ISOLATION AND PRELIMINARY CHARACTERIZATION OF TEMPERATURE-SENSITIVE MUTANTS OF VESICULAR STOMATITIS VIRUS

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ABSTRACT

Ka-Yiu Wong

Isolation and Preliminary Characterization of
Temperature-Sensitive Mutants of Vesicular Stomatitis Virus

Eight temperature-sensitive (ts) mutants of vesicular stomatitis virus (VSV) (Indiana serotype) were isolated using ethyl methane sulfonate (EMS) and nitrous acid as mutagens. The ratio of the number of plaques formed at 38°C and 30°C of these ts mutants was not greater than 1×10^{-4}. At 30°C all ts mutants studied grew almost as well as the heat resistant (HR) parent at 30°C, but somewhat more slowly than did the HR at 38°C. The final titres of the ts mutants at 30°C were generally slightly lower than that of the HR at either temperature. At 38°C, ts1, 2, 4, and 5 showed little or no growth. Some growth was observed with ts3 and 12 at 38°C. The viral growth of ts3 and 12 obtained at 38°C was found to be mostly because of "leak" rather than reversion to HR. Ts1, 2, 3, 5, 8 and 12 were only slightly less heat-stable than the HR but more heat-stable than the wild-type which shows that the inability of the mutants to grow at 38°C is not due to an increase in heat lability of the virion, wheras ts4 was more heat labile than the wild type. The critical temperature-sensitive period of ts4 occurred very early in the growth cycle. An explanation of these facts may be that ts4 contains a heat-labile structural protein which is in some way involved in the uncoating of the virus. Ts1, 3 and 5 all seem to be defective in a function necessary for the synthesis of RNA although the temperature-sensitive events of ts1 and 5 are expressed earlier in the

growth cycle than that of ts3. Ts2 and 12 on the other hand did not show a sharp rise in viral yield no matter when the temperature shift-up took place. It seems that the temperature-sensitive products of ts2 and 12 may be required continuously by the two mutants and these are not produced in a step-wise fashion as in Sindbis virus (Pfefferkorn & Burge, 1967).

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INTRODUCTION

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Viruses are very useful tools for the study of genetics because they are the simplest materials of natural origin thus far available for investigation of genetic phenomena. When a virus infects an animal cell, the virus nucleic acid enters the host cell and dictates the processes by which new virus particles are produced. The viral nucleic acid stops some of the synthetic activities of the host cell, provides a code for the formation of new enzymes, determines the specificity of new virus structural proteins and nucleic acids, and regulates the timing of enzyme synthesis in respect of its onset and duration. If we know the number, constitution and sequence of all the genes of the viral genome, the nature and sequence of their function and the precise composition, function and inter-relationship of all their gene products, we would have a much better understanding of the general question of the genetic control of growth and development. It has also been suggested that the comparison of the types of gene function for each group of viruses when genetic maps become available may provide a sounder basis for viral classification (Cooper, 1967).

Mutant strains are especially useful in the study of gene function because a mutation is generally recognised by a change in a particular character of the virus. By comparing a given feature of the mutant with that of the wild-type under similar conditions, it is possible to obtain information relating to the function of the affected gene. Suitable strains require the following two conditions. First, to avoid ambiguity

in genetic analysis it is essential that comparisons be made between the pure clones of progeny virus which has mutated in a single gene and the unaltered parent (wild-type). Second, to be suitable for experimental investigation, mutants must be easily scored and stable at growth and storage temperatures.

Such mutants are however, not easy to obtain. For if the mutation results in a change of a gene product which is lethal to the virus, the mutant fails to survive and the mutation would not be detected. On the other hand, if the gene product is changed, but remains functional, progeny virus would be produced and the mutation might escape detection. Owing to the lack of suitable mutants, the ability to correlate genetic structure with function was hampered. However, this difficulty was resolved by the discovery of conditional-lethal mutants in the bacteriophage T4 and its successful use in genetic mapping of the phage by Epstein and co-workers (1963).

A conditional-lethal mutant is a mutant which can grow in some conditions and not in others. The better known example is that of the temperature-sensitive mutants (ts); in which a gene product is altered so that it is formed and stable at a lower (permissive) temperature, e.g. $30^{o}C$ in the case of vesicular stomatitis virus (VSV), but it is either not made or is unstable at a higher (non-permissive) temperature, e.g. $38^{o}C$ in the case of VSV. Therefore such mutants will multiply at $30^{o}C$ but not at $38^{o}C$.

Another known example of a conditional-lethal mutant is the

"suppressor-sensitive" mutants, e.g. the amber mutants. The suppressor-sensitive mutants are host dependent mutants recognised so far only in bacteriophages. They can grow in permissive cells, which contain a suppressor gene which compensates for the effect of the phage mutation (Garen, 1968) but they fail to make complete polypeptide in the restrictive host.

It is comparatively easy to select for conditional mutants because one does not have to know which function is altered; any one of the gene functions may be involved, i.e. mutation can occur in most or all genes (Epstein et al., 1963; Edgar & Lielausis, 1964). Hence they are called "universal" markers. A set of ts mutants might therefore include some mutants with altered viral structural protein, others which induce altered enzymes, yet others with changed regulatory functions. But they can all be scored by one type of test, i.e. growth at permissive condition and no growth at non-permissive condition.

The isolation of ts mutants is undertaken as an initial step in the detailed analysis of events during the life cycle, events which may then presumably be explored by biochemical means.

The present study is only concerned with the preparatory step of isolating ts mutants of VSV and preliminary characterization of the mutants. Detailed characterization of the mutants such as studies on the RNA synthesis of the ts mutants (i.e. their ability to induce the synthesis of RNA at the non-permissive temperature in the presence of actinomycin D), prevention of cellular RNA synthesis, synthesis of viral

antigens by ts mutants at the non-permissive temperature, comparison of the structural proteins of ts mutants with HR (heat resistant parent strain) is now in progress.

LITERATURE REVIEW

LITERATURE REVIEW

I. ISOLATION OF ts MUTANTS OF ANIMAL VIRUSES

The ts marker is the only universal one so far obtained with animal viruses. Ts mutants of the following animal viruses have been isolated: poliovirus (Cooper, 1964; Cooper et al., 1966), foot and mouth disease virus (Pringle, 1965; 1968), reovirus 3 (Ikegami & Gomatos, 1968; Fields & Joklik, 1969), Sindbis virus (Burge & Pfefferkorn, 1964; 1965; 1966a), Semliki forest virus (Sambrook, 1965; Tan et al., 1969), vesicular stomatitis virus (Pittman, 1965), influenza virus (MacKenzie cited by Cooper, 1967; Simpson and Hirst, 1968), Newcastle disease virus (Kirvaitis & Simon, 1965), respiratory syncytial virus (Gharpure et al., 1969), rabbit pox virus (Sambrook et al., 1966; Padgett & Tomkins, 1968), vaccinia virus (Basilico & Joklik, 1968), pseudorabies virus (Pfefferkorn & Rutskin cited by Burge & Pfefferkorn, 1966a), and polyoma virus (Fried, 1965; Gershon & Sachs, 1965; Eckhart, 1969; Dimayorca et al., 1969).

II. STEPS TOWARD GENETIC MAPS OF ANIMAL VIRUSES USING ts MUTANTS

Complete genetic mapping of animal viruses using ts mutants has not yet been realised. The following is a brief review of studies on ts mutants of some animal viruses thus far isolated.

PICORNAVIRUSES

Poliovirus Type 1

Genetic mapping has proceded furthest with poliovirus Type 1 whose ts mutants were first isolated in 1964 by Cooper. Cooper and co-workers (1966) separated the ts mutants of poliovirus into four groups (A-D) using three types of physiological defects at the non-permissive temperature, namely defects in prevention of thymidine incorporation into cellular DNA (pti^+ character), prevention of phosphorus incorporation into fraction three of cellular DNA (ppi^+ character) and production of antigen that could bind fluorescent antibody (a^+ character). They found that group A mutants possessed all three types of defects, group B mutants were only defective in ppi^+ and a^+ characters, group C mutants were only defective in the a^+ character, and group D mutants possessed none of the defects.

Wentworth et al. (1968) further showed that the production of infectious RNA and serum-blocking antigen corresponded closely with the above classification of the mutants. No mutant produced fully wild-type yields of either RNA or antigen. In general group A mutants produced the least RNA and antigen, group D mutants produced most and group B and C produced intermediate yields at non-permissive temperature.

Cooper (1967) reported that preliminary recombination tests between some of the mutants showed that the sequence in the genetic map is

$A\text{-}B_1\text{-}B_2\text{-}C,D$, but whether or not this correlation with the physiological map is only coincidental has not been verified. Cooper suspects that since about ten non-structural and four coat proteins are made by the virus (Summers et al., 1965), A-D may represent less than half the genome.

The poliovirus genome was partially mapped (Cooper, 1968) as a result of recombination experiments using mixed infection of wild-type and ts mutants. By using three-factor crosses involving a mutant adapted to guanidine resistance, he showed that recombination frequencies were additive and obtained an additive linear genetic map comprising one linkage group. He further showed that recombination was non-random with time and with most of the mating events occurring early in the growth cycle.

Foot and Mouth Disease Virus (FMDV)

Pringle (1968) isolated 30 ts mutants of foot and mouth disease virus. He showed that certain pairs of these ts mutants complemented one another at the non-permissive temperature and the yields obtained varied markedly and were not related to multiplicity. He also reported that recombination occurred at the permissive temperature between two ts mutants, ts14 (defective in an early function) and ts15 (defective in a late function). Recombination of these two mutants could also be detected when the input of the mutants was not equivalent, provided that one of the mutants was in sufficient excess to infect the majority of the cells. Frequency of non-mutant recombinants was found to be 0.3%.

REOVIRUS

Reovirus 3

All six ts mutants of reovirus 3 isolated by Ikegami & Gomatos (1968) were not virulent for newborn hamsters in marked contrast to the wild-type virus. They possess varying degrees of haemaglutinating activity for bovine erythrocytes. Two mutants, ts-26a and ts-44b, were more sensitive to heating at 52°C then the wild-type virus, while the rest of the mutants were intermediate between that of the wild-type virus and ts-26a and ts-44b.

The growth curves and effect of temperature shift-up of ts-53a and ts-44b were studied. The latent period of both mutants at 30°C was longer than that of the wild-type grown at 37°C but almost similar to that of the wild-type grown at 30°C. In general, the wild-type virus grown at both temperatures reached maximum titre earlier than the two ts mutants.

Both ts-44b and ts-53a were defective in a late function. Shift-up experiments indicated that for ts-53a, temperature sensitive products are needed continuously because viral growth ceased on transfer of infected cells from 30° to 37°C. By contrast, viral growth of ts-44b continued for three more hours at the same rate as when the cultures were maintained at 30°C and then ceased, which suggests that viral components are available which are stable for a short while at the non-permissive temperature and that virus continued to be produced until this pool is depleted.

Recombination studies on the ts mutants of reovirus 3 was made by

Fields and Joklik (1969) who sepsrated the mutants into five recombination groups. Of 35 ts mutants studied, 28 were in group A, four in B and one each in C, D and E. They also found that all group A mutants were able to induce the formation of viral RNA at the non-permissive temperature as efficiently as the wild-type virus. Mutants of group B were less efficient and exhibited a pronounced lag period before inception of viral RNA synthesis, while mutants of group C and D induced only very little viral RNA synthesis. Group E mutants were probably completely inactive. The biochemical characterization using this criterion agrees with the grouping suggested by the recombination test.

ARBOVIRUSES

Sindbis virus

Burge and Pfefferkorn (1965, 1966a) found that the ts mutants of Sindbis virus could be separated into two categories: those that were able to synthesize RNA but not infectious virus at the non-permissive temperature (RNA^+), and those that produce neither infectious RNA nor infectious virus (RNA^-). The RNA^+ mutants were also found by temperature shift-up experiments to be defective in some function expressed "late" in the course of infection, and RNA^- mutants were defective in an "early" function (Burge & Pfefferkorn, 1966a). Pfefferkorn & Burge (1967) found no genetic recombination between the mutants. Furthermore, in the complementation tests, Burge & Pfefferkorn (1966b) showed that the heat-labile RNA^+ mutants fell into two complementation groups C and D and the only heat stable RNA^+

mutant fell into another group (E), while the RNA^- mutants separated into two complementation groups (A and B).

Burge & Pfefferkorn (1967) found that most RNA^- mutants produced the same percentage of RNase-resistant RNA as the HR ancestor during incubation at 31°, 35° and under shift-up conditions at 39°C. One mutant, ts24, produced a higher percentage of RNase-resistant RNA than the HR virus at all incubation temperatures, particularly under shift-up conditions at 39°C. They proposed that viral RNA replication requires two enzymes: Enzyme I which converts input RNA to double stranded RNase-resistant RNA, and Enzyme II which synthesizes plus strands from the double stranded template.

The RNA^+ mutants were further studied by Burge & Pfefferkorn (1968) and Yin & Lockhart (1968). Burge & Pfefferkorn found that group C mutants have a temperature-sensitive defect in the nucleocapsid structural protein, group D mutants have a defect in a membrane protein, whereas group E mutants complete both steps in maturation at the non-permissive temperature, but produce no infectious virus at that temperature. This suggests that a third virus-directed protein, in addition to the two major structural proteins is required for the events of virus maturation subsequent to RNA synthesis. Similar conclusions were arrived at by Yin & Lockhart using different biochemical tests.

Semliki Forest Virus (SFV)

Temperature-sensitive mutants of Semliki Forest virus have been

studied by Sambrook (1965 cited from Cooper, 1967) and Tan et al. (1969). Sambrook showed the occurrence of complementation between various pairs of ts mutants of SFV. Recombination of the mutants was also shown to occur which in some cases gave up to 1% of the wild-type yields. The mutants were grouped into RNA^-, $RNA^\pm$ and RNA^+ according to the amount of viral RNA made at the non-permissive temperature.

Tan et al. (1969) found that the RNA^- mutants were generally as heat stable as the wild-type virus, whereas the RNA^+ mutants were much less heat stable. Temperature shift-up experiments showed that all the RNA^- mutants, except one, were blocked in an early function; and the RNA^+ mutants (and one RNA^- mutant) were blocked in a late function. The RNA^- mutants were prevented from producing viral antigen in cells infected at the non-permissive temperature and also were unable to inhibit host cell RNA synthesis. Among the RNA^+ mutants some were defective in the production of nucleocapsids only, some failed to make membrane only, and some were defective in the synthesis of both nucleocapsid and membrane. A few RNA^+ mutants made both structural proteins, but no infectious virus, and may be defective in another maturation function.

MYXOVIRUSES

Influenza A virus, strain WSN

Simpson & Hirst (1968) isolated 35 induced and six spontaneous ts mutants of influenza A virus, strain WSN and reported the occurrence of

complementation and recombination of certain of their mutants. Complementation test of ts12 and ts25 gave yields of progeny 30 times higher than that expected from the sum of the single infection controls. Some evidence for complementation has been found with other crosses and with some pairs in the absence of detectable recombination.

Results of one pair of mutants, ts12 x ts25 which consistently showed unequivocal evidence for recombination produced recombinants which made up 6% of the eight hour progeny. The same proportion of recombinants were obtained when the combined input multiplicity was reduced ten-fold. High frequency recombination of up to 2% of total virus yield occurs with certain other pairs of ts mutants.

Recombination of ts9 with WSN strain (which forms faint plaques in chick embryo fibroblast cultures at the non-permissive temperature) gave up to 3.7% recombinants which were capable of forming plaques at the non-permissive temperature. The yield obtained was more than 200 times the background of ts9. The WSN strain also recombines at high frequency with other ts mutants. Based on the recombination results the ts mutants were separated into at least five groups. Simpson & Hirst (1968) postulated that if the influenza genome consists of multiple fragments of weakly linked single-stranded RNA as suggested by Pons (1967) which readily exchange during the assembly process, then the number of groups of ts mutants will give the number of fragments of the genome, because ts mutants having defects on the same fragment would give recombination of such low frequencies as to be almost undetectable by methods now used. They further suggested that after the biochemical characters of the mutants are

established it may be possible to correlate certain functions with a genomic fragment and to identify this fragment with those seen by examination of the RNA itself.

MacKenzie (1968, cited from Fenner, 1969) also found that recombination occurred readily when pairs of influenza virus WSN strain were incubated at the permissive temperature. For crosses between many different pairs of the 16 available ts mutants, the recombination frequency of the wild-type varied between 0.4% and 6.6%. It was also found that the recombination frequency was additive. MacKenzie is of the opinion that "these results do not support the hypothesis that the viral RNA replicates in several separate fragments but rather suggest that the mutants studied are arranged in a linear fashion along a single piece of RNA. Lack of additivity between mutants at the two ends of the linear map was resolved by drawing a circular map".

MacKenzie & Dimmock (unpublished results cited from Fenner, 1969) studied several physiological characteristics of the ts mutants of influenza virus strain WSN such as the synthesis of haemaglutinin, the incorporation of a functional neuraminidase into the cell membrane, thermolability, virulence in mice and production of viral RNA polymerase. From the results of these physiological and genetic studies MacKenzie (1968) postulated the existence of 12 cistrons in influenza virus strain WSN.

Respiratory Syncytial Virus (RSV)

Four ts mutants of RSV were isolated and studied (Gharpure et al.

1969). Three of the ts mutants (ts1, 2 and 3) appeared to have "late" temperature-sensitive defects because they produce immunofluorescence and complement-fixing (CF) antigen at the non-permissive temperature. Ts2 produced as much immunofluorescence and CF antigen as the parent virus while ts1 and ts3 produced lessor amounts.

Preliminary complementation studies suggest that the four ts mutants fall into two complementation groups. One group consists of ts1, 3 and 4; the other is represented by ts2. Ts2 was of special interest since it produced atypical non-syncytial plaques at the permissive temperature.

POXVIRUSES

Rabbitpox Virus

Fenner & Sambrook (1966) studied the ability of rabbitpox virus to grow in pig-kidney cells (p^+ character) and showed that all p^- mutants map closely with one u linkage group. In order to extend the genetic map produced, Padgett & Tomkins (1968) isolated 18 ts mutants and examined their physiological properties. They found that all ts mutants synthesized DNA at the non-permissive temperature which indicated that the defect is a function which occurred after the synthesis of viral DNA.

All except three ts mutants produced the same range of soluble antigens at the permissive temperature, and all 18 ts mutants except one produced red ulcerated pocks on the chorioallantoic membrane like those of the wild-type.

In the majority of cases complementation was demonstrated. Six pairs involving seven mutants failed to complement.

Recombination occurred with all combinations of mutants tested with frequencies which varied between 0.02% and 50%.

The authors concluded that the temperature defects in most of the mutants were probably in different cistrons. Although it was not possible to arrange the mutants in order on the basis of the recombination frequencies, it was thought that it would be possible to obtain an ordered genetic map of the mutants using three-factor crosses.

Vaccinia Virus

A number of ts mutants of vaccinia virus were isolated (Basilico & Joklik, 1968). Some, more heat-labile than the wild-type virus were thought to be probably capsid protein mutants. One mutant (ts20.165) which is as heat stable as the wild-type virus was studied in some detail to find out the biosynthetic events which occur following infection with the mutant.

It was found that the rate and extent of absorption and uncoating of ts20.165 was similar to that of the wild-type. Shift-up experiments showed that no significant virus multiplication took place if the infected cells were transferred before two hours and normal yields of virus resulted if shift up took place after six hours. The mutant forms about 25% as much early mRNA as the wild-type at the permissive temperature, although late mRNA was less affected. Synthesis of DNA polymerase at the permissive

temperature was delayed one hour compared to that of the wild-type. However, this time lag disappeared as multiplicity of infection increased. DNA replication of the mutant at the non-permissive temperature was 30-50% less than the wild-type but at the permissive temperature it was only slightly less than the wild-type.

Since ts20.165 recombines efficiently with ts5.72 it indicates that it is not a double mutant. Therefore it is assumed that the decrease in amount of RNA polymerase of ts20.165, the decrease in heat stability of its DNA polymerase, and its inability to yield infectious particles at the non-permissive temperature are all the result of one defect. Two explanations were suggested: "The mutant codes for an altered DNA polymerase which operates with decreased fidelity at the non-permissive temperature. Or it may well be that the primary lesion is concerned in some way with the decreased amount of RNA polymerase associated with the mutant virus particles even though the decreased rate of transcription of early mRNA at the permissive temperature does not result in a detectable decreased virus yield".

The authors however felt that other explanations are possible and concluded that more detailed study on many more ts mutants of vaccinia virus will be necessary before the precise function affecting ts20.165 could be defined.

PAPOVAVIRUSES

Polyoma virus

Eckhart (1969) divided six ts mutants of polyoma virus into three classes on the basis of complementation and their ability to transform cells at the non-permissive temperature. Class I mutants could synthesize infectious viral DNA, but not mature virus particles. They transformed cells normally at the non-permissive temperature. Class II mutants were defective in the synthesis of infectious viral DNA and were also defective in transformation of cells. The single mutant of Class III was defective in the synthesis of infectious viral DNA, but transformed cells normally.

DiMayorca et al. (1969) divided their ts mutants of polyoma virus into four complementation groups and suggested that each complementation group was defective in a gene. It is interesting to note that their first group corresponded to Class II mutants of Eckhart (1969).

VESICULAR STOMATITIS VIRUS

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Vesicular stomatitis infection has been known to occur commonly in domestic animals and occasionally in man. The mode of infection is poorly understood. The possible mode of spread to humans either by direct contact with infected animals or by arthropods has been considered. Both types of spread probably occur, but direct contact appears to be more important (Brody et al., 1967).

CLASSIFICATION

There is indirect evidence that vesicular stomatitis virus (VSV) is a large RNA-containing virus (Chamsy & Cooper, 1963; Prevec & Whitmore, 1963). VSV is now recognised to be the prototype of a new family of viruses, the Rhabdoviruses, because of its peculiar shape and structure (Melnick & McCombs, 1966). Viruses morphologically similar to VSV are found in plants, insects and vertebrates. The following is a list of viruses belonging to this group:

Plant viruses:-

Broccoli Necrotic Yellows Virus (Hills & Campbell, 1968),

Kern Canyon Virus (Murphy & Fields, 1967),

Gomphrena Virus (Kitajima & Costa, 1966),

Lettuce Necrotic Yellow Virus (Harrison & Crowley, 1969; Wolanski et al., 1967; Wolanski & Francki, 1969),

Maize Mosaic Virus (Herold et al., 1960),

Plantain Virus (Hitchborn et al., 1966),

Potato Yellow Dwarf Virus (MacLeod et al., 1966),

Sowthistle Yellow Vein Virus (Sylvester et al., 1968),

Wheat Striate Mosaic Virus (Lee, 1968).

Insect viruses:-

Flanders Hart Park Virus (Murphy et al., 1966),

Sigma Virus (Bekaloff et al., 1965),

Cocal Virus (Ditchfield & Almeida, 1964).

Vertebrate viruses:-

Egtved virus (Zwillenberg et al., 1965),

Marburg virus (Zlotnik et al., 1968) and

Rabies virus (Pinteric & Fenje, 1966; Hummeler et al., 1967; Sokol et al., 1968).

MORPHOLOGY AND BIOCHEMICAL PROPERTIES

The morphology of VSV was first described by Chow et al. (1954). Since then the morphology of the two known serotypes designated New Jersey and Indiana has been studied by the following workers and found to be similar: Bergold & Munz (1967), Bradish & Kirkham (1966), Bradish et al. (1966), Hackett (1964), Howatson & Whitmore (1962), Huang et al. (1966), Huppert et al. (1967), Klimenko et al. (1966), McCombs et al. (1966), Nakai & Howatson (1968), Reczko (1960), Stone et al. (1961), Schulz & Liebermann (1966), Simpson & Hauser (1966) and Thormar (1967).

The virus consists of two types of particles known as B (bullet-shaped) and T (transmissible) particles (Cooper & Bellet, 1959). Fig. 1 shows the

B and T particles of the HR strain of VSV Indiana serotype. The B particle is about 160 mμ long and 60-70 mμ in diameter (Bradish & Kirkham, 1966), rounded at one end and flat at the other. The flat end appears to have a short hole extending along the axis of the particle. Some of the rods also exhibit "trailing filaments" at the flat end (Chow et al., 1954; Bradish et al., 1966; Reczko, 1960; Howatson & Whitmore, 1962). Most of the infectivity of the virus is associated with the B particle (Howatson & Whitmore, 1962; Hackett, 1964).

The T particle is a truncated form of the B particle, similar to the B particle in antigenicity, ultrastructure and width but about one-third its length (Huang et al., 1966). The molecular weight of its RNA was estimated to be 1.1×10^6 daltons (Nakai & Howatson, 1968) which is very similar to the value of 1.2×10^6 daltons obtained by Huang & Wagner (1966b). The T particles are non-infectious and are produced preferentially in cells infected at high multiplicity and undiluted passage (Cooper & Bellet, 1959; Huang & Wagner, 1966a; Huang et al., 1966). They can also interfere with the growth of infectious B particles (Hackett, 1964; Huang & Wagner, 1966a; Huang et al., 1966; Crick et al., 1966; Hackett et al., 1967; Brown et al., 1967).

Although the main external features of the virion of VSV have been established, there is still some uncertainty about the configuration of the internal nucleoprotein component which appears in electron micrographs as a series of striations transverse to the long axis of the particle (Fig. 1). Klimenko et al. (1966) suggested that the nucleoprotein strand may be in the form of a coiled coil, or arranged in four parallel helices. Bergold

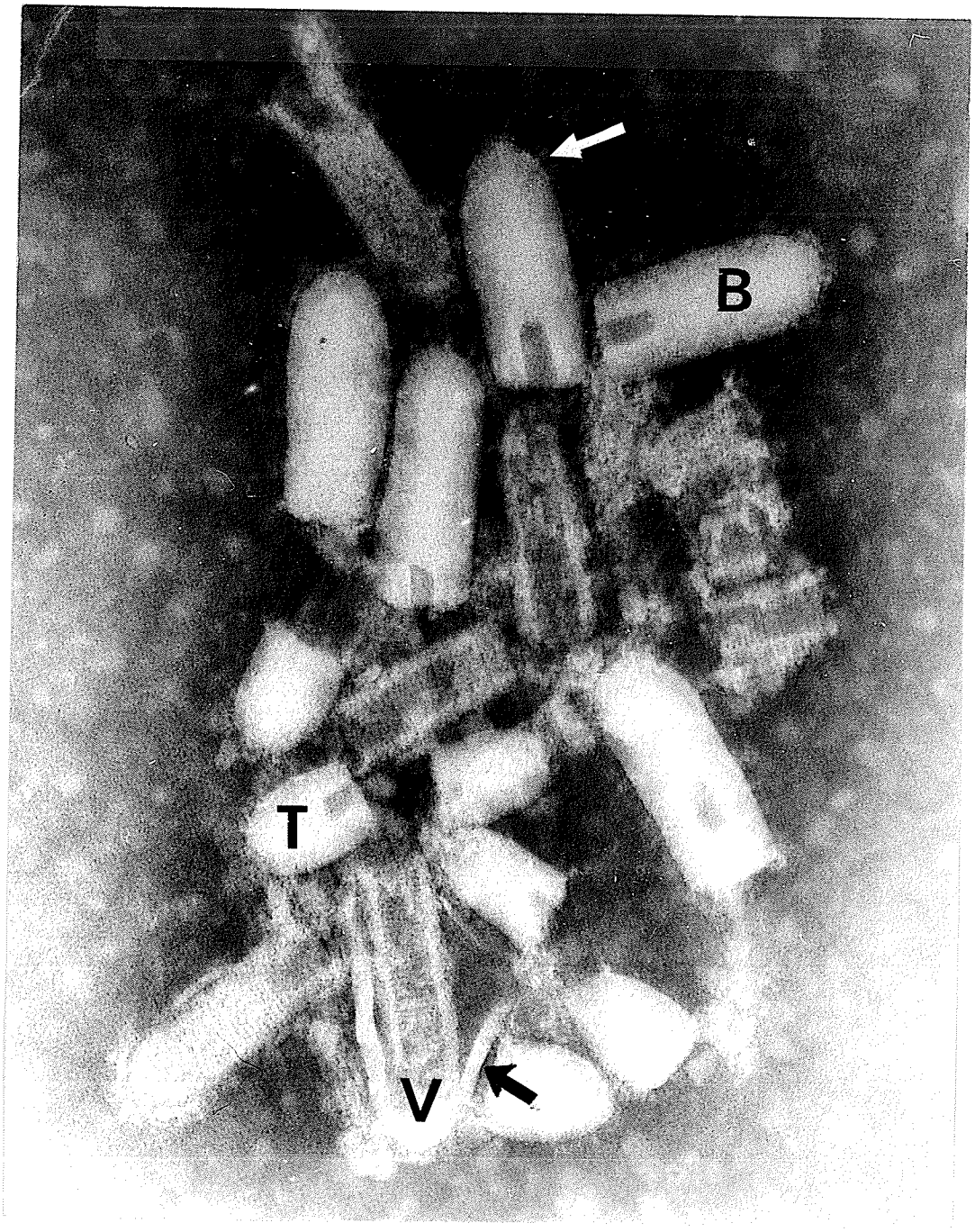

Fig. 1. Electron micrograph of VSV particles negatively stained with 1% phosphotungstic acid with Potassium hydroxide. B: B particles with spikes (white arrow) and short holes extending along their axis. T: Truncated T particles. V: Virion with disrupted coat (arrow) showing transversely striated nucleoprotein core.

& Munz (1967) described the nucleoprotein core as consisting of "an outer looped helix, an inner beaded helix....and a solid main and central core". Simpson & Hauser (1966) discussed two alternative arrangements of the nucleoprotein strands: In one the nucleoprotein strand is a helical structure, 50 mμ in diameter with a smaller helix 17-18 mμ in diameter within it; in the other, the nucleoprotein strand is arranged as a cylindrical array of about five, parallel helical strands along the long axis and immediately adjacent to the viral envelope. Their second arrangement is much like one of the two arrangements proposed by Klimenko et al. (1966). In both arrangements, Simpson & Hauser (1966) suggested that the basic structure in both helices is a single nucleoprotein strand 5 mμ in diameter with subunits spaced at 3.5 mμ intervals. Nakai & Howatson (1968) however believe that the nucleoprotein is in the form of a ribbon-like strand consisting of a series of regular rod-like subunits of dimensions approximately 9 mμ x 3 mμ x 3 mμ. The continuity of the strand is presumed to be maintained by the subunits being attached to the thread of nucleic acid. They observed that in the intact virion, the strand is in the form of a helix of about 30 coils of external diameter 49 mμ, and about four coils of diminishing diameter which form a hemispherical cap at one end. In the helix the long axis of the subunits is radially orientated. Huppert et al. (1967) proposed that the RNA of VSV is a single long molecule, primarily single-stranded, but containing double-stranded sequences at certain points. They further suggested that the junction between single and double-stranded regions constitutes the points of preferential breakage. Huppert et al. also claimed to have morphological evidence which tends to support their proposed model. It is evident from the several models proposed that more work is necessary

before the configuration of the nucleoprotein of VSV can be established.

From the measurements of lengths of isolated strands obtained from disintegrated virions, Simpson & Hauser (1966) deduced a value of 7×10^6 daltons for the molecular weight of the RNA of VSV. However, Nakai & Howatson (1968) using the same method obtained a value of $3.4\text{-}3.8 \times 10^6$ daltons, which is in good agreement with the values of $3.1\text{-}4.0 \times 10^6$ daltons obtained by Huang & Wagner (1966b), and 3.0×10^6 daltons estimated by Brown et al. (1967) by measurement of the sedimentation co-efficient in a linear sucrose gradient. The ratio of the molecular weights of the RNA from B and T particles is consistent with the ratio of particle lengths which is approximately 3 to 1.

Brown & Cartwright (1966) showed that VSV suspensions prepared from cultured baby hamster cells contain at least two complement-fixing antigens, in addition to the virus particles, which stimulated the formation of virus neutralizing antibodies in guinea pigs. Brown et al. (1966) also found that the fractionated virus particles contain three well separated peaks of complement-fixing activity. This study was repeated in 1967 with purified infective components of VSV by treatment with Tween-ether, and three fractions possessing complement-fixing activity and immunogenic activity from disrupted virions were isolated using sucrose gradient centrifugation: 1) A skeleton-like structure of about the same size as the virion but lacking the fringe structure, 2) Rosettes with a sedimentation of 16S and 3) Material of about 3 to 6S which is presumably the fringe-like structures removed from the virion by the Tween-ether treatment.

Kang & Prevec (1969) recently studied the protein constituents of

each antigen of a high-temperature resistant strain of the Indiana serotype of VSV by acrylamide gel electrophoresis. They found that the proteins of B and T particles are identical, each containing one minor and three major proteins. One of the proteins was found to originate from the nucleoprotein core, two from the coat and the origin of one remains unknown.

Wagner et al. (1969) on the other hand identified three major and three minor structural proteins from the Indiana serotype of VSV using the same method. They found that the defective T particles of the Indiana serotype contained the same six proteins in essentially the same proportions as those of the infectious B particles. Six proteins were also isolated from the New Jersey serotype, only one of which could be distinguished from any of the six proteins of the Indiana serotype on the basis of migration in SDS gels.

Besides the structural proteins, a cytoplasmic RNA polymerase has also been isolated from chick-embryo cells infected with VSV (Wilson & Bader, 1965).

REPLICATION

The life cycle of VSV is relatively short for an animal virus. Adsorption and penetration into L cells takes from 15 to 60 minutes (Simpson et al., 1969). The events surrounding the uptake and complete uncoating of VSV appear to occur rapidly because Simpson et al. observed that four-fifths of the inoculated particles present at the start of

incubation at 37°C disappear 15 minutes later. The authors also observed that the virus particles commonly attach to the cell surface at the rounded tip and most of the attached particles are found to be associated with invaginated, thickened areas of the cell membrane. Simpson and co-workers believe that these membrane sites are involved in the phagocytic uptake of the virus but they have found no evidence that there is fusion between viral and host membranes at the cell surface. The coats of the engulfed particles are thought to disintegrate releasing the nucleocapsid helices.

In L cell suspensions the latent period of VSV is about two hours at 38°C and about three hours at 30°C (Fig. 10). Events which occur during the latent period (following engulfment until release of progeny virus) are not known, but cellular changes have been observed. Membrane-bound granular masses appear in the cytoplasmic vacuoles (Reczko, 1960; Hackett et al., 1968). This phenomenon is also associated with mitochondrial changes (Schulz & Liebermann, 1966; Hackett et al., 1968). Whether these structures are sites of early synthetic activity or a cellular reaction resulting from virus multiplication has not been determined. Honeycomb-like intracytoplasmic crystalline structures have also been observed by David-West & Labzoffsky (1968a, 1968b). It is not known whether these inclusions consist of viral precursor material, or progeny virus, or whether indeed they are of viral origin since similar inclusions have been found in uninfected chick-embryo cells (Byers, 1967). David-West & Labzoffsky (1968a, 1968b) believe that replication of the virus takes place in the cytoplasm and not in the nucleus.

On maturation, virions have been observed to bud from membranes of

cytoplasmic vacuoles in infected KB cells (Mussgay & Weibel, 1963) and HeLa cells (Stone et al., 1961), or from cell membranes in the case of L cells (Howatson & Whitmore, 1962), or from both cytoplasmic vacuoles and cell membranes in Chick fibroblast cells (David-West & Labzoffsky, 1968b). Hackett et al. (1968) presented evidence that the virion is released from the cell after maturation within cytoplasmic vesicles. The presence of mature virus particles found free in the cytoplasm could not be confirmed by David-West & Labzoffsky (1968b). We have also observed budding from cytoplasmic vacuoles (Fig. 2, B) and cell membranes of L cells (Fig. 2, A). It has been suggested that the observed differences in the sites of VSV maturation and release may be attributable to the different host cells used or to differences in method and time of observation (Hackett et al., 1968).

Infection with VSV usually results in cytopathic effects. We have observed chromosomal changes in L cell nuclei after infection with VSV (Fig. 3) and the formation of giant cells (Fig. 4). Giant cell formation in an agar overlay monolayer culture was observed to progress in an ever widening circle followed by lysis of cells and ultimately resulted in a plaque.

VSV AS A GENETIC TOOL

VSV possesses certain characteristics which make it a useful agent for genetic studies. It multiplies readily and when the virus is plated on cell monolayer under agar it forms circular plaques rapidly (24 hours at $37^{o}C$, 36 hours at $30^{o}C$). Galasso (1968) demonstrated that it has a

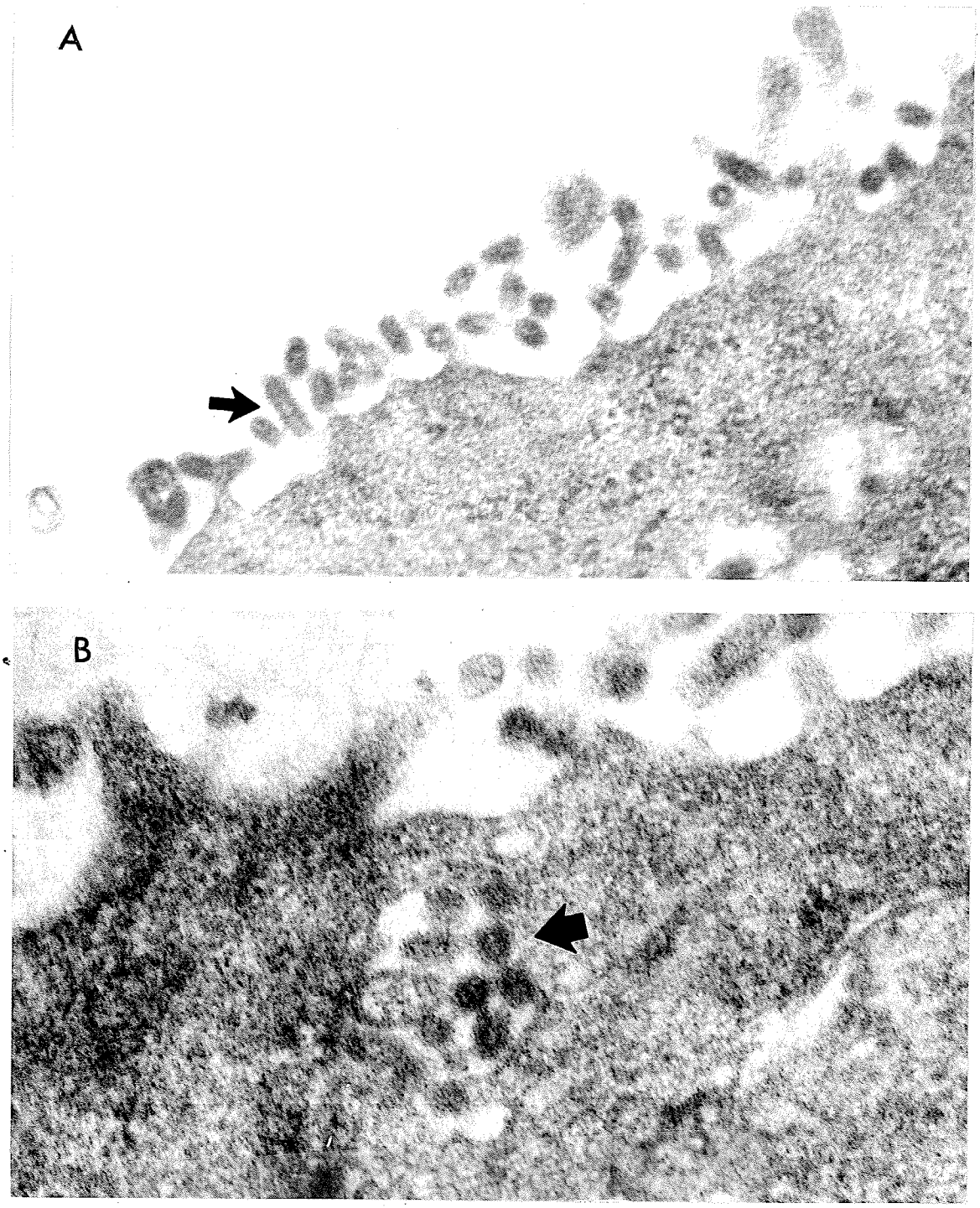

Fig. 2. Electron micrographs of sections of a six-hour VSV infected L cells stained with 1% phosphotungstic acid with potassium hydroxide.

A. Free and budding virus particles at L cell membrane (arrow). Mag. 88,000 x.

B. Mature virus particles in cytoplasmic vacuole of L cell (arrow). Mag. 100,000 x.

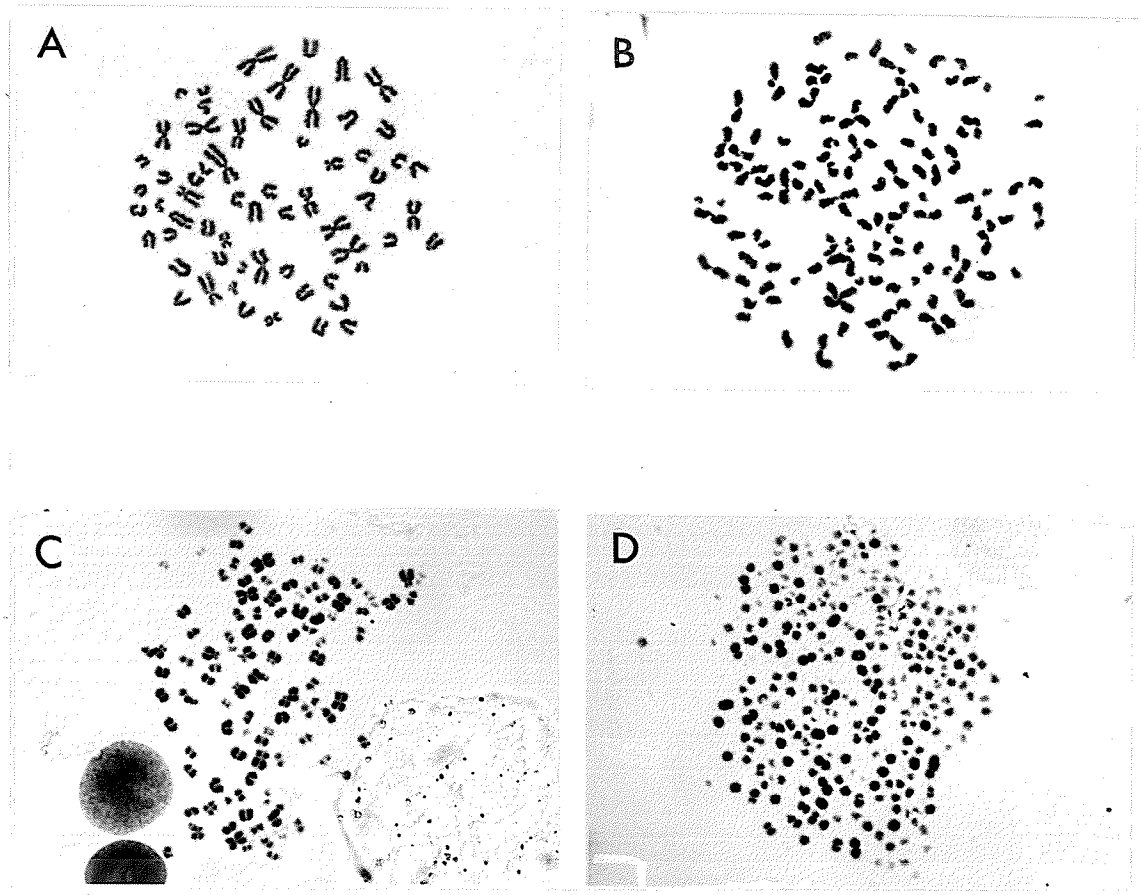

Fig. 3. Chromosomes of normal and VSV infected L 60 cells.

A. Normal morphology of metaphase chromosomes from an uninfected culture of L cells. L cells were treated with 0.5 ug/ml colchicine six hours before harvest. Harvested cells were prepared by the hypotonic pretreatment and aceto-orcein squash technique.

B-D. VSV induced chromosome aberrations in L cells four hours after infection at i.m. of 10. L cells were pretreated with 0.5 ug/ml colchicine two hours before infection. Harvested cells were prepared as in uninfected control.

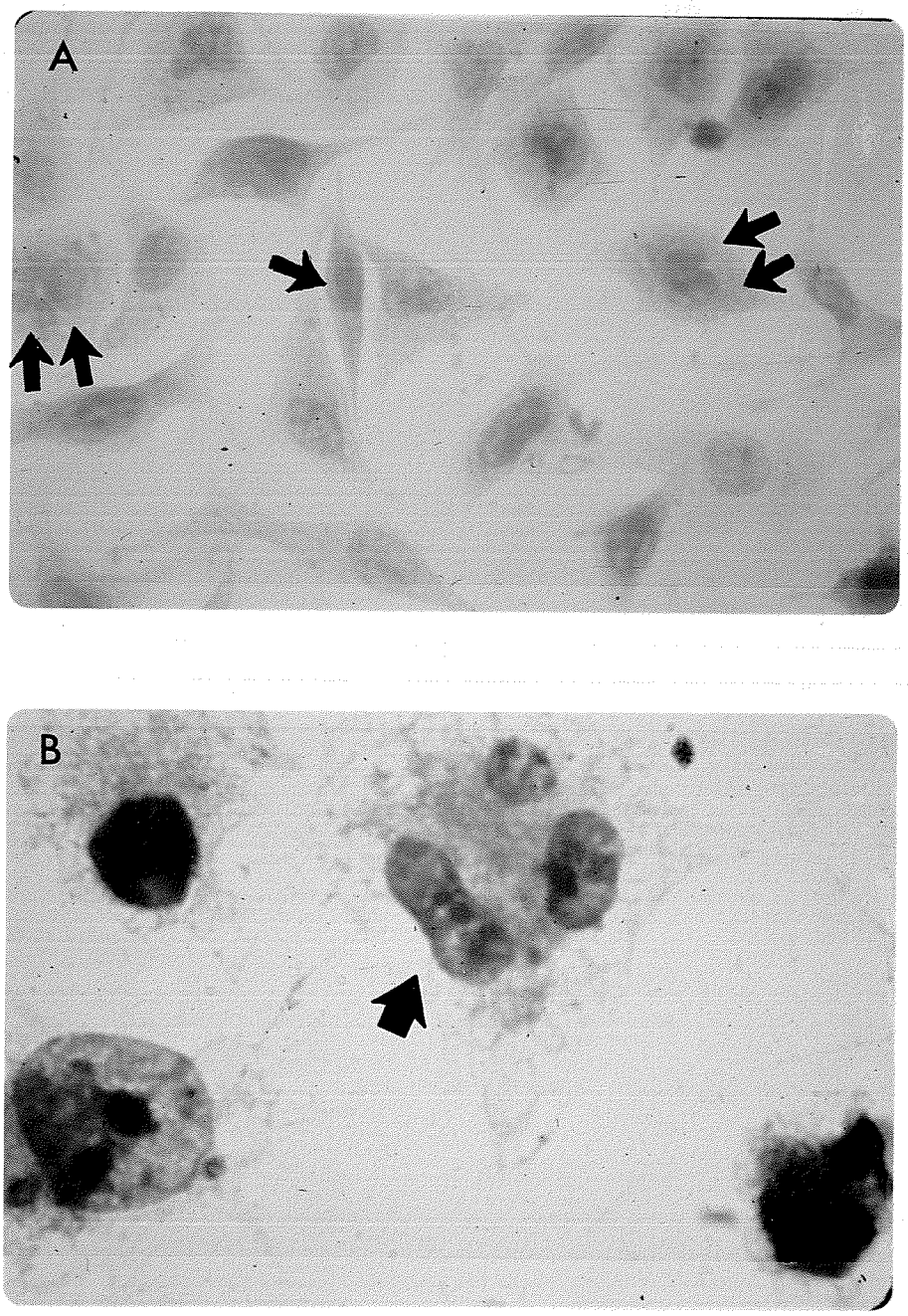

Fig. 4. Giant cells induced by VSV. Monolayer of L cells grown in petri dish were infected with VSV and incubated under nutrient agar at 38°C. 12 hours after infection, cells were fixed with 10% formaldehyde in PBS and then stained with neutral red (1:4500 PBS) and with Giemsa (1:15 double distilled water).

A. Low magnification micrograph of normal (arrow) and giant cells (double arrow).

B. High magnification of a giant cell with three nuclei (arrow).

high degree of plating efficiency and the ratio of virions to "biological unit" is 1:1. Reproducible one-step growth curves can be obtained with relative ease. Recent studies have shown that mutant strains of the virus can be isolated (Pittman, 1965; Shechmeister et al., 1967; Probstmeyer & Shechmeister, 1968). VSV is known to grow in vertebrates and in arthropods. Therefore it must have the ability to multiply over a broad temperature range. This fact facilitates isolation and study of temperature-sensitive conditional-lethal mutants.

Besides, VSV contains a single-stranded RNA of 3-4 x 10^6 daltons (Huang & Wagner, 1966b; Brown et al., 1967; Nakai & Howatson, 1968). The molecular weight of the nucleic acid allows the number of genes for each genome to be estimated. Cooper (1967) estimates that a RNA chain of 3 x 10^6 daltons would contain about 9,000 nucleotides which code for 3,000 amino acids. Since protein contains on the average 200 amino acids one would expect about 15 proteins. The genome of VSV therefore would be expected to contain about 15 genes. This small number of genes in VSV is likely to facilitate a comprehensive analysis of the viral replicative cycle.

ISOLATION OF TEMPERATURE-SENSITIVE (ts) MUTANTS OF VSV

II. VIRUS

Source of Virus

The Indiana serotype of VSV was obtained from Dr. Henle, The Children's Hospital, Philadelphia, Pennsylvania through the Ontario Cancer Institute.

Preparation and Purification of all VSV stocks

A four ounce Brockway bottle was seeded with about 4×10^6 cells. It was incubated at $37^{\circ}C$ for two to six hours until a monolayer was formed. The medium from the bottle was drained just before it was inoculated with a suitably diluted VSV stock (less than one ml) to give an input multiplicity (number of virus particles added per cell, i.m.) of approximately one. The bottle was then incubated at $30^{\circ}C$ for one half to one hour. Ten ml of medium were then added. The bottle was incubated for another 30 hours before harvest.

At harvest, the contents of the Brockway bottle were poured into a test tube and spun in a clinical centrifuge at 2,000 r.p.m. for five minutes. The supernatant containing the virus was plaque purified as follows: The supernatant was serially diluted to 10^6 and the diluted virus suspension plated in monolayers in petri dishes (see following section) and incubated at $30^{\circ}C$ for 36-48 hours. Well isolated plaques were picked using Pasteur pipettes and virus from each plaque resuspended in three ml PBS. One ml of the virus suspension was regrown in a

Brockway bottle containing a monolayer of L cells. At harvest the virus suspension was centrifuged, the cell pellet discarded and the supernatant stored in sealed ampules of one ml each at -196°C.

Assay of VSV stocks by Plaque Technique

Approximately 2 x 10^6 L cells suspended in 5-8 ml of medium were placed in each 60 mm plastic tissue culture petri dish. After two to four hours the medium was removed and 0.1 ml of virus preparation (diluted with PBS - phosphate buffered saline) was added to each plate and allowed to adsorb for one half to one hour at 37°C. The plates were then overlaid with four ml of a mixture of equal parts of 2.2% agar solution and double strength medium and incubated at 37°C for 24 hours. The plaques were made visible by adding one ml of a 1:4,500 PBS diluted neutral red solution, a vital stain which is selectively taken up by living cells. Thus areas where cells have been lysed by the virus are seen as clear areas (plaques) against a red background (Fig. 5).

In order to measure the accuracy of the plaque assay method, an experiment was performed to determine whether plaque count was consistent with dilution. In Fig. 6 we have plotted the apparent titre against the average number of plaques per dish. If y plaques are produced by plating a volume v (in ml) of a dilution x, the titre n of plaque-forming units (PFU) per ml is $n = xy/v$. In this figure the apparent titre is calculated as follows: For the point with 170 plaques per dish, for example, 0.1 ml of a dilution of 10^5 times of

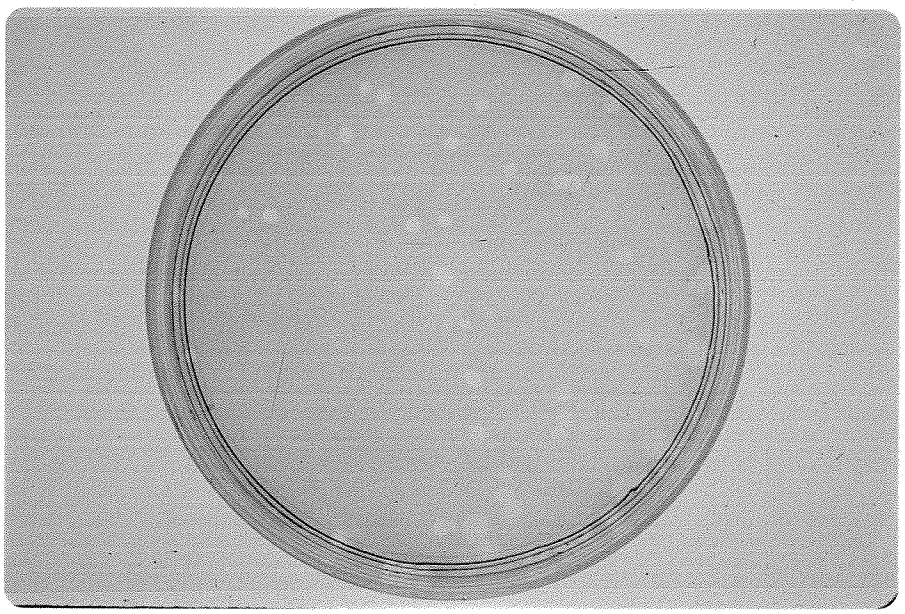

Fig. 5. Plaques caused by VSV. The petri dish shows several p'aques. The living cells were stained with neutral red 20 hours after plating the virus at 38°C. Dead cells in the plaques are unstained.

virus stock gave an average of 170 plaques per dish in four dishes. The titre is therefore $170 \times 10^5 / 0.1 = 1.7 \times 10^8$ PFU/ml. Using a series of N plates and assuming a random distribution, the standard deviation σ should be equal to the square root of the mean number $\bar{n}$ of plaques per dish. The experimental standard deviation $\sigma' = \sqrt{\frac{\Sigma(n - \bar{n})^2}{(N - 1)}}$, compared with σ, provides a test of the randomness of the distribution of particles. Fig. 6 shows that the apparent titre is independent of the number of plaques counted up to 170 plaques per dish. The error bars indicate standard deviation of the determination. The larger the number and the value of $\bar{n}$, the smaller will be the coefficient of variation $\sigma' / \bar{n}$. Of course, when the plaques become too numerous, they are confluent and uncountable. In this experiment we found that within the range of 20 to 170 plaques per dish there will be no effect on the apparent titre. Base on this finding, in all the assays we did throughout this study we used dilution which gave us not more than 170 plaques per dish. Every point showed in the figures of this study represents the average of four counts derived from counting four plates. All the experiments carried out in this study were repeatable.

Selection of Heat-Resistant (HR) Mutant

Wild-type VSV is relatively heat-labile. Since for isolation of ts conditional-lethal mutants a relatively high incubation temperature is required, it is necessary to select as parent stock a strain of VSV that is as stable as possible to heating.

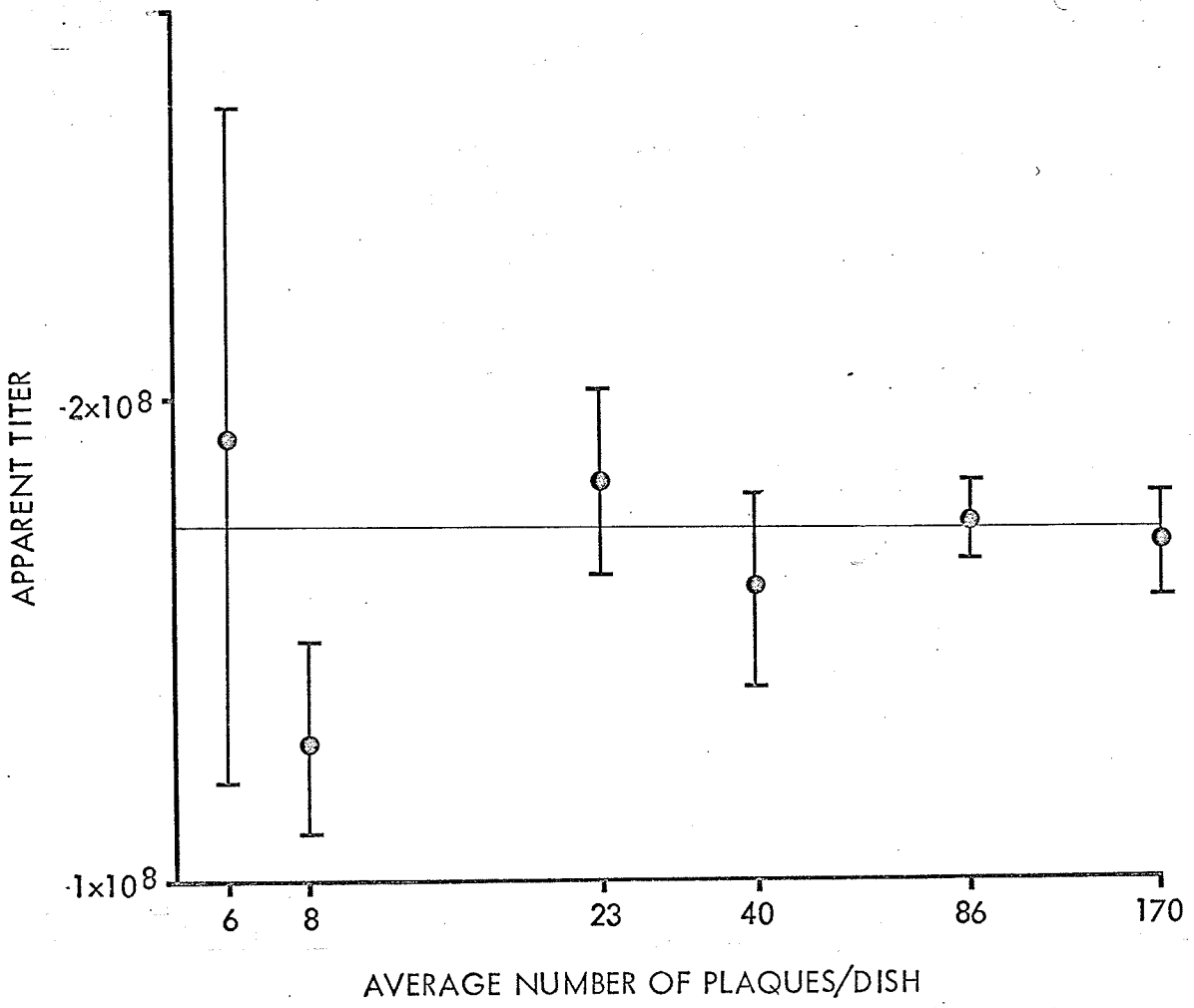

Fig. 6. Relationship between plaque count and apparent titre of VSV. A suspension of VSV was serially diluted. Each dilution was plated on a monolayer in a petri dish. After adsorption for half an hour, the infected cells were incubated at 37°C. About 20 hours later, the plates were stained with neutral red and the number of plaques counted.

Method:

A heat stable mutant was selected by heating the wild-type VSV at 46°C in medium for about four hours and regrowing the survivors to provide another stock for heating at 43°C. This heating cycle at 43°C was repeated several times until the heat-resistance of the mutant could not be further improved.

Result:

Although a single heating cycle failed to select mutants of significantly greater heat stability, selections through several heating cycles finally yielded a mutant (HR) that was substantially more heat stable than the wild-type (Fig. 7). This heat-resistant property of the HR virus is considered a hereditable characteristic that has been preserved through several clonal isolations. The method probably selected a multiple-step mutant with alterations in the proteins that determine the heat stability of the virus. However, with respect to the range of temperature which allows growth, the HR mutant is similar to the wild-type, i.e. it grows well either in cell suspension or in monolayer under agar in the temperature range of 30° to 38°C.

All the ts conditional-lethal mutants described in this report were isolated from this HR strain.

Effect of Input Multiplicity (i.m.) on the growth of VSV

In order to determine whether the yield of virus was dependent on the input multiplicities i.e. the number of virus particles added

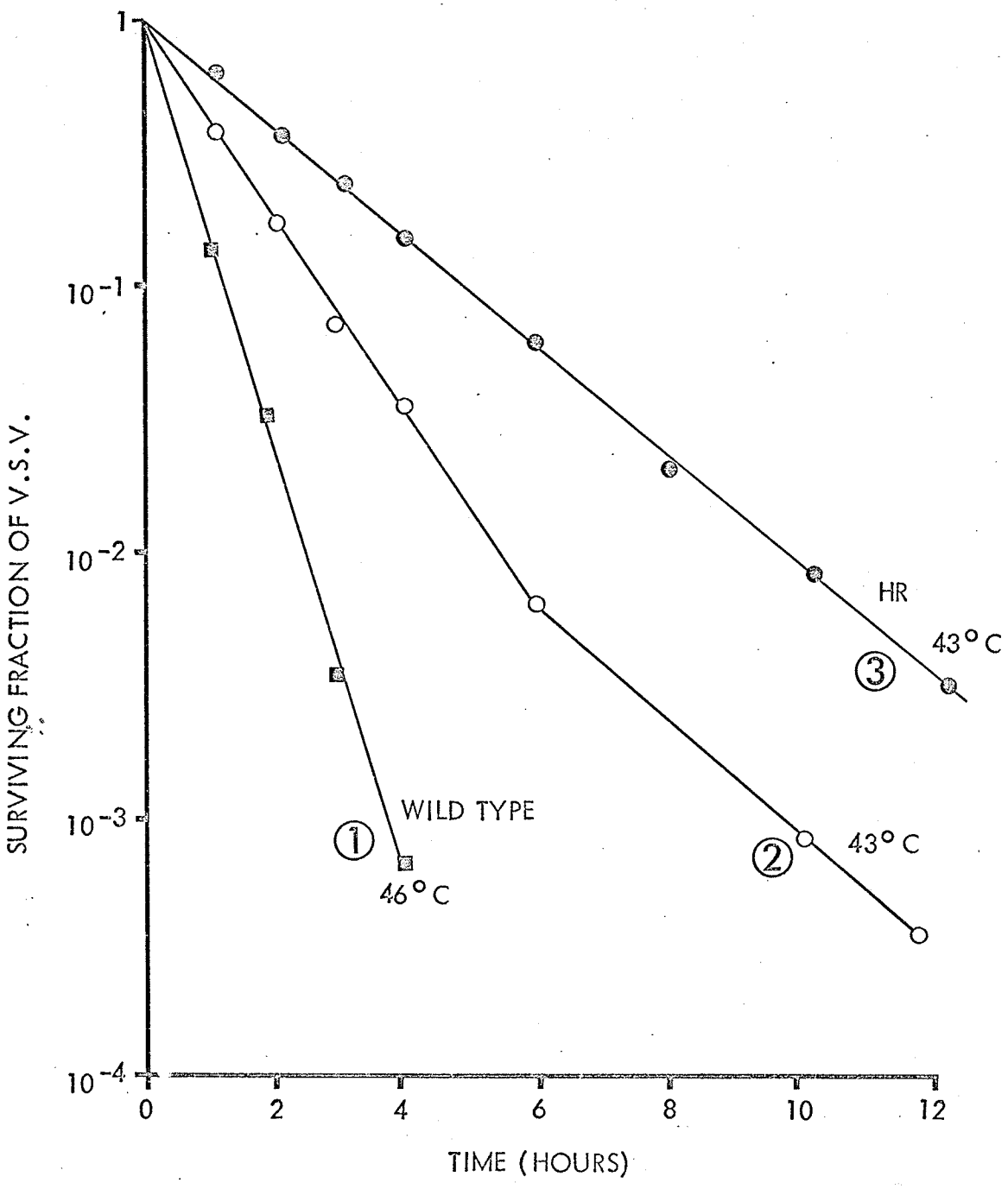

Fig. 7. Surviving fraction of wild type and HR strain of VSV at 43°C and 46°C. Wild type VSV was suspended in medium and incubated at 46°C. At times indicated, 0.1 ml virus suspension was diluted with PBS and assayed at 37°C (1). After four hours incubation at 46°C, the survivors were regrown and incubated in medium at 43°C for 12 hours. The surviving fraction is shown in (2). The heating cycle at 43°C was repeated several times until the heat-resistance of the mutant shown in (3) could not be further improved.

per cell, a series of growth curves were performed using a range of input multiplicities from 1 to 100.

Method:

Cell monolayers were prepared in 45 Brockway bottles with about 4×10^6 cells in each. After two to four hours, the medium was removed from the bottles and 0.5 ml of an appropriately diluted virus suspension was added. After one hour adsorption at 37°C the monolayers were overlaid with 10 ml medium and incubated at 37°C. At various intervals, sample bottles were removed from the incubator and the virus harvested and assayed.

Result:

As shown in Fig. 8, there is a reduced yield of virus with increasing input multiplicity. Highest yields were obtained when the input multiplicity was one. Similar results were obtained by Galasso (1968) with VSV in L cells. Thus the input multiplicity does affect the production of VSV.

Several workers have reported that the non-infectious T particles interfere and inhibit the replication of the virus (Hackett, 1964; Huang & Wagner, 1966a; Huang et al., 1966; Crick et al., 1966; Hackett et al., 1967; Brown et al., 1967). We have also demonstrated with electron microscopy that undiluted passage of the virus results in decreased yield of B particles and enhanced yield of T particles.

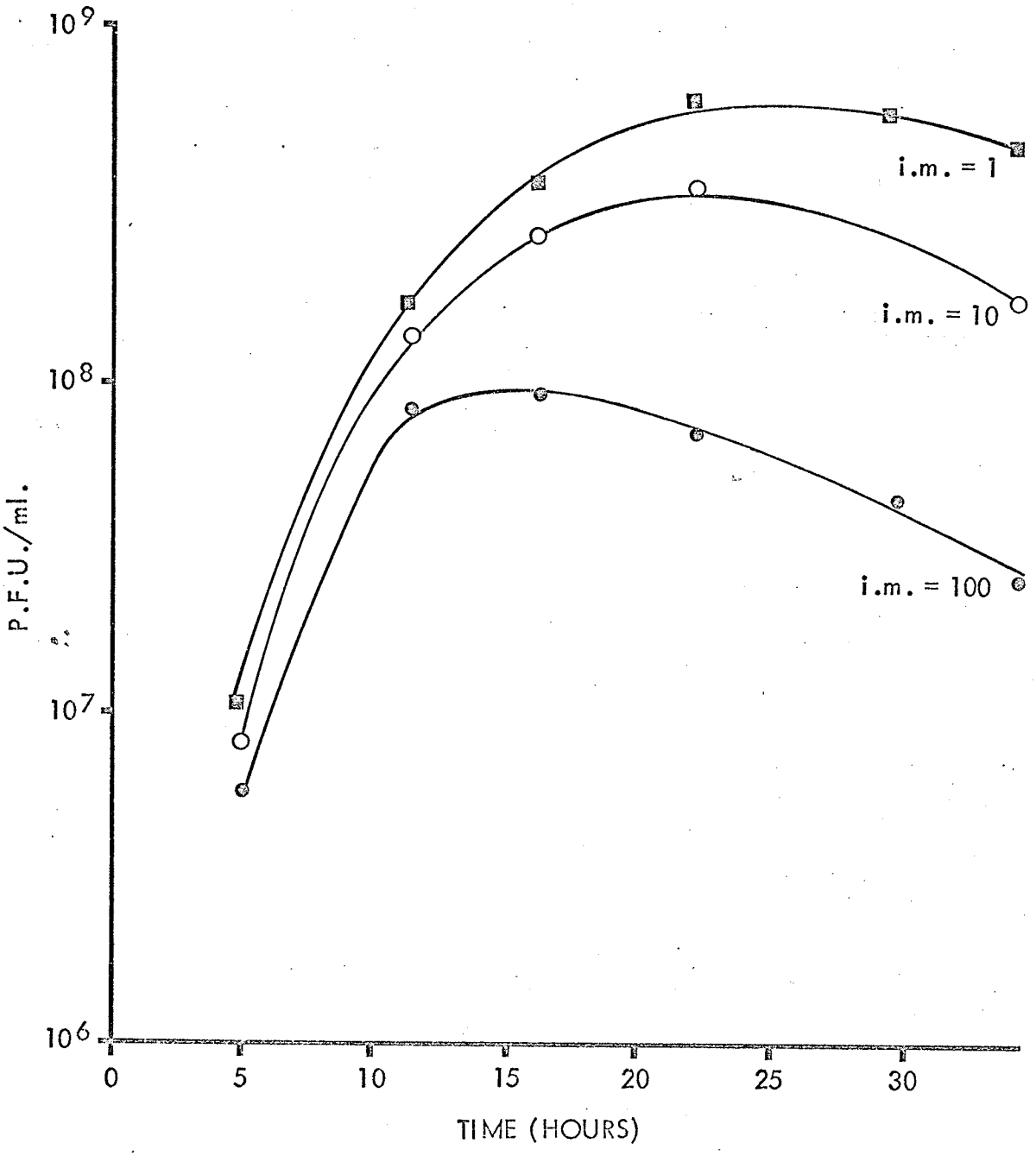

Fig. 8. Growth of VSV in L cells following inoculation with different i.m. at 37°C. Cell monolayers grown in 15, 4 oz Brockway bottles were inoculated with VSV for each i.m. of 1, 10 and 100. After one hour adsorption at 37°C, the monolayers were overlaid with medium and incubated at 37°C. At times indicated, sample bottles were removed and the virus harvested and assayed.

III. MUTAGENIZATION

In order to isolate mutant strains of animal viruses, artificial mutagens are used to increase the mutation rate, thus making it easier to find the mutants. In the present study all mutants were isolated from the heat stable (HR) VSV mutagenized with either ethyl methanesulfonate (EMS) or nitrous acid.

Method:

Mutagenization using EMS

One volume of EMS was added to 150 volumes of PBS (pH 7) containing about 1 x 10^8 PFU of VSV per ml and incubated at 30°C. An aliquot was taken from the virus suspension at the time of mixing (time zero) and at different intervals and assayed at 30°C.

Mutagenization using $NaNO_2$

The virus was exposed to 2.0 M $NaNO_2$ in 0.25 M phosphate buffer* at pH 7 for three to four hours at 27°C. At various time intervals after treatment, an aliquot of the reaction mixture was taken out and assayed at 30°C.

Results:

Figs. 9A & B show the rate of inactivation of VSV by EMS and $NaNO_2$ respectively. It was found that when the surviving fraction was between 10^{-4} and 10^{-5} a suitable number of virus survived for plating. At this time the treated virus was removed and plated directly on L cell

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* 7 ml 0.25 M $NaHPO_4$ solution and
3 ml 0.25 M KH_2PO_4 solution

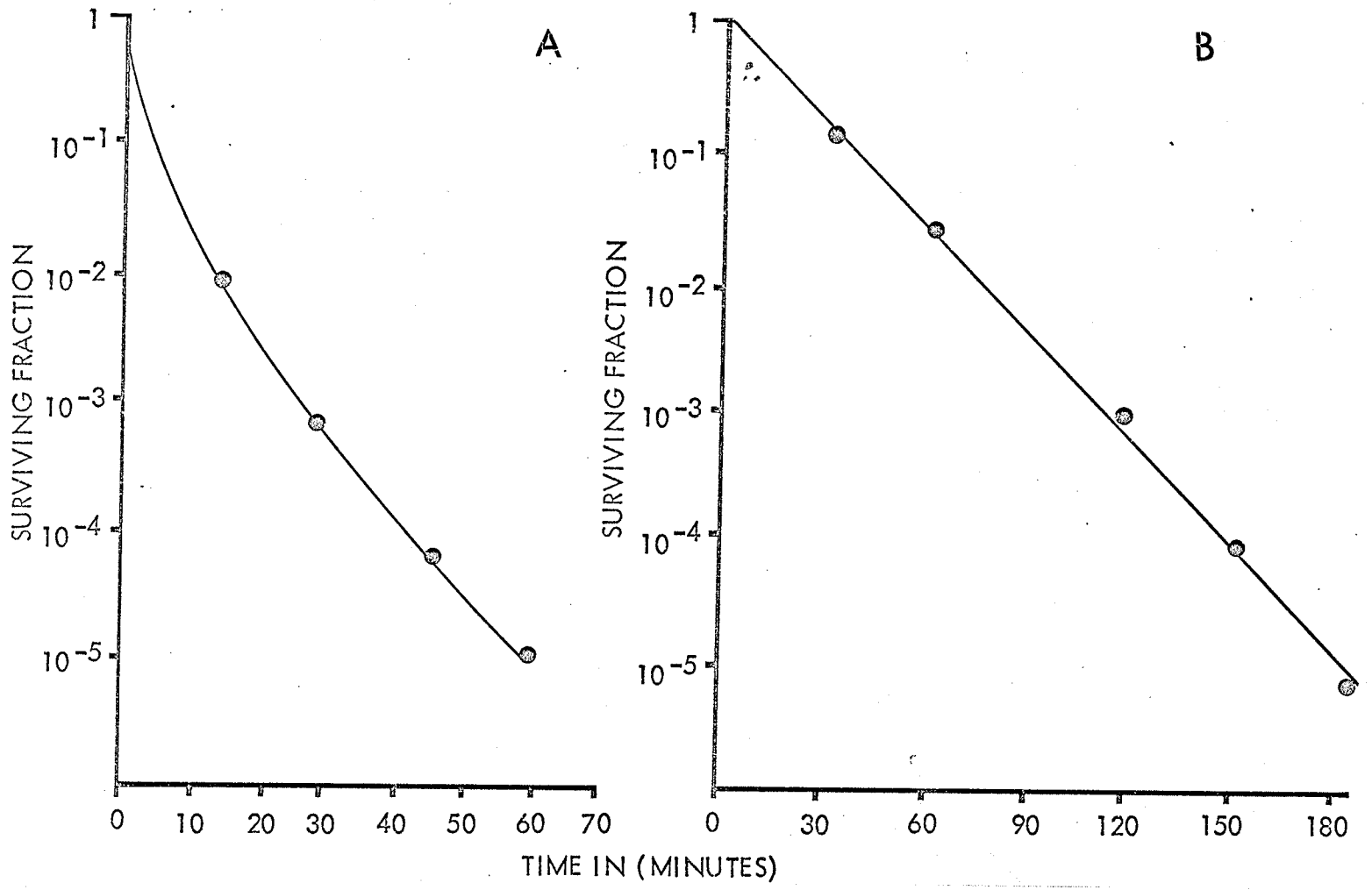

Fig. 9. Inactivation of VSV with chemical mutagens.

A. EMS inactivation of VSV. One volume of EMS added to 150 volumes of PBS virus suspension at pH 7 and incubated at 30°C.

B. Nitrous acid inactivation of VSV. VSV was treated with 2.0M $NaNO_2$ in 0.25M phosphate buffer at pH 7 at 27°C.

monolayers overlaid with an agar medium mixture and incubated at 30°C. After 24 hours the plates were shifted to 38°C for 12-18 hours. Plaques showing little increase in size after the temperature shift-up were considered possible ts mutants and were selected for further testing. Well isolated plaques were picked with a Pasteur pipette and suspended in 2-3 ml of PBS. An inoculum of 0.1 ml of the plaque suspension was then inoculated into each of two 60 mm plates with L cell monolayer, which were subsequently overlaid with agar medium. These plates were incubated at 30° and at 38°C. Virus which produced confluent plaques at 30°C but formed no plaques or a few small plaques at 38°C were regrown in liquid medium for 24 hours at 30°C using virus picked from a single plaque as the inoculum. Thus each mutant stock was cloned at least twice in the process of selection.

Out of about 1,000 plaques picked, eight ts mutants were isolated (Table I).

CHARACTERISTICS OF SOME TEMPERATURE-SENSITIVE (ts) MUTANTS OF VSV

CHARACTERISTICS OF SOME TEMPERATURE-SENSITIVE (ts) MUTANTS OF VSV

I. PLAQUE RATIO

The plaque ratio of the ts mutants as used in this thesis is defined as the ratio of $\frac{\text{No. of plaques formed at } 38^{\circ}C}{\text{No. of plaques formed at } 30^{\circ}C}$. The ratio measured gives some indication of the "leakiness" of the ts mutants and their reversion probability to wild-type during the multiplication of the virus stock. Therefore, knowledge of the plaque ratio is not only important in recombination studies but also gives some indication of the stability of the mutant strains.

To determine the plaque ratio of the ts mutants, a monolayer culture was infected with virus derived from a single plaque and then incubated at $30^{\circ}C$ for 24 hours. The resulting viral stock was titrated at $30^{\circ}C$ and at $38^{\circ}C$.

Results:

As shown in Table I, the plaque ratio of all the ts mutants tested was not greater than 2×10^{-4}. However, as in other investigations it was found that the plaque ratio of VSV varied from stock to stock of the same mutant strain. This may be due to different factors such as input multiplicity, growth conditions and temperature. Ts mutants with a plaque ratio greater than 1×10^{-3} have been isolated but these were considered unsuitable for further biochemical tests and were not further studied.

TABLE I

SUMMARY OF SOME BIOLOGICAL PROPERTIES OF HR STRAIN AND ts MUTANTS OF VSV

Virus	Mutagen used	No. of plaques formed at		Plaque ratio	Surviving fraction after 3 hrs at 43°C	Temperature-sensitive period of growth (hrs. after adsorption
HR	none	$1x10^{9}$	$1x10^{9}$		$2x10^{-1}$	none
ts1	$NaNO_2$	$1x10^{9}$	$7x10^{4}$	$6x10^{-5}$	$8x10^{-2}$	½
ts2	EMS	$5x10^{8}$	$5x10^{4}$	$1x10^{-4}$	$8x10^{-2}$	note 1
ts3	EMS	$5x10^{8}$	$1x10^{4}$	$2x10^{-5}$	$1x10^{-1}$	2
ts4	$NaNO_2$	$2x10^{8}$	$8x10^{3}$	$3x10^{-5}$	$1x10^{-3}$	0-¼
ts5	EMS	$4x10^{8}$	$1x10^{5}$	$2x10^{-4}$	$2x10^{-1}$	½
ts6	$NaNO_2$	$4x10^{8}$	$2x10^{4}$	$5x10^{-5}$	note 2	note 2
ts8	EMS	$2x10^{8}$	$1x10^{3}$	$5x10^{-5}$	$2x10^{-1}$	note 2
ts12	$NaNO_2$	$5x10^{8}$	$1x10^{3}$	$2x10^{-6}$	$1x10^{-1}$	note 1

Note 1: Ts2 and 12 did not show a particular critical temperature-sensitive period during growth. Viral yield slowly diminishes whenever temperature shift-up occurred over the entire experimental period.

Note 2: Not tested.

The plaque ratio as derived from the above formula does not take into consideration viral replication during growth of the stock. This can however be estimated. A plaque starts with a single cell. We know from the one-step growth curve that at 30°C, within eight hours virus particles are liberated and begin to infect cells around it. From the release of viral particles to re-infection probably takes less than an hour. Therefore, in a 40 hour plaque growth at 30°C about five generations of virus have been produced. The plaque is then picked and grown in a bottle at 30°C for 24 hours. It appears that at least one generation of virus must be produced during the 24 hours. Therefore from plaque to final titrations at 30° and 38°C about six generations of virus must have been produced. The plaque ratio per virus generation is therefore probably considerably lower than the figures given in Table I.

II. ONE-STEP GROWTH CURVES

Method:

One-step growth curves at the permissive and non-permissive temperatures were obtained for the HR and ts mutants. Sixty ml of cell suspension containing about 4×10^5 cells/ml were spun down in a clinical centrifuge. The supernatant was discarded and the cell pellet infected with 1 ml of VSV to give an input multiplicity of about ten. After 20 minutes adsorption, the cells were washed in pre-warmed PBS. The infected cells were spun down again and resuspended in pre-warmed medium in a small spinner flask and incubated in a 38°C bath. A second sample of cells were treated in the same way except that they were incubated at 30°C.

At set intervals, aliquots were taken from both spinners and immediately assayed at 30°C.

Results:

When the one-step growth curves of the ts mutants were compared with that of the HR, it was found that at 30°C all mutant strains grew almost as well as the HR at 30°C but somewhat more slowly than did the HR at 38°C (Fig. 10). The final titres of the ts mutants at 30°C were generally slightly lower than that of the HR at either temperature. At 38°C, ts1, 2, 4 and 5 showed little or no growth. But the initial growth rates of ts3 and 12 at 38°C were somewhat less than at 30°C, and the final viral yields were substantially lower than that obtained at 30°C. When the viral yields of ts3 and 12 obtained at 38°C were assayed at 30° and at 38°C, very few plaques were formed at 38°C in contrast to the many plaques formed at 30°C. This result shows that the viral growth at 38°C was mainly due to "leak" rather than to reversion to HR.

III. HEAT STABILITY

Method:

The ts mutants were placed in a 43°C water bath. At intervals aliquot samples were taken out and assayed at 30°C.

Results:

Fig. 11 shows that the mutants ts1, 2, 3, 5, 8 and 12 are only slightly less heat stable than the HR from which they were derived and less heat-labile than the wild-type which can form plaques normally at

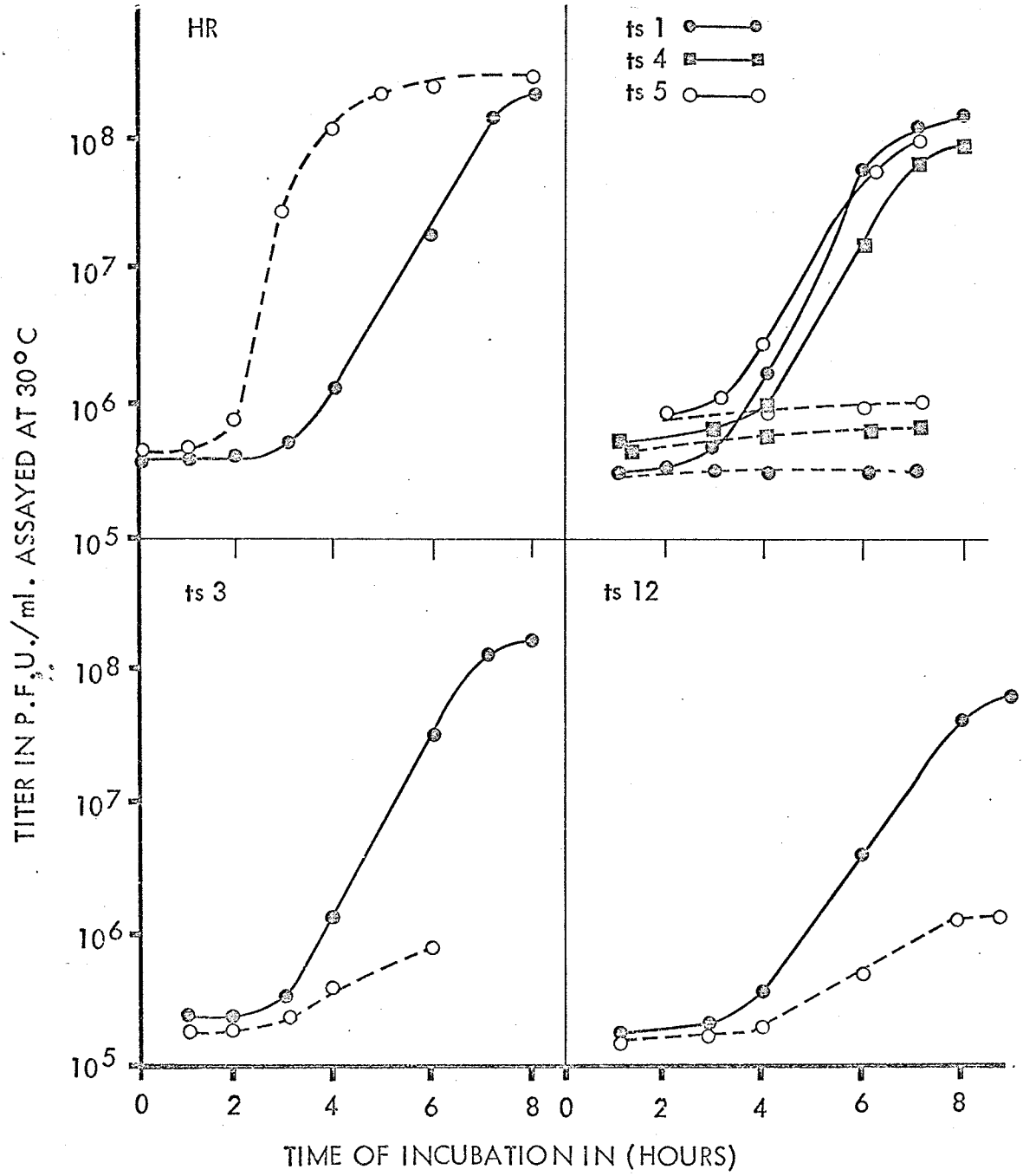

Fig. 10. One-step growth curves of HR strain and ts mutants of VSV in L cells at 30°C (solid line) and 38°C (dotted line). L cells infected with HR strain or one of the ts mutants at i.m. of 10-20 were adsorbed for 20 minutes, washed and then suspended in medium in a 100 ml spinner. Infected cells were incubated and samples removed at times indicated and assayed for viral yield.

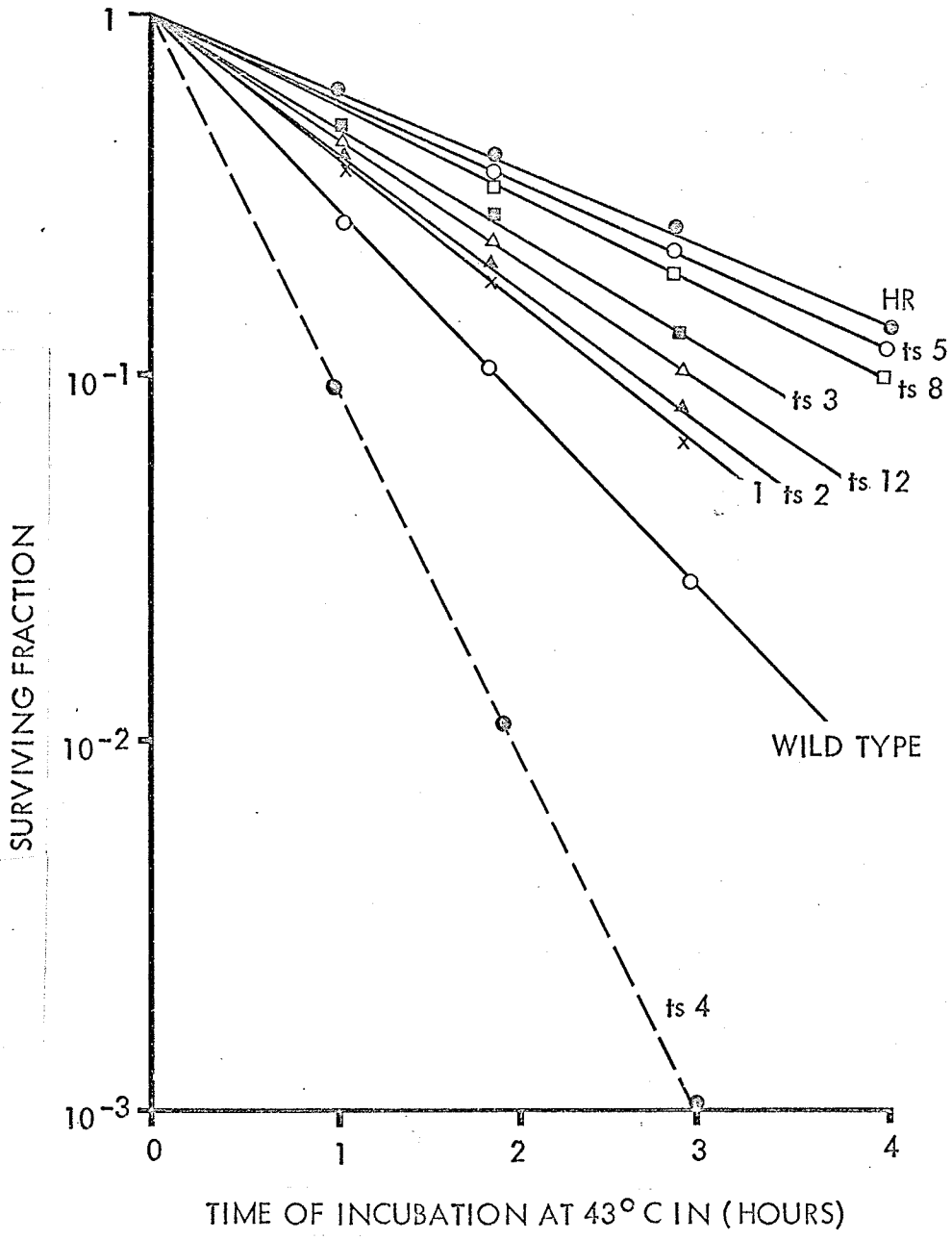

Fig. 11. Surviving fraction of HR strain and ts mutants of VSV at 43°C. Virus stock was suspended in medium and incubated at 43°C. At times indicated, 0.1 ml of virus suspension was diluted with cold PBS and assayed at 30°C.

38°C, whereas ts4 was more heat-labile than the wild-type. The results suggest that the inability of the mutants ts1, 2, 3, 5, 8 and 12 to grow at 38°C is not due to an increase in heat lability of the virus particles.

IV. EFFECTS OF TEMPERATURE SHIFT-UP

Temperature shift-up experiments were carried out in order to investigate the time-dependence of the temperature-sensitive function during the growth cycle expressed by each of the ts mutants isolated.

Method:

One hundred ml of L cells were spun down to one ml. The L cells were then infected with a ts mutant at an input multiplicity of approximately 20. After adsorption for about 15 minutes at 30°C the cells were washed in order to remove unadsorbed virus. The infected cells were then placed in a 100 ml spinner with complete medium and made up to 100 ml. The spinner was placed in a 30°C water bath. Twenty ml of the infected cell suspension were taken out immediately and put into a new spinner which was pre-warmed in a 43°C water bath. This new spinner was then transferred to the 38°C bath for eight hours. After one half hour, 20 ml of the infected cell suspension were again transferred to 38°C and incubated in another pre-warmed spinner. This was repeated at intervals. Viral yield in all spinners was measured at the eighth hour. In addition, the number of infectious centres was measured at the time of each shift-up.

Results:

Fig. 12 shows the results of temperature shift-up on ts mutants 1, 2, 3, 4, 5, and 12. Mutant ts4 seems to have a critical temperature-sensitive period very early in the growth cycle. There was a sharp rise in viral yield when shift-up took place within 15 minutes after adsorption. Both ts1 and 5 show no significant virus multiplication if the infected cells were transferred to 38^{o}C before half an hour, and in the case of ts3, there was no increase in viral yield if shift-up occurred before two hours. Ts2 and 12 however did not show a sharp rise in viral yield no matter when the shift-up took place.

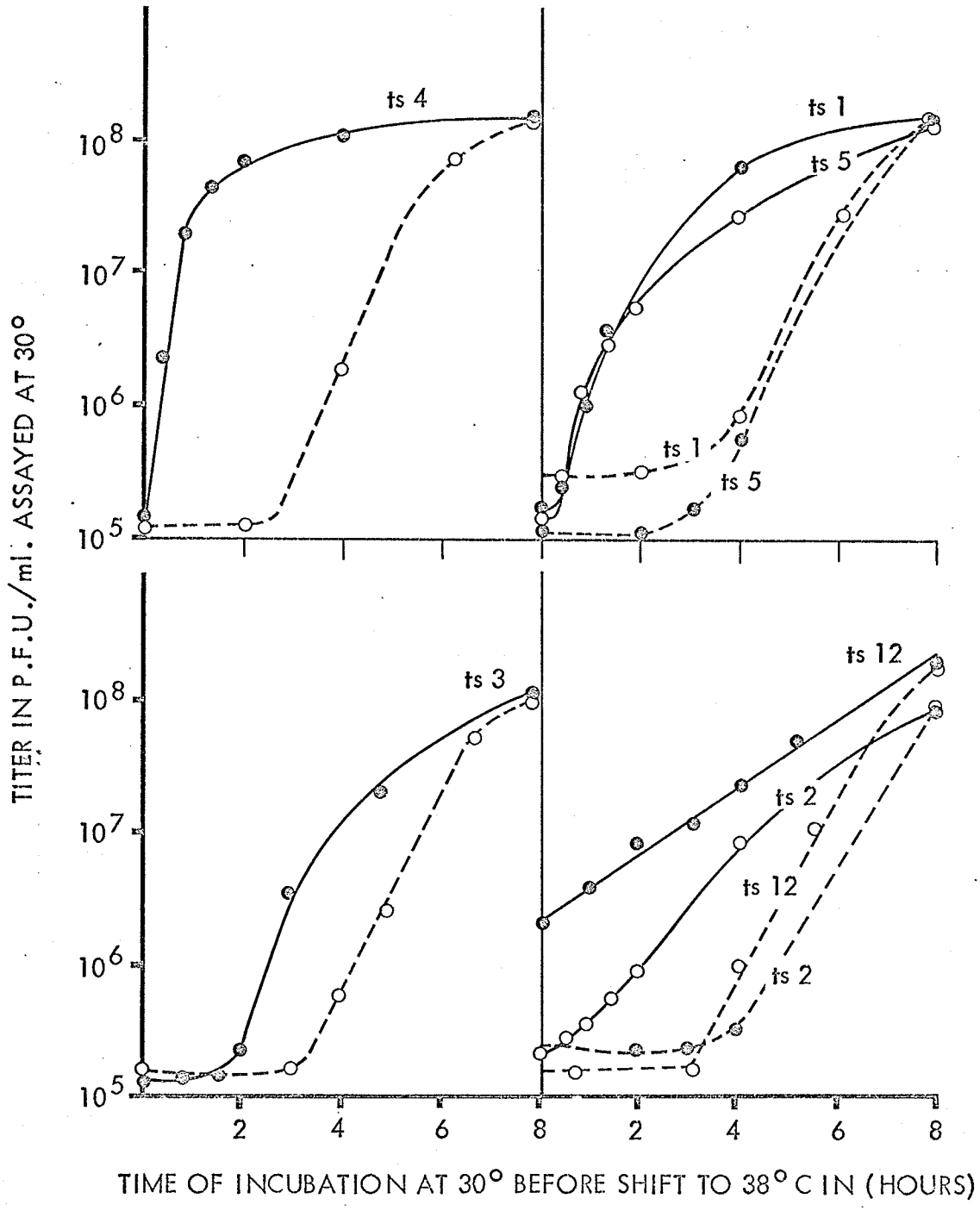

Fig. 12. Temperature shift-up on ts mutants of VSV. L cells were infected with one of six ts mutants at i.m. of about 20. After 15 minutes adsorption at 30°C, the infected cells were washed and placed in a 100 ml spinner with medium made up to 100 ml and incubated at 30°C. Twenty ml of the infected cell suspension was removed immediately and transferred to a 38°C water bath. This was repeated at times indicated and viral yields in all spinners were measured at the eigth hour (solid line). The number of infectious centres was also measured at the time of shift-up (dotted line).

DISCUSSION

DISCUSSION

The work described in this thesis represents an attempt to isolate and to find out the characteristics of some of the temperature-sensitive mutants of VSV (Indiana serotype) which have been isolated in this laboratory using EMS and $NaNO_2$ as mutagens.

The highest mutation frequency of ts mutants so far recorded is 10% of plaques picked after mutagenization (Cooper, 1968). However, Cooper subsequently found that most of the poliovirus ts mutants isolated were double mutants. In our experiments, out of about 1,000 plaques picked, eight mutants were isolated with a plaque ratio less than 2×10^{-4} (Table I). Therefore less than 1% of the survivors of either mutagenic treatment were found to be stable ts mutants. This low value may be because some ts mutants were rejected as a result of plaque formation due to failure to maintain a constant non-permissive temperature of $38^{o}C$ during the plaque forming period. It was subsequently found that mutant ts2 for which the effect of incubation temperature on the plaque forming capacity of the mutants was tested formed plaques equally well at $35^{o}C$ and at $30^{o}C$. However, the number of plaques formed at $35^{o}C$ was 5×10^{4} greater than at $38^{o}C$. Poor temperature control of the incubator when isolation of ts mutants was first carried out in our laboratory probably produced a spuriously high plaque count with some of the rejected mutants. The rate of spontaneous mutation is unknown but is thought to be low. The extent of the genetic change which occurred in the ts mutants cannot be inferred from the available data. It is probable that in the mutants produced, only one gene is affected because of the low mutation frequencies of VSV obtained when treated

with EMS and $NaNO_2$.

In the heat inactivation experiment it was found that ts4 was more heat-labile than the wild-type. It seems probable that ts4 may contain a structural protein which is non-functional at the non-permissive temperature as has been suggested for the heat-labile virions of ts mutants of Sindbis virus (Burge & Pfefferkorn, 1966) and SFV (Tan et al., 1969). However, it was found that for the rest of the ts mutants studied, ts1, 2, 3, 5, 8 and 12, the mature virus particles are as heat stable or almost as heat stable as the HR strain, which suggests that each mutant is defective either in an enzyme or a structural protein and the mutants are sensitive to temperature only during growth. As suggested by Fenner (1969), the temperature sensitivity of such mutants may be due to the fact that the viral proteins synthesized during the growth cycle may be unable to assume their correct configurations or to maintain functional configurations at the non-permissive temperature.

Temperature-shift experiments are carried out in order to determine the time at which the temperature-sensitive defect of ts mutants is expressed. Thus mutants with a "late" function are those which do not produce any significant amount of virus at the non-permissive temperature even when the early part of its growth cycle is maintained at a permissive temperature. In contrast, mutants with an "early" function are those which after a shift from the permissive to the non-permissive temperature go on to produce a nearly normal yield of virus (Pfefferkorn & Burge, 1967). Pfefferkorn & Burge (1967) and Tan et al. (1969) were able to correlate the RNA phenotype of Sindbis and SFV ts mutants respectively

with the time at which the defect was expressed. Thus, the RNA^+ mutants of Sindbis and the $RNA^{\pm}$ and RNA^+ mutants of SFV were defective in some "late" function and the RNA^- mutants (except one in the case of SFV) were defective in some "early" function. Pringle (1968) reported one ts mutant of FMDV with an "early" function and one with a "late" function. Basilico & Joklik (1968) reported one ts mutant of vaccinia virus defective in an "early" function. Of the two ts mutants of reovirus defective in a "late" function, the viral growth of one of them continued for another three hours on shift-up temperature and then ceased (Ikegami & Gomatos, 1968). The authors suggest that viral components may be available which are stable for a short while at the non-permissive temperature, so that virus would continue to be produced until this pool is depleted. Cooper et al. (1967) found that 16 of 17 ts mutants of poliovirus were defective in a "late" function which they define as an event occurring subsequent to synthesis of progeny viral RNA. They however pointed out that "temperature-shift experiments on their own cannot show whether given functions are in fact early or late because heat-defective gene products may or may not be thermo-labile". They are of the opinion that the simplest interpretation of their temperature-shift data is probably that the defective gene products are all made throughout the growth cycle, that their continued functioning is needed for further replication even after some maturation has begun, but that some products are stable and others are unstable at the non-permissive temperature.

We found that for six of the ts mutants of VSV tested, the time at which the defect is expressed in four mutants varied from 15 minutes to two

hours after adsorption, and for two mutants no sharp rise in viral yield was observed no matter when the shift-up occurred (Fig. 12). Unlike Sindbis and SFV ts mutants, there was no correlation of the RNA phenotype with the time of expression of temperature-sensitive defect. Apart from ts12, all the mutants tested are RNA^- (unpublished results). The temperature-sensitive event of ts4 occurred very early in the growth cycle. In the heat inactivation experiment it was found that ts4 was more heat-labile than the wild-type. It has been suggested that the heat-labile virions of ts mutants of Sindbis (Burge & Pfefferkorn, 1966) and SFV (Tan et al., 1969) contain altered structural proteins which may be non-functional at the non-permissive temperature. However, unlike those found in Sindbis and SFV, ts4 is RNA^- and its temperature-sensitive event is expressed within 15 minutes after adsorption. It seems possible that ts4 may contain a heat-labile structural protein which is in some way involved in the uncoating of the virus. Ts1, 3 and 5 seem to be defective in a function necessary for the synthesis of RNA although the temperature-sensitive events of ts1 and 5 are expressed earlier in the growth cycle than that of ts3. The failure to produce a sharp rise of viral yield in ts2 and 12 may be because temperature-sensitive products or functions are required continuously throughout the growth cycle as suggested for poliovirus ts mutants by Cooper et al. (1967), instead of the temperature-sensitive products being produced in a stepwise fashion as in the case of Sindbis (Pfefferkorn & Burge, 1967) and SFV (Tan et al., 1969).

Differences observed in characteristics of the ts mutants of VSV and those of Sindbis, SFV, poliovirus and reovirus can be expected. Compared

with other viruses VSV appears to be intermediate in complexity in the size of its RNA molecule and its protein composition. The molecular weight of the RNA of VSV is 3-4 x 10^6 daltons which is intermediate be-between those of Sindbis (2 x 10^6 daltons) ((Wecker, 1959)), polio (2 x 10^6 daltons) ((Fenner, 1967)) and reovirus (10 x 10^6) ((Fenner, 1967)). While reovirus contains at least seven structural polypeptides (Loh & Shatkin, 1968), poliovirus four (Maizel & Summers, 1968), Sindbis two (Strauss et al., 1968), VSV contains four (Kang & Prevec, 1969) to six (Wagner et al.,1969). As pointed out by Wagner et al. (1969), the protein structure of Sindbis virus is as simple as that of small RNA phages. It can be expected therefore that its replication pattern is more similar to the small RNA phages. Besides the greater complexity of its protein composition, the presence of T particles in VSV may make its replication more complicated than Sindbis virus but perhaps also more interesting.

APPENDIX

APPENDIX

MOLECULAR BASIS OF CHEMICAL MUTAGENIZATION

Mutation in animal viruses can occur spontaneously or by mutagenic agents. Spontaneous mutations appear only during the course of intracellular virus replication and not during the extracellular resting stage. (Hirst, 1965). Different spontaneous mutations occur at characteristic frequencies which are generally low, and it is possible to alter these rates by the inclusion of mutagenic compounds in the growth media. Mutations have been induced in animal viruses by adding mutagens to the cellular growth medium at the time of infection and also by treating the extracellular particles with the mutagen directly. The method employed depends on the mutagen used.

Mutagens act in a number of different ways. Table II lists the mode of action of some of the mutagens so far used for isolation of ts conditional-lethal mutants of animal viruses.

Mutagens like 5-bromodeoxyuridine (5BDU) or 5-fluorouracil (5FU) which act on the virus during the course of viral replication cause mistakes in the replication of the nucleic acid chain, sometimes by becoming incorporated in place of a regular base or sometimes by causing the omission of a base.

The other type of mutagens act directly on the DNA or RNA in vitro and are examplified by nitrous acid or EMS. Nitrous acid is known to deaminate adenine, guanine and cytosine (Freese, 1963). This deamination

TABLE II

MODE OF ACTION OF MUTAGENS USED FOR THE ISOLATION OF ts MUTANTS OF ANIMAL VIRUSES

Mutagenic agent	Mode of Action	Reference	Virus Treated
Nitrous acid	Deamination of adenine cytosine & guanine: Adenine → hypoxanthine Cytosine → uracil Guanine → xanthine	Freese (1963)	rabbitpox, influenza A, Sindbis, polyoma, Vesicular Stomatitis virus.
5-Bromodeoxyuridine (5-BDU)	Base substitution in the replicating DNA strand	Freese (1963)	rabbitpox, vaccinia WR.
5-Fluorouracil (5-FU)	Base substitution in the replicating RNA strand	Freese (1963)	polio, reovirus 3, influenza A, foot-and-mouth disease virus, Semliki Forest virus
Ethyl methanesulfonate (EMS)	Alkylating agent with several possible modes of action Mutate all four bases to varying degree	Bautz & Freese (1960), Lawley & Brooks (1961) Tessman et al. (1964)	Sindbis, Vesicular Stomatitis virus.
N-methyl-N'-Nitro-N-Nitroso guanidine (NTG)	Acting on guanine through intercalation of NTG molecules between the guanine rings	Singer & Fraenkel-Conrat (1967)	Sindbis, Semliki Forest virus, respiratory syncytial virus.
Proflavin	Intercalation in the replicating nucleic acid strand	Freese (1963)	rabbitpox

results in changed base pairing characteristics (Fig. 13) so that when the affected chain is used as a template for construction of a complementary chain, the resulting base sequence may be expected to differ from the normal one. If the new chain is then recopied, the base sequence alteration is perpetuated. Deamination of cytosine resulting in its replacement by uracil is termed a transition mutation in which a pyrimidine replaces another pyrimidine. When adenine is deaminated the ultimate result is to replace it with cytosine, a transversion mutation in which a purine is replaced by a pyrimidine.

The mutagenic effects of EMS have been attributed mainly to the formation of the major product of nucleotide alkylation: 7-alkylguanine (Bautz & Freese, 1960; Lawley & Brooks, 1961). Tessman et al. (1964) found that EMS mutates all four bases but to varying degrees. Wong et al. (in press) showed that 7-methylguanine (7MG) and N-methylguanine (NMG) function as normal bases in their pairing with cytosine, whereas 1-methylguanine (1MG), dimethylguanine (DMG), methyladenine (MA) and dimethyladenine (DMA) cannot pair with cytosine and uridine (Fig. 14). These findings suggest that mutation with EMS may also be due to the formation of such non-pairing bases, as well as to the enhanced ionisation at N-1 of 7-alkylguanine as suggested by Tessman et al. (1964).

It is probable that some "missense" mutations produced by mutagens result in defective gene products whose defects are apparent only at high temperatures. The decrease in thermo-stability or inability to polymerise with other proteins or incorrectness of folding during

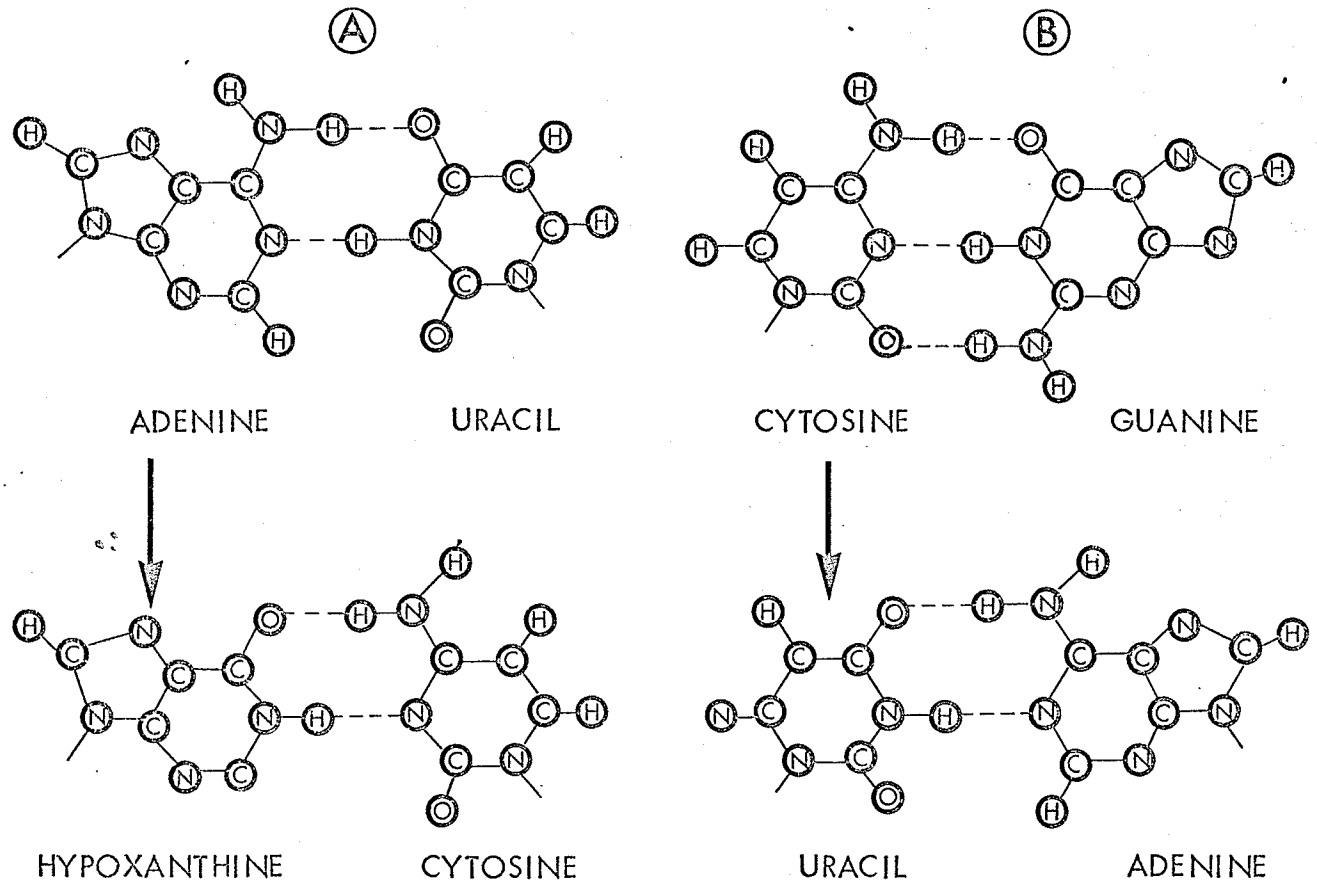

Fig. 13. MUTAGENIC EFFECT OF NITROUS ACID

Deamination of adenine to hypoxanthine (A) and cytosine to uracil (B) resulting in changed base pairing characteristics.

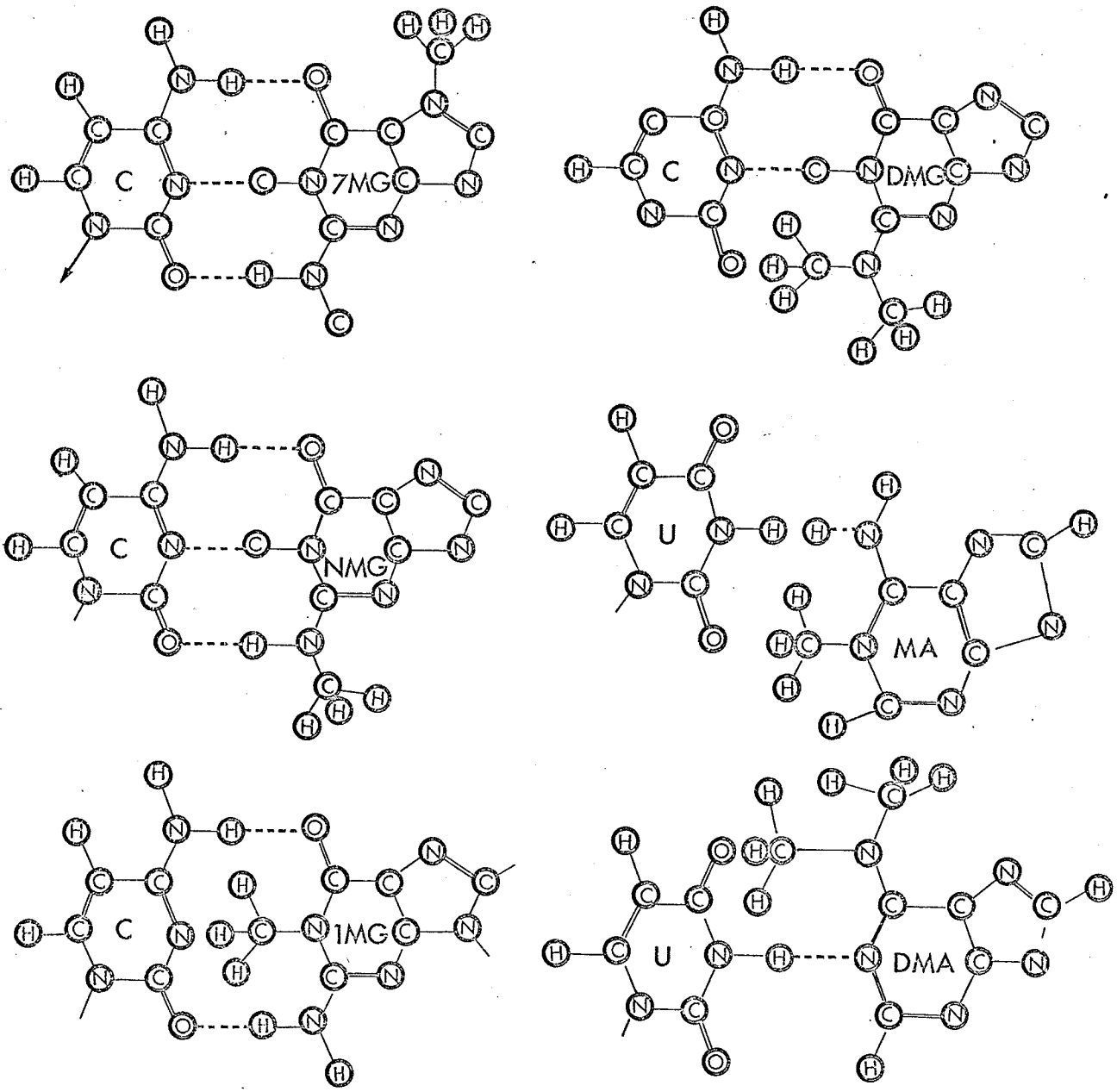

Fig. 14. Pairing characteristics of alkylated bases of nucleic acids. 7MG and NMG function as normal bases in their pairing with cytosine, whereas 1MG, DMG, MA and DMA cannot pair with cytosine and uridine.

synthesis of the proteins seems to be supported by the fact that the configuration of proteins depends upon critically placed hydrophobic bonds, together with salt and disulfide linkages between various amino acids (Kauzmann, 1959). Cooper (1964) pointed out that "a replacement of any amino acid by another will be unlikely to result in the same type of cross-linkage and accordingly will usually weaken the non-primary structures. The structures will then become more dependent on the hydrogen bonding that exists between peptide links, and hence more easily changed by a small rise in temperature. Only a very small change in non-primary structure, perhaps originating at points distant from the "active site" may suffice to alter the molecules' metabolic efficiency".

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