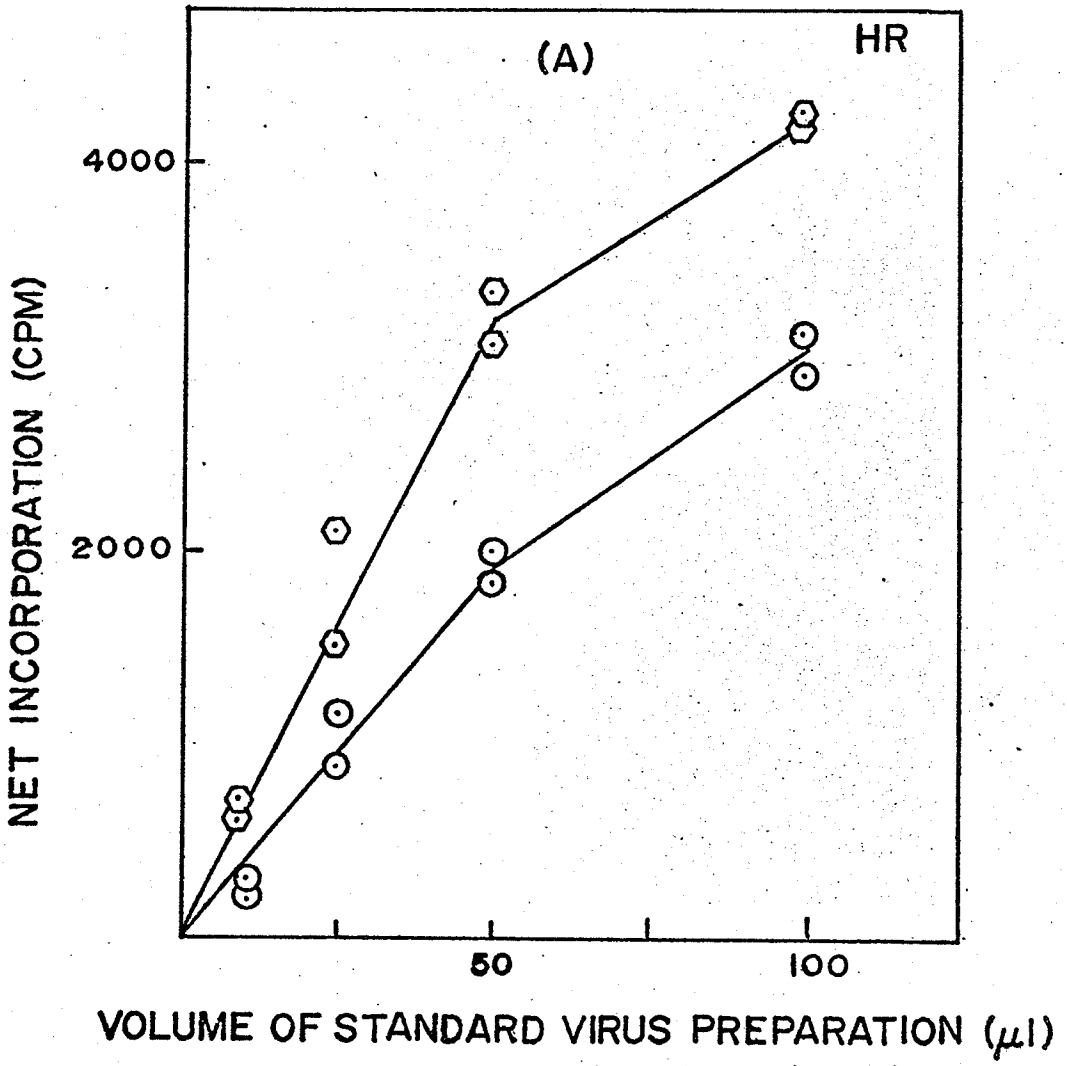
non-specific background have a strong influence on the variation of the TDI. This is probably the reason why the range of the 95% confidence limits of the TDI of ts11 is relatively large. A similar difficulty arises in assigning an average TDI to ts4 and ts14, both of which have been tested only in two experiments. Perhaps in such cases, the ratio which indicates the relative ability of a mutant to synthesize RNA at 30°C is more meaningful to describe an altered polymerase than the TDI.

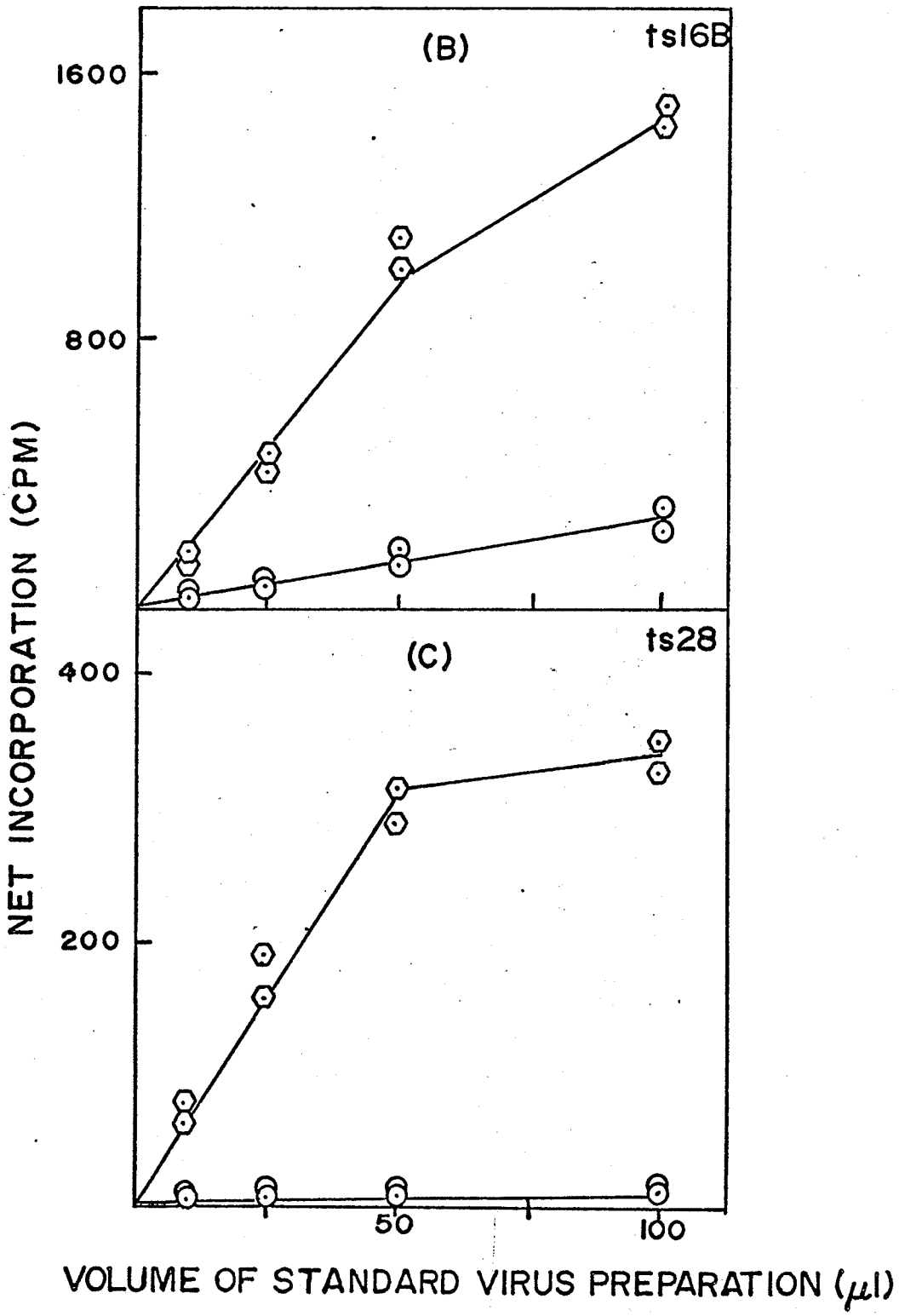
Table V indicates that even for those mutants which can synthesize RNA at 30°C approximately as well HR, there was considerable variation in the amount of RNA synthesized per PFU. However, in compiling the data for Table V, it was noticed that there was little variation in the amount of incorporation per PFU, when the same preparation was tested in different experiments. That is to say, the variation in Table V appears mainly to be due to differences in the preparations. For example, the results used in Table V for ts 29 were from the 6 experiments referred to in Table 1V. In one of these experiments, the value of the ratio describing the ability of ts29 to synthesize RNA relative to HR was 2.7. When the experiment was repeated on the following day, the value was again 2.7. However, when a different set of HR and ts29 preparations were tested in another experiment, the value was 0.37. In another experiment with these preparations the ratio was essentially the same (0.36).

III Relationship between Virus Concentration and Polymerase Activity

Fig. 4 shows the polymerase activity at 30°C for various concentrations of HR, ts16B, and ts28 preparations. These concentrations were obtained by adding to a series of test tubes, various volumes of a standard virus preparation (see Materials and Methods) and bringing the volume up to 100 μl with 0.1M tris buffer. Two hundred μl of a stock reaction mixture (see Materials and Methods) were then added to each tube. It will be noted that the polymerase activity increases linearly with the amount of virus added, up to 50 μl per 300 μl of reaction mixture. Seven other standard virus preparations have been studied at 30°C, and for all the preparations, the incorporation was proportional to the volume of virus added up to 50 μl. The volume of standard virus preparation used in each complete 300 μl reaction mixture was normally 50 μl, that is, in the linear region of the concentration curve. However, as Fig. 4 illustrates, the polymerase activities of ts16B and ts28 are more temperature sensitive than that of HR, even at concentrations beyond the linear regions.

Fig. 4 Relationship between incorporation and the volume of standard virus preparation used in the polymerase assay. To 200 μl of a stock polymerase reaction mixture, 10, 25, 50, or 100 μl of a standard virus preparation (see Materials and Methods) of either HR (A), ts16B (B), or ts28 (C) were added, and the final volume was brought to 300 μl. The reaction mixtures containing virus were then incubated for 20 min at 30°C (⊙——⊙) or at 38°C (⊙——⊙). The standard virus preparations contained the following PFU/ ml; HR (7×10^{10}); ts28 (3×10^{10}); ts16B (1×10^{10}).


(A)
HR
4000
2000
NET INCORPORATION (CPM)
50
100
VOLUME OF STANDARD VIRUS PREPARATION (μl)


(B)
ts16B
1600
800
(C)
ts28
400
200
50
100
NET INCORPORATION (CPM)
VOLUME OF STANDARD VIRUS PREPARATION (μl)

1V Temperature Shifts from 30°C to 38°C

Temperature shift-up experiments were done in order to investigate whether the temperature-sensitive polymerase activity of some of our mutants might be due to a defect in a different activity which must occur before the virion-associated polymerase can function. For example, perhaps the polymerase requires activation by the virion-associated protein kinase (Strand and August, 1971). In these experiments, virus preparations in polymerase reaction mixtures were shifted to 38°C after the virion-associated polymerase had been allowed to function at 30°C for 10 min. If a mutant's only defect were in a function which must precede the polymerase activity, the mutant's polymerase should be able to function like HR after shift. Fig. 5 shows an example of an experiment which indicates that the polymerase activities of ts16B and ts28 but not HR cease shortly after shift. The behaviour of HR and of ts16B has been confirmed by four additional experiments, while ts28's behaviour has been confirmed by 2 additional experiments. Fig.6 shows that the virion-associated polymerase activity of ts11 also shuts off after shift, while Fig. 7 shows an example of 2 experiments which demonstrate that the behaviour of ts29 after shift is like HR. Fig. 8 shows an example of 3 experiments which demonstrate that ts10 synthesizes some RNA after shift. However, since the rate of ts10's polymerase activity drops after shift to

that of the same preparation of ts10 incubated continuously at 38°C, it appears that a defective activity prior to polymerase activity is not responsible for the increased temperature sensitivity of ts10 shown in Fig. 2, Table III, and Table IV.

Fig. 5 Comparison of the effect of a temperature shift-up on the polymerase activities of HR, ts16B and of ts28. Reaction mixtures containing virus were incubated for 10 min at 30°C and then shifted to a 38°C water bath. The PFU in each reaction mixture were as follows: HR (7×10^8 PFU); ts16B (1×10^8 PFU); ts28 (5×10^9 PFU).

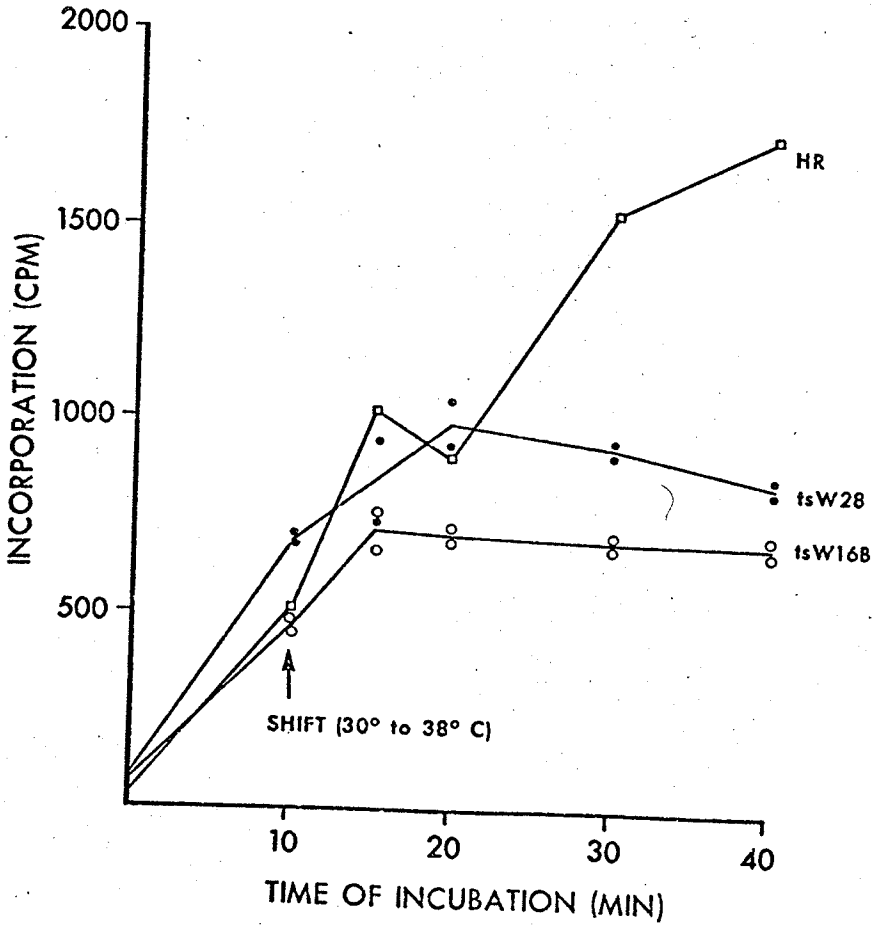

2000
1500
1000
500
INCORPORATION (CPM)
HR
tsW28
tsW168
SHIFT (30° to 38° C)
10
20
30
40
TIME OF INCUBATION (MIN)

Fig. 6 Effect of a temperature shift-up on the polymerase activities of tsll and of HR.

 incorporation by tsll;

 incorporation by HR

The PFU in each reaction mixture were as follows: tsll (9×10^9 PFU); HR (4×10^9 PFU).

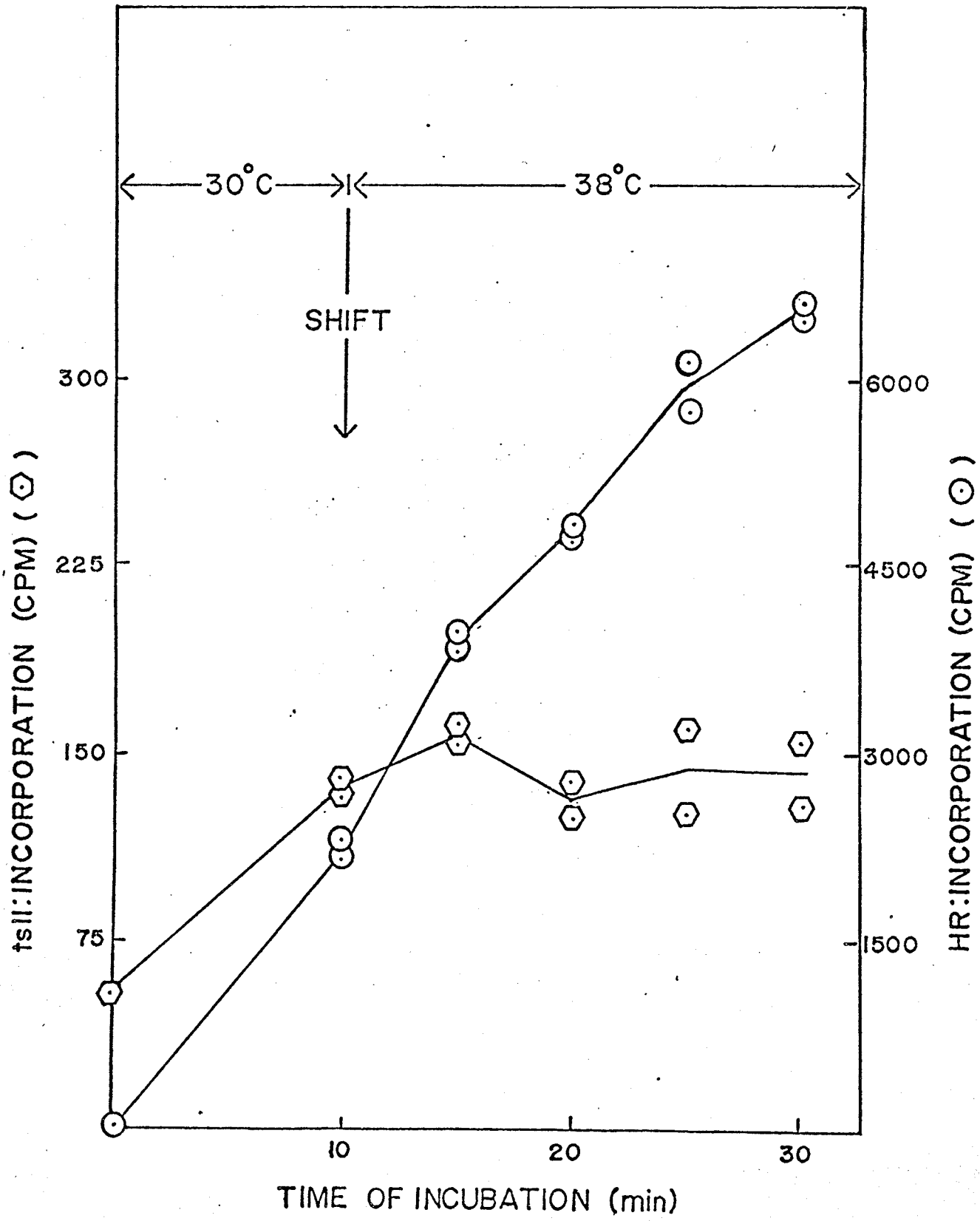

30°C
38°C
SHIFT
300
225
150
75
6000
4500
3000
1500
10
20
30
tsII:INCORPORATION (CPM) (⊙)
HR:INCORPORATION (CPM) (⊙)
TIME OF INCUBATION (min)

Fig. 7 Effect of a temperature shift-up on the polymerase activities of ts29 and of HR.

⊙——⊙ incorporation by ts29;

⊙——⊙ incorporation by HR.

The PFU present in each reaction mixture were as follows: ts29 (4×10^9 PFU); HR (1×10^{10} PFU).

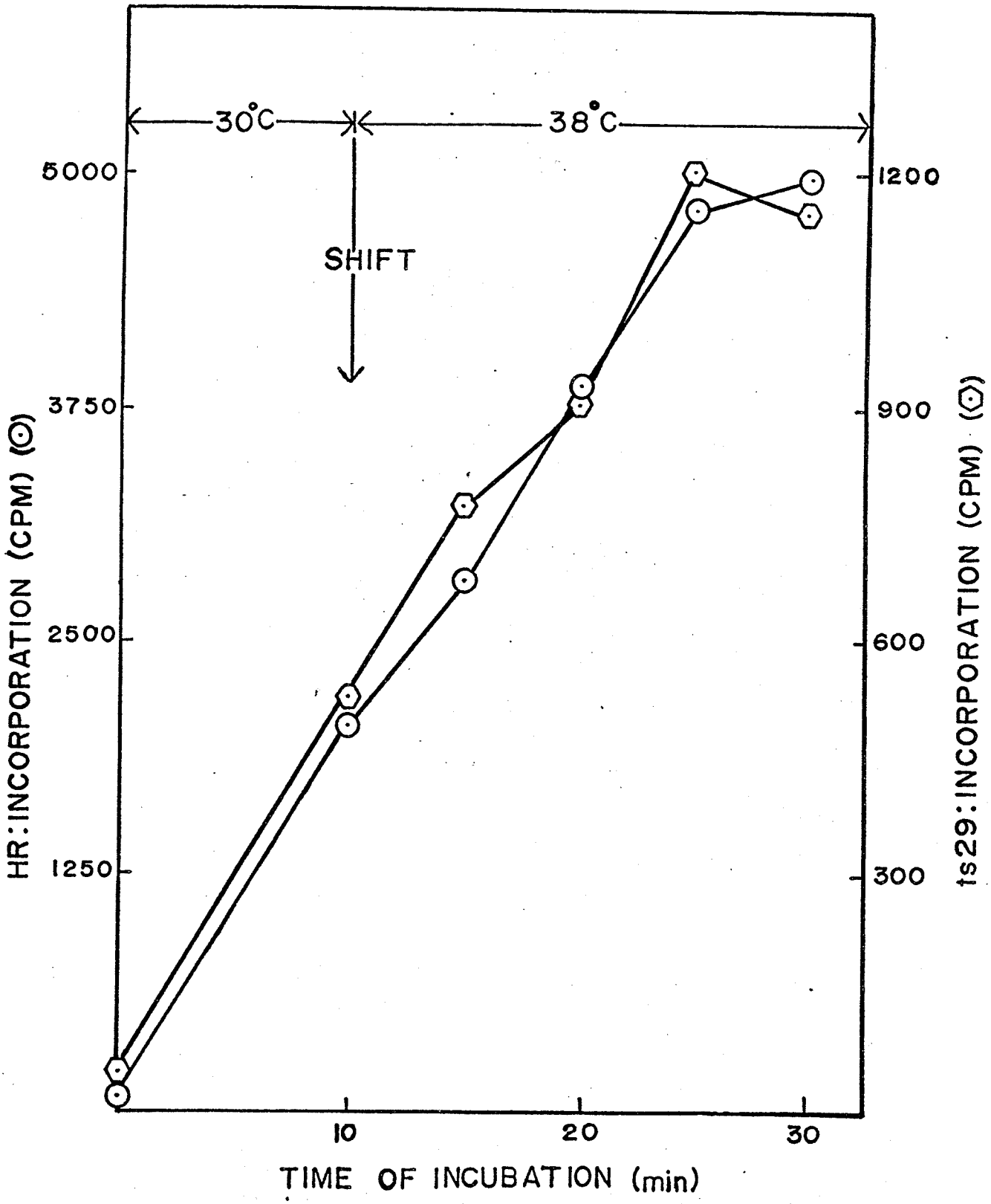
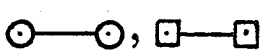

30°C
38°C
SHIFT
5000
3750
2500
1250
1200
900
600
300
HR: INCORPORATION (CPM) (⊙)
ts29: INCORPORATION (CPM) (⊙)
10
20
30
TIME OF INCUBATION (min)

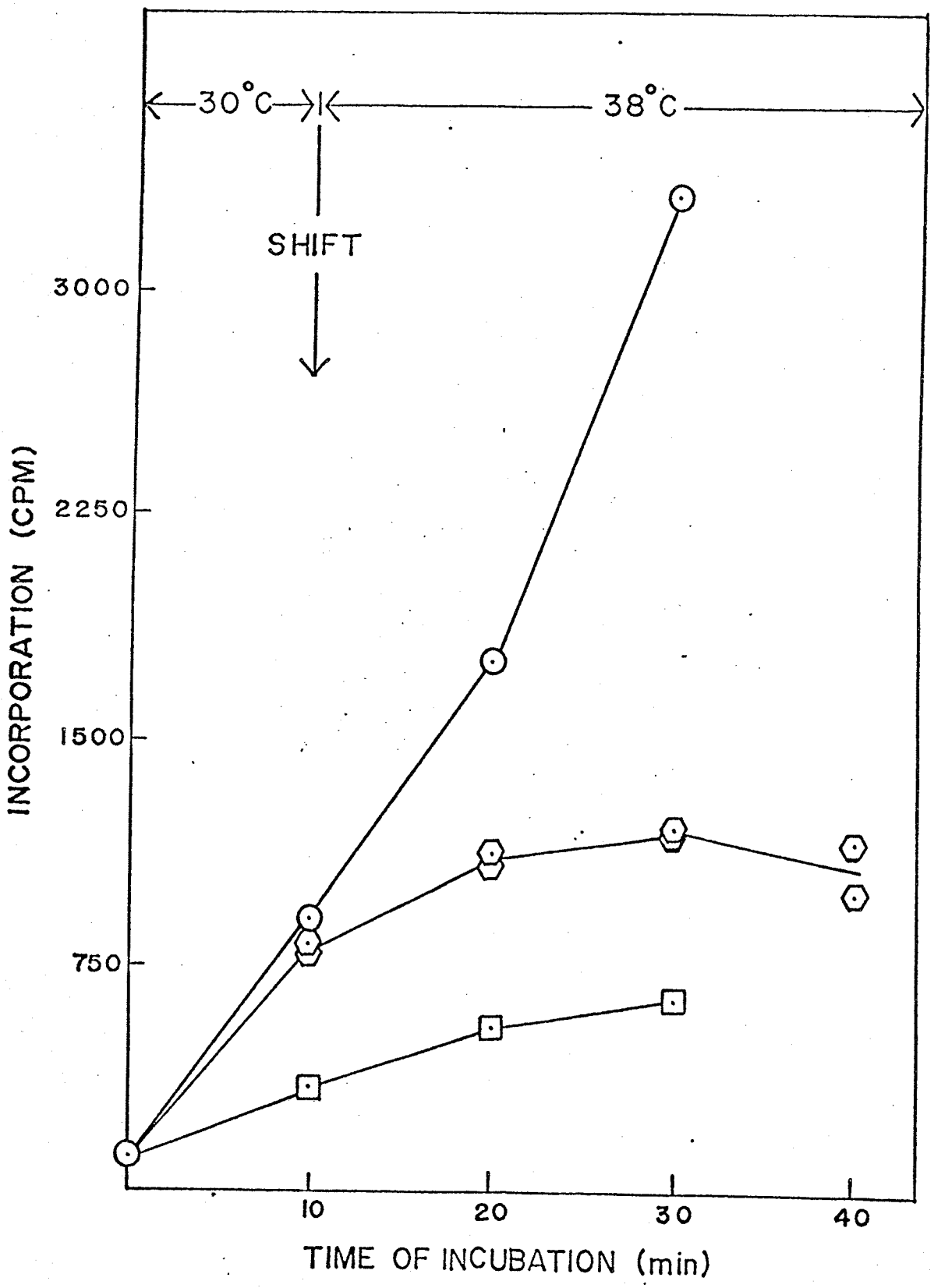
Fig. 8 Effect of a temperature shift-up on the polymerase activity of ts10.



incorporation by ts10 incubated continuously at 30°C and at 38°C, respectively;

incorporation by ts10 before and after temperature shift-up.

The PFU in each reaction mixture were 1.5×10^9 PFU.


30°C
38°C
SHIFT
3000
2250
1500
750
INCORPORATION (CPM)
10
20
30
40
TIME OF INCUBATION (min)

V Polymerase Activity in Mixtures of Mutant Virions and HR Virions

Experiments in which mutant preparations were mixed with HR preparations were done to imvestigate the possibility that the polymerase activity of the preparations of some mutants appeared temperature sensitive because, unlike HR, they contained cellular RNase and/or proteases which were more active at 38°C than at 30°C. If a mutant preparation appeared to have a temperature-sensitive polymerase because of such enzymes, then it would be expected that the amount of polymerase activity at 38°C, in a mixture of HR and such a mutant, would be reduced, by these cellular enzymes, to approach the activity seen for the mutant alone. Table Vl (a), shows the incorporation after 20 min incubation at 38°C, when 200 μl of a stock reaction mixture were added to 100 μl of the following types of mixtures: 50 μl of HR plus 50 μl of tris buffer; 50 μl of a ts mutant plus 50 μl of tris buffer; 50 μl of HR plus 50 μl of the ts mutant. This table demonstrates that no reduction of the polymerase activity occurs in mixtures of HR with ts28 or with ts11, while there is a slight reduction in the mixture of HR with ts16B. In order to facilitate the comparison of the results of several experiments, an inhibition index (I.I.) was calculated as follows:

TABLE VI

(a) POLYMERASE ACTIVITIES IN MIXTURES OF HR VIRIONS WITH MUTANT VIRIONS

COMPONENT #1		COMPONENT #2		MIXTURE (#1+2)
ts no.	NET CPM-38°C	ts no.	NET CPM-38°C	NET CPM-38°C
HR	1790	ts16B	230	1490
HR	1790	ts11	20	1920
HR	1790	ts28	50	1870

The net acid-insoluble cpm given in the table were obtained after the reaction mixtures had been incubated for 20 min at 38°C. Component #1 or #2 consisted of 50 μl of a standard virus suspension plus 50 μl of tris buffer, while the mixtures contained 50 μl of component #1 plus 50 μl of component #2. The virus preparations were assayed on L cell monolayers at 30°C and gave the following titres: HR (4×10^{10} PFU/ml); ts16B (2×10^{10} PFU/ml); ts11 (6×10^{10} PFU/ml); ts28 (2×10^{10} PFU/ml).

(b) I.I. VALUES

MIXTURE:	HR+ts11	HR+ts28	HR+ts16B
EXP. 1	0.9	1.0	1.4
11	0.8	0.9	1.7
111	1.0	1.0	---
1V	0.9	---	1.7

$$\text{I.I.} = \frac{\text{(incorporation by HR alone)+(incorporation by mutant alone)}}{\text{incorporation by mixture}}$$

The experimental data of experiment I have been shown in part (a).

$$\text{I.I.} = \frac{\text{(incorporation by HR alone)} + \text{(incorporation by ts mutant alone)}}{\text{incorporation by mixture}}$$

Inhibition (that is, the reduction in incorporation which occurs in a mixture) is considered to be absent if the value of the I.I. is less than or equal to unity. Table Vl (b) compares the results of 4 experiments. The experimental data used to derive the results of experiment I (Table Vl (b) are those shown in Table Vl (a). The results of Table Vl (b) are consistent in showing that no inhibition occurs in mixtures of HR with ts28 or with ts11, while there is some inhibition in mixtures of HR with ts16B. This inhibition could be due to the presence, in the ts16B preparation, of a cellular inhibitor. This is unlikely for the following reasons. First, since in these experiments, the HR preparation and the ts16B preparation had been grown at the permissive temperature in cells which had been grown and harvested under the same conditions it would be expected that any cellular material present would be the same in both preparations. Second, in Fig. 5, after the temperature shift-up there is no apparent degradation of the RNA that has already been made at 30°C by ts16B. Third, data to be presented in the next section show that, after an incubation at 38°C, a reaction mixture containing ts16B still has polymerase activity at 30°C. If a protease were causing ts16B's polymerase to appear temperature sensitive, such a recovery would not occur. Finally, Table,VII shows that the TDI of HR or

of ts16B is essentially unchanged by further purification by rate zonal and isopycnic centrifugation (details about the purification are described in the appendix). It appears more likely that altered components of ts16B interfere with the polymerase activity of HR.

TABLE VII

TDI VALUES AFTER FURTHER PURIFICATION

VIRUS	DEGREE OF PURIFICATION[a]	PFU/RM	NET CPM-30°	NET CPM-38°	TDI
HR*	DIF.	1.5×10^9	2490	1970	79%
	(DIF.R.Z.(K)ISO.)-TOP	3.5×10^9	480	310	65%
	(")-BOT.	4.5×10^9	1160	720	62%
ts16B*	DIF.	1.1×10^9	1150	150	13%
	(DIF.R.Z.(K)ISO.)-TOP	1.1×10^9	200	10	5%
	(")-BOT.	1.5×10^9	150	20	13%
ts16B	DIF.	2.0×10^9	1390	110	8%
	(DIF.R.Z.(S)G.ISO.)-TOP	7.0×10^8	370	50	14%
	(")-BOT.	1.0×10^9	520	50	10%
ts16B	DIF.	9.0×10^8	2830	340	12%
	(DIF.R.Z.(S')ISO.)-TOP	2.0×10^7	50	1	indeterminate
	(")-BOT.	7.0×10^7	790	80	10%

RM = reaction mixture

* These preparations were grown, harvested, and tested for polymerase activity at the same time under identical conditions.

(a) where

DIF. = differential centrifugation

R.Z. = rate zonal centrifugation (K) = 9-29% potassium tartrate gradient

(S) = 9-33% sucrose gradient

(S') = 9-29% sucrose gradient

ISO = isopycnic centrifugation

V1 Some Characteristics of the Temperature-sensitive Polymerase Activities Observed in Various Mutants

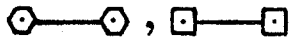
(A) Temperature shifts from 38°C to 30°C

Reversibility of the defective polymerase activity observed *in vitro* at 38°C in ts10, ts16B, and ts28 has been tested by incubating these mutants in polymerase reaction mixtures for five minutes at 38°C, and shifting the virus-containing reaction mixes to a 30°C water bath. Fig. 9 demonstrates that both HR and ts10 recover after shift to an activity similar to the sample kept continuously at 30°C. On the other hand, the polymerase activity of ts28 hardly recovers after shift, while ts16B recovers to a large extent. (These results have been confirmed by repeating the experiment once for ts10, ts28, and HR, and twice for ts16B.) Hence, according to this criterion, ts28 differs from ts16B and ts10.

(B) Polymerase activity in reaction mixtures containing puromycin

Since the virion-associated polymerase was tested *in vivo* in this laboratory by infecting actinomycin D-treated cells in the presence of puromycin, it was of interest to investigate whether puromucin would have any effect on the polymerase activity *in vitro*. Fig. 10 (a) demonstrates that puromycin has little effect on the polymerase activity

Fig. 9 Effect of a temperature shift-down on the polymerase activities of HR, ts10, ts16B, and ts28. Reaction mixtures containing virus were incubated for 5 min at 38°C, and then shifted to 30°C.

 incorporation when reaction mixtures were incubated continuously at 30°C and at 38°C, respectively.

 incorporation before and after the temperature shift-down.

The PFU present in each reaction mixture were as follows: HR ($1x10^{10}$ PFU); ts10 ($2x10^{9}$ PFU); ts16B ($1x10^{9}$ PFU); ts28 ($5x10^{9}$ PFU).

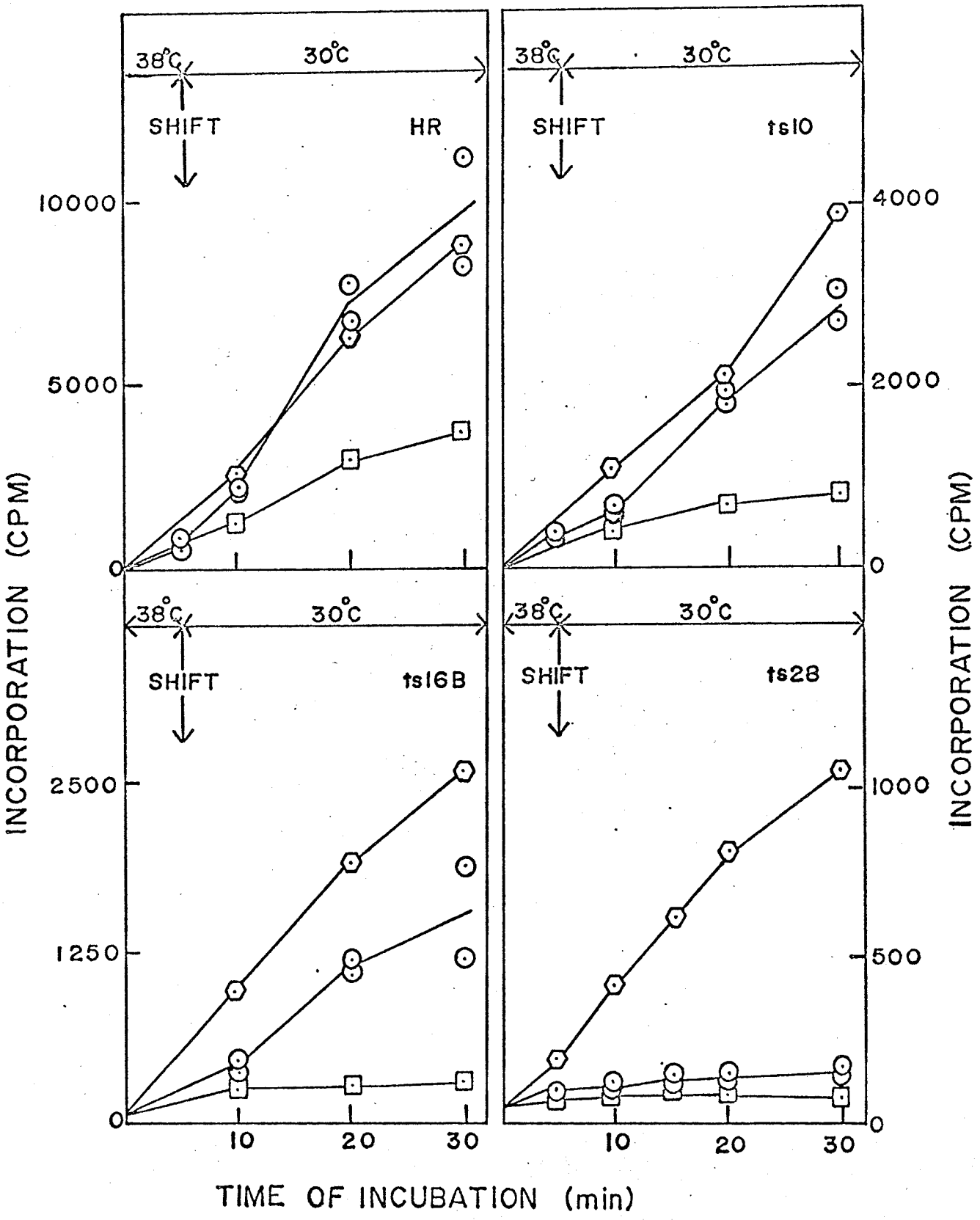

38°C
30°C
SHIFT
HR
ts10
ts16B
ts28
10000
5000
0
4000
2000
2500
1250
1000
500
10
20
30
INCORPORATION (CPM)
TIME OF INCUBATION (min)

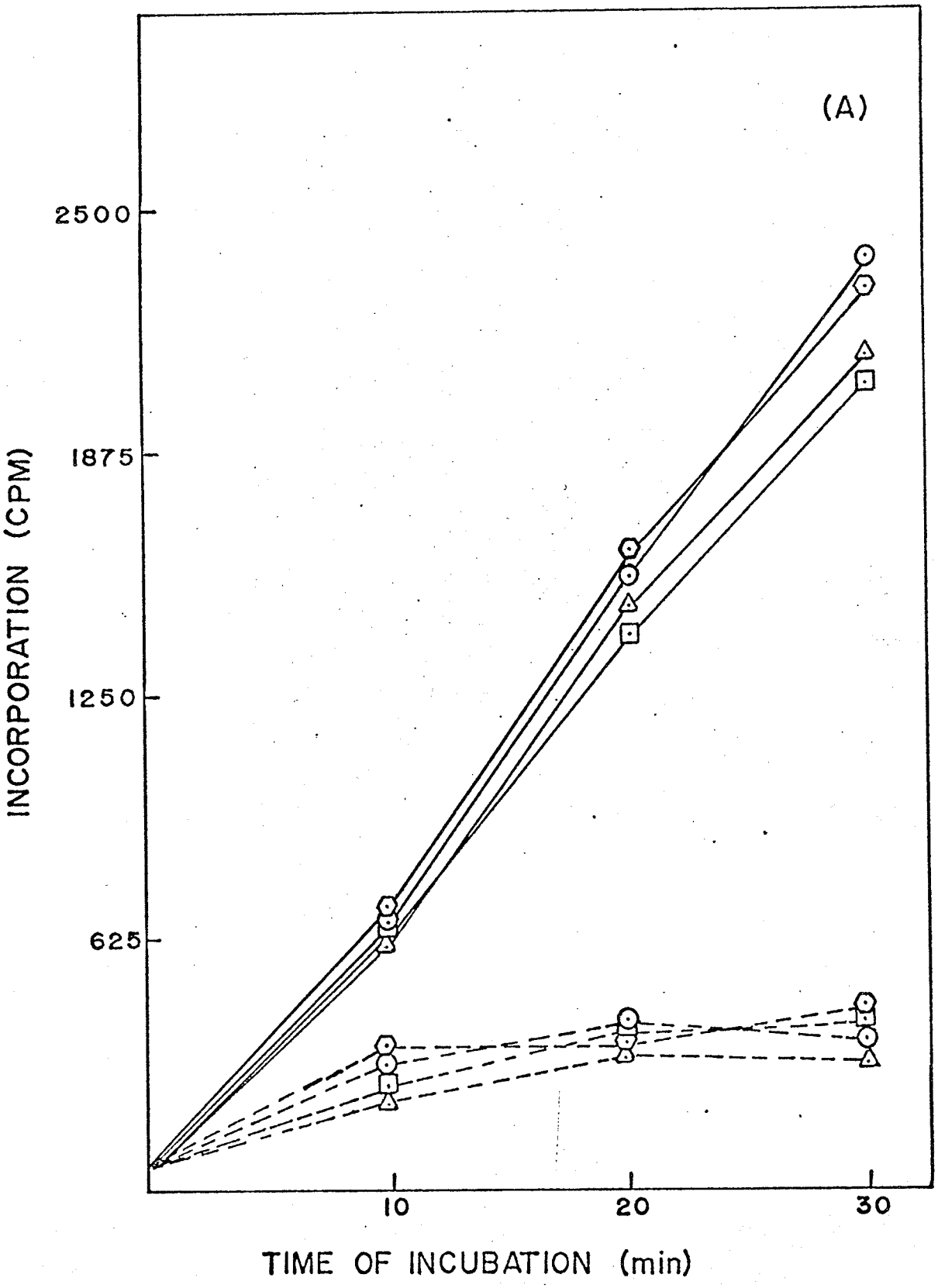
Fig. 10 (A) The effect of various concentrations of puromycin on the kinetics of incorporation by the polymerase of ts16B at 30°C and at 38°C. The incorporation at 30°C is indicated by a solid line, while the incorporation at 38°C is indicated by a dotted line. Incorporation was measured in the presence of 0 (⊙), 30 (⊙), 60 (⊡), and 120 (△) μg/ml of puromycin. The number of PFU present in each reaction mixture was 3×10^9 PFU.

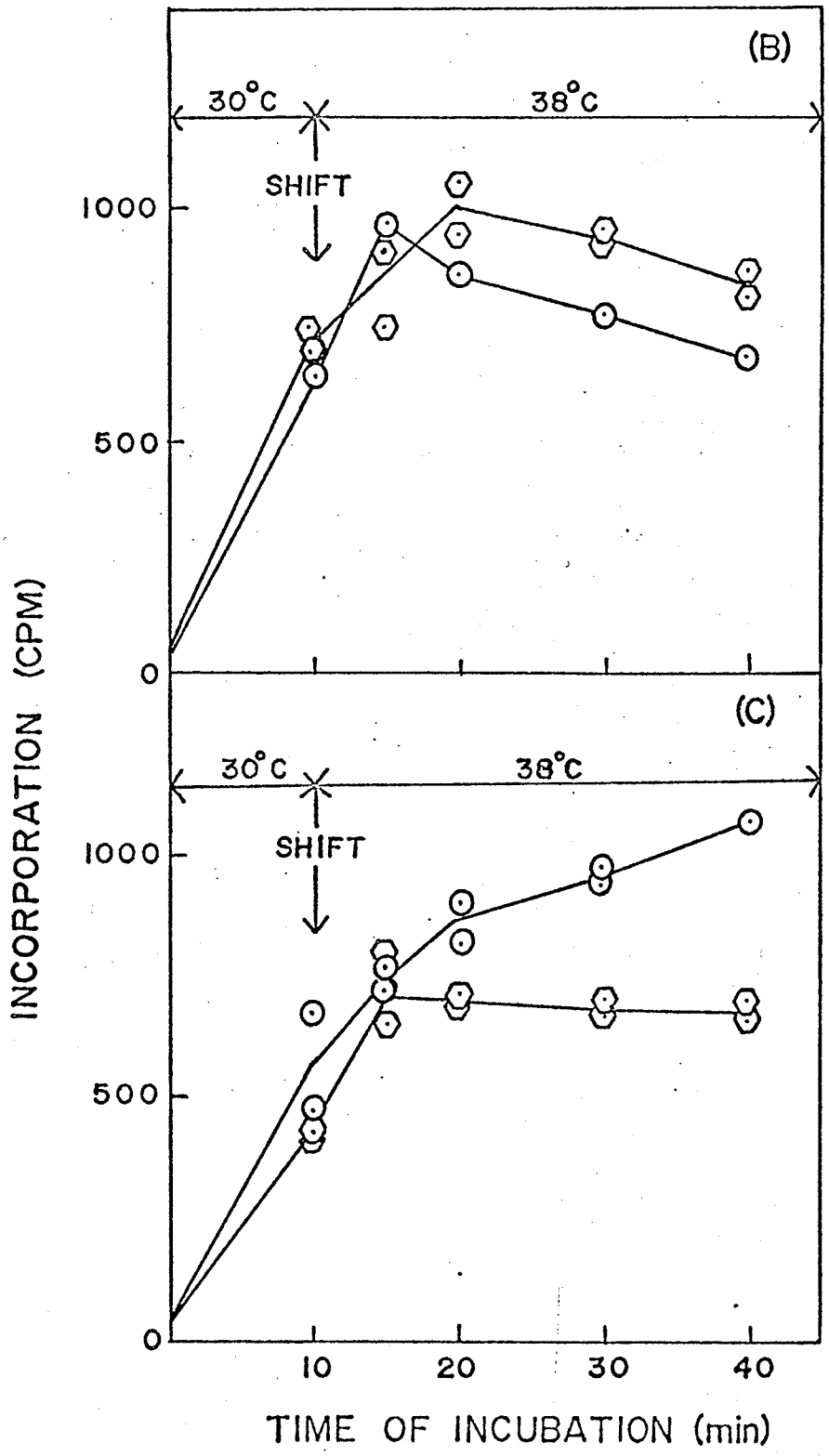
(B) (C) Effect of puromycin (60 μg/ml) on the polymerase activities of ts28 (B) and ts16B (C) after a temperature shift-up. The reaction mixtures containing virus were incubated for 10 min at 30°C and then shifted to a 38°C water bath.

⊙——⊙ incorporation with no puromycin in the reaction mixture;

⊙——⊙ incorporation with puromycin in the reaction mixture.

The PFU present in each reaction mixture were as follows : ts16B (5×10^8 PFU); ts28 (5×10^9 PFU).


(A)
2500
1875
1250
625
INCORPORATION (CPM)
10
20
30
TIME OF INCUBATION (min)


(B)
30°C
38°C
SHIFT
1000
500
0
(C)
30°C
38°C
SHIFT
1000
500
0
10
20
30
40
INCORPORATION (CPM)
TIME OF INCUBATION (min)

of ts16B (even at concentrations of 120 μg/ml). To investigate if puromycin might have had an effect on a mutant's polymerase at 38°C after its polymerase had first been permitted to function at 30°C, a temperature shift-up experiment as previously described was performed in the presence of puromycin. Fig. 10 (b) and Fig. 10 (c) show the results for an experiment which demonstrates that the polymerase of ts16B but not of ts28 appears to function after shift in the presence of puromycin (60 μg/ml), while in the absence of puromycin, both mutants cease to synthesize RNA approximately five minutes after shift. This type of experiment has been repeated two other times for ts16B and the results, summarized in Table VIII, are consistent in showing increased RNA synthesis after shift in the presence of puromycin. The significance of this effect of puromycin to the interpretation of the in vivo experiments is presented in the Discussion. However, it is interesting to note that this is another characteristic in which ts16B and ts28 differ.

(C) Complementation of in vitro RNA polymerase activities in mixtures of mutants

The observation that some pairs of our mutants complement each other at the non-permissive temperature in L cells to yield infectious progeny, prompted an investigation of the ability of mutants to complement each other's in vitro polymerase activity. Furthermore, there is evidence of

TABLE VIII

TEMPERATURE SHIFT-UP EXPERIMENTS OF ts16B WITH AND WITHOUT PUROMYCIN IN THE REACTION MIXTURE (1)

	AVERAGE RATE OF INCORPORATION (CPM/MIN)	
	NO PUROMYCIN(3)	+ PUROMYCIN(4)
EXP. 1(2)	- 1.3 ± 3.2	12.5 ± 4.5
EXP. 11	1.7 ± 3.4	7.9 ± 6.1
EXP. 111	-14.6 ±15.9	6.4 ±15.2

(1) Reaction mixtures containing ts16B (with or without puromycin) were shifted to 38°C after an incubation of 10 min at 30°C. Five min were allowed for thermal equilibrium to be achieved, and the average rate of incorporation with 95% confidence levels was calculated from the incorporation observed in the following 25 min (Exp.1) or 15 min (Exp.11 & 111).

(2) The kinetics of incorporation in this experiment are shown in Fig.X(b).

(3) A typical complete reaction mixture was used in obtaining these results.

(4) A complete reaction mixture containing puromycin (60 μg/l) was used in obtaining these results.

in vitro complementation in other enzyme systems (for example, Schlesinger, 1964; Nealson *et al*, 1970; Case *et al*, 1971; Yeh & Tessman, 1972). *In vitro* complementation of virion-associated RNA polymerase activity was tested by comparing the polymerase activity of a mixture of a pair of mutants with the polymerase activity of each member of the pair alone. Fig. 11, which shows the polymerase activity at 38°C of mixtures of ts16B and ts11 in various proportions, demonstrates that the polymerase activity observed in the mixtures is several times greater than the activity of observed for either mutant alone. The polymerase activities of other mixtures of mutants are shown in Table IX. Complementation is considered to have occured if the incorporation shown by a mixture of a pair of mutants is greater than the sum of the two mutants alone. Table IX shows that ts16B complements not only ts11, but also ts14 and ts28, while ts11, ts14, and ts28 cannot complement each other's polymerase activities. The mutant ts10 also appears to complement the polymerase activities of ts11 and ts28, but the complementation is less than the complementation which occurs in the mixtures of ts16B with ts28 and ts11. This is not surprising since ts10's polymerase is not very temperature sensitive (It has an average TDI of 34 %, as shown in Table IV.) In addition, the polymerases of ts10 and ts16B fail to complement each other. Those mutants which complement each other to give increased polymerase activity at 38°C also show increased

Fig. 11 Polymerase activity in mixtures of ts11 with ts16B. A constant volume (100 μl) of a mixture of standard virus preparations was mixed with 200 μl of a stock reaction mixture and incubated at 38°C for 20 min. The standard virus preparation of ts16B had 1×10^{10} PFU/ ml, while ts11 preparation had 5×10^{10} PFU/ ml.

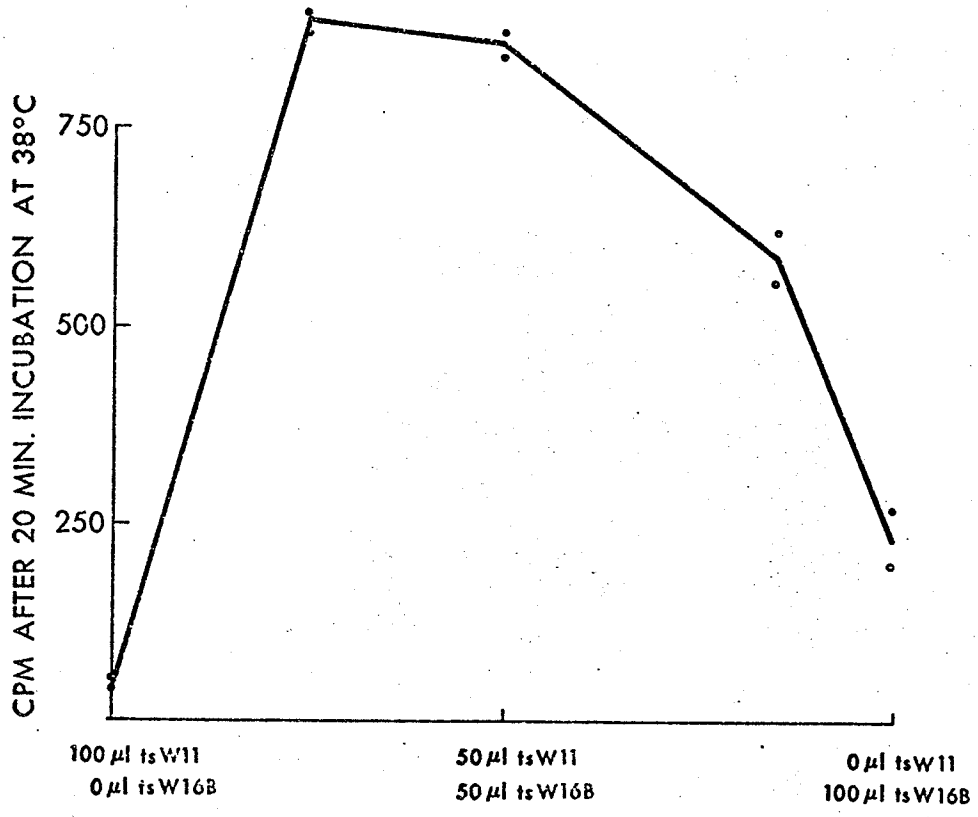

CPM AFTER 20 MIN. INCUBATION AT 38°C
750
500
250
100 µl tsW11
0 µl tsW16B
50 µl tsW11
50 µl tsW16B
0 µl tsW11
100 µl tsW16B

TABLE IX

IN VITRO COMPLEMENTATION OF VIRION-ASSOCIATED RNA POLYMERASE ACTIVITY IN MIXTURES OF MUTANTS(a)

COMPONENT #1(b)	COMPONENT #2(c)	NET INCORPORATION (CPM)-30°C			NET INCORPORATION (CPM)-38°C		
		#1	#2	#1+2(d)	#1	#2	#1+2(d)
ts16B	ts11	2000	70	3140	230	20	1200
ts16B	ts28	2000	460	3560	230	50	1300
ts16B*	ts11*	1310	120	2030	230	40	930
ts16B*	ts28*	1310	600	2385	230	40	1160
ts16B*	ts14*	1310	120	2380	230	90	1150
ts16B	ts10	2000	4320	4820	230	1230	1270
ts10	ts11	4320	70	4680	1230	20	1710
ts10	ts28	4320	460	4900	1230	50	2070
ts11	ts28	70	460	490	20	50	40
ts11*	ts28*	120	600	920	40	40	40
ts11*	ts14*	120	120	260	40	90	20
ts14*	ts28*	120	600	1050	90	40	50

(a) 200 μl of a stock reaction mixture were added to 100 μl of a virus mixture and acid-insoluble cpm given in the table were obtained after the reaction mixtures had been incubated for 20 min. The results are examples from two experiments. The suspensions tested in one experiment are marked with an asterisk (*), while the suspensions tested in the other experiment are unmarked. The data of the former

experiment are from single samples while those of the latter experiment are averages of duplicate samples. The titres of the standard virus suspensions used in these experiments were as follows: ts16B(2×10^{10} PFU/ml); ts11(6×10^{10} PFU/ml); ts28(2×10^{10} PFU/ml); ts10(7×10^{10} PFU/ml); ts16B*(6×10^{10} PFU/ml); ts11*(9×10^{10} PFU/ml); ts14*(1×10^{11} PFU/ml); ts28*(7×10^{10} PFU/ml).

(b) Component #1 consists of 50 μl of a standard virus suspension plus 50 μl of tris buffer.

(c) Component #2 consists of 50 μl of a different standard virus suspension plus 50 μl of tris buffer.

(d) Mixtures consist of 50 μl of the standard virus suspension of component #1 plus 50 μl of the standard virus suspension of component #2.

polymerase activity at 30°C. This suggests that in the in vitro environment, some of the polymerase is defective even at 30°C in some of these mutants.

In order to obtain a quantitative measure for complementation at 38°C, complementation levels of polymerase activity (C.L.P.) were calculated as follows:

$$\text{C.L.P.} = \frac{\text{incorporation by mixtures of component \#1 with \#2}}{\text{incorporation by component \#1} + \text{incorporation by component \#2}}$$

Table X shows the C.L.P. values for all the complementation experiments which have been done except for the mixtures not involving ts10 or ts16B. When neither ts10 nor ts16B were present, the net incorporation at 38°C did not exceed 50 cpm. (Mixes of ts11 with ts28 have been tested in 4 experiments, while the mixtures with ts14 (that is, ts14 with ts28 and ts14 with ts11) have been tested only in the experiment shown in Table IX.) The results in Table X demonstrate that the complementation behaviour of the mutants shown in Table IX is consistent in all the experiments performed.

TABLE X

COMPLEMENTATION LEVELS OF IN VITRO POLYMERASE ACTIVITY (C.L.P.)[a] IN MIXTURES OF PAIRS OF MUTANTS

EXPERIMENT	VIRUS MIXTURE					
	ts10+ts11	ts10+ts28	ts16B+ts11	ts16B+ts28	ts16B+ts14	ts10+ts16B
1	-	-	3.8	-	-	-
11	-	-	>10.0	-	-	-
111	1.6	-	-	-	-	0.6
1V	-	-	2.6	-	-	-
V[b]	-	-	3.5	6.0	3.3	-
V1	-	-	-	2.1	-	-
V11	-	-	5.7	-	-	-
V111	1.2	1.2	2.7	4.7	-	0.7
1X	-	-	3.5	-	-	0.7
X[b]	1.4	1.6	4.7	4.5	-	0.7

(a) $$C.L.P. = \frac{\text{(INCORPORATION BY MIXTURE OF COMPONENT \#1 WITH COMPONENT \#2)}}{\text{(INCORPORATION BY COMPONENT \#1) + (INCORPORATION BY COMPONENT \#2)}}$$

(b) These experiments are shown in Table 1X. The preparations tested in experiment V were marked with an asterisk (*) while the preparations tested in experiment X were unmarked.

V11 Temperature Dependence of the Virion-associated Polymerase in Ts+ revertants

If the temperature sensitivity of the virion-associated polymerase of a mutant is related to its inability to replicate at 38°C, then, the virion-associated polymerase of a mutant which has regained its ability to replicate at 38°C (ie., reverted to ts+ phenotype) would be expected to be less temperature sensitive. Ts+ revertants of two of our mutants, ts11 and ts16B, were isolated by picking HR-like plaques on L cell monolayers at 38°C. The material isolated from each plaque was resuspended in 2 ml of PBS. This suspension was used to infect cell monolayers in 16 oz. Brockway bottles at 30°C (as described in Materials and Methods) in order to obtain stocks of these revertants. The polymerase activity of one of these revertant stocks of ts11 and of 18 different revertant stocks of ts16B were tested in vitro for polymerase activity at 30°C, and at 38°C, and the TDI was calculated for each of them. A plaque assay, as described in Materials and Methods was done on the ts11 revertant and on the 4 revertants of ts16B which had the lowest TDI's. The results demonstrated that these preparations had equal numbers of plaques in the 30°C and 38°C assays. Examples of some of the experimental data of the ts11 revertant, 11R1, and a ts16B revertant, 16R6, are shown

in Table XI. A frequency distribution of the TDI's of all the ts16B revertants is shown in Fig. 12. The TDI of the ts11 revertant is also indicated. The figure shows, in addition, the 95% confidence limits of the average TDI's of HR and some of the mutants with temperature-sensitive polymerases. The TDI's of all the revertants are greater than those of the mutants, and this indicates that the reason why ts11 and ts16B fail to replicate at the nonpermissive temperature is associated with their temperature-sensitive behaviour in the in vitro polymerase assay.

TABLE XI

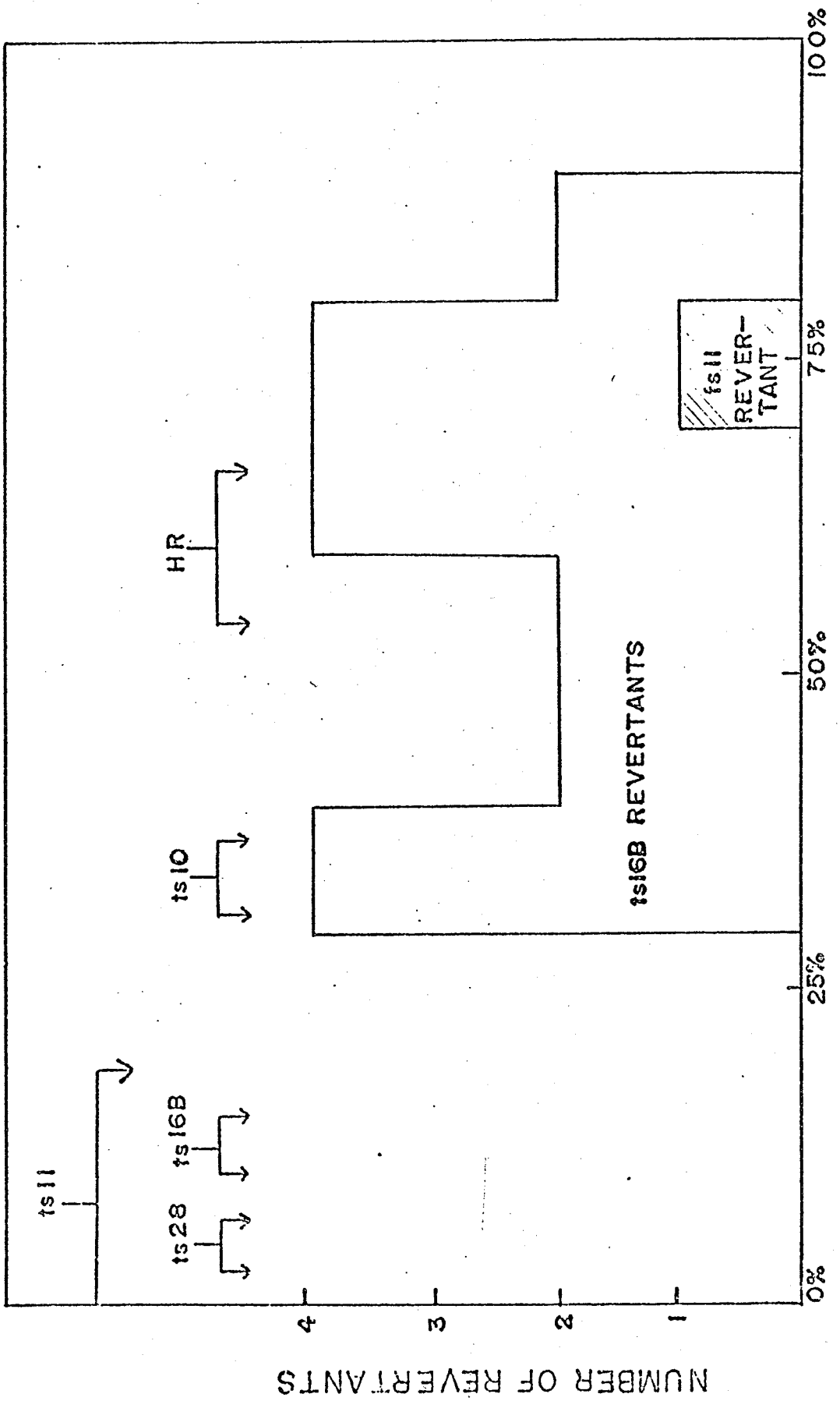
TEMPERATURE DEPENDENCE OF REVERTANTS OF tsl1 AND OF tsl6B

	PFU/REACTION MIX	PLAQUE RATIO(b)	NET CPM(a)		
VIRUS			30°C	38°C	T.D.I.
tsl1	$1x10^9$	$1x10^{-3}$	125	13	10%
11R1	$2x10^9$	1	1420	1020	72%
tsl6B	$4x10^9$	$5x10^{-4}$	620	70	11%
16R6	$8x10^9$	1	1590	480	30%

(a) After 20 min incubation.

(b) Ratio of the number of plaques formed in 24 hours at 38°C to that formed in 48 hours at 30°C.

Fig. 12 Frequency distribution of TDI values of revertants of ts16B. The position in the distribution of the TDI of the revertant of ts11 is shown in the cross hatched area. The arrows at the top of the graph show the range of the average values of the TDI's of HR and of some of the ts mutants.


100%
75%
50%
25%
0%
ts11 REVER-TANT
HR
ts10
ts16B REVERTANTS
ts11
ts16B
ts28
4
3
2
1
NUMBER OF REVERTANTS
T.D.I.

DISCUSSION

The data shown in Fig. 1-3 and Tables III and IV indicate that all of the mutants except ts29 have a virion-associated polymerase activity which is more temperature-sensitive *in vitro* than their parent, HR. The temperature shift-up experiments indicate that this increased temperature sensitivity is in the polymerase itself rather than in some preceding function.

Furthermore, the *in vitro* complementation of the virion-associated RNA polymerase activity in mixtures of certain pairs of mutants (Table IX) suggests that, in VSV, the virion-associated polymerase system consists of at least two components and that there can be an exchange of components amongst detergent-treated virions. According to this model, when two mutants which have defects in different components of the polymerase system are mixed together, increased incorporation occurs. However, when two mutants have a defect in the same component, no complementation of polymerase occurs. Hence, if this model is correct, ts11, ts14, and ts28 have a defect in a different component of the transcriptase system than do ts10 and ts16B. Perhaps the two components involved in this complementation are the NS and the N proteins (see Literature Review, p. 8).

It may be possible that complementation of polymerase

activities also occurs *in vivo* by a similar mechanism. Although *in vivo* complementation of RNA polymerase activities *per se* has not been studied by our laboratory, the mutants have been tested for their ability to complement each other in mixed infections of L cells to produce infectious progeny at the non-permissive temperature (see Literature Review, Table Ia). By comparing Table IX and Table Ia, it can be seen that those pairs of mutants which complement each other in the *in vitro* polymerase test also complement each other *in vivo* to produce infectious virus at the non-permissive temperature, while those pairs of mutants which fail to complement each other's polymerase activity *in vitro*, also fail to complement each other *in vivo*.

This correspondence of the *in vitro* complementation of virion-associated polymerase activities and the *in vivo* complementation of infectivity suggests that the temperature-sensitive polymerase activity of these mutants observed *in vitro* is associated with their inability to replicate in L cells at the non-permissive temperature. The observation that the polymerase activity of all the revertants of ts16B and ts11 have a decreased temperature sensitivity also supports this idea.

Although neither revertant nor complementation studies have thus far been done for ts^4, one would predict on the basis of its complementation behaviour *in vivo* (Table XII),

TABLE XII

^{14}C-CELLULAR CONTAMINANTS OBSERVED AT VARIOUS STAGES OF PURIFICATION[a]

SOURCE OF 25 μl SAMPLE	VOLUME OF SOURCE (ML)	NET[b] CPM IN 25 μl SAMPLE	CPM EXPECTED IF SOURCE HAD BEEN CONTAINED IN 1.0 ML
(1) PELLET OF CELLS+VIRUS	1.0	2610	2610
(2) PELLET OF CELLS ONLY	1.0	2460	2460
(3) PELLET OF VIRUS ONLY	1.0	250	250
(4) BAND FROM RATE ZONAL CENTRIFUGATION	2.5	5	12
(5) PELLETED VIRUS FROM GRADIENT BAND	0.55	1	0.5
(6) TOP BAND FROM ISOPYCNIC GRADIENT	0.5	1	1
(7) BOTTOM BAND FROM ISOPYCNIC GRADIENT	0.5	1	1

(a) The stages at which the samples were removed are indicated in FIG 23.

(b) The experimental results were corrected for background activity by subtracting 25 cpm (the cpm seen for a vial containing cocktail D only). The data shown in the table are an average of duplicate samples. Duplicates were ±10% of the average or ±5cpm whichever is the greater. The net cpm seen in 25μl of supernatant at stage #1 (pellet of cells + virus) was 260 cpm.

that it has similar properties to ts11, ts14, and ts28. Hence, the indications are that in ts4, ts11, ts14, ts28, ts10, and ts16B, but not in ts29, there is a defect in the virion-associated polymerase system which prevents the replication of these mutants in L cells at the non-permissive temperature. In order to confirm these indications, studies are in progress on the nature of the RNA made by the mutants in infected L cells at the permissive and non-permissive temperatures.

The above interpretation is, in general, supported by in vivo studies performed in this laboratory (Wong et al, 1972). For example, the temperature dependence of the virion-associated polymerase activity of ts29 (of complementation group III-see Literature Review) appears similar to HR not only in vitro (Table IV, Fig. 1) but also in vivo.

Three other observations agree with the idea that ts29 does not have a defect in its virion-associated transcriptase activity. First, as indicated by temperature shift-up experiments, ts29 appears to have a defect in a late function. Second, heat inactivation experiments give no evidence that ts29 has a defect in a structural component critical to virion infectivity. Third, in mixedly infected cells at 38°C ts29 can complement the six mutants which appear to have a temperature sensitive transcriptase in the in vitro study.

Moreover, the temperature dependence of the virion-associated polymerase activity of the mutants of complementation group I (ts^4, ts11, $ts1^4$, ts28) *in vivo* is similar to the *in vitro* behaviour in that the virion-associated transcriptases of these mutants appear to have an increased temperature sensitivity relative to HR. In addition, *in vivo* studies such as temperature shift-up experiments, which suggest that the defect in these mutants is an early function, and the heat inactivation experiments, which show an increased heat lability of these mutants relative to HR, are consistent with the idea that these mutants have a virion-associated transcriptase which is temperature-sensitive.

There are also many similarities in the *in vitro* and *in vivo* studies with ts10 and ts16B (complementation group 1V). As discussed previously, there is a parallel in the complementation behaviour. The observation that these mutants do not synthesize RNA at 38°C in the presence of actinomycin D with low i.m.'s (10-50 PFU/ cell) indicates that there is a temperature-sensitive defect associated with the RNA synthesis of these mutants. In addition, the temperature shift experiments which indicate that the defect is in an early function, support the idea that these mutants have a defect in RNA synthesis. The *in vitro* polymerase studies similarly indicate a defect in the RNA

polymerase activities of these mutants (Fig. 2, Table III and Table IV).

Another similarity is seen in the heat inactivation studies. The in vivo studies indicate that ts10 and ts16B have heat inactivation rates similar to HR, while Fig. 9 demonstrates that the degree to which the in vitro polymerase activities of ts10 and ts16B recover from an incubation at 38°C is more like HR than ts28 (which is a member of the complementation group whose mutants are more heat labile than HR).

However, when cells are infected in the presence of puromycin with high i.m.'s (approximately 1000 PFU/ cell), the temperature dependence of the virion-associated polymerase activity of ts16B and ts10 appears similar to HR, while, as it was mentioned above, this polymerase activity in vitro appears more temperature sensitive than HR. It is interesting to note, that the presence of puromycin appears to enhance the RNA synthesis of ts10 and ts16B in vivo. An enhancing effect of puromycin in vitro has been shown for ts16B in Fig. 10, (b). This enhancing effect of puromycin does not explain entirely the difference between the in vivo and in vitro observation because both ts10 and ts16B have some virion-associated RNA polymerase activity at 38°C in vivo, even in the absence of puromycin. This discrepancy is especially surprising in ts16B, since, as it was discussed previously, the in vitro studies of

the revertants suggest that the temperature-sensitive behaviour of ts16B's *in vitro* polymerase is related to the mutant's inability to replicate at 38°C. Furthermore, the *in vitro* complementation studies suggest that ts10 has a similar defect to that of ts16B.

Perhaps, there is a critical temperature-sensitive defect in the virion-associated polymerases of ts16B and ts10, but this defect is expressed differently *in vivo* than *in vitro*. For example, perhaps both *in vivo* and *in vitro*, at the nonpermissive temperature, the transcriptase dissociates from the template before synthesizing a complete messenger RNA molecule, and returns to an initiation site on the template to synthesize more RNA. Hence, the RNA produced would be non-functional. However, perhaps at 38°C, the transcriptase dissociates sooner or more often from the template *in vitro*. Moreover, the efficiency of re-initiation of transcription may be less *in vitro* than *in vivo*. This model could explain why the virion-associated transcriptase of a mutant like ts16B appears temperature sensitive *in vitro* but not *in vivo* but yet this temperature sensitivity of the virion-associated transcriptase of the mutant decreases when reversion to a ts+ phenotype occurs.

The analyses of the RNA made *in vivo* at the permissive and non-permissive temperatures in the presence and absence of puromycin by ts10 and ts16B, which are currently being performed, may indicate whether or not the above model is correct. On the basis of this model, one would predict that, at the non-permissive temperature, the species of messenger RNA of ts16B or ts10 would be smaller than that of HR.

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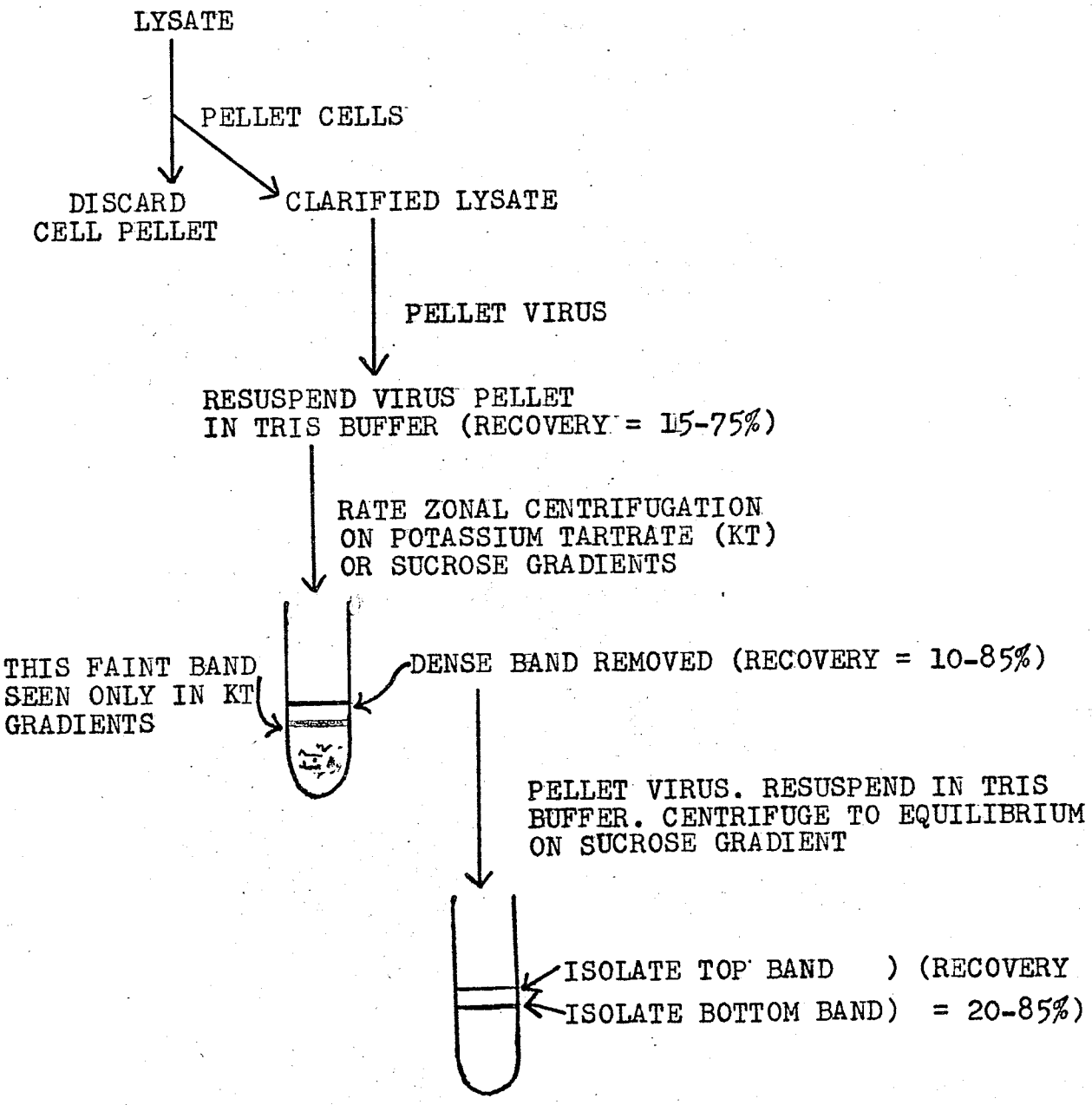
APPENDIX

A flow diagram of the basic procedure used in purifying VSV preparations is shown in Fig. 20. The percent recoveries indicated in the parentheses represent the recovery of PFU from one step to another. The details of the procedure are as follows: The virus in approximately 500-1000 ml of clarified lysate of HR or ts 16B, obtained as described in Materials and Methods, was pelleted by centrifugation in an International centrifuge for 1 hour at 16,000 rpm and then resuspended in approximately 40 ml of tris buffer. The virus was pelleted again and resuspended in 1 ml of tris buffer. The concentrated virus was then purified further by rate zonal and isopycnic centrifugation. All gradients contained β-mercaptoethanol at a concentration of 3mM. Also, all gradients were made in a two cylinder gradient maker which mixed the gradient solutions by means of a small magnetic stirring bar. Isopycnic centrifugation followed the rate zonal centrifugation since the isopycnic centrifugation also concentrated the virus to the extent necessary for the polymerase test.

Several conditions were tested for the rate zonal centrifugation but, except where indicated, the centrifugations were done in an SW27 rotor with 35 ml gradients.

One condition that was used was a 9 to 33% (solute weight% of solution weight) linear sucrose gradient. Centrifugation was done at 23,000 rpm for sixty min. After centrifugation, only one band was consistently seen in the gradient. This band appeared to contain most of the virus since, when a gradient

Fig. 13 Outline of procedures used for purification of VSV preparations. Details are given in text. Recoveries observed from step to step are given in parentheses.


LYSATE
PELLET CELLS
DISCARD CELL PELLET
CLARIFIED LYSATE
PELLET VIRUS
RESUSPEND VIRUS PELLET IN TRIS BUFFER (RECOVERY = 15-75%)
RATE ZONAL CENTRIFUGATION ON POTASSIUM TARTRATE (KT) OR SUCROSE GRADIENTS
THIS FAINT BAND SEEN ONLY IN KT GRADIENTS
DENSE BAND REMOVED (RECOVERY = 10-85%)
PELLET VIRUS. RESUSPEND IN TRIS BUFFER. CENTRIFUGE TO EQUILIBRIUM ON SUCROSE GRADIENT
ISOLATE TOP BAND) (RECOVERY
ISOLATE BOTTOM BAND) = 20-85%)

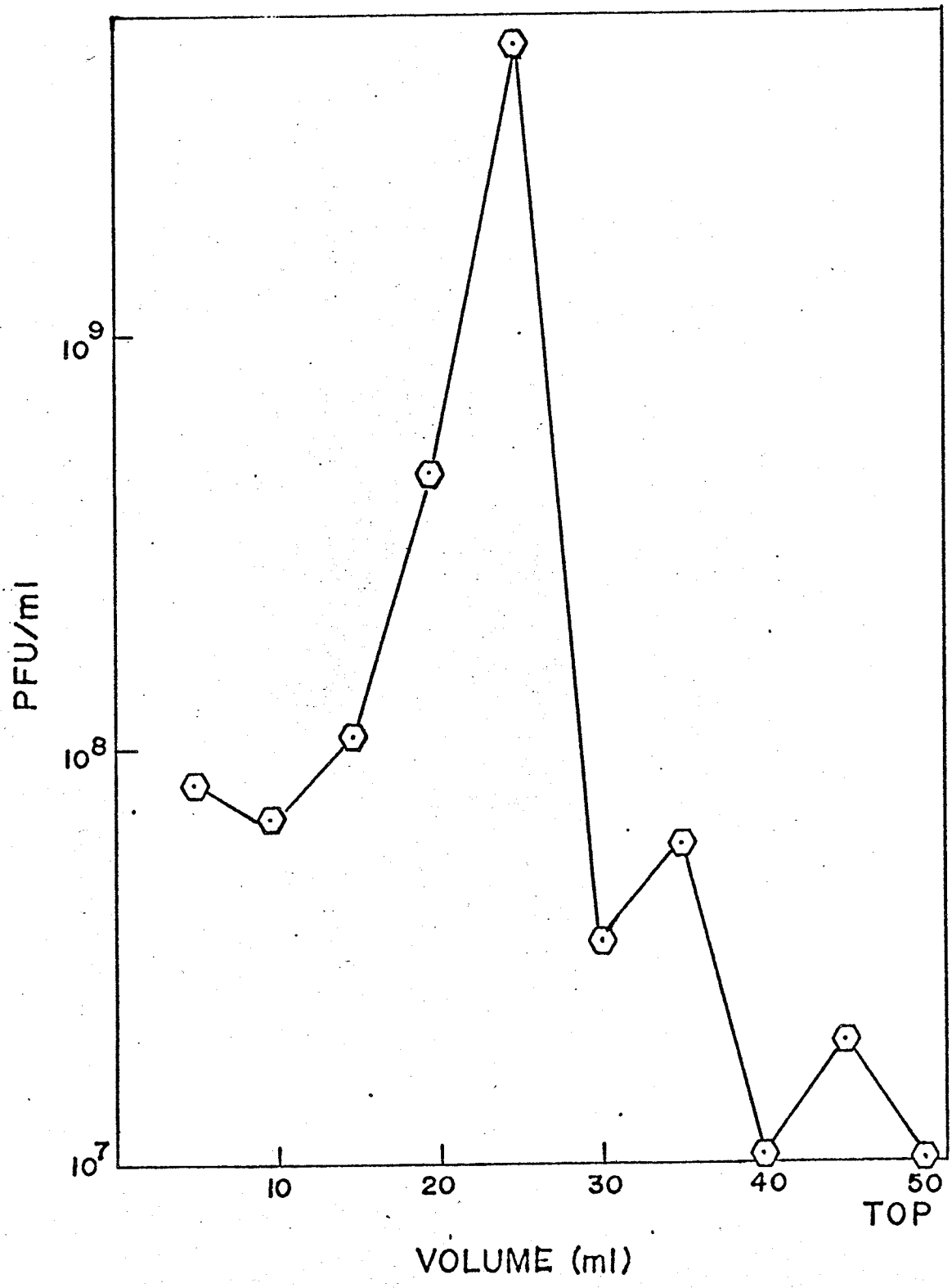
which was run under essentially the same conditions (An SW25.3 rotor was used instead of an SW27 rotor) was fractionated, the region which contained the visible band also contained no less than 70% of the PFU. An example of a gradient profile obtained under these conditions is shown in Fig. 21.

If the virus was centrifuged for 60 min at 23,000 rpm in a gradient of 9-29% (solute weight % of solution weight) potassium tartrate (KT), two bands appeared consistently. The uppermost band was more dense and contained at least 80% of the total PFU seen in the two bands. Only the dense band was further purified.

Floating below the virus bands in either the KT or sucrose gradients were clumps of material, presumably cellular fragments, since they were not infectious. Furthermore, these fragments were of varying size because they did not form discrete bands.

Except in the cases where a gradient fractionation was performed, the virus bands were isolated by using the P→L method (in piercing needle, out drain tube) of a Beckmann fraction recovery system. The band was contained in 3-5 ml. The isolated virus band was then diluted approximately ten fold in tris buffer and the virus was pelleted in the International centrifuge for 1 hour at 16,000 rpm (In one case, the virus was collected by polyethylene glycol (PEG) precipitation by mixing into the diluted virus suspension, 5 gm of PEG and 1 gm of NaCl and allowing the mixture to stand for 1 hour at 4°C prior to pelleting). This procedure, however, seemed to make no apparent

Fig. 14 Distribution of infectivity (PFU/ ml) after rate zonal centrifugation. Centrifugation was done with a 50 ml gradient of 9-33% (solute weight % of solution weight) sucrose in an SW 25.3 rotor for 60 min at 23,000 rpm. After centrifugation, the tube was punctured at the bottom and 5 ml fractions were collected and assayed at 30°C for PFU.

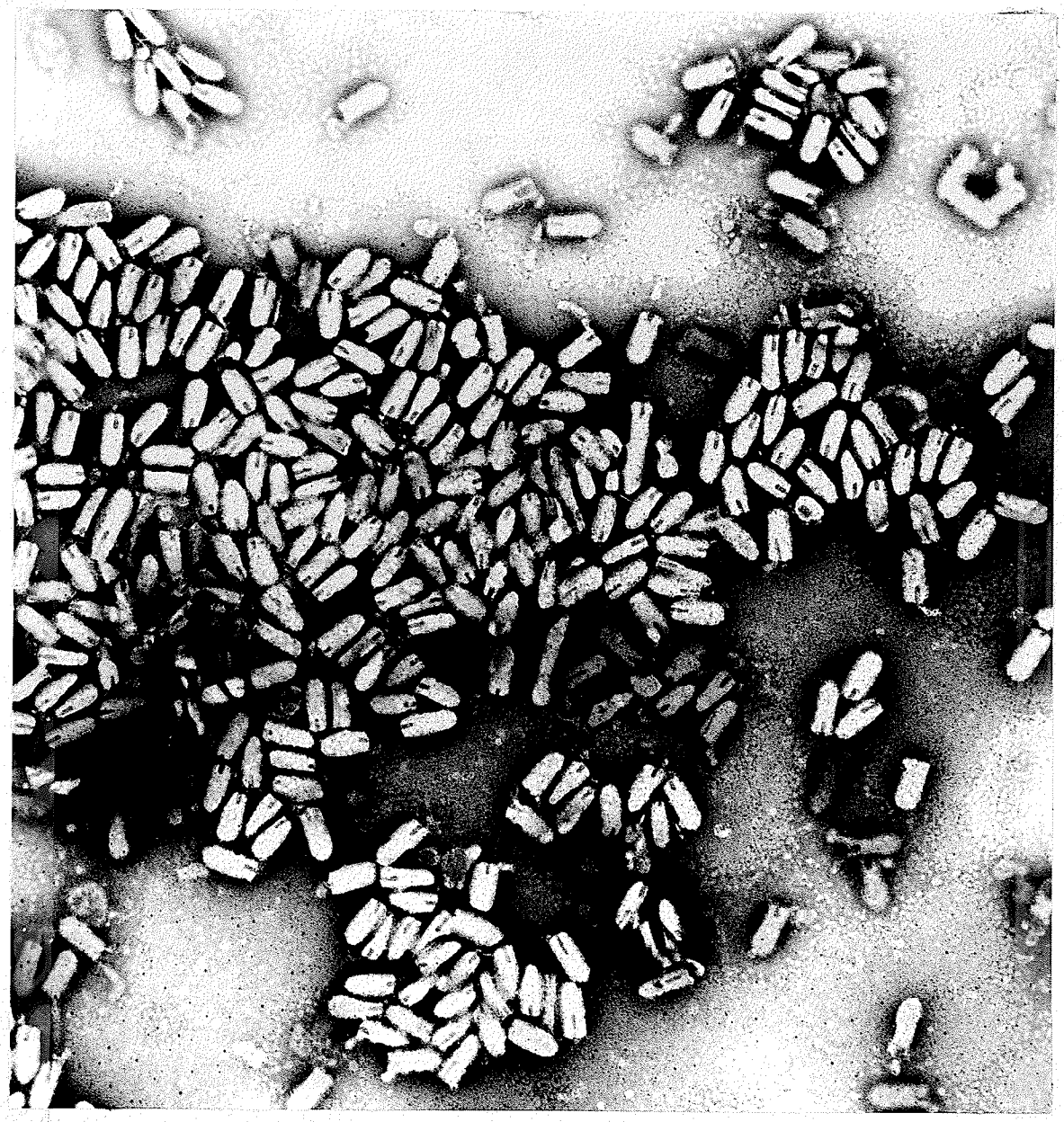

PFU/ml
10^9
10^8
10^7
10
20
30
40
50
TOP
VOLUME (ml)

difference in the recovery. The pellet was resuspended in 0.5 ml of tris buffer and the resuspended virus was layered on a 5 ml linear sucrose gradient (16.5-62.5% (solute weight % of solution weight)) and centrifuged to equilibrium for twenty hours at 50,000 rpm in an SW65 rotor. CsCl was not chosen for a gradient material for isopycnic centrifugation because, on the one attempt of using CsCl, the total recovery of the infectious input was only 0.01%. Recoveries of infectious virus using KT or sucrose were similar to eachother. However, if KT gradients were used, it was obligatory to dialyze out the gradient material since the concentration of KT in the virus band would probably inhibit the VSV associated polymerase, while polymerase activity can be demonstrated in VSV virions isolated immediately from a sucrose gradient (Baltimore et al (1970)). Hence, sucrose offered the most flexible system: If the virus bands observed after centrifugation appeared relatively faint, only a small amount of virus was probably present. When this occurred, the virus bands were isolated and immediately tested for polymerase activity without prior dialysis. In such a case, dialysis is undesirable, since the resulting dilution of the virus would probably be sufficient to dilute the polymerase activity of the preparation to an undetectable level. However if all of the virus was not going to be used immediately, and there appeared to be a relatively large amount of virus, dialysis was performed to reduce possible

disruption of the virus by sucrose (McCombs et al (1966). Dialysis was done overnight against 1 litre of 0.1M tris-HCl, pH 7.9, which contained 3mM of β-mercaptoethanol. In the absence of β-mercaptoethanol, a loss of up to 99.9% of the PFU occured, while recovery was virtually 100% in the presence of β-mercaptoethanol.

Whether the isopycnic centrifugation followed rate zonal centrifugation in KT gradients or in sucrose gradients, two bands appeared after centrifugation. Both bands contained PFU and polymerase activity (see Results, Table VII). If any of the material from the bands remained after the assays, the material was examined on the electron microscope. An electron micrograph of the bottom band, shown in Fig. 15, appears to be a relatively homogeneous preparation of B particles. The nature of the top band is unknown. Two possible explanations for the appearance of two bands are that the top band consists of T particles contaminated with B particles (since PFU were present) or that the top band consists of B particles altered by the effect of the gradient material (McCombs et al (1966). If the top band did consist of T particles, it must mean that there was an inadequate separation of T and B particles in the rate zonal centrifugation step. Rate zonal centrifugation with a less dense sucrose gradient (9-29%) was investigated in attempt to improve such a separation. The virus suspension was centrifuged through the gradient for 75 min at 20,000 rpm in the SW27 rotor (These conditions are essentially those of McSharry & Wagner (1971)). Only a single visible band was

Fig. 15 Electron micrograph of VSV virions isolated from the bottom band of a sucrose isopycnic gradient. The virions were negatively stained with 1% PTA, pH 7.2, and the magnification is 48,300 X.



present and the cellular debris, instead of being suspended below the virus band, had been pelleted to the bottom of the tube. When the virus was isolated from this band and centrifuged to equilibrium in a sucrose gradient as described previously, two bands which had PFU and polymerase activity were present again.

An investigation of the nature of the difference of the virus in the two bands was not continued. The appearance of two bands was probably not significant to our studies because this phenomenon occurred for both HR and ts16B preparations and the temperature dependence of the polymerase of the virus from the top band was essentially the same as the bottom band.

However, it appeared important to determine if the additional purification procedures removed cellular material from the pelleted virus preparations that were usually used in the polymerase test.

McSharry & Wagner (1971) have shown that the degree of purity of VSV preparations can be measured either by an increase in specific infectivity (PFU/μg protein) or by the disappearance of ^{14}C labelled cellular proteins. The latter method was chosen since it was found that the former required relatively large volumes of lysate to obtain a sufficient amount of viral protein necessary to measure the specific infectivity easily. Morever, the results of the former method were obscured by any loss of infectivity of the virions during purification. This loss has been reported

to be high (at least 90%) in all the investigations in which the recovery of input infectivity has been indicated (Huang et al, McSharry & Wagner, 1971; Bishop, 1971).

Although McSharry & Wagner (1971) used the radioactively labelled debris from cells disrupted by sonication, it might be possible that this debris is not representative of the kind of cellular contaminants that are present in the lysate of a VSV infection. To circumvent this possibility, a method was used which was based on the observation of Kang & Prevec (1969) that progeny VSV from cells which had been prelabelled with ^{14}C amino acids had no detectable amount of label incorporated into them. Hence, the lysate of prelabelled cells should offer a source of labelled contaminants with no appreciable amount of label in the virus. The experiment was carried out as follows:

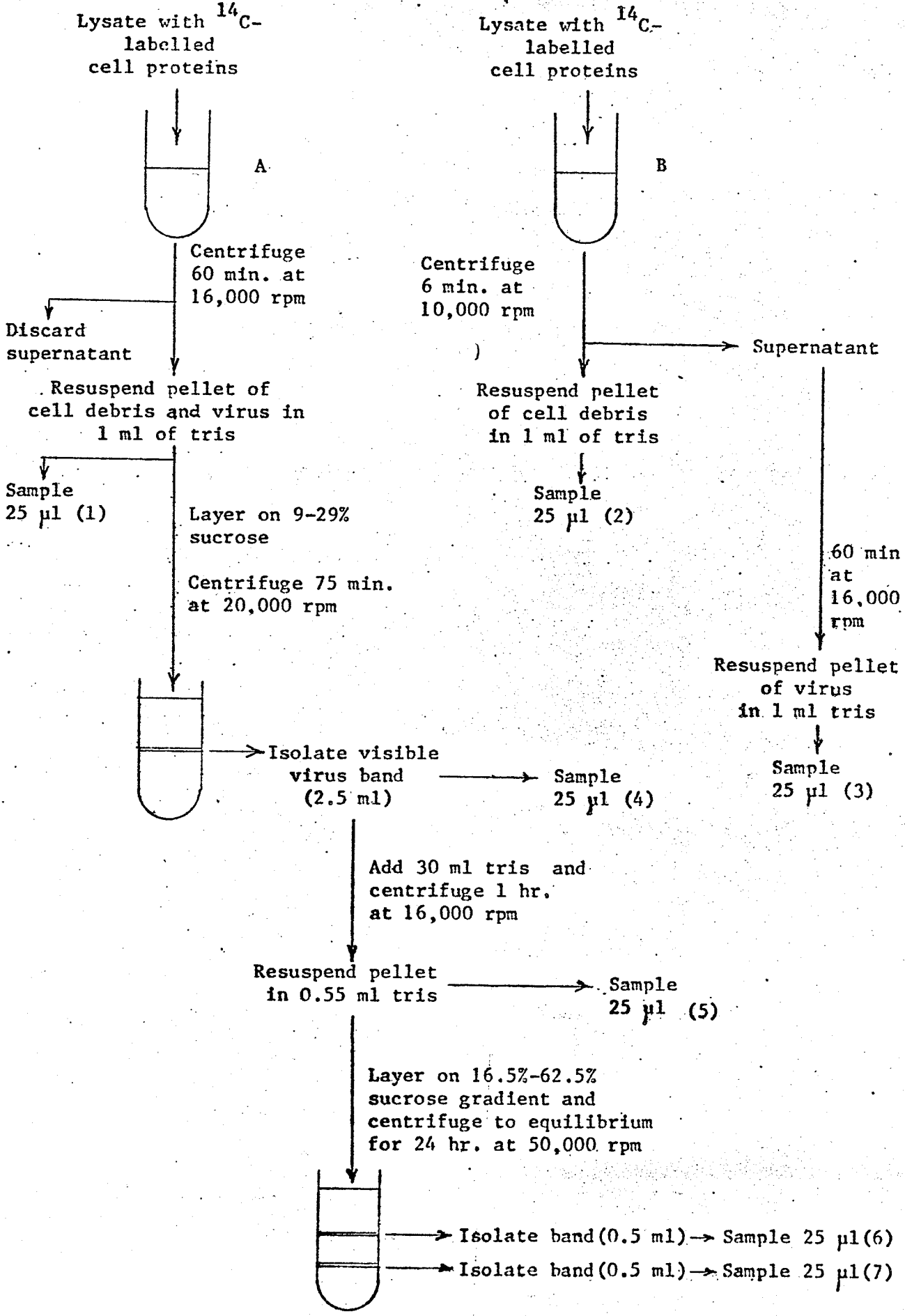
Two 16 oz. Brockway bottles were each seeded with 12.5 ml of an L cell spinner culture containing 3.8×10^5 cells/ml. After the cells had attached to the glass of the bottles, 0.5 ml of ^{14}C-labelled amino acids (approximately 3μCi) were added to each bottle. The cells were incubated for 24 hours at 37°C in a CO_2 incubator. Since 24 hours is longer than the doubling time for L cells in log phase (18 hours), the monolayer had now become thicker and the proteins of the newly formed cells ought to be radioactively labelled. The medium was then removed and the monolayer was washed three times with 25ml of PBS,pH7.0. After the wash, 25 ml

of non-radioactive medium were added to each bottle and the bottles were incubated for an additional 4 hours at 37°C. After this incubation, each monolayer was infected with $7x10^9$ PFU of HR as described in Materials and Methods. After this incubation, each monolayer was infected with $7x10^9$ PFU of HR as described in Materials and Methods. After 24 hours at 30°C in a CO_2 incubator, the monolayers had been completely lysed and the bottles were vigorously shaken to suspend the cellular debris. The lysate of the two bottles was pooled, divided into two halves designated A and B and run through the purification procedure as shown in Fig. 23. Before centrifugation approximately $5x10^9$ PFU were added to part A in order to serve as a marker in case the amount of original virus present was insufficient to make the virus band visible. As indicated in Fig. 23, 25 μl samples were taken with a capillary pipette at various stages in order to monitor the disappearance of labelled cellular protein.

Table XIV demonstrates that after pelleting the virus, approximately 10% of the label is still present, while after the rate zonal and isopycnic centrifugation, there was no detectable radioactivity in the samples. Hence, this procedure appears to remove cellular proteins from the viral preparations.

Fig. 16 Flow diagram of the experiment used to determine if cellular protein was being removed from the virus preparations during the purification procedure. Samples were removed at various stages (#1-7). The cpm measured in these samples are shown in Table XIV.

Fig. 16


Lysate with ^{14}C-labelled cell proteins
A
Centrifuge 60 min. at 16,000 rpm
Discard supernatant
Resuspend pellet of cell debris and virus in 1 ml of tris
Sample 25 µl (1)
Layer on 9-29% sucrose
Centrifuge 75 min. at 20,000 rpm
Isolate visible virus band (2.5 ml)
Sample 25 µl (4)
Add 30 ml tris and centrifuge 1 hr. at 16,000 rpm
Resuspend pellet in 0.55 ml tris
Sample 25 µl (5)
Layer on 16.5%-62.5% sucrose gradient and centrifuge to equilibrium for 24 hr. at 50,000 rpm
Isolate band (0.5 ml) → Sample 25 µl (6)
Isolate band (0.5 ml) → Sample 25 µl (7)
Lysate with ^{14}C-labelled cell proteins
B
Centrifuge 6 min. at 10,000 rpm
Supernatant
Resuspend pellet of cell debris in 1 ml of tris
Sample 25 µl (2)
60 min at 16,000 rpm
Resuspend pellet of virus in 1 ml tris
Sample 25 µl (3)